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Genetic and morphological identification of formalin fixed, preserved larval fishes; can we have the best of both worlds?

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ABSTRACT

Surveys of larval fishes require accurate identifications of larvae, which are essential to understand early life history of fish, fish ecology and fisheries. However, the identification of larval fishes requires microscopic examination that is substantially more difficult than that of juvenile and adult fishes, as many larval stages remain undescribed. Furthermore, the traditional, formalin fixation of larval fishes were previously thought to prevent genetic sequencing compared to ethanol preserved larvae. In this study, we used an integrative taxonomic approach based on morphology, imaging and DNA barcoding of the mitochondrial (mtDNA) cytochrome *c* oxidase subunit (COI) gene. We used this approach in both cultured yellowtail kingfish, *Seriola lalandi* and wild sourced fish larvae that had been fixed in 5% formalin. Based on controlled and in-field formalin treatments, DNA barcoding and genetic species identification was 100% successful in cultured yellowtail kingfish fixed in formalin for up to 6 months, while barcoding of wild caught fish larvae enabled species identification of 93% of up to 8-weeks formalin fixed specimens. Furthermore, we demonstrated the viability of using either whole larval individuals or a single eyeball (<1 mm diameter, thus retaining the specimen intact) from formalin fixed larval fish for genetic species identification. While COI genetic identifications from the in-field experiments were patchier than the controlled experiments, our study highlights the possibility of recovering suitable DNA from larvae that have been fixed in formalin for up to six months. This was achieved by applying DNA extraction methods that use de-cross-linking steps and species identification based on both full-length reference and mini-barcodes. Our study provides the larval fish research community with a practical framework for undertaking both morphological and genetic identifications of larval fish assemblages, particularly when geographic relevant reference sequence databases (based on vouchered adult fishes) are available for interrogation. It also simplifies field-based collection of samples allowing their preservation in formalin without compromising the genetic identification of species.

1. Introduction

Information obtained from larval fish surveys, coupled with the accurate identification of larvae, is essential for our understanding of early life stage biology and ecology of fishes. Such information supports biodiversity and fisheries resource management, identification of fish spawning areas, ecosystem monitoring and the tracking of fish population dynamics and trends (Hallerman, 2020; Hou et al., 2021; Ko et al., 2013; Marancik et al., 2020; Mateos-Rivera et al., 2020; Panprommin et al., 2021; Smith et al., 2018). However, the identification of larval fishes is substantially more difficult than that of juvenile and adult fishes due to the small size of specimens, a general lack of diagnostic morphological and meristic characters in larval fishes, reliance on morphometric analyses and overlapping fish spawning areas. Because of this, generally when larvae are morphologically identified under the microscope, taxonomic classification to the level of family, is more likely

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achieved than to genus or species level (Cunningham et al., 2000; Hou et al., 2021; Ko et al., 2013; Panprommin et al., 2021; Silva-Segundo et al., 2021; Steinke et al., 2016). Moreover, of the approximately 34,000 species of living teleosts (Fricke et al., 2022), less than 10% have larval stages described to genus or species. Leis (2015) reported that for many Indo-Pacific fish families, <50% of species have described larvae, with only a small proportion of species descriptions based on full development series of larvae. Therefore, the possibility of misidentification of a known species with undescribed members of the genus or family remains high.

To enable the microscopic examination of larval fishes for identification, specimens are usually fixed in a 5% dilution of concentrated formaldehyde (a 37% saturated solution of formaldehyde gas), colloquially known as 5% formalin, and then transferred into 70% ethanol for long term storage. The time spent in formalin and the formalin concentration used may vary depending on the size of the specimen(s), field sampling logistics and institutional processes (Gordeeva et al., 2019; Appleyard et al., 2021). Formalin fixation preserves the pattern of melanophores and morphological integrity of larval fishes to enable identification and possible determination of age and growth, providing the solution is buffered to conserve the otoliths (Cunningham et al., 2000). Generally, larval fixation in a 5–10% buffered formalin solution results in less shrinkage and damage to morphological features than in concentrated ethanol (Cunningham et al., 2000; Hanahara et al., 2021; Hou et al., 2021; Mateos-Rivera et al., 2020) and because of this, the use of formalin for fixation of plankton is commonplace on research vessels. However, formalin fixation was not usually recommended for genetic techniques given that resulting DNA yields may be low and substantially degraded (Klanten et al., 2003; Lewis et al., 2016), thereby hindering the ability of using molecular techniques for species identification. To date, typical sample or tissue preservation is either fresh, frozen or storage in a suitable preservative (e.g., 95% ethanol) to support genetic identifications. The downside to using alcohol for larval preservation is that alcohol solutions may not prevent degradation of animal tissue (Gagliano et al., 2006) and may damage the morphological features of fish larvae, making studies of larval fishes difficult over time (Hou et al., 2021).

Since the early 2010s, an increasing number of studies have combined larval fish morphology, imaging and DNA barcoding to identify species and elucidate larval taxonomy (Hanahara et al., 2021; Ko et al., 2013; Marancik et al., 2020; Mateos-Rivera et al., 2020; Nonaka et al., 2021; Panprommin et al., 2021; Silva-Segundo et al., 2021). Indeed, Ko et al. (2013) has stated that many of the issues with larval fish identification can be resolved with the use of molecular techniques, with DNA barcoding as the main method used for genetic species identification. While fish specimens fixed in formalin were assumed to be unsuitable for genetic or genomic analyses, recent studies have proven this not to be the case (Appleyard et al., 2021; Gordeeva et al., 2019; Hanahara et al., 2021; Hou et al., 2021; Richardson et al., 2016; Vivien et al., 2016); although the extraction of useable DNA from formalin fixed specimens and subsequent successful genetic identifications, remains challenging. Success depends on several factors including size of the specimen, duration of fixation, formalin concentration, the DNA extraction method, and the DNA sequencing approach. Recently, Richardson et al. (2016) used diagnostic morphological characters and sequencing of 5% formalin (buffered with seawater) fixed tuna larvae ($n = 24$). While some of the larvae were in formalin for approximately one year, 83% of the tuna larvae were genetically identified using single nucleotide polymorphisms in short sequences of the mtDNA NADH dehydrogenase 5 gene. Hanahara et al. (2021) demonstrated that short-term (24 h) fixation in 10% seawater formalin of gobioid larvae (followed by fixation in 70% ethanol for up to 7 years) enabled both morphological and genetic identifications. Similarly, Hou et al. (2021) successfully DNA barcoded fixed (<10 days in formalin) larval fish from the South China Sea, although they were not able to obtain high-quality sequences from larvae fixed in formalin for >30 days (Hou et al., 2021). Appleyard et al. (2021) identified whole (up to 10 mm) larval fish specimens from Tasmanian waters fixed in 10% formalin for 6 weeks, with either Sanger sequenced full-length reference COI barcodes or Illumina sequenced mini-barcodes obtained from several specimens. The DNA extraction method used in Appleyard et al. (2021) was based on a de-cross-linking step to liberate useable DNA. Where DNA barcoding was not successful, the relatively small size of the larvae, rather than formalin exposure, was deemed the reason for poor sequencing (Appleyard et al., 2021).

DNA barcoding is a method of species identification based on a short section of mitochondrial DNA (mtDNA). The global research community adopted the mtDNA COI gene region (≈650 base pairs) as the barcode standard for the animal kingdom (Chambers and Hebert, 2016; Hajibabaei et al., 2007; Hebert et al., 2003; Shokralla et al., 2015). Shorter sections of the COI gene region, or mini-barcodes are also deployed (see Leray et al., 2013; Shokralla et al., 2015; Appleyard et al., 2021) for fish metabarcoding, environmental DNA analyses, authentication of fish projects and in degraded DNA studies. As of January 2022, the international Barcode of Life Data System (BOLD) contains over 23,000 species of fishes with COI barcodes (from Class Actinopterygii) – these barcodes are accessible for fish species comparisons and genetic identifications.

Australia's Integrated Marine Observing System (IMOS) established in 2014, the Larval Fish Monitoring Program, where monthly sampling occurs at five national reference stations (Smith et al., 2018). Samples were initially formalin fixed at sea, but since mid-2016, half the plankton samples were fixed in 5% formalin while the other half are preserved in 95% ethanol for genetic identification. The logistics of sampling at sea and transporting ethanol samples around the country is a complex process as these samples are classified as dangerous goods. Additionally, ethanol must be replaced in the ethanol preserved samples within 48 h of sampling, thereby complicating the process further. Collecting information on larval fish assemblages and their accurate identification for ecological and fisheries studies is essential. This requires extensive resources for sample collection, preservation and maintenance and the identification of larval fish. Any improvement in this process can help to optimise the use of resources and logistics (Asch, 2015; Peabody et al., 2018; Hinchliffe et al., 2021; Matis et al., 2014; Schilling et al., 2020, 2021). Currently, the largest Australian larval fish collection is housed in the Australian Museum with over 50,000 lots (Leis, 2015) including many of the samples detailed in Smith et al. (2018).

With a view to ensuring that collected Australian larval fish assemblages continue to provide information for societal benefits, the study's authors, alongside the CSIRO Australian National Fish Collection (ANFC) are undertaking a multi-disciplinary approach to larval fish identification by combing alpha taxonomy, larval imagery, and extensive metadata curation with COI barcoding of larval fishes. The ANFC currently manages and curates a COI reference sequence library in BOLD for Australian fishes and has recently succeeded with the genetic identification of adult and larval fishes based on degraded DNA sourced from specimens exposed to formalin (Appleyard et al., 2021). We actively support the need for maintaining taxonomic expertise and knowledge of regional (in this instance, Australian) fish species diversity; therefore in this study, we use an integrated taxonomic approach that uses barcoding (with pairwise identity of ≥98% for recognising that two samples are likely from the same species, as per Ward et al. (2009) and Knebelsberger et al. (2014)), imagery, spatial occurrence data and sampling information for reaching consensus on species identifications.

The current study aimed to (a) determine the optimal time that larval fish samples (whole specimens and or single eyeballs (approximately 0.3–0.5 mm diameter) removed from larvae), can be exposed to 5% formalin while still enabling successful sequencing of DNA (i.e., the 'sweet spot'); and (b) investigate the effect of formalin fixation on COI DNA barcoding for fish species identifications from a controlled and an in-field experiment. This new knowledge will enable the application of optimal fixation and preservation treatments for larval fishes and

voucher specimens, that enable both morphological and genetic techniques for species identification.

2. Materials and methods

Two experimental approaches were used in this study. The first experiment consisted of laboratory-controlled formalin fixation of hatchery reared larval fish, and the second experiment was based on in-field tests of formalin fixation of wild collected larval fishes. Irrespective of the length or type of formalin fixation or experimental approach, larval fishes were processed in the same way for genetic identification in the laboratory. Fig. 1 is a schematic of the laboratory processing pipeline that was followed for each larval specimen.

2.1. Controlled formalin fixation experiment

Two replicate collections were made of yellowtail kingfish (*Seriola lalandi* Valenciennes 1833) larvae at 31 days post hatching after an induced mass spawning event on 21.10.2020 at the New South Wales Department of Primary Industries, Port Stephens Fisheries Institute. Larvae were cultured in tanks following methods described in Field and Heasman (2011). Larvae ranged in size from 5.3 to 8.3 mm notochord length and were fixed in 5% formalin (using freshwater as the diluent). Larval fish were processed after a series of exposure times to formalin fixation (1-day, 1-week, 2-weeks, 4-weeks, 8-weeks, 12-weeks, 16-weeks, 20-weeks, and 24-weeks) (Table 1). Within each fixation treatment, there were 8 whole larvae and 8 paired right-side eyeballs – thereby 16 samples per treatment. For each replicate, four larvae from the bulk sample of larvae, the largest, smallest and two intermediate specimens were selected for analysis.

After each period of formalin fixation, the *S. lalandi* larvae were placed into 100% absolute ethanol, before being imaged and a single eye removed from each larva. Following imaging (see below), individuals and accompanying eyes were placed into 96-well plates, overlaid with a drop of ethanol, and sent to the CSIRO marine laboratories in Hobart. On arrival in the genetics laboratory, the *S. lalandi* 96-well plates were stored at −20 °C until DNA extraction.

2.2. In-field formalin fixation experiment

Fixed and ethanol preserved larval fish from the IMOS Maria Island (MAI) National Reference Station (NRS) (Lat/Lon 42.597 S, 148.233 E), collected in 2018–2020 were used in this experiment. Sampling for larval fish from the pelagic environment was undertaken as per Smith et al. (2018). Eighty-four archival fish larvae were fixed and kept in 5% formalin in seawater for different time periods (approximately 1-day, 1-week, 3-weeks, 4-weeks, and 8-weeks) before transferring to 70% analytical grade ethanol. Larval fish were identified by one of the authors and taxonomic expert (A.G. Miskiewicz) where possible to either family, genus or species level using morphological characters. After morphological identification, specimens were sent in jars containing ethanol to the CSIRO marine laboratories in Hobart. On arrival in the genetics laboratory, jars were stored at room temperature, larvae were individually imaged (see below) and placed into 96-well plates in preparation for DNA extraction.

Table 1
5% formalin fixation treatment of mass spawned *S. lalandi* larvae (31 days post hatching), following spawning on 21.10.2020 at New South Wales Fisheries Research Institute at Port Stephens.

Date	Day	Week	Label	Sample sizes per replicate
24.11.2020	1		Day1	Day1R1 (4 larvae +4 eyeballs); Day1R2 (4 larvae +4 eyeballs)
01.12.2020	7	1	Week1	Week1R1 (4 larvae +4 eyeballs); Week1R2 (4 larvae +4 eyeballs)
08.12.2020	14	2	Week2	Week2R1 (4 larvae +4 eyeballs); Week2R2 (4 larvae +4 eyeballs)
22.12.2020	28	4	Week4	Week4R1 (4 larvae +4 eyeballs); Week4R2 (4 larvae +4 eyeballs)
19.01.2021	56	8	Week8	Week8R1 (4 larvae +4 eyeballs); Week8R2 (4 larvae +4 eyeballs)
16.02.2021	84	12	Week12	Week12R1 (4 larvae +4 eyeballs); Week12R2 (4 larvae +4 eyeballs)
16.03.2021	112	16	Week16	Week16R1 (4 larvae +4 eyeballs); Week16R2 (4 larvae +4 eyeballs)
13.04.2021	140	20	Week20	Week20R1 (4 larvae +4 eyeballs); Week20R2 (4 larvae +4 eyeballs)
11.05.2021	168	24	Week24	Week24R1(4 larvae +4 eyeballs); Week2R2 (4 larvae +4 eyeballs)

2.3. Imaging of larvae

Prior to DNA extraction (and simultaneous specimen destruction), whole larval images were taken for each specimen to retain a morphological identification reference. Using fine larval forceps, specimens were transferred to an initial wash of 100% ethanol to remove any debris carrying exogenous DNA or obscuring characteristic features. Larval specimens from the in-field experiment at MAI were photographed using a Leica Microscope M250C (Leica, Germany) with an LED5000 HDI™ illuminator set to maximum light setting, a Leica FlexiDome™ attachment, and associated software (Leica Application Suite LAS V3.6). The 'focus stack' application was used to ensure detailed capture of the entire surface of the specimen and the resulting montage image was retained.

The *S. lalandi* larvae were photographed using a digital Olympus Tough TG6 camera using the microscope and focus stacking settings.

For the *S. lalandi* larvae, a lateral image was taken (representative images of yellowtail kingfish are included below, see Fig. 2). For the MAI larvae, various views, including dorsal, ventral, and lateral views were imaged for each specimen where possible. Total lengths of each specimen (and diameter of eye if being used) was measured and recorded in millimetres. The images for both studies were edited using the IrfanView 64 and Paint 3D and a scalebar (in mm) was added to images using the scale bar tool. Montage images of the *S. lalandi* were exported, saved as .

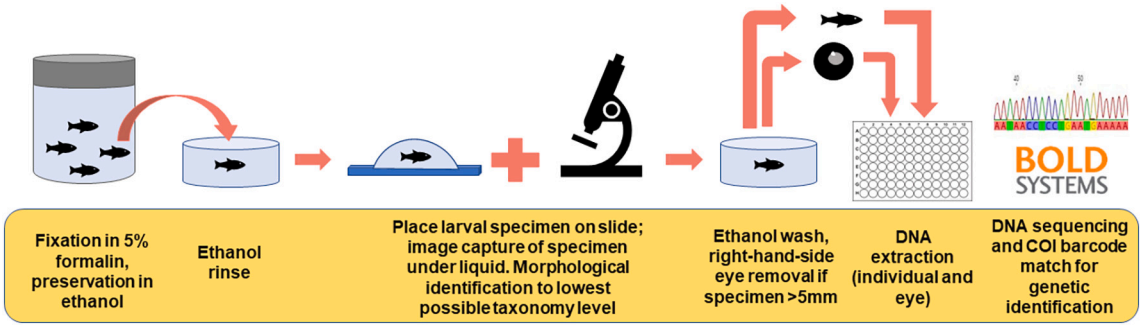

Fig. 1. Schematic of larval fish processing, imaging and genetic identification following formalin fixation.

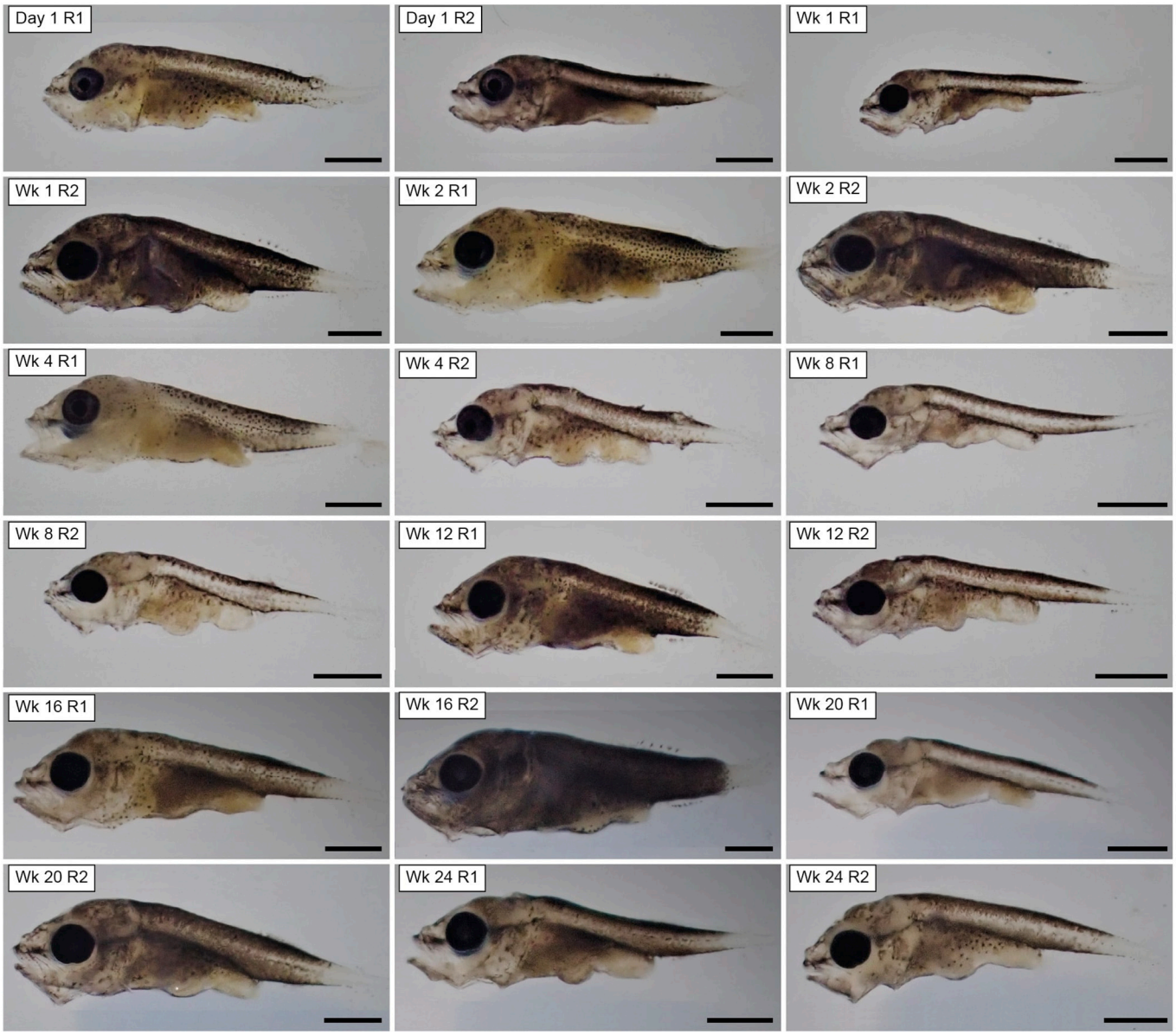

Fig. 2. Lateral images of *S. lalandi* larvae (two per treatment, R1 & R2) following 5% formalin fixation at 1-day (Day 1 R1, Day 1 R2); 1-week (Wk 1 R1, Wk 1 R2); 2-weeks (Wk2 R1, Wk R2); 4-weeks (4Wk R1, 4Wk R2); 8-weeks (Wk8 R1, Wk8 R2); 12-weeks (Wk12 R1, Wk12 R2); 16-weeks (Wk16 R1, Wk16 R2); 20-weeks (Wk20 R1, Wk20 R2); and 24-weeks (Wk 24 R1, Wk24 R2) time points. Scale bar = 1 mm.

jpg files and are stored on CSIRO Data Access Portal (Appleyard et al., Strategic ANFC Research, CSIRO Data Access Portal, 2022, https://data.csiro.au/collection/csiro:54021). Representative images of the MAI larval fish are included below (see Fig. 3).

2.4. DNA extraction of larval specimens

Following imaging, DNA was extracted from the *S. lalandi* and MAI larval fish specimens (whole individuals and right-side eyeballs) using two different methods; – (a) modified small volume Wizard® SV Genomic DNA Purification system (Promega, Australia) for larvae that had been fixed in formalin for 1-day and 1-week; or (b) reduced volume ReliaPrep™ FFPE gDNA Miniprep system (Promega, Australia) for larvae that had been fixed in formalin for 2–24-weeks. Both extraction methods were based on a fifth (1/5) volume reduction from that used for adult fish tissues (see Appleyard et al., 2018, 2021) with digestions and extractions undertaken in 96-well plates where total volume capacity of each well was 200 µl. Plates were always capped with strip caps.

For the modified small volume Wizard genomic DNA extraction, whole individuals and eyes were digested overnight at 55 °C (as per manufacturer's instructions using a 1/5 reduced volume and including 20 µl of 20 mg/ml Proteinase K) and then lysed in 96-well plates. The contents of each well were then loaded onto the 96-well lysate clearing plates. Three rounds of washing with column wash buffer (300 µl per round) were undertaken and DNA was precipitated by two rounds of elution with 40 µl of water per round.

For the reduced volume ReliaPrep gDNA extraction, whole individuals and eyes were digested overnight at 55 °C (as per manufacturer's instructions using a 1/5 reduced volume and including 20 μl of 20 mg/ml Proteinase K) and then lysed in 96-well plates. After overnight digestion, samples were de-crosslinked at 80 °C for two hours and then cooled to room temperature. Following de-crosslinking, 1/5 volumes of the ReliaPrep BL buffer and 95% ethanol were added to each well. Samples were vortexed briefly and spun down. The contents of each well were then loaded onto 24 individual binding columns, attached to a vacuum manifold. The vacuum was applied until the lysate passed

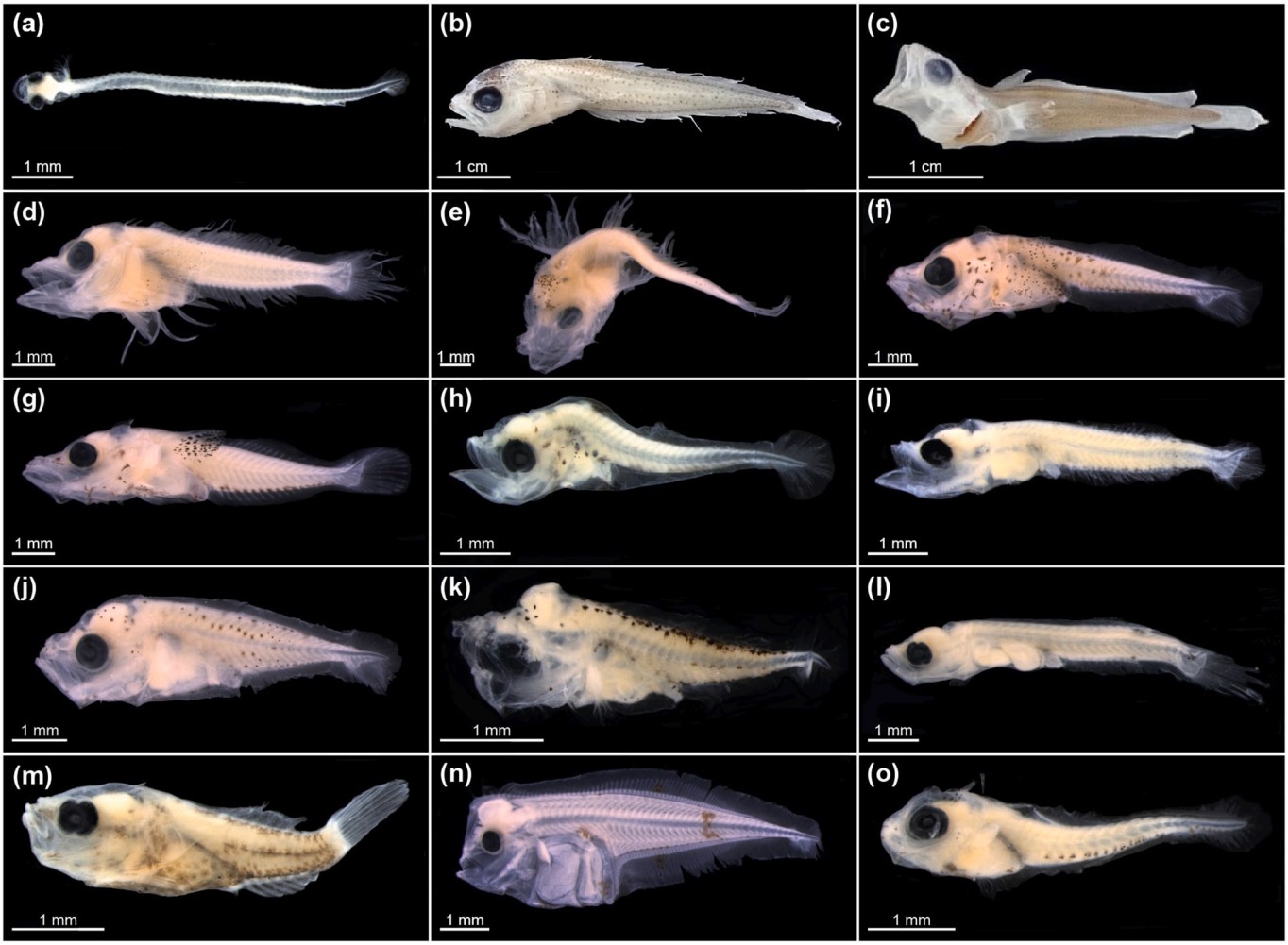

Fig. 3. Lateral images of a. *Sardinops sagax;* b. *Pseudophycis breviuscula;* c. *Pseudophycis bachus* - now *Pseudophycis palmata* (Gomon et al. (2021)); d. *Lepidotrigla mulhalli;* e. *Lepidotrigla* sp.; f. *Platycephalus richardsoni;* g. *Platycephalus bassensis;* h. *Caesioperca lepidoptera;* i. *Sillago flindersi;* j. *Trachurus declivis;* k. *Pseudocaranx georgianus;* l. *Notolabrus tetricus;* m. *Synchiropus papilio;* n. *Lophonectes gallus;* o. *Thamnaconus degeni* sampled at Maria Island National Reference Station and fixed in 5% formalin.

through the silica columns. Two rounds of washing with column wash buffer (500 μl) were undertaken and DNA was precipitated by two rounds of elution with 40 μl of water per round.

DNAs from both extraction methods were stored at 4 °C overnight (to ensure DNA was fully dissolved); with a sub-aliquot of each DNA quantified on a Nanodrop 8000 (Thermofisher, USA). Aliquots of DNA from each method are also archived at −80 °C in the CSIRO genetics laboratory. DNA aliquots were used for COI amplification and sequencing at either the CSIRO genetics laboratory or at the Ramaciotti Centre for Genomics at the University of New South Wales.

2.5. COI barcoding

The reference COI mtDNA gene fragment (approximately 650 base pairs, referred to here as full-length) was amplified by BCL and BCH primers (Baldwin et al., 2009) with an annealing temperature of 50 °C (see Appleyard et al., 2018). Bi-directional cycle sequencing was undertaken using the abovementioned PCR primers and BigDye® Terminator v3.1 Cycle sequencing kit (Life Technologies, USA). Cycle sequenced products were run out on either an ABI3130XL Autosequencer (Applied Biosystems, USA) at CSIRO (see Appleyard et al., 2018) or an ABI3730XL Autosequencer (Applied Biosystems) at Ramaciotti following dye terminator removal with CleanSEQ® (Beckmann Coulter, Australia) magnetic beads or ExoSAp-IT (Ramaciotti commercial in-confidence protocol, see Core Prep) respectively.

Due to the likely need to undertake both full-length COI barcoding and bespoke mini-barcoding of a region of the COI gene in the degraded, formalin impacted DNA, amplification and sequencing of the COI_E region (approximately 225 bp – referred to here as mini-barcode) as per Appleyard et al. (2021) (and Shokralla et al., 2015) was also undertaken (with an annealing temperature of 50 °C). This occurred in DNA samples if the full-length COI barcoding did not work in the first instance. In these instances, PCR and sequencing was undertaken at CSIRO.

Following either full-length or mini-barcode COI sequencing, raw forward and reverse sequences were de novo assembled in Geneious Prime 2020.0.5 (Biomatters Ltd. Auckland, New Zealand). Sequences were visually inspected and where required, base calls were manually recorded. Consensus sequences were generated for each specimen (either full-length or shorter length mini-barcodes) and submitted to BOLD via the Identification System to determine homology between the COI sequences from this study and those in the database. Genetic identification was based on a $\geq$ 98% pairwise match to sequences in BOLD. For the in-field fixation experiment, the validity of the species occurrences at the Maria Island NRS was also confirmed by the Australian Faunal Directory online distribution.

Representative barcode sequences (in FASTA formats – both full-length and shorter length mini-barcodes) for specimens from the *S. lalandi* controlled and the in-field formalin fixation experiments and

relevant metadata from the MAI NRS have been deposited at the CSIRO Data Access Portal https://data.csiro.au/collection/csiro:54021 (Appleyard et al., Strategic ANFC Research, CSIRO Data Access Portal, 2022) and are also given here as Supplementary material (Table S1 and COI text). Additionally, the full-length reference COI barcodes for the identified fish larvae from the MAI NRS have been submitted to BOLD, under dataset DS-IFLFORM with a DOI of dx.doi.org/10.5883/DS-IFLFORM (Appleyard, DS-IFLFORM FIMOS formalin fixed larval specimens, BOLD Systems, 2022). Representative images of larval fish in this dataset have also been submitted to BOLD.

3. Results

The results of sequencing of specimens from the various formalin treatments are presented in Supplementary material Table S1. Across both experiments, of the 227 formalin fixed samples (including whole larvae and eyeballs) that were extracted, 217 (96%) were successfully amplified, sequenced (at either the full-length reference or the mini-barcode COI region) and returned a genetic identification. Cultured fish larvae were produced at the land-based hatchery as part of the controlled formalin fixation experiment, while wild fish larvae were caught as part of the in-field, at sea formalin fixation experiment.

3.1. Controlled formalin fixation experiment

All imaged *S. lalandi* larvae were morphologically identified as *S. lalandi* (Fig. 2) and of the 144.

S. lalandi samples, 142 samples returned a COI genetic identification (Table 2).

Two larvae (one individual from the 1-day and one individual from the 4-weeks formalin treatment) did not sequence, however their corresponding eyeballs were successfully sequenced. Larvae (and paired eyeballs) from the 20- and 24-weeks formalin exposure did not sequence for the COI reference barcode, however the COI_E mini-barcode was successfully sequenced in these samples (see Table 2). From a species perspective, the 72 individuals from the mass spawning event were confirmed genetically (by COI reference and or COI mini barcoding) as *S. lalandi*.

There was no impact on COI reference barcoding from the various formalin treatments in the 1-day to 16-week samples. In all instances, *S. lalandi* larvae that were exposed to 5% formalin for one day, sequenced and returned the correct genetic identification as did those that were exposed to 5% formalin for up to 16 weeks. The ability to amplify and sequence the COI reference fragment however was impacted in the 20- and 24-weeks exposed larvae and a full-length sequence was not obtained from any of these individuals (see Table 2). In contrast, we were able to amplify and sequence the 225 bp COI_E mini barcodes in these 16 specimens and all returned high level matches to *S. lalandi*.

Additionally, within this experiment, there were 72 instances of individuals and their paired single eyeball extractions. Following sequencing, 70 of the paired instances resulted in concordant species identifications (all *S. lalandi*) (see Supplementary material table S1). Therefore, in these *S. lalandi* specimens, single eyeballs were as effective as a source of DNA for genetic identifications as the whole individual, irrespective of formalin treatment.

Table 2
Outcomes of COI reference and mini-barcode sequencing in *S. lalandi* larvae across nine formalin treatment groups undertaken at the New South Wales Fisheries Research Institute at Port Stephens.

Formalin treatment	*S. lalandi* sample size (individuals + eyeballs) in treatment group	*S. lalandi* samples identified with COI_reference barcode	*S. lalandi* samples identified with COI_E mini-barcode[a]
1-day	16	15	0
1-week	16	16	0
2-weeks	16	16	0
4-weeks	16	15	0
8-weeks	16	16	0
12-weeks	16	16	0
16-weeks	16	16	0
20-weeks	16	0	16
24-weeks	16	0	16

[a] Where samples sequenced for the full-length COI_reference barcode, COI_E mini-barcodes were not required.

3.2. In-field formalin fixation experiment

Based on morphology and physical identification, prior to sequencing, 81% of the MAI specimens were identified to family and 18% were identified to species level (see Supplementary material table S1). Following extraction, the majority (90%) of the MAI samples amplified, sequenced, and returned a genetic identification to species level irrespective of the formalin fixation time (Table 3).

Unlike the controlled exposure experiment, the success rate of genetic identifications of the MAI larvae per treatment was patchier (see Supplementary material table S1). Some specimens that were labelled as being exposed to formalin for 1-day or 1-week did not sequence for the full-length COI reference barcode or the mini-barcode fragment. By contrast, in the *S. lalandi* experiment, a full complement of genetically barcoded samples (based on either larva, eyeball, or both) was achieved irrespective of formalin exposure time. Based on a combination of reference and mini-barcode sequencing, 78.5% of the 1-day, 94% of the 1-week, 100% of the 3-weeks and 71% of the 4-weeks exposed larvae were genetically identified. Moreover, 93% of larval fish that were fixed in formalin for 8-weeks were successfully extracted, reference sequence barcoded and genetically identified to species. Table 4 outlines all the species which were detected genetically (with species occurrences confirmed from the AFD) at Maria Island from the 84-formalin fixed larval fish specimens. Fig. 3 presents representative images for each of these confirmed species.

4. Discussion

Larval fish specimens are traditionally fixed in 4–5% formalin which preserves body tissues for future morphological examination (Cunningham et al., 2000; Hanahara et al., 2021; Hou et al., 2021; Mateos-Rivera et al., 2020). While this is advantageous for species identifications using microscopy and morphology, the use of formalin is not recommended for genetic techniques given that the extracted DNA may be degraded and characterised by DNA:protein cross links with low DNA yields. However, in this study, we showed the extracted DNAs from larval fish fixed in 5% formalin for up to 6 months could be successfully extracted, amplified, and sequenced for the COI gene fragment, thereby enabling practical integrative taxonomy for species identification of larval fish assemblages.

Table 3
Outcomes of COI reference and mini-barcode sequencing in Maria Island National Reference Station larval fish specimens collected in 2018–2020 across five formalin treatment groups.

Formalin treatment	Mixed larvae sample size in treatment group	Mixed larvae identified with COI_reference barcode	Mixed larvae identified with COI_E mini-barcode[a]
1-day	14	0	11
1-week	17	13	3
3-weeks	18	10	8
4-weeks	7	2	3
8-weeks	28	26	0

[a] Where samples sequenced for the full-length COI reference barcode, COI_E mini-barcodes were not required.

Table 4
Morphological (prior to barcoding) and COI identification from the BOLD Identification Engine (following barcoding) of formalin fixed fish larvae from Maria Island National Reference Station, collected 2018–2020.

Morphological_taxonomic identification[a]	COI_genetic identification[b]
Sardinops sagax	*S. sagax* (Jenyns 1842)
Moridae	*Pseudophycis breviuscula* (Richardson 1846)
Moridae	*Pseudophycis bachus* – now *Pseudophycis palmata*[c] (Klunzinger 1872)
Triglidae	*Lepidotrigla mulhalli* (Macleay 1884)
Triglidae	*Lepidotrigla* sp.
Platycephalidae: other	*Platycephalus richardsoni* (Castelnau 1872)
Platycephalidae: other	*Platycephalus bassensis* (Cuvier 1829)
Anthiinae	*Caesioperca lepidoptera* (Forster 1801)
Sillago flindersi	*S. flindersi* (McKay 1985)
Trachurus declivis	*T. declivis* (Jenyns 1841)
Carangidae: *Pseudocaranx*	*Pseudocaranx georgianus* (Cuvier 1833)
Labridae	*Notolabrus tetricus* (Richardson 1840)
Callionymidae	*Synchiropus papilio* (Günther 1864)
Lophonectes gallus	*L. gallus* (Günther 1880)
Monacanthidae	*Thamnaconus degeni* (Regan 1903)

[a] Prior to COI sequencing and barcoding, see also Supplementary Material Table S1.
[b] Across formalin treatments of 1-day to 8-weeks.
[c] At the time of sequencing, identification in the BOLD Identification Engine and manuscript preparation, this species was listed as *P. bachus*. In a recent review of the Australasian genus Pseudophycis, Gomon et al. (2021) stated *P. bachus* is only found in New Zealand waters, and Australian listed *P. bachus* should be called *P. palmata*.

4.1. Formalin fixed specimen sequencing

Our modified (reduced volume) DNA extraction methods (i.e., Wizard extraction method for samples fixed in formalin from 1-day to 1-week; ReliaPrep FFPE extraction method for samples fixed in formalin from 2-weeks to 24- weeks) were again confirmed (see also Appleyard et al., 2021) as suitable for use with formalin fixed fish specimens, when mtDNA barcode identifications are required. The ReliaPrep method which includes a de-crosslinking step (80 °C for two hours) was used for samples that had been fixed in 5% formalin for >1 week, as it was expected that these samples would be characterised by higher levels of protein: DNA cross linking (although this was not explicitly tested).

While other studies have demonstrated successes in genetic identification of formalin fixed fish specimens (e.g., Hanahara et al., 2021; Appleyard et al., 2021; Hou et al., 2021), exposure times in previous studies were considerably shorter than in our current study, and formalin concentrations varied. Hou et al. (2021) reported sequencing success in larvae exposed to formalin for 10 days or less, while Raja et al. (2011) extracted total DNA from short-term (1 month) 10% buffered formalin fixed samples of *Danio aequipinnatus* and *Puntius tambraparniei*. Hanahara et al. (2020) reported success with a short-term (24 h) 10% seawater formalin fixation technique which enabled identification of gobioid larvae. Our current study is one of few that has attempted COI species identifications in specimens fixed in formalin for more than a month.

Specimens fixed in 5% formalin for up to 16-weeks (i.e., 4 months) provided good sources of DNA for COI reference sequence barcoding, while COI mini-barcode sequences were generated in degraded DNA from specimens from the 20 or 24-weeks (5–6 months) exposure treatments. Based on these and earlier results (Appleyard et al., 2021), we suggest that the cut-off point for generating full length COI reference barcodes in 5–10% formalin fixed fish specimens to be approximately four months (although this may depend on formalin concentration). The DNA from the 2-weeks to 16-weeks exposed samples were obtained using the ReliaPrep method which is a more time-consuming extraction method than that used in the 1-day and 1-week exposure samples. The ReliaPrep method uses individual columns and a de-cross linking step, while the Wizard method is based on a 96-well protocol with no de-cross linking. However, the ability to generate COI reference sequence barcodes in these degraded DNA samples makes the extra processing time worthwhile. While we used the ReliaPrep method for the 20 and 24-weeks formalin treated larval fish, and full-length COI reference barcodes were not achievable, we were able to generate mini-barcodes which resulted in species identification. While our objective was not to identify an absolute decay rate outside of the timeframe of this study (i.e., does sequencing length reduce further as formalin fixation time increases post 6 months), we would recommend attempting COI mini-barcoding of formalin fixed specimens post 6 months if the specimens are expected to be informative or valuable, as useful shorter sequences are more likely to be generated than full-length barcodes. A downside to the use of the mini-barcodes is that only approximately 220 bp of sequence is achieved, in comparison to the 650 bp of the full length sequence and in some specimens and for some closely related species, this shorter fragment may not enable species delineation. Nevertheless, in the current study, and given the higher-level morphological identifications to family, this did not impact our ability to genetically identify the larval fish from the field (MAI NRS) or the controlled (mass spawned *S. lalandi*) experiments.

4.2. Fish eyeballs for DNA barcoding

Importantly, in this study we showed that single eyeballs from larval fish provided suitable DNA sources for genetic identification irrespective of formalin fixation treatment. Paired comparisons between whole larval individuals and their right-side eyeball showed no differences in amplification and barcoding in the yellowtail kingfish specimens. While the use of eyeballs for the genetic identification of ethanol preserved larval fish has been shown previously (Hyde et al., 2005; Marancik et al., 2020; Richardson et al., 2007), our study demonstrates the utility of formalin fixed eyeballs and our successes in moving to non-destructive genetic identification of larval fish. The use of eyeballs for DNA barcoding allows for the whole larval fish to be maintained as a voucher specimen, while having a good source of DNA for genetic identification.

4.3. Controlled and in-field experimental outcomes and implications

The success of the genetic species identifications differed between the controlled and in-field experiments, with patchier results from the field. In the controlled experiment, *S. lalandi* larvae from 1-day to 16-weeks of 5% formalin fixation were successfully sequenced at the full-length COI barcode. However, in the MAI 1-day fixed larvae, we were only able to generate mini-barcode sequences; we were not able to generate the 650 bp COI sequence. We attribute this difference to field conditions - the 1-day fixation sample bottle may have been mislabelled; the field fixation may have been inadequate to prevent autolysis (which may affect DNA extraction, see Kiernan, 2000); or the field-based preparation of the formalin solutions may have varied considerably, particularly when made up with seawater in the field.

In this study, we have shown how our integrated taxonomy of larval fishes' approach, which combines traditional morphological techniques with DNA barcoding (see also Marancik et al., 2020; Mateos-Rivera et al., 2020) in 5% formalin fixed (up to six months) specimens is an effective and accurate method for species identification. From a logistical perspective for cross country transportation and for our specific project needs, our technique simplifies the field collections of zooplankton samples (which likely contain larval fishes) by fixing samples in formalin without compromising future genetic identification. This allows us to increase the efficiency of our sampling regime by eliminating the need to collect and fix a separate sample for genetic identification and improves safety at sea by reducing the need to transport 2 l of 95% ethanol in small vessels. It also reduces the additional processing requirements of field acquired ethanol preserved samples, which need to be drained and replaced with fresh ethanol to

prevent degradation. Our new method enables robust preservation of material in the field by fixing samples in formalin which do not shrink or distort as much compared to samples preserved directly in ethanol. The method, however, still requires the larval fish to be transferred to ethanol within 6 months, and formalin requires careful handling with gloves and safety glasses as it is a carcinogen. We also highlight that the outcomes from our study and the applications may not transfer successfully to all larval fish studies, due to confounding factors such as storage conditions, temperatures, and source and quality of available formalin and ethanol.

Therefore, future research in larval fish barcoding from the field, should consider (a) the effect of different dilutions of formaldehyde and (b) the possible effect of diluting in fresh or salt water. We note that in Tucker and Chester (1984) shrinkage, distortion and deterioration of preserved fish larvae was minimised by the judicious choice of preservative solutions. The authors recommended that for minimising larval shrinkage, distortion and retaining larval pigments in southern flounder larvae, fixation and preservation should be undertaken in 4% formalin in distilled water buffered with 1% sodium acetate (Tucker and Chester, 1984). For the *S. lalandi* controlled experiment, we used 5% formalin diluted with freshwater. At the IMOS national reference stations (including at the Maria Island reference station) where seawater is used at sea as the diluent, we expect seawater to have carbonate and other ions acting as buffers in the formalin for at least a week; however longer periods of time in buffers can cause the breakdown of melanin of chromatophores (which impacts morphological identifications). In the current study, we did not specifically aim to test the impact of buffered formalin (or using freshwater or saltwater as the formalin diluent) on the ability to extract useable DNA, however we believe these conditions should be tested in future research.

The effect of refrigeration should also be considered to determine if lower temperatures delay DNA: protein cross linking, or if hot conditions (as encountered at tropical sampling locations) increases the rate. Researchers that undertake irregular, in-field larval fish sampling activities, particularly onboard larger research vessels where fixation capacity is likely to be greater than on the IMOS NRS small vessels, should also reflect at the outset on the likelihood of obtaining rare specimens and consider the risk of solely using formalin fixation and ethanol preservation without taking genetic samples first. Additionally, if larval fishes are to be sampled for long-read genome sequencing, we do not recommend using formalin or ethanol for fixation and preservation, rather specimens should be flash frozen in liquid nitrogen and maintained at very low temperatures (see Blom, 2021).

4.4. DNA barcoding of larval fish from IMOS

More broadly, the basis of undertaking fish larvae surveys is the ability to accurately identify larvae to species, especially for multi-species families and genera. Unfortunately for Australian waters, although the larvae of many species have been described (Neira et al., 1998), larvae of many taxa are only identified to the family level (Smith et al., 2018) due to a lack of species level descriptions. The use of barcoding for specific identification of larvae allows the assembly of developmental series with reference to confirmed specimens, that can then be used to prepare detailed species descriptions. The MAI samples detailed in this study were part of the larval collection that the IMOS program intentionally archived from five NRS stations around southern Australia since 2014 (Smith et al., 2018). These larval collections are preserved in either formalin and or 95% ethanol.

Combined with our ability to now produce either full length or mini-COI barcodes in the MAI larval samples from either formalin fixed (and ethanol preserved, see Appleyard et al., 2021) larvae, future species identification of larvae based on barcoding will allow finer scale assessment of spatial and temporal distribution patterns of larval fish from the IMOS samples in relation to oceanographic and other phenomena. Enhanced species larval descriptions will also allow review of archived historical samples to identify larvae of target species that are commercially or recreationally important. Revised identifications can then be used to update the Australian Ocean Data Network (AODN) larval fish database (Smith et al., 2018). This database has been used to assess large scale patterns in larval communities and temporal and spatial changes in species distributions due to climate change (Hinchliffe et al., 2021).

Barcoding results presented in this study and an ongoing program of larval fish barcoding from the Australian NRSs will facilitate the creation of long-term time series from the region. This will also assist with the ability to understand long-terms trends in larval fish communities including fish species of commercial and recreational value and that are currently identified only to family level. Outcomes from this will be ongoing and will be three-fold. The first will be documentation using images and descriptions of the larval development of currently unidentified larvae. When larvae can be accurately identified, this will allow a review of larvae in samples from the NRS program and archived historical samples to assess in finer detail, patterns of spatial and temporal variation in larvae distribution. Secondly, this information will be useful for fisheries managers, by informing the assessment of patterns in larval communities and temporal and spatial changes in species distributions due to climate change (Hinchliffe et al., 2021). Thirdly, larval fish barcodes will contribute to the ongoing COI reference sequence library of Australian marine fishes.

4.5. Conclusions

Where formalin fixation has occurred for less than six months, we encourage curators and managers of larval fish collections to trial DNA barcoding in their formalin fixed and ethanol preserved specimens, particularly where geographic relevant reference sequence databases are available for interrogation. We recommend screening more recent formalin fixed specimens. As part of quality control, the sample metadata should also be checked and where possible, information obtained about the timeframes of when larval fish specimens were collected, fixed and subsequently sorted and transferred to ethanol, as this can vary depending on available sorting resources and the number of specimens. We believe the methods outlined in this study underpin the future of monitoring larval fish for climate and fisheries research, particularly with respect to the continuing loss of taxonomic ability and where both morphological and genetic identifications are required. Based on the outcomes of this study, we reiterate previous findings in the literature (Hanahara et al., 2021; Ko et al., 2013; Marancik et al., 2020; Panprommin et al., 2021; Silva-Segundo et al., 2021; Steinke et al., 2016) that describe DNA barcoding of larval fishes to be the best method to confirm species identification. Barcoding provides a genetic identification to confirmed samples that are then used as reference points for examining current and historic formalin fixed specimens – this enables the assembly of a development series for larval descriptions. We clearly improved species identification rates from 18% (morphologically identified to species) to 93% (genetically identified to species) in specimens from the Maria Island NRS, that had been fixed in 5% formalin for $\leq$8 weeks and successfully identified *S. lalandi* from a controlled 6 month formalin fixation experiment This was achieved by applying a specific DNA extraction method that included de-cross-linking steps and using DNA barcoding and our Australian fishes reference sequence COI library in BOLD.

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Animal Ethics statement

Larval spawning and handling were undertaken at the Port Stephens research institute under the NSW Department of Primary Industries (Fisheries), Animal Care and Ethics Permits 93–1 and 93–3 for Larval Fish Rearing and Marine Fish Breeding respectively. Larval fish collections at the IMOS national reference stations were collected under ethics permit UNSW ACEC 19/96B.

CRediT authorship contribution statement

Sharon A. Appleyard: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Resources. **Safia Maher:** Methodology, Investigation, Data curation. **Anthony G. Miskiewicz:** Methodology, Investigation, Data curation, Writing – review & editing. **Ana Lara-Lopez:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Paloma Matis:** Conceptualization, Methodology, Writing – review & editing. **D. Stewart Fielder:** Investigation, Writing – review & editing. **Iain M. Suthers:** Methodology, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest or personal relationships that could have appeared to influence the study outlined in this paper.

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Appendix A. Supplementary data

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