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Research Article

Establishment of a taxonomic and molecular reference collection to support the identification of species regulated by the Western Australian Prevention List for Introduced Marine Pests

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This study was first presented at the 9th International Conference on Marine Bioinvasions held in Sydney, Australia, January 19–21, 2016 (http://www.marinebioinvasions.info/previous-conferences). Since their inception in 1999, ICMB series have provided a venue for the exchange of information on various aspects of biological invasions in marine ecosystems, including ecological research, education, management and policies tackling marine bioinvasions.

Abstract

Introduced Marine Pests (IMP, = non-indigenous marine species) prevention, early detection and risk-based management strategies have become the priority for biosecurity operations worldwide, in recognition of the fact that, once established, the effective management of marine pests can rapidly become cost prohibitive or impractical. In Western Australia (WA), biosecurity management is guided by the "Western Australian Prevention List for Introduced Marine Pests" which is a policy tool that details species or genera as being of high risk to the region. This list forms the basis of management efforts to prevent introduction of these species, monitoring efforts to detect them at an early stage, and rapid response should they be detected. It is therefore essential that the species listed can be rapid and confidently identified and discriminated from native species by a range of government and industry stakeholders. Recognising that identification of these species requires very specialist expertise which may be in short supply and not readily accessible in a regulatory environment, and the fact that much publicly available data is not verifiable or suitable for regulatory enforcement, the WA government commissioned the current project to collate a reference collection of these marine pest specimens. In this work, we thus established collaboration with researchers worldwide in order to source representative specimens of the species listed. Our main objective was to build a reference collection of taxonomically vouchered specimens and subsequently to generate species-specific DNA barcodes suited to supporting their future identification. To date, we were able to obtain specimens of 75 species (representative of all but four of the pests listed) which have been identified by experts and placed with the WA Government Department of Fisheries and, where possible, in accessible museums and institutions in Australasia. The reference collection supports the fast and reliable taxonomic and molecular identification of marine pests in WA and constitutes a valuable resource for training of stakeholders with interest in IMP recognition in Australia. The reference collection is also useful in supporting the development of a variety of DNA-based detection strategies such as real-time PCR and metabarcoding of complex environmental samples (e.g. biofouling communities). The Prevention List is under regular review to ensure its continued relevance and that it remains evidence and risk-based. Similarly, its associated reference collection also remains to some extent a work in progress. In recognition of this fact, this report seeks to provide details of this continually evolving information repository publicly available to the biosecurity management community worldwide.

Key words: Introduced Marine Pests (IMP), taxonomic voucher, cytochrome c oxidase I, COI, The Barcode of Life Data System, BoLD, marine biosecurity

Introduction

Species have been historically transported and introduced around the globe in increasing numbers due to global trade and anthropogenic activities (Elton 1958; di Castri 1989; Carlton 2011). Although only a small percentage of introduced species actually become pests (Williamson and Fitter 1996; Lockwood et al. 2013), their impacts can be dramatic and are often irreversible, making them one of the greatest environmental concerns globally (UNEP 2011; Lockwood et al. 2013; Bellard et al. 2016; McGeoch et al. 2016). In the marine environment, introduced pests have been considered one of the most significant threats to biodiversity (Bax et al. 2003; Molnar et al. 2008), with over 1781 marine and estuarine species having been transported and introduced by human-mediated activities around the world (Hewitt and Campbell 2010; Katsanevakis et al. 2013; Galil et al. 2014; http://www.marinespecies.org/introduced). Within Australia, at least 250 species have been reported as introduced (Hewitt and Campbell 2010; http://www.marinepests.gov.au). The introduction of such a high number of species (and diversity of taxa) poses a serious challenge for scientists and policy makers seeking to comprehensively understand, predict and manage introductions and potential impacts (Simberloff et al. 2013; Ojaveer et al. 2015).

Prevention strategies are the main focus of biosecurity operations worldwide due to their relative cost-effectiveness. When prevention fails, early detection and rapid response are the next lines of defense against Introduced Marine Pests (IMP, = non-indigenous marine species) (Simberloff et al. 2013; Simberloff 2014). Due to the "out of sight" aspect of the marine environment, effective surveillance and rapid identification are essential to ensure introductions are caught early enough for effective management. Examples of successful eradication efforts of marine pest species worldwide are few, and to date have proven feasible only at a very early stage of introduction or establishment (Crombie et al. 2008; Simberloff et al. 2013; Summerson et al. 2013), or with established spatially restricted populations (Williams and Schroeder 2004; Hopkins et al. 2011). In the face of invasion biology complexity and limited human and financial resources, IMP prevention and management is recognised to be best achieved through stringent biosecurity, based on risk assessments and prioritisation (UNEP 2011; Simberloff et al. 2013; Davidson et al. 2015; Ojaveer et al. 2015; McGeoch et al. 2016). In order to achieve this, the introduction and impact potential of known pest species can be inferred from intrinsic characteristics such as reproductive strategy, growth rate, environmental tolerances and diet specificity. Extrinsic characteristics can also be incorporated, including habitat matching, propagule pressure, invasion history (including human health, economic and environmental impacts elsewhere), and vector analysis such as ship movement (Simberloff et al. 2013; Bridgwood and McDonald 2014; Ojaveer et al. 2015).

Biosecurity strategies, worldwide, are often focussed on developing "lists" of pests of concern in order to focus prevention, early detection efforts including awareness-raising to encourage pest reporting and rapid response should they be detected. Examples include comprehensive online databases like the International Union for Conservation of Nature (IUCN) Global Invasive Species Database (GISD), including the "100 of the World's Worst Invasive Alien Species" list (http://www.issg.org/database/species/search.asp?st=100ss), and the World Register of Introduced Marine Species (http://www.marinespecies.org/introduced). Due to their recognised value in increasing awareness and facilitating prevention and management, online pest species lists and databases are also available and growing within regions and countries, such as in Europe (EASIN http://easin.jrc.ec.europa.eu/, NOBANIS https://www.nobanis.org, AquaNIS http://www.corpi.ku.lt/databases/aquanis), in New Zealand (http://marinebiosecurity.org.nz), in USA (National Invasive Species Information Center http://www.invasivespeciesinfo.gov/index.shtml, NEMESIS http://invasions.si.edu/nemesis/index.jsp, Introduced Marine Species of Hawaii http://www2.bishopmuseum.org/HBS/invertguide/index.htm), or Jamaica (Jamaica Invasive Species Database http://apps.licj.org.jm/jamaica-invasives). Most of the worldwide databases and lists are however general (terrestrial, marine and freshwater) and/or refer to species already introduced in the areas they cover.

In Australia, increased detection of marine pest species during the 1980s and 1990s led in 2000 to the establishment of a National Introduced Marine Pests Coordination Group to develop the National System for the Prevention and Management of Introduced Marine Pest Incursions (NIMPCG 2010, http://www.marinepests.gov.au). Fifty five species were listed as target biosecurity species in Australia under the National System (http://www.marinepests.gov.au/marine_pests/publications/Documents/Monitoring_Guidelines-lowres.pdf). In Western Australia (WA), this list was adopted as a basis for focusing IMP management efforts, with the addition of species identified in the review by Hewitt et al. (2011) and

a small number of species of particular concern to the State, to form the "Western Australian Prevention List for Introduced Marine Pests" (Department of Fisheries 2016, http://www.fish.wa.gov.au/Documents/biosecurity/epa_introduced_marine_pests.pdf). In October 2014, all but one of these species were prescribed as noxious fish in Schedule 5 of the *Fish Resources Management Regulations 1995*. The Prevention List and associated noxious fish list therefore provide the Department, risk creators like vessel managers, biofouling inspectors and the public with a focus for prevention, early detection and rapid response activities. The main rapid response activity so far has been to manage vessels on which listed species have been detected to ensure that they do not spread into the environment. However, regulatory actions may also occur.

Fundamental to efficient detection and rapid response is the ability to rapid and reliably identify the species in the Prevention List. This task is not without its challenges including the fact that taxonomic identification of the listed species requires very specialist expertise, which may be in short supply and not readily accessible, significantly hampering response efforts. Also, the fact that most listed species are not present in Australia (so comparative specimens may be unavailable), some specimens may not have diagnostic morphological features (eg larvae, juveniles, incomplete or damaged) and the presence of analogous native or cryptic species, can confound reliable taxonomic identification. Partly for these reasons, WA biosecurity managers have elected to promote the development of DNA barcoding as one of their tools for supporting confident and timely identification. Molecular tools and particularly DNA barcoding have become popular in recent years as they offer a reliable, accessible, cost-effective and relatively fast alternative to traditional morphological taxonomic identification (Darling and Blum 2007; Comtet et al. 2015; Trivedi et al. 2016). DNA-based identification also allows for identification when the whole specimen is not available or diagnostic morphological features are absent (Darling and Blum 2007; Neigel et al. 2007; Bott et al. 2010; Comtet et al. 2015).

Most species in the Prevention List have a history of invasion somewhere in the world and at the start of this study only five species—*Anomia nobilis* Reeve, 1859, *Solidobalanus fallax* (Broch, 1927), *Gelliodes fibrosa* Dendy, 1905, *Cliona thoosina* Topsent, 1888 and *Chaetoceros convolutus* Castracane, 1886—did not have a DNA barcode available in reference databases such as GenBank (Benson et al. 2013) or BoLD (Barcode of Life Data System, Ratnasingham and Hebert 2007). However, the accuracy of the data provided in online databases is only as good as the identification ability of the person or agency depositing the barcode. Recognising a paucity of global data to support the DNA-based identification of species on the Prevention List and the fact that much publicly available data is not verifiable or suitable for rapid response, the WA government commissioned the current project to collate a taxonomic and molecular reference collection of these species.

The taxonomic verification of a reference specimen corresponding to a suitable species-specific short DNA sequence or "barcode" is a prerequisite to validating any subsequent molecular identification of the same species (Darling and Blum 2007; Comtet et al. 2015). However, once this vouchering process has been completed, DNA barcoding is able to free taxonomists from the time-consuming identification of previously described species (Darling and Blum 2007; Comtet et al. 2015). DNA barcode libraries linked to voucher collections of specimens have been recognised to foster the development and routine implementation of molecular tools for detection of new IMP, correct any mis-identification of IMP and monitoring of biodiversity and potential pest species (Hebert et al. 2003, McGlashan et al. 2008, Puillandre et al. 2012, Comtet et al. 2015). Such libraries have proven essential in identifying highly diverse taxonomically challenging groups of important terrestrial (deWaard et al. 2011) and aquatic (Serrao et al. 2014) invasive species and in empowering biosecurity agencies to identify high-risk fish species in the aquarium trade (Collins et al. 2012). Given the increased rate of introductions, they have been recognised as the way forward to rapidly detect and manage IMP and timely inform policy makers and stake holders in a timely manner (Comtet et al. 2015; Darling 2015; Coissac et al. 2016).

In the present work, we set out to build an in-house readily available taxonomic and molecular reference collection of specimens, sourced from across the globe. The collection has been developed at the WA Government Department of Fisheries, which is the lead agency for marine biosecurity research, monitoring, policy and compliance in the State. The collection is supported by the deposition of parallel samples at museums and institutions in Australasia to support accessibility and availability to the biosecurity research and management community. With this collection, we intend to build a resource for training and support of fast and reliable taxonomic and molecular identification of IMP in Australia. We also aim to develop marine biosecurity molecular capability in WA including early-detection and monitoring strategies based on DNA barcoding,

real-time PCR and metabarcoding of complex environmental samples. Importantly, because the reference collection can provide crucial support for rapid and effective biosecurity emergency responses, we believe it is of foremost importance to make details on the reference specimens in the collection publicly available.

Materials and methods

2.1 Sourcing of specimens

The work towards sourcing specimens representative of species in the Prevention List was initiated in 2011 with preserved specimens donated by colleagues (from collections already maintained by experts in Australasia, e.g. at the Museum and Art Gallery of the Northern Territory) or opportunistically collected, preserved and transported by members of the WA Government Department of Fisheries biosecurity team during work trips in the region. During 2014–2016 resources were specifically allocated for this task. Experts with established research and publications on the listed species in Australia and abroad were contacted to provide, where possible, a minimum of 2 whole specimens per species. Specimens were donated from private research collections worldwide or freshly collected using a variety of methods (e.g. by hand, snorkelling, scuba diving, sediment core sampling) from locations within 23 countries. Instructions regarding collection, preservation, shipping and import of samples to Australia were provided where necessary.

The great majority of animal specimens were preserved in 60–100% ethanol, drained off and shipped in small amounts of this preservative (e.g. wrapped in tissue soaked in the preservative), in order to comply with transport of dangerous goods requirements and the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) conditions under which no permit was required for the import of invertebrate specimens into Australia. Samples of the colonial tunicates *Didemnum perlucidum* and *Didemnum vexillum* were relaxed in seawater with menthol crystals for 2 hours and each colony was split into two portions: one preserved in 95% ethanol to allow for molecular identification, and the other in formalin-seawater 4% to allow for taxonomic identification. Fish specimens were the only animal vertebrate species on the list and were imported in small amounts of ethanol under permit from DAFF.

Seaweed specimens were preserved and transported dry in silica gel in order to comply with DAFF conditions under which no permit was required for the import of dried seaweed into Australia. Samples of seven out of the ten listed species of dinoflagellate and diatoms were obtained from pure cultures available commercially from the Australian National Algae Culture Collection (ANACC) and the US National Center for Marine Algae and Microbiota (NCMA). Samples of *Dinophysis norvegica* Claparède and Lachmann, 1859 and *Alexandrium monilatum* (J. F. Howell) Balech, 1995 were obtained from pure cultures maintained and preserved in research institutions. A mixed-species sample of *Pseudo-nitzschia seriata* (Cleve) H. Peragallo, 1899 was collected from a bloom of this species in the natural environment. Samples were preserved and transported in 1% lugol solution in order to comply with DAFF conditions under which no permit was required for the import of plankton into Australia.

All samples were transported to the Marine Research Laboratories of the WA Government Department of Fisheries and processed on arrival for labelling, database entry, storage, preservation (ethanol added for long tern preservation of animal specimens) and DNA Barcoding. Following successful DNA barcoding, specimens were provided in batches (e.g. molluscs, polychaetes, crustaceans) to experts in specific taxa at museums and institutions holding taxonomical collections in Australasia. A few other specimens were received but because they consisted of partial specimens (either hard parts e.g. shells or tissue only) or had been preserved in formalin, they did not allow for taxonomy to be verified or for DNA barcodes to be amplified from the same individual, and hence could not be used as reference specimens. For details on individual reference specimen collection, preservation, identification and storage refer to BoLD records (http://www.barcodinglife.org) listed in Supplementary material Table S1.

2.2 Identification of voucher specimens

Identification of specimens was firstly made by the local collectors (researchers that have extensive experience with the species) and where possible confirmed by expert taxonomists. Where more than one specimen per species (or per sex within species if sexual dimorphism was clearly present, as often the case for crustaceans) was available, and successfully barcoded, they were vouchered and lodged in museum and institutional taxonomic collections for long term care and maintenance, and a matching identified reference specimen deposited in the WA Government Department of Fisheries reference collection.

2.3 DNA Barcoding

DNA extraction from animal tissue was performed using a FavorPrep Tissue Genomic DNA Extraction Mini Kit, following the manufacturer's instructions (Fisher Biotec). For the great majority of animal specimens, barcodes were generated for the COI region with primers LCO1490 / HCO2198 developed by Folmer et al. (1994). Alternative primers were used for the COI region whenever taxon-specific primers were available and successfully routinely used at the Department of Fisheries molecular lab or where the Folmer primers are not successful. Tunicate-specific primers developed by Stefaniak et al. 2009 have proven effective in the identification of colonial ascidians and are routinely used for the identification of *D. perlucidum* in Australia (Bridgwood et al. 2014; Dias et al. 2016). Fish specific primers developed by Ward et al. (2005) were used to amplify the COI region of all seven fish species listed. The Folmer primers failed to amplify the COI gene region from the three species of Annelida and the one species of Echinodermata listed, but amplification proved possible with alternative primers developed for invertebrate groups by Lobo et al. (2013) and Geller et al. (2013).

From seaweed material, genomic DNA was extracted using a modified CTAB procedure described by Doyle and Doyle (1987). PCR amplification of seaweed DNA was performed using multiple sets of primers targeting COI, rbcl and tufA gene regions for red, brown and green macroalgae. Primers used for each taxon are listed in Supplementary material Table S1 and PCR reactions and conditions given in Table S2. TufA is the preferred gene for barcoding green macroalgae, as the presence of introns in CO1 makes it an unsuitable barcode marker for green macroalgae (Saunders and Kucera 2010).

All PCR reactions were conducted in an Applied Biosystems (ABI) 2720 thermal cycler. A negative control, with no template DNA added, was included in all PCR assays. PCR products were separated by electrophoresis using 1.5% agarose (Fisher Biotec) gels stained with GelRed (Biotium) alongside a 100 base pair (bp) molecular weight marker (Axygen Biosciences) and visualised under UV light. Sequencing of unpurified PCR products was performed using the service provided by the Australian Genome Research Facility (AGRF) in Perth. All samples were sequenced in both directions. Sequences were aligned, edited (checked for errors) and consensus sequences generated using the Sequencher® 5.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA). High quality sequences were used to interrogate the BoLD and NCBI Genbank (using the Basic Local Alignment Search Tool, BLASTN; http://blast.ncbi.nlm.nih.gov) databases to confirm independent taxonomic species identifications.

All work was performed at the WA Government Department of Fisheries molecular laboratory which has attained independent accreditation (NATA; ISO/IEC 17025) for species identification based on DNA-barcoding. All DNA barcodes generated for reference specimens were deposited alongside collection, preservation, identification and storage details in BOLD under project "OZIMP – Reference Specimens of Introduced Marine Pests on the Western Australia Prevention List" (Table S1).

Results

During 2011–2013 we obtained specimens of 17 of the species on the Prevention List, mostly representative of the 19 listed species which are known to be present in at least one area of Australia (Table S1). Specimens of four species—*Perna perna* (Linnaeus, 1758), *Brachidontes pharaonis* (P. Fischer, 1870) species complex, *Mytilopsis sallei* (Récluz, 1849) and *Charybdis japonica* (A. Milne-Edwards, 1861)—were collected during incursions to Australia and identified by expert taxonomists. During 2014–2016 additional specimens were obtained from across world resulting in a reference collection of a total of 75 of the species on the Prevention List. From the dozens of researchers contacted by email, only a very small minority did not respond as willing to contribute specimens to the reference collection, or suggest an alternative contact. Specimens sourced from outside Australia took from 2 days to 2 years to source depending on whether they were transported opportunistically by the authors or using commercial courier services; the ready availability of adequately preserved specimens from published research work or the need to collect them; the need for collecting, exporting or importing permits to be issued; weather conditions and seasonality of occurrence of the species to be collected; and ultimately the availability of researchers to do this. Samples obtained using international courier cost from AUD 30–300 depending on the size and weight of the package and if a drop-off or scheduled pick up service (necessary at more remote locations) was used.

We were unable to obtain specimens representative of only four species in the list: the barnacle *Amphibalanus eburneus* (Gould, 1841), the sponges *G. fibrosa* and *C. thoosina* and the ctenophore *Beroe ovata* Bruguière, 1789 (Table S1). Also, although the Prevention List makes reference to the necessary monitoring of all exotic Didemnidae species with

invasive characteristics, specimens were obtained and barcodes generated for only the two listed species, *Didemnum vexillum* Kott, 2002 and *Didemnum perlucidum* Monniot F., 1983, that have recognised worldwide invasive potential. Similarly, all *Mytilopsis* species and *Congeria* species are indicated for monitoring, despite only certain specific species of concern being listed. The reference specimen and barcode deposited under the record OZIMP075-15 belongs to *Marenzelleria arctia* (Chamberlin, 1920) and to date we have been unable to acquire specimens of the remaining listed species within this genus reported as pests, namely *Marenzelleria neglecta* Sikorski and Bick, 2004, *Marenzelleria viridis* (Verrill, 1873), *Marenzelleria bastropi* Bick, 2005 and *Marenzelleria wireni* Augener, 1913 (Maximov 2011).

DNA barcodes were generated for 65 of the species. All, except three, of the DNA barcodes obtained from the specimens deposited to date in the reference collection and available online through the BoLD project "OZIMP – Reference Specimens of Introduced Marine Pests on the Western Australian Prevention List" matched (≥98% similarity at species level) at least some DNA barcodes previously deposited in GenBank and BoLD. The exceptions were the barcodes obtained for the specimens identified by collectors as *A. nobilis* and *S. fallax* for which species-specific DNA barcodes were not available, the closest match being therefore at the genus level, and S*abella spallanzani* (Gmelin, 1791). The barcode available in BoLD for *S. spallanzani* (GBAN0214-06), mined from GenBank and obtained from a specimen collected in the Azores Islands, Portugal, largely differs from our vouchered specimen barcode (OZIMP074-15).

To date, for 34 species there were at least two whole, adequately preserved, specimens sourced with matching barcodes that allowed at least one specimen to be vouchered and permanently lodged in alternate museums and institutional taxonomic collections, and matching identified specimens to be returned to the reference collection of the WA Government Department of Fisheries (Table S1).

Discussion

In the present work, we report on the assembly of a reference collection of specimens listed on the Western Australian Prevention List for Introduced Marine Pests and their associated DNA barcodes. We believe the establishment of a comprehensive and simultaneous taxonomic and molecular collection of IMP aimed at enhancing marine biosecurity, to be a world first. During 2011–2013 it was clear that we largely underestimated the time associated with sourcing dozens of specimens, mainly exotic to Australia. To date, we were able to obtain specimens representative of 75 species in the list (all except four) and although sourcing of specimens of the remaining four species is still possible, it seems highly unlikely. The species *A. eburneus* and *G. fibrosa* have been flagged as introduced species of potential concern in Hawaii (http://www2.bishopmuseum.org/HBS/invertguide/index.htm), but despite attempts by researchers in Hawaii to sample these species on request, they were not commonly found at sites from where they had been previously described. Molnar et al. (2008) reported *C. thoosina* as introduced in the Aegean Sea and Alaska (http://www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=170479), but we were not able to find sponge experts that were aware of this species being a pest. Given that this species is native from the Mediterranean, synonym with *C. cretensis*, and lack of studies available in the literature, the listing of this species as a potential IMP needs to be reviewed. Obtaining specimens of *B. ovata* was made difficult due to this planktonic species being commonly encountered only during short seasonal summer blooms in the Black Sea, where it has been introduced, and difficulties encountered in exporting samples from Russia to Australia. Also, although more specimens of exotic Didemnidae, *Mytilopsis*, *Congeria* and *Marenzelleria* species can still be added to the reference collection, specimens were obtained from the most common pest species and these should be useful as reference for the genus. To date, barcodes were generated for 65 of the 75 species obtained during this work, which include all but the ten listed dinoflagellate and diatom species. Although we maintain representatives of these ten species in the collection for taxonomic reference, attempts to generate barcodes from these samples failed and were not repeated. The identification of some species (e.g. *Alexandrium tamarense* species complex) can only be done through morphology as the DNA barcode is instead indicative of the species geographic region (Dias et al. 2015). Also, efficient monitoring and response methods for the control and management of these species are unrealistic. This has been recognised by the Australian Priority Marine Pests Task Group which has agreed to recommend their deletion from the National List to the Marine Pest Sectoral Group of the Commonwealth Department of Agriculture. Their removal from the Western Australian Prevention List for Introduced Marine Pests can possibly follow and therefore we decided to focus our work on the remaining species on the list.

The following reference specimens and associated barcodes deserve a particular note because they are native to some region of Australia, are freshwater species, part of "species complexes", have taxonomies that are under review, belong to a broader listed genus including multiple similar IMP, or possess both native and exotic strains. The WA Prevention List makes reference to the monitoring of the seaweed *Caulerpa taxifolia* (M. Vahl) C. Agardh, 1817 in the southern states of Australia only, as this species is considered native in northern Australia. Two species, *Dikerogammarus villosus* (Sowinsky, 1894) and *Dreissena* sp. Van Beneden, 1835, although included in the WA Prevention List, are freshwater species and are notorious pests in freshwater rather than marine ecosystems (Bacela-Spychalska et al. 2012; Nalepa and Schloesser 2014). These species are listed and indicated for monitoring because they are known to survive in ballast waters and fouling of vessels during transoceanic trips, until they reach a freshwater ecosystem where they may become pests (Bacela-Spychalska et al. 2013; Nalepa and Schloesser 2014). Record OZIMP021-15 refers to a marine mussel specimen of *Brachidontes* sp. collected in Australia, and should be treated with care as it is a representative of the native *Brachidontes ustulatus* (Lamarck, 1819). Because of the inability to morphologically distinguish species included in the *Brachidontes pharaonis* species-complex (including the invasive Red Sea *Brachidontes pharaonis*, the Indo-Pacific *Brachidontes variabilis* (Krauss, 1848) and the Australian native *B. ustulatus*) these species were considered synonyms in the most recent review (Huber 2010). The species within the complex can, however, be distinguished genetically through barcoding of the mitochondrial COI gene region (Terranova et al. 2007). Invasive COI haplotypes from the Red Sea and the rest of the world appear to be geographically structured and significantly different (Terranova et al. 2007). A comprehensive systematic review of the genus is needed that would include Australian representatives. One outcome of such work may result in *B. ustulatus* conclusively removed from current synonymy with the invasive *B. pharaonis* (Terranova et al. 2007). Records OZIMP065-15 and OZIMP066-15 refer to the marine crab *Eriocheir sinensis* H. Milne Edwards, 1853, the most notorious invasive species of this crab genus worldwide. However, there are other species within this genus, which if introduced to Australia may be of equal concern, including *Eriocheir japonica* (De Haan, 1835) and *Eriocheir hepuensis* Dai, 1991 (Naser et al. 2012). Although the *E. sinensis* specimen information is deposited in the reference collection, it should be noted that *Eriocheir* spp. are under taxonomic revision and that all are exotic to Australia and recommended for monitoring. Records OZIMP069-15 and OZIMP070-15 refer to specimens of *Hemigrapsus takanoi* Asakura and Watanabe, 2005 that were genetically identified as this species is morphologically indistinguishable from *Hemigrapsus penicillatus* (De Haan, 1835) (Markert et al. 2014), the latter of which is also listed.

Validation of identification of specimens sent to us as belonging to species like *A. nobilis* are complicated as they would involve the review of this genus, which is highly undescribed. The small size of specimens received as *S. fallax* has not allowed us to date to successfully barcode this in a way to allow for specimens to remain intact for taxonomic identification. In the case of our *S. spallanzani* specimen though, it was possible to validate the identification and DNA barcode through collaboration with the Australian Museum which is currently conducting a phylogeographic study on the species. Phylogeographic and population genetic studies are typically expensive and time-consuming, and in the case of invasive species are further challenged by the inevitable need to source specimens worldwide, and from often unknown or unstudied native ranges (Nunez and Pauchard 2010; Gaither et al. 2013). Indeed, most specimens of listed IMP were obtained from researchers studying these species in their introduced range in USA, Europe, Australia and New Zealand. The availability of reference specimens, tissue and DNA is crucial for a posteriori identification of IMP undergoing taxonomic revision or re-classification. For instance, it is only very recently that the worldwide invader tunicate *Ciona intestinalis* has been reclassified as two distinct species, namely *C. intestinalis* and *C. robusta* (Brunetti et al. 2015).

The reference collection has a range of applications, most of which have proven useful throughout its assembly in the last six years. Access to reference specimens in-house provides valuable support to the initial non-expert taxonomic examination of morphological characteristics of suspected IMP, collected during monitoring surveys from vessels or during ad hoc collection, and specimens from the reference collection have already been used in taxonomic workshops aimed at training marine biosecurity personnel in WA. The in-house access to whole reference specimens further allowed for considerable marine biosecurity molecular capability to be developed in WA, including the development of accredited diagnostic barcoding and early detection strategies based on real-time PCR and metabarcoding of complex environmental samples (e.g. biofouling communities). Real-time PCR assays were developed for species of high concern in WA, like *Perna* spp.

(Dias et al. 2013) and *D. perlucidum* (Schenk et al. 2016), and are routinely used to help verify suspected detections of these species. Other real-time PCR assays available for the detection of target IMP (Deagle et al. 2003; Gunasekera et al. 2005; Smith et al. 2012) were also implemented for routine screening in WA, using reference specimens as positive controls to help validate detections. Such assays can provide results within 24 h of sample reception, representing an advantage over the 2–3 days turnaround expected from barcoding. This is particularly important from a marine biosecurity perspective as it allows for the timely establishment of emergency responses and/or establishment of control strategies (Bott et al. 2010). Further, the in-house access to tissue, DNA and barcodes of reference specimens has also been used in the development, testing and validation of metabarcoding strategies from complex environmental samples (e.g. whole biofouling communities from settlement arrays). DNA metabarcoding based on high-throughput sequencing (HTS) is an emerging technology that promises to be most useful for the early detection of IMP due to the sensitivity associated with its "deep-sequencing" capacity, and for an important ecosystem insight given the background biodiversity information resulting from the thousands of sequences generated (for more detail on emerging biosecurity molecular tools see Bott et al. 2010; Wilson et al. 2011; Appeltans et al. 2012; Pochon et al. 2013; Comtet et al. 2015; Coissac et al. 2016).

We do acknowledge that DNA barcoding based identification is only as robust as the underlying taxonomy and obtaining specimens and DNA barcodes from closely related species and extensively researching phylogenies is outside the resources and scope of this work. This is why it is worth noting that regulatory decisions (e. g. rapid responses for containment and/or eradication, vessel management) are not made based on non-expert or DNA-based identification alone, these are used conservatively, as an early-warning system. In this sense, the reference collection can provide crucial support for timely and effective rapid responses in the event of an incursion of a listed IMP in Australia. Reference specimens and DNA-based methodologies have allowed for rapid and confident identification during incursions of *P. viridis* and *C. japonica* in WA. This is why, despite the Western Australian Prevention List for Introduced Marine Pests and its associated reference collection being under continual revision, we are making details on the reference collection publicly available through a regularly updated Barcode of Life Database (BoLD) project and this publication. We hope the present study motivates the establishment and sharing of similar reference collections around the world, fostering the development of essential taxonomic and molecular expertise on notoriously challenging marine invertebrate groups.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Phylum, family and name of species listed in the WA Prevention List for Introduced Marine Pests.

Table S2. Polymerase Chain Reaction (PCR) amplification primers details including name, approximate fragment size, reference, target gene region and PCR reaction and cycling conditions.

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