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Using DNA barcoding and phylogenetics to identify Antarctic invertebrate larvae: Lessons from a large scale study

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ABSTRACT

Ecological studies of the diversity and distribution of marine planktonic larvae are increasingly depending on molecular methods for accurate taxonomic identification. The greater coverage of reference marine species on genetic databases such as GenBank and BoLD (Barcoding of Life Data Systems; www.boldystems.org); together with the decreasing costs for DNA sequencing have made large scale larval identification studies using molecular methods more feasible. Here, we present the development and implementation of a practical molecular approach to identify over 2000 individual marine invertebrate larvae that were collected in the Ross Sea, Antarctica, during the austral summer over five years (2002–2007) as part of the LGP (Latitudinal Gradient Project). Larvae for molecular ID were morphologically identified to belong to the Phyla Mollusca, Echinodermata, Nemertea and Annelida (Class Polychaeta), but also included unidentified early developmental stages which could not be assigned a specific taxon (e.g., eggs, blastulae). The use of a 100 μm mesh plankton net makes this one of the first larval identification studies to simultaneously consider both embryos and larvae. Molecular identification methods included amplification of up to three molecular loci for each specimen, a pre-identification step using BLAST with GenBank, phylogenetic reconstructions and cross-validation of assigned Molecular Operational Taxonomic Units (MOTUs). This combined approach of morphological and molecular methods assigned about 700 individuals to 53 MOTUs, which were identified to the lowest possible taxonomic level. During the course of this long-term study we identified several procedural difficulties, including issues with the collection of larvae, locus amplification, contamination, assignment and validation of MOTUs. The practical guidelines that we describe here should greatly assist other researchers to conduct reliable molecular identification studies of larvae in the future.

1. Introduction

The planktonic larval stages of marine animals, the meroplankton, are a crucial component in the functioning of marine ecosystems through their pivotal role in dispersal and recruitment. Recent studies have also indicated that meroplankton diversity may be a leading indicator of climate change (Kirby et al., 2007, 2008). However, ecological studies on the diversity and distribution of marine planktonic larvae require accurate taxonomic identification, which can be a challenging task for the meroplankton. At present, most larvae can be morphologically identified with ease only to Phylum, or Class, and rarely to Order and Family. Traditionally, the link between larval and adult form has been achieved either through rearing larvae collected from plankton, or from spawning adults in the laboratory (Shanks, 2001). Culturing larvae is, however, particularly difficult in cold-water environments such as the poles and the deep-sea, where these labour-intensive approaches take even longer due to slow rates of development, and there is a general uncertainty about appropriate foods for planktotrophic (feeding) forms.

An alternative approach is to use molecular methods, as first applied by Olson et al. (Olson et al., 1991), who used mitochondrial 16S rRNA sequences to differentiate between the morphologically similar larvae of temperate sea cucumbers. With great optimism the paper ends with the statement: "With the establishment of libraries of mitochondrial DNA sequences, the problem of identifying morphologically similar invertebrate larvae will at last be solved" (Olson et al., 1991).

Unfortunately, progress in applying molecular methods to larval identification has been slower than anticipated for two reasons. Firstly, to identify an unknown larva from a particular location requires a comprehensive reference collection of species (adults, DNA and/or sequences) against which the larval DNA from a particular location can be compared. Construction of such a reference collection is particularly problematic in marine habitats where there is a high diversity of adults from which any one larval morphological form could be derived. Secondly, for a molecular method to succeed as an identification tool it must be highly discriminatory and, ultimately, DNA sequencing is likely

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to be required to construct reference collections, except in small well-defined taxonomic groups (Deagle et al., 2003; Hare et al., 2000). Until relatively recently, sequencing costs and the degree of effort involved made the development of "libraries of mitochondrial DNA sequences" (Olson et al., 1991) both costly and time-consuming. Lowered costs through greater automation and growing reference collections from DNA sequencing initiatives (such as the Barcoding of Life Initiative (www.barcoding.si.edu), the Census of Marine Life (CoML, www.coml.org) and Census of Antarctic Marine Life (CAML, www.caml.aq) are now finally making this approach more feasible.

The Antarctic meroplankton provides a particularly good example of the difficulties faced in the species identification of marine invertebrate larvae. In all marine habitats, matching of larval to adult forms has to consider two potential constraints: (1) that morphological changes during larval development may make identification of all stages of a single species difficult (Thompson, 1828; Mortensen, 1921) and, (2) that there may be considerable morphological similarity of larvae between related species (Thompson, 1828; Winsor, 1976). In the Antarctic, larval identification may be especially difficult because extended developmental times (Pearse et al., 1991; Peck, 1993; Peck and Robinson, 1994) increase the time over which developmental changes can occur, and there is high diversity in several benthic taxa (Clarke and Johnston, 2003). In addition, logistic constraints, such as the time available for sampling, a deep continental shelf, and a poor knowledge of biodiversity (Brandt et al., 2007), has resulted in limited availability of the Antarctic reference sequences required for large scale molecular identification of larvae.

Consequently, although there has been a long history of morphological description of larvae in the Ross Sea (MacBride and Simpson, 1908; MacBride, 1920; Murray, 1895), few invertebrate larvae have been identified to the species level. Recent studies have, however, used a molecular identification approach to assign larvae to specific taxa. Whereas most studies have concentrated on specific phyla (Barber and Boyce, 2006; Goetze, 2010; Hart et al., 2003; Janosik et al., 2008; Mahon et al., 2009; Puillandre et al., 2009) only one study has tried to identify Antarctic larvae on a broader scale so far, although with limited success [14/64 larvae, 22% success; (Webb et al., 2006)]. Recent work, including our own, has expanded the scale of DNA identification of both holo- and meroplankton (Bucklin et al., 2010; Machida et al., 2009; Sewell et al., 2006).

In this study we had three initial aims: 1) the *discrimination* of Ross Sea meroplankton into distinct 'species' — either to operational taxonomic units (Stanwell-Smith et al., 1999; OTUs) based on morphology and/or molecular operational taxonomic units (Blaxter et al., 2005; MOTUs) based on DNA sequence, 2) the *comparison*, where possible, between morphological and molecular OTUs, and 3) the *identification* of these OTUs to species (or higher taxon) by comparison to adult and other reference sequences (e.g., from Genbank). Our long-term goal is to be able to use the accurate species identification provided to address broad-scale ecological questions regarding patterns in larval diversity and abundance within the Ross Sea and contribute to a better understanding of larval dispersal and recruitment patterns in the Antarctic benthos.

In this paper our aim is to present the reality and some of the complexities of taking a DNA sequencing approach to larval identification, and to test the feasibility of large scale molecular methods to identify planktonic larva. Obtaining a clean DNA sequence from a generally small (<200 μm) embryo or larvae, that is potentially contaminated with other organisms during plankton net collection, is a non-trivial task, and there are procedural issues that may not be immediately apparent in the brief descriptions found in methods sections of scientific papers. By sharing the "wisdom of our experience", taking special note of the mistakes we made and the resulting improvements to procedures, we hope to provide a set of guidelines for researchers who might want to conduct reliable molecular identification studies of larvae in the future.

2. Material and methods

2.1. Plankton collection

Meroplanktonic samples were collected from five different coastal sites on Ross Island, and along the Victoria Land Coast of the Ross Sea, Antarctica, during the austral summer months of November and December in 2002–2004, and 2006–2007. The initial development of the protocols described here was completed in 2002 while based at Scott Base, on Ross Island, and then sampling continued at two sites of Antarctica New Zealand's Latitudinal Gradient Project at Cape Hallett (2003–2004: 72°18.412′S, 170°11.290′E) and Terra Nova Bay (2006–2007: 74838.474′S, 164812.473′E; see map in Howard-Williams et al., 2006). At all sites, meroplankton samples were collected with a 100 μm mesh collapsible plankton net from a single hole drilled through the annual sea ice; as described in detail in (Sewell, 2005). In 2002, we also obtained larvae from the filter bag on the seawater intake of the Scott Base reverse osmosis plant as described in Sewell and Jury (Sewell and Jury, 2009).

Plankton samples were sorted and enumerated under a dissecting microscope in the laboratory and representative larvae photographed on clean depression slides using an Olympus C4040 camera at either 40× or 100× magnification. Larvae for DNA sequencing were then transferred by pipette directly to an individual well of a 96-well PCR plate with minimal seawater. 100–200 μl of 95% ethanol was then added, and the well was sealed with a silicon rubber cap. Based on their morphological identification, conducted by the same investigator (MAS) in all years, individuals were preserved in sample trays specific to one of four main phyla: Echinodermata, Mollusca, Annelida (Class Polychaetea), Nemertea. When the phylum was morphologically not identifiable, which included very early stages of larval development such as egg, blastulae and gastrulae, the larvae were collected in sample trays labelled as Unknowns. Sample plates were stored, depending on location, at room temperature (2002, 2006, 2007), or in semi-cool conditions (in a laboratory tent, 2003, 2004) until the end of the summer sampling season. The PCR plates were then transported at 4 °C to the University of Auckland where they were stored at −20 °C until analysis. The initial protocol development was based on the Ross Island 2002 samples, in techniques developed in the laboratory in 2003 and 2004 (Sewell et al., 2006). DNA extraction and molecular processing for 2003, 2004, 2006 and 2007 samples were all completed during 2008 after minor modifications to the protocols (see Results section).

2.2. Adult collection

Adult Antarctic marine invertebrates were collected for DNA analysis through sampling programmes already underway in the Ross Sea. Specimens were kindly provided through the following sources: SCUBA diving for benthic specimens by the New Zealand National Institute for Water and Atmosphere (NIWA, A. Norkko, V. Cummings), incidental by-catch from fish-traps (G. Hofmann, UC Santa Barbara), by removing a few tube-feet from starfish collected for spawning experiments (D. Ginsberg, University of Southern California) and through collections made by the Italian benthic programme at Terra Nova Bay (M. Chiantore). Depending on size, after photographing, adults were either stored whole in 95% ethanol, or first a tissue subsample was taken before storing in ethanol. Subsequent storage and transport to the University of Auckland was as per the meroplankton samples.

2.3. DNA extraction

Genomic DNA was extracted from planktonic larvae from each sample plate. After evaporating the ethanol by placing the sample plate in a thermocycler at 90 °C until the wells were dry, 25 μl of a solution of 10 mg/ml proteinase-K and 5% chelex in ddH_20 was then