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Species diversity in the marine microturbellarian *Astrotorhynchus bifidus* sensu lato (Platyhelminthes: Rhabdocoela) from the Northeast Pacific Ocean

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ABSTRACT

Increasing evidence suggests that many widespread species of meiofauna are in fact regional complexes of (pseudo-)cryptic species. This knowledge has challenged the 'Everything is Everywhere' hypothesis and also partly explains the meiofauna paradox of widespread nominal species with limited dispersal abilities. Here, we investigated species diversity within the marine microturbellarian *Astrotorhynchus bifidus* sensu lato in the Northeast Pacific Ocean. We used a multiple-evidence approach combining multi-gene (18S, 28S, COI) phylogenetic analyses, several single-gene and multi-gene species delimitation methods, haplotype networks and conventional taxonomy to designate Primary Species Hypotheses (PSHs). This included the development of rhabdocoel-specific COI barcode primers, which also have the potential to aid in species identification and delimitation in other rhabdocoels. Secondary Species Hypotheses (SSHs) corresponding to morphospecies and pseudo-cryptic species were then proposed based on the minimum consensus of different PSHs. Our results showed that (a) there are at least five species in the *A. bifidus* complex in the Northeast Pacific Ocean, four of which can be diagnosed based on stylet morphology, (b) the *A. bifidus* complex is a mixture of sympatric and allopatric species with regional and/or subglobal distributions, (c) sympatry occurs on local (sample sites), regional (Northeastern Pacific) and subglobal (Northern Atlantic, Arctic, Northeastern Pacific) scales. Mechanisms for this co-occurrence are still poorly understood, but we hypothesize they could include habitat differentiation (spatial and/or seasonal) and life history characteristics such as sexual selection and dispersal abilities. Our results also suggest the need for improved sampling and exploration of molecular markers to accurately map gene flow and broaden our understanding of species diversity and distribution of microturbellarians in particular and meiofauna in general.

1. Introduction

Marine meiofauna are an important component of earth's biodiversity and play critical roles in ecosystem functioning (Zeppilli et al., 2015). Yet, only a fraction of meiofaunal diversity is known to science (Carugati et al., 2015; Fonseca et al., 2010; Snelgrove, 1999). Current knowledge on their diversity and distribution is heavily biased toward specific sampling localities and efforts, and even well-studied areas can contain relatively high numbers of undescribed species (Curini-Galletti et al., 2012). For many groups, taxonomic expertise is limited and many taxa are hard to identify because of the time-consuming microscopy to recover morphological characters. More recently, DNA taxonomy based mostly on COI and/or ribosomal genes has emerged as a new tool for species identification and species delimitation. As a result, it became clear that many nominal species of meiofauna, including representatives of cycliophorans (Baker et al., 2007), copepods (Garlitska et al., 2012), polychaetes (Westheide and Schmidt, 2003), flatworms (Scarpa et al., 2016), rotifers (Gómez et al., 2002), gastropods (Jörger et al., 2012), gastrotrichs (Kieneke et al., 2012), nematodes (Derycke et al., 2008) and nemerteans (Leasi et al., 2016), are in fact complexes of cryptic (morphologically indistinguishable) and pseudo-cryptic (*a posteriori* morphologically distinguishable) species. This hidden diversity at the molecular level will significantly increase estimates of biodiversity for many marine taxa and influence our understanding of ecosystem functioning and priorities for conservation (Appeltans et al., 2012).

Although DNA taxonomy is an exciting tool to assess biodiversity, DNA-based species delimitation has methodological challenges (Fontaneto et al., 2015). Most species delimitation studies on meiofauna have used either single-gene or gene cluster datasets and single-gene delimitation methods. Puillandre et al. (2012b) developed a standardized workflow for large-scale species delimitation of hyperdiverse animal groups using the COI barcode as a starting point to formulate primary species hypotheses (PSHs). These PSHs can then be

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corroborated with additional information from ribosomal genes, morphology and reciprocal monophyly, leading to SSHs and formal species delimitations. This approach allows for an efficient workflow when sampling coverage is high. Fewer studies have used multi-gene data and multi-gene delimitation methods to generate PSHs. Secondary species hypotheses (SSHs) can then be inferred based on a minimum consensus between independent molecular markers, morphology and phylogeny (Fontaneto et al., 2015). This multiple-evidence approach provides more resolution to smaller datasets with under-sampled taxa and rare specimens (i.e., singletons) (Jörger et al., 2012). It also improves diagnosability and can uncover problems with contamination, pseudogenes, hybridization or introgression. Combining evidence from phylogeny, molecular species delimitation, morphology and other sources (e.g., ecology) allows taxa to be recognized under a unified species concept (De Queiroz, 2007), rather than just one of the primary species concepts (e.g., biological, phylogenetic, morphological) (Adams et al., 2014).

Distribution patterns of meiofauna are poorly understood. Many nominal species of meiofauna seem to either be cosmopolitan or have wide geographical ranges and therefore fit within the Everything is Everywhere' (EiE) hypothesis (Fenchel and Finlay, 2004; Finlay, 2002). However, they represent a unique case known as the 'meiofauna paradox' (Giere, 2009), because, contrary to many prokaryotes and protists, meiofauna often lack active dispersal propagules and dormancy capabilities. The meiofauna paradox and EiE hypothesis are now challenged by DNA taxonomy and phylogeography, providing accumulating evidence of (pseudo-)cryptic species complexes with biogeographical ranges (Fontaneto, 2011; Fontaneto et al., 2015; Leasi and Norenburg, 2014). However, the biogeographical patterns within these complexes are not always clear. Indeed, several studies have shown that (pseudo-)cryptic meiofauna can be composed of allopatric species (Westheide and Schmidt, 2003), sympatric species (Derycke et al., 2008) or, most often, a mix of sympatric and allopatric species (Garlitska et al., 2012; Jörger et al., 2012; Kieneke et al., 2012; Leasi et al., 2016; Meyer-Wachsmuth et al., 2014; Scarpa et al., 2016). In addition, species within the same complex can either be widespread or have more restricted geographical ranges (Tessens, 2012). The lack of a generalized biogeographical concept could be partly explained by the taxonomic and ecological variation of meiofauna. The majority of animal phyla has meiofaunal representatives with their own life history characteristics, such as reproduction, absence/presence of larval stages, absence/presence of dormancy stages, and ecological tolerances associated with different habitats. These can influence their ability to disperse and adapt to new environmental conditions. As such, (pseudo-) cryptic species and biogeographical patterns need to be carefully looked at taxon by taxon.

Here we examine species diversity in the marine microturbellarian *Astrotorhynchus bifidus* sensu lato (s.l.). Microturbellarians occur abundantly in the interstitial habitats of sediments and epiphytically on macro-algae worldwide. Knowledge on their diversity and distribution is still scarce and heavily biased by sampling efforts (Artois et al., 2011). Exact numbers of the global diversity of marine microturbellarians are not available, but should at least surpass 2000 described species. More intensively studied groups of microturbellarians, such as rhabdocoels and proseriates, are known to harbor high levels of undescribed diversity, both among traditional morphospecies (Appeltans et al., 2012; Curini-Galletti et al., 2012) and cryptic species complexes (Casu and Curini-Galletti, 2004; Curini-Galletti and Puccinelli, 1998; Delogu and Curini-Galletti, 2009; Scarpa et al., 2016; Tessens, 2012).

In this study, we provide insight into the *Astrotorhynchus bifidus* species complex from the coasts of British Columbia and Washington. The nominal species is the only representative of *Astrotorhynchus* within Paramesostominae (Rhabdocoela, Dalytyphloplanida). It was previously recorded from the intertidal and subtidal zones in a wide area spanning from the Northwest Atlantic to the White Sea. As such, this is the first record of *A. bifidus* s. l. in the Pacific Ocean. We uncover 5 species in the Northeastern Pacific by combining single-gene and multigene species delimitation analyses, haplotype networks, molecular phylogenetic analyses and comparative morphological data. The molecular data include DNA sequences from two ribosomal genes, 18S rRNA and 28S rRNA, and the COI barcode gene. To obtain the latter, new rhabdocoel-specific COI primers were developed. Four species could be formally diagnosed based on stylet morphology, two of which correspond to the previously recognized subspecies *A. bifidus bifidus* and *A. bifidus regulatus*. Insights into patterns of co-occurrence and biogeography are discussed with reference to life history and passive dispersal of meiofaunal flatworms.

2. Material and methods

2.1. Collection and morphological examination of the taxa

Specimens of *A. bifidus* s. l. were collected in 2015–2016 from seaweeds in the rocky intertidal along the Pacific coast of British Columbia (Canada) and Washington (USA), including the Juan de Fuca Strait (Victoria), the Vancouver Island Shelf (Bamfield), the Strait of Georgia (Friday Harbor, Quadra Island), and the North Coast Fjords (Calvert Island) (Fig. 1).

Live animals were isolated from algae using the $MgCl_2$ decantation method (Schockaert, 1996). Specimens were studied alive with the aid of a stereoscope and DIC optics, photographed, and subsequently whole mounted with lactophenol to preserve the stylet of the male genital system. The stylet was then photographed, measured and drawn to examine its morphological details. All pictures were taken with a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera, except for the ones from Calvert Island, which were taken with a Leica DMIL inverted microscope. Pictures of whole mounts in the taxonomic account (Appendix A) were produced in Helicon Focus (HeliconSoft) by stacking series of micrographs. Schematic line art diagrams were drawn freehand based on series of micrographs, scanned and retouched with Inkscape (www.inkscape.org) and GIMP (www.gimp.org). Measurements were taken from whole-mounted and live specimens using ImageJ software (www.imagej.net). From each population (except for Quadra Island), specimens were frozen in a few µL of seawater for DNA extraction.

All type material was deposited in the Beaty Biodiversity Museum (BBM, University of British Columbia, Vancouver, Canada). Metadata on all the specimens of *A. bifidus* s. l., including collection information, DNA, voucher and type material, and access to pictures on morphobank (www.morphobank.org), are given in Table S4.

2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from entire specimens with the DNeasy Blood & Tissue kit (Qiagen). Extractions followed manufacturer's instructions except that (a) the AE elution buffer was heated to 60 °C before elution, and (b) DNA was eluted twice for every sample in reduced volumes of 60 µL and 30 µL, respectively.

The COI barcode region (655 bp) of specimens of *A. bifidus* s. l. and all outgroup taxa was amplified with Phusion® Hot Start Flex DNA polymerase (New England Biolabs, Inc.) and the newly developed primers RhCo1F and RhCo1R. In addition, the new COI primers were also tested on 17 other species of rhabdocoels (Table S1). Nearly complete 18S rDNA (1693–1695 bp) and partial 28S rDNA (1568–1656 bp) sequences from all Pacific specimens of *A. bifidus* and the two species of *Promesostoma* were amplified using Illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) and several different sequencing primer pairs. The 18S and 28S rDNA sequences from the two Atlantic specimens of *A. bifidus* and the other outgroup taxa were mined from Genbank (Table 1). All primers and thermocycling conditions are listed in Table S2. Amplicons were visualized on 1.5% agarose gels stained with