
# A MORPHOLOGICAL AND MOLECULAR STUDY OF *POLYDORA* CF. *WEBSTERI* (ANNELIDA: SPIONIDAE): ACCURATE IDENTIFICATION TOWARDS IMPROVED MANAGEMENT OF A GLOBALLY DISTRIBUTED PEST OF CULTURED MOLLUSCS

by

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# DECLARATION

I, **Nicola Rodewald**, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

3 MAY 2020

**Signed** **Date**

# ABSTRACT

*Polydora websteri* Hartman, 1943, a member of the *Polydora*-complex, commonly known as polydorids (Annelida: Spionidae), is one of the most wide-spread polydorid pests of mollusc culture in the world. Specimens resembling *P. websteri* were first recorded in South Africa, in 2009, boring into shells of oysters in a culture facility in Port Elizabeth and since then, has been recorded on cultured oysters from Namibia, Kleinzee and Paternoster on the west coast of South Africa. A preliminary investigation showed that specimens collected in South Africa, Australia and Japan were genetically different from *P. websteri* specimens collected in Rhode Island in the United States of America (USA), near the type locality of *P. websteri*. The possible wide-spread distribution of this shell-boring polydorid species emphasises the importance of timeous and accurate identification, for improved management and even eradication. The specimens in South Africa, Australia and Japan have therefore been referred to as *P.* cf. *websteri* until its taxonomy could be clarified using morphological and molecular information, which was the main aim of this study. Additionally, for improved management of pest polydorids locally and internationally, this study aimed to contribute to the sequence library for polydorid species and develop a taxonomic key of shell-boring polydorid pest species in South Africa for mariculture practitioners. Sequencing of cytochrome c oxidase subunit I (COI), cytochrome *b* (Cyt *b*) and 18S rRNA markers conducted in this study, yielded thirty-three sequences of specimens from South Africa, seven from Australia and seven from wild molluscs collected in Rhode Island on the east coast of the USA. Forty-three additional COI, four Cyt *b* and 24 18S rRNA sequences from South Africa, Namibia, Australia, China, Japan, Hawaii, west and east coasts of the USA were obtained from GenBank. Bayesian and maximum likelihood analysis of COI, Cyt *b* and 18S rRNA markers indicated that *P.* cf. *websteri* from South Africa and sequences of specimens from all other locations form a monophyletic group (share a common ancestor); but do not group with specimens collected from wild molluscs in Rhode Island near the type locality in the USA. Instead, the specimens from Rhode Island clustered with *Polydora onagawaensis* Teramoto, Sato-Okoshi, Abe, Nishitani & Endo, 2013. The interspecific distances between specimens from Rhode Island and sequences of *P. websteri* from all other regions were one or two orders of magnitude (10 or 100 times) higher than the intraspecific distances of either *P. websteri* or *P. onagawaensis*. However, the interspecific distance between specimens from Rhode Island and *P. onagawaensis* was 0 %. These results were also confirmed by species delimitation analysis and haplotype networks. Additionally, South African specimens were similar to the *P. websteri* lectotype and matched morphological descriptions of those from Australia, China, Japan, New Zealand, the east, west and gulf coast of the USA, but not the specimens collected from Rhode Island which were morphologically similar to *P. onagawaensis* from Japan. It is therefore concluded that

specimens from South Africa are *P. websteri*, a new non-indigenous record, and that the specimens collected from Rhode Island had been misidentified. The confusion between *P. websteri* and *P. onagawaensis* in the USA reinforces the difficulty in identifying pest polydorids using only morphology and highlights that such misidentifications may impede accurate identifications of non-indigenous polydorid pests, which is problematic for implementing effective management strategies. Additionally, haplotype sharing and lack of genetic differentiation among populations of *P. websteri* from geographically distant locations confirm anthropogenic transportation of the species into South Africa. This haplotype sharing has undoubtedly resulted from the repeated movement of molluscs making it difficult to identify the source of the population in South Africa. The results strongly suggest that *P. websteri* arrived in South Africa via the transport of infested oysters for mariculture and has been spread between farms through the intraregional movement of infested oyster stock. Confirming the identity of *P. websteri* in Australia, China, Japan, South Africa and the east, west and gulf coasts of the USA shows that it is the second most widespread polydorid pest of mariculture known. The distribution may be even wider if the identity of *P. websteri* from South America and Europe is confirmed; this may help to better understand the global route of invasion and subsequently assist with preventing, or at least minimising further spread. It is recommended that the mariculture industry in South Africa implement a monitoring plan to facilitate the rapid identification of shell-boring polydorid pests, by using a combination of the taxonomic key provided in this study and genetic methods to identify new non-indigenous pests timeously.

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*There is no limit to what we, as women, can accomplish – Michelle Obama*

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# GLOSSARY

| Terms | Definition/ Explanation |
|---|---|
| Adelphophagic | Larvae develop within the brood capsule to an advanced stage and settle soon after emerging from the maternal burrow (Radashevsky, 1994; David et al., 2014). |
| Caruncle (Spionidae) | A dorsal extension of the prostomium, separating the nuchal organs (Wong et al., 2014). |
| Chaeta (pl. chaetae) (hence Polychaeta, 'with many hairs') | Bristle or seta made of chitin protruding from the body wall (Wong et al., 2014). |
| Chaetiger | A segment bearing chaetae (Wong et al., 2014). |
| Cryptic species | Genetically distinct species, that cannot interbreed, but are morphologically identical to each other (Radashevsky & Pankova, 2006). |
| Extralimital | Species whose indigenous range falls within the boundaries of a country, but whose presence in another part of the same country is due to anthropogenic transport across biogeographical barriers (Robinson et al., 2016). |
| Falcate spines (Spionidae) | Modified, robust chaetae with processes resembling teeth; in polydorids characteristically present on the 5$^{th}$ chaetiger (Wong et al., 2014). |
| GenBank ® | The National Institutes of Health (NIH) genetic sequence database, a collection of all publicly available DNA sequences (Nucleic Acids Research, 2013 Jan;41(D1): D36-42). GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at National Center for Biotechnology Information (NCBI) (GenBank, 2020). |
| Indigenous (synonym: native) | Species within their native range (Robinson et al., 2016). |
| Invasive | Non-indigenous species that have spread from their point of introduction and have self-replacing populations over several generations (Robinson et al., 2016). |
| Mollusc culture (synonym: mollusc aquaculture) | Farming members of the phylum Mollusca, in an aquatic (including marine) environment for food (Haupt et al., 2010a). |
| Monophyletic | Describes a group of organisms that are classified in the same taxon and share a common recent ancestor. A monophyletic group includes all descendants of that common recent ancestor (Lee, 2000). |
| Neuropodium (pl. Neuropodia) | Ventral branch or ramus of a parapodium (Wong et al., 2014). |

| | |
|---|---|
| Non-indigenous (synonym: alien) | Species present in a region due to human actions; these have enabled the species to overcome important biogeographical barriers (Robinson et al., 2016). |
| Notopodium (pl. Notopodia) | Dorsal branch or ramus of a parapodium (Wong et al., 2014). |
| Occipital antenna (Spionidae) | A short intermediate appendage on the prostomium (Wong et al., 2014). |
| Palps | A pair of feeding and/or sensory appendages attached to the head or anterior end of body (Wong et al., 2014). |
| Planktotrophic | Larvae emerge from burrows and actively feed on plankton in the water column for up to 85 days (Blake, 1969; David et al., 2014; Simon & Sato-Okoshi, 2015). |
| Poecilogonous | When individual females or different individuals within the same population produce different types of larvae (planktotrophic and adelphophagic) (David et al., 2014). |
| Polydorids | A grouping of species from the *Polydora* – complex of the family Spionidae and the phylum Polychaeta that have a modified chaetiger five (Blake, 1969). |
| Polymerase chain reaction (PCR) | A technique used in which a specific region of DNA or RNA is replicated repeatedly to produce many copies (i.e. amplification) of a particular gene. PCR requires DNA polymerase, Taq polymerase and primers designed to target a specific region of DNA or RNA. In PCR, the reaction is repeatedly cycled through a series of temperature changes (Friedheim, 2016). |
| Primer | A short strand of DNA that serves as a starting point for DNA synthesis. Primers are required for amplification because DNA polymerase can only add nucleotides to an existing strand of DNA (Friedheim, 2016). |
| Prostomium | Anterior-most region of the body, before the mouth; usually bearing radioles and sensory organs such as palps, antennae, nuchal organs and eyes (Wong et al., 2014). |
| Pygidium | Post-segmental terminal part of the body surrounding the anus (Wong et al., 2014). |
| Ship ballast water | A ballast tank is a compartment within a floating structure that holds water providing stability for a vessel. Ballast water is taken up or discharged when cargo is unloaded or leaded. Ships often take up ballast water in ports and coastal regions which usually contains many forms of organisms. Discharging this ballast water at ports and coastal regions releases these organisms into new areas where they can become marine pests. To date, the International Convention for the Control and Management of Ships' Ballast Water and Sediments ("the Ballast Water Management Convention") 2004, introduced global regulations to control the transfer of potentially invasive species. This convention requires international traffic to manage their ballast water and sediments to a certain standard, |

| | |
|---|---|
| | according to a ship specific ballast management plan (Robinson et al., 2020). |
| Ship hull fouling | Organisms can attach themselves to the hulls of ships, also known as biofouling, or live within this fouling community. These organisms are then transported from one port or area to the next and may be released into new areas where they can become marine pests (Robinson et al., 2020). |

# CHAPTER ONE
# INTRODUCTION

## 1.1. Statement of the research problem

Polychaete worms of the *Polydora*-complex (commonly known as polydorids) are among the species most frequently translocated via both oyster mariculture (marine aquaculture) and ship ballast water (Simon et al., 2006; Sato-Okoshi et al., 2008; Simon, 2011; Çinar, 2013; Abe et al., 2019). The worldwide establishment of non-indigenous polydorids makes it very important that these pests are identified timeously and accurately, for improved management and or eradication in both recipient and donor regions. *Polydora websteri* Hartman, 1943 is one of the most wide-spread polydorid pests of mariculture in the world (Simon & Sato-Okoshi, 2015). Preliminary evidence by Williams (2015) suggested that a new species found on oyster farms in South Africa match the description of *P. websteri*, but that these and specimens collected in Australia and Japan were genetically different from specimens of *P. websteri* collected near the type locality from the United States of America (USA). The specimens in South Africa, Australia and Japan should therefore be referred to as *P.* cf. *websteri* until its taxonomy has been clarified using morphological and molecular information (Simon & Sato-Okoshi, 2015), which is the main aim of this study. The molecular analysis includes sequence data generated in this study and obtained from GenBank from; mitochondrial DNA markers cytochrome c oxidase subunit I and cytochrome *b*, and the nuclear DNA marker 18S rRNA. Comparing *P*. cf. *websteri* from South Africa with those from other places around the world may help identify the origin and introduction of the species, which can facilitate informed management strategies for *P*. cf. *websteri* populations. Additionally, comparing the molecular structure of *P*. cf. *websteri* from different sites in South Africa can provide evidence to support the theory that movement of oysters facilitates the movement of *P*. cf. *websteri* within South Africa. Comparing the molecular structure among populations of *P*. cf. *websteri* in South Africa can indicate if the introduction to the region occurred recently and possibly be eradicated before populations become invasive. The study will, therefore, contribute towards informed management strategies of *P.* cf. *websteri* populations in South Africa.

## 1.2. Literature review

### 1.2.1. Background to the research problem

Polydorids include some of the most common pests of cultured molluscs (Blake, 1969; Simon et al., 2006; Walker, 2011; Simon & Sato-Okoshi, 2015; Williams, 2015). Polydorids comprise nine genera, but most belong to the genera *Polydora* Bosc 1802 and *Dipolydora* Verrill 1879, with fewer species belonging to *Pseudopolydora* Czerniavsky, 1881, *Boccardia* Carazzi, 1893, *Polydorella* Augener, 1914, *Tripolydora* Woodwick, 1964, *Boccardiella* Blake & Kudenov 1978,

*Carazziella* Blake & Kudenov 1978 and *Amphipolydora* Blake, 1983 (Simon, 2011; Walker, 2011). Many polydorids are capable of boring into non-calcareous and/or calcareous substrates, such as mollusc shells and corals, often resulting in simple U-shaped burrows, complex branching burrows, shallow depressions or mud-blisters (Blake & Evans, 1973). The boring activity compromises the integrity of mollusc shells, making them more brittle (Blake & Evans, 1973; Clements et al., 2017). However, mud-blisters do not only make the mollusc shells brittle, but can distort the shape of the shell and possibly foul the mollusc flesh (Read, 2010). Formation of blisters can be initiated either by (1) worm larvae settling on the outer surface of the mollusc valves and excavating U-shaped burrows into the shell matrix until the burrows penetrate the inner surface of the shell, or (2) worms entering between the mantle and the inner shell surface. In response to this, the molluscs secrete a thin sheet of conchiolin followed by calcite layers, leaving U-shaped burrows (Lauckner, 1983). Blister formation is subsequently followed by worms compacting mud to fill the space in the burrows (Lunz, 1941; Haigler, 1969; Blake & Evans, 1973). The polydorids then occupy the mud-filled chambers that were formed (Blake & Evans, 1973).

The shell-boring polydorids do not usually harm molluscs under natural conditions (Loosanoff & Engle, 1943; Radashevsky, 1999; Read, 2010), but high infestations can become a problem when culturing molluscs, as it may lead to reduced growth rate, meat yield and increased mortality of molluscs, especially during the grading and handling processes (Simon et al., 2006; Sato-Okoshi et al., 2008). Additionally, if the blisters are broken during processing or shucking for consumption, the smell and the look can be unpleasant to the consumers (Read, 2010). This reduces the commercial quality and increases production time of the molluscs, therefore reducing the profit of the associated farm (Simon & Sato-Okoshi, 2015). Pest polydorids have therefore been investigated quite intensively (e.g. Blake & Evans, 1973; Sato-Okoshi, 1999; Sato-Okoshi & Okoshi, 2000; Radashevsky & Olivares, 2005; Simon et al., 2006; Sato-Okoshi et al., 2008; Simon et al., 2009; Simon, 2011; Sato-Okoshi & Abe, 2012; Simon & Sato-Okoshi, 2015; Rice et al., 2018; Martinelli et al., 2020).

Modern culture of molluscs, particularly oysters, is often associated with large-scale movement of stock, which becomes a problem when shell-boring polydorids are moved together with the molluscs (Ruesink et al., 2005; Haupt et al. 2010a, b, 2012). Polydorids have therefore invaded many parts the world (Simon & Sato-Okoshi, 2015), with at least 13 shell-infesting and pest polydorid species associated with cultured or commercially harvested molluscs having been transported outside of their indigenous ranges. Of these, *Polydora hoplura* Claparède, 1870, *Boccardia proboscidea* Hartman, 1940 and *P.* cf. *websteri* are the most important non-indigenous pest species infesting cultured molluscs in South Africa (Radashevsky & Olivares, 2005; Simon, 2015; Simon & Sato-Okoshi, 2015; Williams et al., 2016).

Packaging of animals for mariculture, ship ballast water and hull fouling may also play a role in polydorid introductions (Bailey-Brock, 1990; Simon et al., 2006; Haupt et al., 2010b). For example, *P. hoplura* was first recorded in South Africa in 1947 (Mead et al., 2011). It was, however, suggested that ship ballast water and/or hull fouling was the original vector for the transportation of *P. hoplura* into South Africa (Mead et al., 2011; Williams, 2015), as transport to South Africa precedes mariculture here (Haupt et al., 2010a, b). It is therefore unlikely that the farming of molluscs was the source of the original introduction (Van Niekerk, 2014).

Irrespective of the original source of introduction of a species to a new region, once it is there and makes its way onto the farm, it can be moved among farms with infested molluscs, which is the most important mechanism for intraregional transport of polydorids in South Africa (Simon et al., 2009; Haupt et al., 2010a; Williams et al., 2016). For example, Simon et al. (2009) indicated that *B. proboscidea* was mainly spread within South Africa through the transportation of infested abalone among farms. Similarly, in Chile Moreno et al. (2006) suggested that the continuous movement of molluscs, particularly oysters, has resulted in the secondary spread of shell-boring polychaetes. Moreno et al. (2006) and Williams et al. (2016) also reported that some polychaete species (host generalists) spread through mariculture were able to infest nearby populations of indigenous host species, which further promotes the spread of the introduced worms. These shell-boring polychaete pests can therefore become established, as non-indigenous species (NIS), in their new environments. Thus, non-indigenous shell-boring polydorid pests not only pose a threat to mollusc farms, but also are a source of ecological concern, as shown in Chile and Australia (Sato-Okoshi & Takatsuka, 2001; Moreno et al., 2006; Sato-Okoshi et al., 2008). The presence of polydorids in wild hosts near farms puts molluscs on farms at risk of re-infestation, exacerbating the problem. In South Africa, *P. hoplura* infests wild mollusc species, including those that occur close to commercial mollusc farms (Williams et al., 2016).

To date, five alien polychaeta species have been identified from wild populations in South Africa, including three pest polydorids, *P. hoplura*, *Polydora neocaeca* Williams & Radashevsky, 1999 and *B. proboscidea* (Robinson et al., 2016; Robinson et al., 2020; Malan et al. 2020). The increasing number of alien species in South Africa increases the need to appropriately manage and prevent further invasions (Mead et al., 2011; Robinson et al., 2020). However, because different management strategies apply to different stages of the invasion process (Blackburn et al., 2014), it is important to understand the invasion potential of a NIS. Understanding the invasive history of a recently introduced NIS and using invasive species that are closely related as ‘predictors’ for assessing the invasive potential, could aid in future

management strategies of these newly introduced NIS (e.g. David & Simon, 2014; David et al., 2016).

*Polydora websteri* is one of the most wide-spread (Figure 1.1) polydorid pests of mariculture in the world (Simon & Sato-Okoshi, 2015). *P. websteri* was first described in Connecticut on the east coast of USA (Loosanoff & Engle, 1943). Since then, reports that include detailed morphological descriptions recorded *P. websteri* along the east coast of North America from Newfoundland in Canada to the Gulf of Mexico in the USA (Blake, 1969, 1971; Foster, 1971). Similar publications subsequently recorded this species in the Gulf of California in Mexico (Blake, 1969, 1971; Foster, 1971), Australia (Blake & Kudenov, 1978), Brazil (Bonifácio, 2009), China (Sato-Okoshi et al., 2013), Japan (Sato-Okoshi, 1999; Sato-Okoshi & Abe, 2012), New Zealand (Read, 2010), Romania and Ukraine (Surugiu, 2005, 2012). More recently, reports including detailed morphological descriptions accompanied by genetic information, recorded *P. websteri* on the east coast of the USA, close to its type locality, in Maine, Maryland, Massachusetts, Alabama (Rice et al., 2018) and New York (Martinelli et al., 2020), as an extralimital species on the west coast of the USA in Washington (Martinelli et al., 2020) and further afield in Australia (Sato-Okoshi & Abe, 2013), China (Sato-Okoshi et al., 2013; Ye et al., 2017), Japan (Sato-Okoshi & Abe, 2013) and Hawaii (Rice et al., 2018). Reports of *P. websteri* from Argentina (Diez et al., 2011), Brazil (Netto & Gallucci, 2003; Breves-Ramos et al., 2005; Sabry & Magalhães, 2005; Diez et al., 2011; Amaral et al., 2013), British Columbia and New Brunswick in Canada (Bergman et al., 1982; Bower et al., 1992; Clements et al., 2017), Chile (Basilio et al., 1995), Ecuador, Peru (Blake, 1983), Galapagos Islands (Keppel et al., 2019), Red Sea (Abd-Elnaby, 2019), South Island in New Zealand (Handley, 1995) and Tasmania in Australia (Nell, 2001), are not accompanied by detailed morphological descriptions nor genetic information, making it difficult to confirm these reports. Additionally, *P.* cf. *websteri* was reported in Venezuela (Díaz-Díaz & Liñero-Arana, 2009) and Brazil (Barros et al., 2017), and it is not known if these species are the 'true' *P. websteri* or if they are morphologically similar, but different species. Recently, specimens resembling *P.* cf. *websteri* were first recorded in South Africa in 2009 from oysters in a culture facility in Port Elizabeth (as *Polydora* cf. *ciliata* (Simon, 2011)). Since then, it has been recorded on farmed oysters from Namibia, Kleinzee and Paternoster on the west coast of South Africa (as *P.* cf. *websteri* (Williams, 2015)) (Figure 1.2).

This possible wide-spread distribution of non-indigenous *P. websteri* and of specimens that resemble it, makes it very important that this shell-boring pest is identified timeously and accurately, for improved management and even eradication. However, accurate species identification is hampered if the taxonomy of the species is unresolved, or if several species are morphologically similar to each other. This is the case for *P. websteri*; it is morphologically

similar to several other polydorid species; *Polydora ciliata* Johnston, 1838, *Polydora agassizii* Claparède, 1869, *Polydora brevipalpa* Zachs, 1933, *Polydora limicola* Annenkova, 1934, *Polydora aggregata* Blake, 1969, *Polydora haswelli* Blake & Kudenov, 1978, *Polydora curiosa* Radashevsky, 1994, *P. neocaeca* and *Polydora onagawaensis* Teramoto, Sato-Okoshi, Abe, Nishitani & Endo, 2013 (Radashevsky & Pankova, 2006; Read, 2010; Sato-Okoshi & Abe, 2012, 2013; Teramoto et al., 2013). Therefore, combining morphological and molecular data would enable more accurate species identification. Williams (2015) found preliminary evidence to suggest that *P.* cf. *websteri* collected in South Africa, Australia and Japan were genetically different from specimens from near the type locality in the USA, suggesting that the identity of this widespread pest needs to be resolved. Simon & Sato-Okoshi (2015) recommended that specimens in South Africa, Australia and Japan should be referred to as *P.* cf. *websteri*.

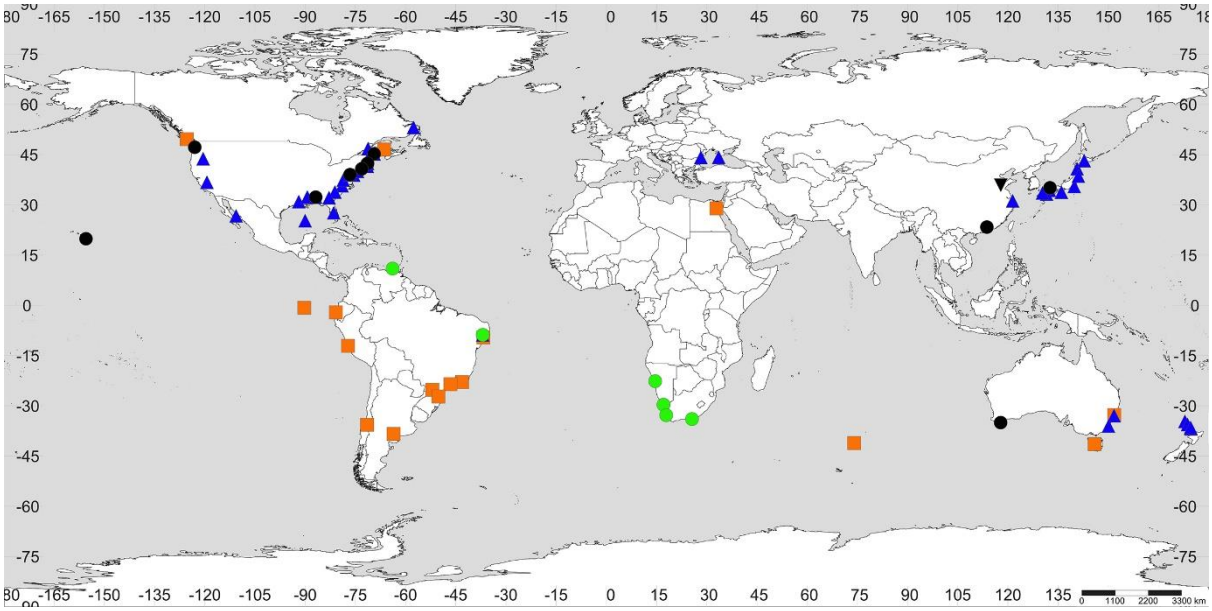


**Figure 1.1:** Map showing worldwide distribution of farmed and wild *Polydora websteri*: reports with detailed descriptions (blue triangle), detailed descriptions accompanied by genetic information (black circle), genetic information (black inverted triangle), not accompanied by detailed descriptions nor genetic information (orange squares) and *Polydora* cf. *websteri* reports (green circle)

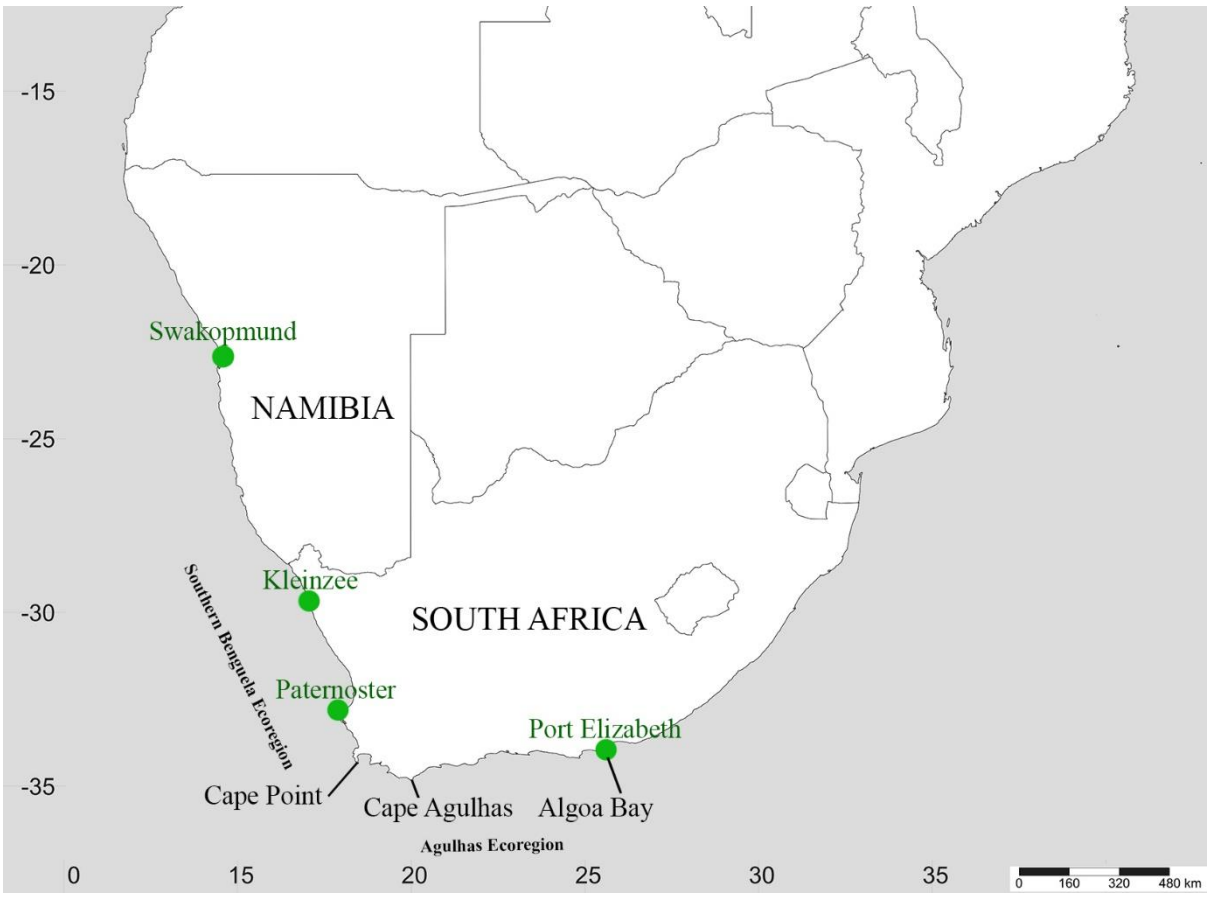


**Figure 1.2:** Map showing southern African *Polydora* cf. *websteri* distributions on farms (green circle), ecoregions and phylogeographic breaks between farms

### 1.2.2. Oyster culture and associated species

Oyster culture and trade date to Roman times (Andrews, 1980) and the continued demand for oysters led to the overexploitation and depletion of natural stocks (Simon & Sato-Okoshi, 2015). To keep up with the demand, adult oysters were imported from various countries for immediate consumption. To replenish stocks, either oyster spat was imported from various countries and/or indigenous oyster species were used to set up oyster culture facilities (Ruesink et al., 2005; Haupt et al., 2010a; Simon & Sato-Okoshi, 2015). One of the most widely introduced oyster species is the Japanese or Pacific oyster *Crassostrea gigas* Thunberg, 1793, translocated to at least 66 countries outside its indigenous range compared to 17 other oyster species translocated to 13 countries outside their indigenous ranges (Ruesink et al., 2005). As the demand for oysters increased, larger quantities of oysters were translocated worldwide (Haupt et al., 2010a; Cinar, 2013; Teramoto et al., 2013). This translocation and importation of oysters for commercial purposes can facilitate the accidental introduction of NIS species since oysters host a diverse community of epi-and infaunal fouling organisms. Haupt et al. (2010b, 2012) showed that cleaning oysters before translocation did not successfully remove all the fouling organisms on the oysters. This is particularly important for a country like South Africa, whose oyster industry is based entirely on the importation of *C. gigas* spat from Chile, France, Namibia, United Kingdom and the USA (Haupt et al., 2010a). As such, Haupt et al. (2010b)

recorded *Ostrea edulis* Linnaeus, 1758, *Tetrapygus niger* Molina, 1782, *Xantho hydrophilus* Herbst, 1790 (as *Xantho incisus* Leach, 1814 (Haupt et al., 2010b)) and *Discinisca tenuis* Sowerby, 1847 as NIS in South Africa that appear to have been introduced as a result of oyster importation. Once a NIS has been introduced, the local translocation or movement of commercial oyster stock and spat, between nurseries and grow-out facilities of mariculture farms in different areas, may further facilitate their spread within the boundaries of the country (Haupt et al., 2010b; Van Niekerk, 2014; Williams et al., 2016).

Polydorids are frequently translocated and consequently, at least 13 species have been recorded as non-indigenous pests (Simon et al., 2006; Sato-Okoshi et al., 2008; Simon, 2011; Simon & Sato-Okoshi, 2015; Sato-Okoshi et al., 2017; Abe et al., 2019; Ye et al., 2019). For example, *P. websteri* and *B. proboscidea* (host generalists) were possibly introduced to Hawaii with oysters from North America (Bailey-Brock, 1990, 2000) and *Polydora rickettsi* Woodwick, 1961 was possibly introduced to Chile from Mexico with *C. gigas* (Moreno et al., 2006). Simon & Sato-Okoshi (2015) identified *P. hoplura* and *P. websteri* as two of the most widely spread polydorid pests in the world, especially since the synonymisation of *P. hoplura* and *Polydora uncinata* Sato-Okoshi, 1998, which increased the known distribution range of *P. hoplura* from 17 to 20 countries (Radashevsky et al., 2017; Sato-Okoshi et al., 2017).

### 1.2.3. Polydorid morphology

Polydorid polychaetes belong to the family Spionidae, commonly known as spionids. Spionids are mainly characterised by the presence of a pair of long, grooved peristomial palps used for feeding, and dorsal branchiae that extend posteriorly along the length of the worm (Blake, 1971). Polydorids are further characterised by a modified fifth chaetiger with modified spines (Blake, 1969; Walker, 2011). Individual genera can be distinguished by prostomial shape and caruncle, which may be outlined by a nuchal organ, the chaetiger on which the hooded hooks begin and the angle between the teeth of the hooded hooks, and whether the branchiae start at chaetiger two or after chaetiger five. The fifth chaetiger is usually enlarged with one or two rows of large modified notopodial spines, while the type of modified spines and the presence or absence of the notopodial or neuropodial lobes on chaetiger five also distinguishes individual genera (Blake, 1971; Sato-Okoshi & Okoshi, 2000; Read, 2010; Walker, 2011).

Many morphological characteristics of polydorids are not species specific and are therefore shared across species. It is therefore the variation in the combination of these characteristics that define individual species (Van Niekerk, 2014). For example, *B. proboscidea* and *Boccardia polybranchia* Haswell, 1885, have similar types of modified spines on chaetiger five and dark pigmentation along the margin of the caruncle and prostomium. However, they differ with respect to the shape of the prostomium, branchiae, presence of notochaetae on the first

chaetiger in *B. proboscidea* and pigmentation patterns on the posterior end of the body (Simon et al., 2010), making it easy to distinguish between them. However, some species are morphologically very similar or even indistinguishable from one another and can only be distinguished molecularly (Rice et al., 2008). Thus, to avoid misidentification and confusion, detailed descriptions are essential for polydorid identifications (Read, 2010), but using a detailed morphological description alone can be difficult, and this can have major consequences for management if the species in question are pests and/or non-indigenous.

### 1.2.4. Identification difficulties

Traditional identification of polydorids has mainly relied on the morphological identification of distinguishing characteristics as described above (Blake, 1971; Walker, 2011), but the accurate identification of polydorids using morphological characteristics alone can be complicated by several factors which will be explored below.

Firstly, two or more species may be mistaken for a single species. This occurs when a high degree of overlap in characters among different species makes it difficult to distinguish between them (Blake & Kudenov, 1978; Van Niekerk, 2014). For example, Read (2010) found that it is difficult to distinguish between morphologically similar *P. websteri* and *P. haswelli*. In extreme cases, the overlap in characters is so high that species cannot be distinguished using morphological features and can only be distinguished molecularly (Radashevsky & Pankova, 2006). Such species are called cryptic species and their presence has complicated polydorid species identification (Rice et al., 2008; Radashevsky & Pankova, 2013).

Secondly, one species may be mistaken for multiple species. This occurs when species have a high degree of intraspecific morphological variation (Read, 2010; Sato-Okoshi & Abe, 2013; Teramoto et al., 2013), which can impede the accurate identification, as was the case with *P. ciliata* and *Polydora calcarea* Templeton, 1836 (Radashevsky & Pankova, 2006). One species may also be mistaken for multiple species when a new NIS is recorded, and mistaken for an indigenous species, as happened when *P. hoplura* was mistakenly described as *P. uncinata* in Japan (Sato-Okoshi et al., 2017).

Thirdly, preservation methods may change the shape of morphological characteristics. This was shown by Bick (2001) where the shape of the prostomium of *Dipolydora armata* Langerhans, 1880, varied from rounded to incised. Poorly preserved material may also increase difficulty of identification (Van Niekerk, 2014).

These complications may impede accurate identifications of non-indigenous pest species, which is problematic for implementing effective management strategies. To overcome these

difficulties in morphological identification, modern species identifications have combined morphology with genetics (e.g. Sato-Okoshi et al., 2017; Ye et al., 2017), which is further facilitated by the development of a library of sequences for easy identification using molecular data only (Williams et al., 2017). With a reliable library of sequences for pest species, specimens can be identified much faster and more accurately, even when taxonomic expertise are lacking (Stoeckle & Hebert, 2008). However, Williams et al. (2017) suggested that using only molecular information to accurately identify polydorids may not be as easy as was originally considered, especially when using only the barcoding marker (Hebert et al., 2003; Stoeckle & Hebert, 2008). It is therefore vital that sequences are linked to reliably identified species and be made available to facilitate the accurate identification of pests, otherwise if sequences of incorrectly identified species are available, species will never be identified accurately.

### 1.2.5. Genetic approaches

Molecular data have proven invaluable in facilitating the distinction of closely related species, especially for the identification of introduced cryptic polychaetes that cannot be identified using morphology alone (Sun et al., 2016, 2017). However, using molecular data alone is not viable either, because it is expensive to run DNA sequences (da Silva & Willows-Munro, 2016). Furthermore, limited or incomplete databases make it difficult to identify a species, especially if there are no data on that species. The number of polydorid species for which genetic data are available is disproportionately small (38, of which 15 are known pests) relative to the more than 150 polydorid species described to date (GenBank, 2020). Finally, if sequences in the databases are linked to species that were identified incorrectly, it can further complicate phylogenetic analysis and subsequent taxonomic annotation (Friedheim, 2016; Sun et al., 2016). Therefore, using both morphological and molecular data has become increasingly important for accurate species identification (Sun et al., 2016, 2017).

Genetic markers that have been used for polydorids include the mitochondrial gene fragments (mtDNA) cytochrome c oxidase subunit I (COI), 12S rRNA, 16S RNA and cytochrome *b* (Cyt *b*), and nuclear gene fragments (nuDNA) 18S rRNA and 28S rRNA (Rice et al., 2008; Sato-Okoshi & Abe, 2012, 2013; Sato-Okoshi et al., 2017; Williams et al., 2017; GenBank, 2020). The COI marker has been proposed as a barcoding marker for animals to enable rapid and accurate identification of species (Hebert et al., 2003). However, when Williams et al. (2017) analysed the phylogenetics and sequence divergences, to compare COI, Cyt *b* and 18S rRNA markers to be used for polydorid identifications, the former showed such high intraspecific variation, that *Boccardia pseudonatrix* Day, 1961 from east and west coasts of South Africa were retrieved as separate species. This separation was not supported by the Cyt *b* and 18S rRNA markers, suggesting that COI may be too variable to be used on its own as there is a

risk of overestimating diversity. Although mtDNA markers are used for polydorids (Rice et al., 2008; Williams et al., 2017), they may be less efficient compared to the nuDNA markers such as 18S rRNA that has been effectively used to confirm genetic distinction and delineate taxonomic relationships among shell-boring polydorid species (Sato-Okoshi & Abe, 2012, 2013; Sato-Okoshi et al., 2013; Abe et al., 2016; Sato-Okoshi et al., 2017; Williams et al., 2017). Williams et al. (2017) found that the 18S rRNA marker showed the least intra- and interspecific variation, and was the most accurate for polydorid species identifications compared to the mtDNA markers COI and Cyt *b*.

Mitochondrial gene fragments are also effective for investigating population structure, and therefore may be able to infer the origin of the species in South Africa relative to the populations elsewhere. This was done previously when Simon et al. (2009) showed that *B. proboscidea* in South Africa probably came from populations in California and Washington on the west coast of the USA. Compared to COI, Cyt *b* has been used to effectively resolve intraspecific relationships among polydorids (Simon et al., 2009; Oyarzun et al., 2011; Williams, 2015; David et al., 2016; Sun et al., 2016; Williams et al., 2017). Therefore, COI, Cyt *b* and 18S rRNA markers were used together to determine whether *P.* cf. *websteri* from South Africa, Australia, China and Japan is the same as *P. websteri* from the USA.

### 1.2.6. *Polydora websteri* species account

A species tentatively identified as *P. websteri* was recently found on oysters in South Africa (Simon, 2015; Williams, 2015). The name *P. websteri* was originally proposed by Hartman (Loosanoff & Engle, 1943) to replace *Polydora caeca* Webster, 1879. Webster's holotype, thought to be a junior homonym of *P. websteri* from Virginia, was lost (Loosanoff & Engle, 1943; Radashevsky & Williams, 1998; Radashevsky, 1999). As such, Hartman re-described and illustrated *P. websteri* in Loosanoff & Engle (1943) based on material collected by Mr. J. B. Engle, Milford Wildlife Laboratory in 1943 from the mouth of the Milford River, Long Island Sound, Connecticut in the USA. It is now known that *P. websteri* is not the same species as Webster's *P. caeca* (Radashevsky & Williams, 1998; Radashevsky, 1999). Hartman's material of *P. websteri* was deposited in the Los Angeles County Museum of Natural History and one of the specimens, with the catalogue number LACM-AHF POLY 1628, was designated as the lectotype (i.e. a specimen designated as the type if no holotype was indicated at the time of publication, lost or destroyed (Turland et al., 2018)), for the species (Radashevsky & Williams, 1998; Radashevsky, 1999).

Lunz (1941) was the first to draw attention to *P. websteri* (misidentified as *P. ciliata*) as a problem for oyster culture. *P. websteri* is morphologically similar to *P. ciliata* and has probably been mistaken for it on numerous occasions (Blake, 1971). *Polydora ciliata* is, however, not a

shell-borer, and it was therefore suggested that *P. ciliata* reported as infesting cultured molluscs on the east coast of North America and in Australia were probably *P. websteri* (Blake, 1969, 1971; Blake & Kudenov, 1978). Since then, *P. websteri* has been reported widely infesting bivalves and limestone (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Evans, 1973; Díaz-Díaz & Liñero-Arana, 2003; Surugiu, 2005; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Ye et al., 2017; Rice et al., 2018).

Comprehensive morphological descriptions of *P. websteri* (Appendix A, Table A1) from different sources (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Sato-Okoshi et al., 2008; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018; Martinelli et al., 2020) suggest that there may be more than one species involved as characters varied, such as the shape of chaetae, especially the spines on chaetiger five, caruncle length and the pigmentation patterns. However, some characters did overlap (Appendix A, Table A1), such as the presence of up to four eyes in a trapezoidal arrangement (Loosanoff & Engle, 1943; Blake, 1971; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Sato-Okoshi et al., 2008; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Ye et al., 2017). The prostomium shape ranged from rounded to bilobed, extending posteriorly into the caruncle (Appendix A, Table A1). The caruncle length varies with body size (Williams & Radashevsky, 1999), extending between mid chaetiger two to end of three (Loosanoff & Engle, 1943; Blake, 1969, 1971; Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Sato-Okoshi et al., 2008; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Barros et al., 2017), or even to chaetiger four (Foster, 1971; Ye et al., 2017; Rice et al., 2018). The absence of the occipital antenna was evident (Appendix A, Table A1).

Blake (1971), Sato-Okoshi (1999) and Sato-Okoshi and Abe (2013) found dark pigmentation on the anterior and posterior regions of some *P. websteri* specimens. Sato-Okoshi (1999) noted that while specimens from the Okhotsk Sea Coast of Hokkaido did not have any pigmentation, specimens inhabiting Miyagi Prefecture had dense pigmentation along the caruncle, the anterior prostomium, the posterior chaetigers and the pygidium. Pigmentation on palps were noted as continuous (Sato-Okoshi, 1999; Surugiu, 2005; Read, 2010; Surugiu, 2012) or discontinuous black lines (Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017) along the feeding grooves. This variation in palp and body pigmentation of *P. websteri* make identification difficult. Read (2010) shows that similar morphology of *P. websteri* and *P. haswelli* makes identification difficult, especially with regards to pigmentation on palps, if the material is in poor quality. Palps with distinct and independent black bars are considered to be one of the most important morphological characteristics of *P. haswelli,* whereas in *P. websteri*

there are no bars or bands but there is a very narrow black line along the palp groove edge (Read, 2010; Sato-Okoshi & Abe, 2013).

Loosanoff & Engle (1943) and Foster (1971) stated that the hooded hooks start on chaetiger 8, however, the re-description of *P. websteri* (Radashevsky, 1999) and Blake (1969) noted that this was incorrect as the hooded hooks start at chaetiger seven which corresponds to most descriptions of *P. websteri* (Blake, 1969, 1971; Blake & Kudenov, 1978; Handley & Bergquist, 1997; Sato-Okoshi, 1999; Surugiu, 2005; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018). The shape of the hooded hooks, bidentate with a constriction at the shaft, overlapped in the descriptions of *P. websteri* (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Kudenov, 1978; Handley & Bergquist, 1997; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018).

All the descriptions recognise chaetiger five as greatly modified and enlarged, having falcate spines with companion chaetae (Appendix A, Table A1). Falcate spines on chaetiger five were recorded to have a tooth and/or a sheath in some descriptions (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Sato-Okoshi & Abe, 2013; Ye et al., 2017) and others only recorded a flange (Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Barros et al., 2017; Rice et al., 2018; Martinelli et al., 2020). The shape of the companion chaetae on chaetiger five were described as pennoned (Loosanoff & Engle, 1943; Blake, 1969, 1971; Radashevsky, 1999; Sato-Okoshi, 1999; Sato-Okoshi et al., 2008; Surugiu 2012; Sato-Okoshi & Abe, 2013; Ye et al., 2017) or as frayed or hastate (Foster, 1971; Read, 2010; Barros et al., 2017; Rice et al., 2018; Martinelli et al., 2020). The presence of superior and inferior chaetae on chaetiger five were noted (Radashevsky, 1999; Read, 2010; Barros et al., 2017; Rice et al., 2018), while other descriptions expanded on this and recorded them as winged (Blake, 1971; Blake & Kudenov, 1978; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Martinelli et al., 2020).

The descriptions agreed with branchiae commencing at chaetiger seven, increasing in length with the longest pair found between chaetiger 9 and 13, then shortening posteriorly and are absent on the last third of the body (Loosanoff & Engle, 1943; Blake, 1969, 1971; Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Sato-Okoshi et al., 2008; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018). The pygidium varied from cup- to disc-shaped, however, a dorsal notch was always apparent (Appendix A, Table A1).

Differences in larval development also raise questions whether all these reports are of the same species. For example, *P. websteri* from Japan, New Zealand and *P*. cf. *websteri* from Brazil were observed producing only planktotrophic larvae (Sato-Okoshi, 1999; Read, 2010; Barros et al., 2017), which was also observed in *P. websteri* from North America (Blake, 1969). Populations of *P. websteri* from Virginia in the USA and populations of *P*. cf. *websteri* from South Africa were observed to be poecilogonous (Haigler, 1969; Simon, 2015). It is, however, important to note that larval development in species can be flexible, as the same species can produce different larval development types or combinations of types in different or within the same populations. For example, on the west coast of South Africa, *P. hoplura* was found producing mainly broods of planktotrophic larvae together with broods of adelphophagic larvae in the same mollusc shell (David et al., 2014; David, 2015), whereas *P. hoplura* broods from Australia, Brazil, California and Chile with adelphophagic larvae were observed (Sato-Okoshi et al., 2008; Radashevsky & Migotto, 2017). Similarly, Gibson (1997) found *B. proboscidea* was able to produce broods that are either planktotrophic, adelphophagic or poecilogonous.

Producing different types of larvae by the same individual or different individuals within the same population (i.e. poecilogonous species (David et al., 2014)), is advantageous to pest polydorids as they benefit from dispersal of planktotrophic larvae, following the development and maintenance of local populations by the adelphophagic larvae (David & Simon, 2014; David et al., 2014). If a polydorid pest is non-indigenous, depending on larval developmental mode, together with the movement of polydorid pests together with cultured molluscs, these could increase the rate of spread of the pest in the wild around the country (as seen with *P. hoplura* and *B. proboscidea* (David et al., 2016)). For example, if a non-indigenous polydorid pest species only occurs on oysters in Kleinzee in South Africa, and then escapes to the wild, there is only one source of invasion. However, for example if these pests are moved with infested stock to oyster farms in Paternoster and Port Elizabeth in South Africa and then also spread into the wild, there are three points of invasion, thus increasing the rate of spread into the wild along the South African coast.

### 1.2.7. Significance of the research

Accurate identification of pest species is important to determine whether the species is new to science, indigenous or non-indigenous. If a non-indigenous pest species is identified and still restricted to farms, it could be more easily controlled or possibly eradicated through the culling of infested stock (Simon & Sato-Okoshi, 2015). By contrast, if the pest species is indigenous, cultured oysters are at constant danger of re-infestation from wild populations and farmers need to manage their oyster stock accordingly. It is, therefore, vital that these pests are identified timeously and accurately. Conclusions of Williams (2015) were based on small sample sizes and few markers, and results were preliminary but indicated that there is a new

NIS on oyster farms in South Africa, and that species designation of *P.* cf. *websteri* is in need of revision. To confirm the results of this preliminary study, additional morphological and molecular data are needed.

The exact origin and introduction of the South African *P*. cf. *websteri* is unknown (Williams, 2015). If the species is non-indigenous, clarifying its taxonomic status is essential to understand invasion source and the spread of the invasive species (Sun et al., 2017). Comparing specimens from South Africa with *P. websteri* and *P.* cf. *websteri* from other places around the world may help identify the origin and introduction of the species, which can also facilitate management by amending importation legislation. Additionally, comparing the genetic variation of *P*. cf. *websteri* from different sites in South Africa can support the suggestion that the movement of oysters facilitates the movement of this pest within South Africa, as well as indicate whether it is a recent introduction into South African waters that should be appropriately managed.

The preliminary evidence by Williams (2015) using only 18S rRNA sequence data, revealed that the species, then identified as *P. ciliata*/*calcarea* (Simon, 2011) from South Africa is very similar to *P. websteri* from Australia and Japan. However, the specimens from South Africa, Australia and Japan all differed markedly from *P. websteri* collected close to the type locality in the USA, using the same marker. This suggested a paraphyletic clustering for the two *P. websteri* lineages, and that the species designation needs revision. To confirm the earlier preliminary study, additional sequence data, especially from mtDNA markers such as COI and Cyt *b* (Rice et al., 2008; Sato-Okoshi & Abe, 2012, 2013; Ye et al., 2017) are needed.

### 1.2.8. Hypotheses

$H_0$ Specimens identified as *Polydora* cf. *websteri* in South Africa are reciprocally monophyletic with those from Australia, China and Japan; and with *P. websteri* collected near the type locality in the USA.

$H_1$ Specimens identified as *P.* cf. *websteri* in South Africa are reciprocally monophyletic with those from Australia, China and Japan; and not with *P. websteri* collected near the type locality in the USA.

$H_2$ Specimens identified as *P.* cf. *websteri* in South Africa are reciprocally monophyletic with *P. websteri* collected near the type locality in the USA; but not with *P.* cf. *websteri* from Australia, China and Japan.

$H_3$ Specimens identified as *P.* cf. *websteri* in South Africa are not reciprocally monophyletic with those from Australia, China and Japan; nor with *P. websteri* collected near the type locality in the USA.

### 1.2.9. Aims and objectives of the research

This study aims to (1) clarify the taxonomy of *Polydora* cf*. websteri* from South Africa, Australia, China and Japan using morphological and molecular information. For improved management of pest polydorids locally and internationally, this study also aims to (2) contribute to the sequence library for species in the *Polydora*-complex and to (3) develop a taxonomic key of shell-boring pest species in the *Polydora*-complex in South Africa for mariculture practitioners.

The specific research objectives of the proposed research are;

To determine whether specimens identified as *Polydora* cf. *websteri* from South Africa are reciprocally monophyletic (share a single common ancestor) with those from Australia, China and Japan, and with *P. websteri* collected near the type locality in the USA.

To determine whether specimens identified as *P.* cf. *websteri* from South Africa are morphologically identical to those from Australia, China and Japan; and with *P. websteri* collected near the type locality in the USA.

To contribute towards improved management of shell-boring polydorid pests of cultured molluscs in South Africa.

To contribute to the sequence library for species in the *Polydora*-complex.

To develop a taxonomic key of shell-boring pest species in the *Polydora*-complex in South Africa for mariculture practitioners.

# CHAPTER TWO

# METHODS AND MATERIALS

## 2.1. Specimen collection and storage

The preserved specimens of *Polydora* cf. *websteri* from South Africa, used in this study, came from the private collection of Professor Carol A. Simon. These specimens were extracted from broken shells of molluscs of cultured oysters, *Crassostrea gigas* from Kleinzee (29°39′59″ S, 17°04′60″ E) in November 2012 and from the Knysna Oyster Company in Nelson Mandela Bay, Eastern Cape (33°50′0″ S, 25°50′0″ E) in August 2017. At the time of collection, specimens used for morphology were anaesthetised by placing them in 7 % magnesium chloride in tap water and then fixed in a 4 % formalin and seawater solution. Specimens used for molecular analysis were directly fixed in 96 % ethanol. Preserved specimens of *P. websteri* collected from wild molluscs, *Crepidula fornicata* (Linnaeus, 1758) shells, in November 1996 from Narrow River, Rhode Island, United States of America (USA) (41°31′8.7″ N, 71°26′43.77″ W), were provided by Professor Jason D. Williams at Hofstra University, New York, USA. Extracted DNA of *P.* cf. *websteri* from Australia, collected by Professor Carol A. Simon, was provided by an earlier study (Williams et al., 2017) (Figure 2.1).

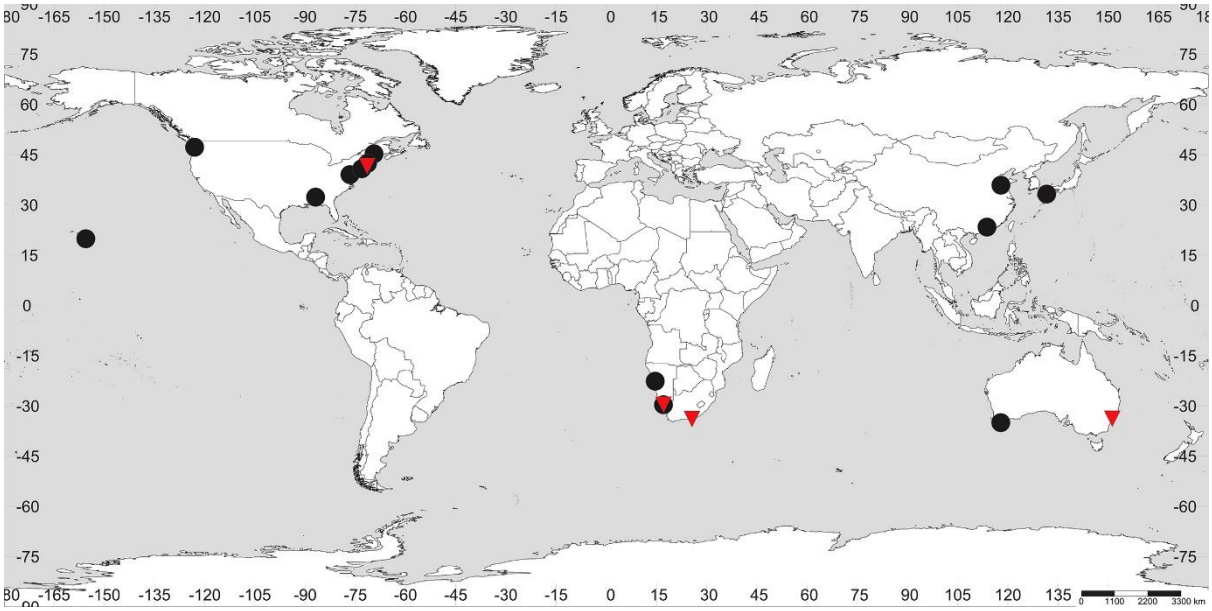


**Figure 2.1:** Map showing distribution of sequences produced in the current study (red triangles) and sequences obtained from GenBank (black circles)

## 2.2. DNA extraction and amplification

Genomic DNA was extracted from the tissue of *P.* cf. *websteri* from South Africa using the Quick-DNA™ Miniprep Plus Kit (Zymo Research) following the standard protocol as specified by the manufacturer. As the worms were small, the entire specimen was used to make up approximately 25 mg of tissue. The specimens were rinsed with distilled water to remove

excess ethanol which could potentially prevent proteinase-K activity. The tissue was then cut into small pieces to provide the best possible DNA yield.

For the last step of extraction, the spin column was put into a clean eppendorf tube and 50 µl of DNA Elution Buffer was added to each sample and left to incubate for approximately 5 minutes at room temperature. The tubes were then centrifuged at high speed for 30 seconds to allow for the elution of DNA. This step was repeated but with 100 µl of DNA Elution Buffer, to ensure the extraction of as much DNA as possible.

The integrity of the extracted DNA was determined by using 3 µl of the DNA and 1 µl of DNA Gel Loading Dye (Thermo Scientific™) added to a 1 % agarose gel (1 g agarose powder and 100 ml of 1X TBE buffer) with ethidium bromide for electrophoresis at 100 V for 60 minutes. Labnet Enduro™ GDS imaging system was used to take images of the DNA bands in the gel. If the DNA was extracted successfully it was then stored in a –26 °C freezer until used for Polymerase Chain Reaction (PCR).

The molecular markers used include the mitochondrial DNA markers (mtDNA) cytochrome c oxidase subunit I (COI) and cytochrome b (Cyt *b*) and nuclear DNA (nuDNA) 18S rRNA (Williams et al., 2017). Primers used were genus specific and are listed in Appendix B, Table B1 and B2. Genomic DNA was amplified using PCR for South African, Australian and USA specimens.

The isolated genomic DNA was amplified using a total PCR reaction volume of 25 µl, containing 12.5 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs), 0.5 µl of forward and 0.5 µl of reverse primers concentrated at 10 µM and 1 µl of Bovine Serum Albumin. To make up 25 µl, the volume of template DNA and molecular biology grade water differed according to the primer and quality of the DNA, these different volumes are listed in Appendix B, Table B1. PCR products were stored in a refrigerator at 12 °C.

The PCR products were run on a 1 % agarose gel with ethidium bromide at 100 V for 60 minutes using 2 to 15 µl of the PCR product and 3 µl of 100 bp DNA Ladder (Solis BioDyne), then visually inspected under an ultraviolet light and images of the PCR product bands in the gels were taken. The PCR products of COI and Cyt *b* from South African samples were extracted and purified using the Biospin Gel Extraction Kit (BioFlux, Bioer Technology), following the standard protocol as specified by the manufacturer. Lastly, PCR products were sequenced at the Central Analytical Facility at Stellenbosch University.

### 2.3. Genetic analysis

DNA sequences were edited and aligned in MEGA7® ver. 7.0 (Kumar et al., 2016) with MUSCLE (Edgar, 2004). All newly generated sequences were BLASTed (Basic Local Alignment Search Tool) to query their authenticity against the online sequences on GenBank. All newly generated sequences were aligned with consensus sequences from GenBank (listed in Figure 2.1 and Appendix B, Table B2). The 18S rRNA fragments from three primers were aligned according to sequences of *P.* cf. *websteri* from South Africa (GenBank acession numbers: KY677904, KY677905) and generated using the same primers (Nishitani et al., 2012; Williams et al., 2017).

Phylogenetic trees were constructed for COI, Cyt *b* and 18S rRNA datasets, to determine evolutionary relationships among species. The Bayesian and maximum likelihood trees were rooted with *Boccardia proboscidea* for which sequences were obtained from GenBank (accession numbers listed in Appendix B, Table B2). The best-fit model of evolution for each sequence dataset (listed in Appendix B, Table B3) was chosen according to the Akaike Information Criterion (AIC) and used to construct maximum likelihood phylogenetic trees in MEGA7. Parameters were set to default and were run for a 1000 bootstrap replicates. Nodes with ≤ 50 % bootstrap support were collapsed. Bootstrap probabilities in maximum likelihood analysis are slightly conservative, therefore values ≥ 90 % were considered as a cut-off for "good" support and values ≥ 80 % were considered as "moderate" support (Hillis & Bull, 1993; Buzan & Krystufek, 2008).

The best-fit model of evolution for the Bayesian trees were calculated in MrModelTest ver. 2.3 (Nylander, 2004). The AIC selected the Symmetrical model and Gamma distributed (SYM+G) as the most appropriate model to construct the Bayesian tees for COI, Cyt *b* and 18S rRNA using MrBayes ver. 3.2.6 (Ronquist et al., 2012). Four Markov chains were run for 1 million generations each and every 10th tree was sampled. The first 25 % of trees were excluded as burn-in and the remaining trees were used to construct a 50 % majority-rule consensus tree with Bayesian posterior probability (BPP) support for each clade. As BPP are considered less conservative than bootstrap probabilities in maximum likelihood analysis, values of BPP ≥ 95 % were considered as a cut-off for "good" support (Lee, 2000; Buzan & Krystufek, 2008). Trace plots using Tracer ver. 1.7.1 (Rambaut et al., 2018) were inspected to test convergence and effective sample sizes (ESS). As ESS > 200 were obtained, the results were accepted. Consensus trees were viewed and edited in FigTree ver. 1.4.3 (Rambaut, 2016).

Species delimitation was analysed by the automatic barcode gap discovery method (Puillandre et al., 2012), to complement the phylogenetic analysis and to split the sequence dataset into species groups. The minimum intraspecific pairwise distance (Pmin) and the maximum

intraspecific pairwise distance (Pmax) values were calculated using Kimura 2-parameter model in MEGA7. Pmin was set to 0.001 or 0.005 and Pmax was set between 0.015 and 0.207 (Appendix B, Table B4). The barcode gap threshold (X) was set to 1.5 and was run for 40 steps.

To estimate the degree of genetic distance among and between populations, the intraspecific and interspecific distances were generated in MEGA7 using Kimura 2-parameter model and gamma distributed (parameter = 1) with 1000 bootstrap replications (Williams et al., 2017). The intraspecific and interspecific distances were determined for each geographic region in the COI, Cyt *b* and 18S rRNA datasets. The regions included South Africa, Namibia, Australia, China, Japan, Hawaii, west coast of the USA, east coast of the USA and specimens collected from wild molluscs on the east coast of the USA.

To determine the population structure and evolutionary relationship among haplotypes, DnaSp ver. 6 (Rozas et al., 2017) was used to generate a haplotype data file for each marker. Diversity indices including the number of haplotypes, nucleotide diversity and haplotype diversity were calculated in DnaSp for each region (as mentioned above) in the COI, Cyt *b* and 18S rRNA datasets. Unrooted TCS haplotype networks were generated at a 95 % cutoff criterion and edited for each marker using PopART ver. 1.7 (Clement et al., 2002).

### 2.4. Morphological methodology

Samples from South Africa and Rhode Island, USA were examined under a Leica MZ 75 stereomicroscope (Leica, Wetzlar, Germany). Previous descriptions of *P. websteri* (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Surugiu, 2005; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018) were consulted regarding characters such as pigmentation patterns, chaetal structure and pygidium shape. Formalin-fixed specimens were stained with a saturated solution of methyl-green in ethanol to increase contrast, which enhances distinguishing features (Wong et al., 2014) and to assess species specific staining patterns (Read, 2010). Permanent slides of chaetal structures were prepared by mounting sections on microscope slides using Aqua-Tex®, which was dried and sealed with clear nail varnish. The slides were viewed under 40 x and 100 x magnification on a Leica DM 1000 light microscope. Whole specimens and slides were photographed with a Leica EC3 camera attachment and the Leica application suite, LasEs software ver. 3.3 (2016) and was stacked in Helicon Focus ver. 6.8. The lectotype specimen of *P. websteri* (LACM-AHF POLY 1628), described in Radashevsky (1999) was photographed under a Leica M165C microscope using a Nikon D610 camera by Adam Wall (Los Angeles County Museum of Natural History,

August 2019) and photographs were stacked in Helicon Focus ver. 6.7.1. All stacked photographs were edited in Adobe Photoshop CC (2019).

# CHAPTER THREE
# RESULTS

## 3.1. Molecular

### 3.1.1. Sequence yield

Sequencing of COI, Cyt *b* and 18S rRNA markers yielded 33 sequences of specimens from South Africa (SA), seven from Australia (AU) and seven from Rhode Island (RI), on the east coast (EC) of United States of America (USA) collected from wild molluscs (WILD). Forty-three additional COI, four Cyt *b* and 24 18S rRNA sequences from SA, Namibia (NAM), AU, China (CH), Japan (JP), Hawaii, west coast (WC) and EC of NA were obatined from GenBank (Figure 2.1 and Appendix B, Table B2).

Alignment of the COI dataset yielded 864 base pairs (bp) of which 587 counted as missing data, 227 were monomorphic (invariable) sites and 50 were polymorphic (variable) sites. There were 54 mutations with 49 parsimony informative sites and 1 singleton variable site. The large amount of missing data in the data set is due to various primers used to produce the COI sequences on GenBank (Appendix B, Table B2), therefore, a second COI dataset was trimmed to 277 bp with no missing data. The trimmed COI dataset was analysed separately and presented when results differed to the untrimmed COI dataset.

Alignment of the Cyt *b* dataset yielded 367 bp of which 38 counted as missing data, 275 were monomorphic (invariable) sites and 54 were polymorphic (variable) sites. There were 57 mutations with 54 parsimony informative sites and no singleton variable sites.

Alignment of the 18S rRNA dataset yielded 1737 bp of which 1181 counted as missing data, 546 were monomorphic (invariable) sites and 10 were polymorphic (variable) sites. There were 10 mutations with 8 parsimony informative sites and 2 singleton variable site. The large amount of missing data in the dataset is due to the USA 18S rRNA sequences on GenBank, consisting only of 569 to 643 bp compared to all other 18S rRNA sequnces with > 1700 bp (Appendix B, Table B2), therefore, a second 18S rRNA dataset without the USA sequences from GenBank was trimmed to 1700 bp with no missing data. The trimmed 18S rRNA dataset was analysed separately and presented when results differed to the untrimmed 18S rRNA dataset.

### 3.1.2. Species delimitation and genetic distances

The automatic barcode gap discovery (ABGD) method consistently returned two putative species groups (PSG) for the COI, Cyt *b* and 18S rRNA datasets. PSG1 contained sequences from SA, NAM, AU, CH, USA (WC), USA (EC), HAWAII and JP, with a maximum distance of 0.2 % within PSG1 (Table 3.1, Figures 3.1 – 3.3). PSG2 contained all sequences from USA (EC RI WILD) in the COI and Cyt *b* dataset, as well as a *Polydora onagawaensis* sequence in the 18S rRNA dataset, with a maximum distance of 0.4 % within PSG2 (Table 3.1, Figures 3.1 – 3.3). The distances between PSG1 and PSG2 for COI and Cyt *b* datasets were > 18 %, but 1.5 % for the 18S rRNA dataset. However, the distances between PSG1 and PSG2 were one to two orders of magnitude bigger than the within-group distances for all datasets (Table 3.1), thus great enough to separate USA (EC RI WILD) from SA, NAM, AU, CH, USA (WC), USA (EC), HAWAII and JP. The ABGD method could not separate SA sequences from NAM, AU, CH, USA (WC), USA (EC), HAWAII and JP sequences.

**Table 3.1:** Intraspecific and interspecific distances of *Polydora websteri* and *Polydora* cf. *websteri* for COI, Cyt *b* and 18S rRNA datasets for two putative species groups (PSG); PSG1: South Africa, Namibia, Australia, China, west and east coast of North America, Hawaii and Japan; PSG2: Rhode Island on the east coast of the USA collected from wild molluscs and *Polydora onagawaensis*; calculated using Kimura 2-parameter model; intraspecific distances are along the diagonal in italics ± standard error estimates, interspecific distances with standard error estimates are below the diagonal for each dataset; trimmed data set with no missing data (T)

| Marker | | PSG 1 | PSG 2 |
|---|---|---|---|
| COI | PSG 1 | *0.001±0.001* | |
| | PSG 2 | 0.197±0.029 | *0.003±0.002* |
| COI (T) | PSG 1 | *0.002±0.001* | |
| | PSG 2 | 0.207±0.017 | *0.003±0.002* |
| Cyt *b* | PSG 1 | *0.002±0.001* | |
| | PSG 2 | 0.190±0.024 | *0.003±0.002* |
| Cyt *b* (T) | PSG 1 | *0.002±0.002* | |
| | PSG 2 | 0.180±0.026 | *0.004±0.002* |
| 18S rRNA | PSG 1 | *0.001±0.001* | |
| | PSG 2 | 0.015±0.003 | *0.000±0.000* |
| 18S rRNA (T) | PSG 1 | *0.000±0.000* | |
| | PSG 2 | 0.015±0.003 | *0.000±0.000* |

### 3.1.3. Phylogenetic analysis

Models selected for the sequence datasets are listed in Appendix B, Table B3. The maximum likelihood and Bayesian trees for COI, Cyt *b* and 18S rRNA (trimmed) recovered similar topologies and a monophyletic clustering of SA, NAM, AU, CH, USA (WC), USA (EC), HAWAII and JP specimens with strong nodal support, supporting the grouping of PSG1 and its separation from PSG2 (Figures 3.1 A, 3.2 and 3.3 B). The Bayesian trees for COI (trimmed) and 18S rRNA each recovered monophyletic clustering of SA, NAM, AU, CH, USA (WC), USA (EC), HAWAII and JP specimens with maximum support. Similarly, the maximum likelihood trees support the separation of PSG1 and PSG2, but not as pronounced as the Bayesian trees, as the grouping of PSG1 recovered 94 % support for COI (trimmed) and 82 % support for 18S rRNA (Figures 3.1 B and 3.3 A). For both the trimmed and untrimmed 18S rRNA trees, the

maximum likelihood and Bayesian trees recovered strong support for the monophyletic clustering of USA (EC RI WILD) with *P. onagawaensis*, supporting the grouping of PSG2 (Figures 3.3 A, B).

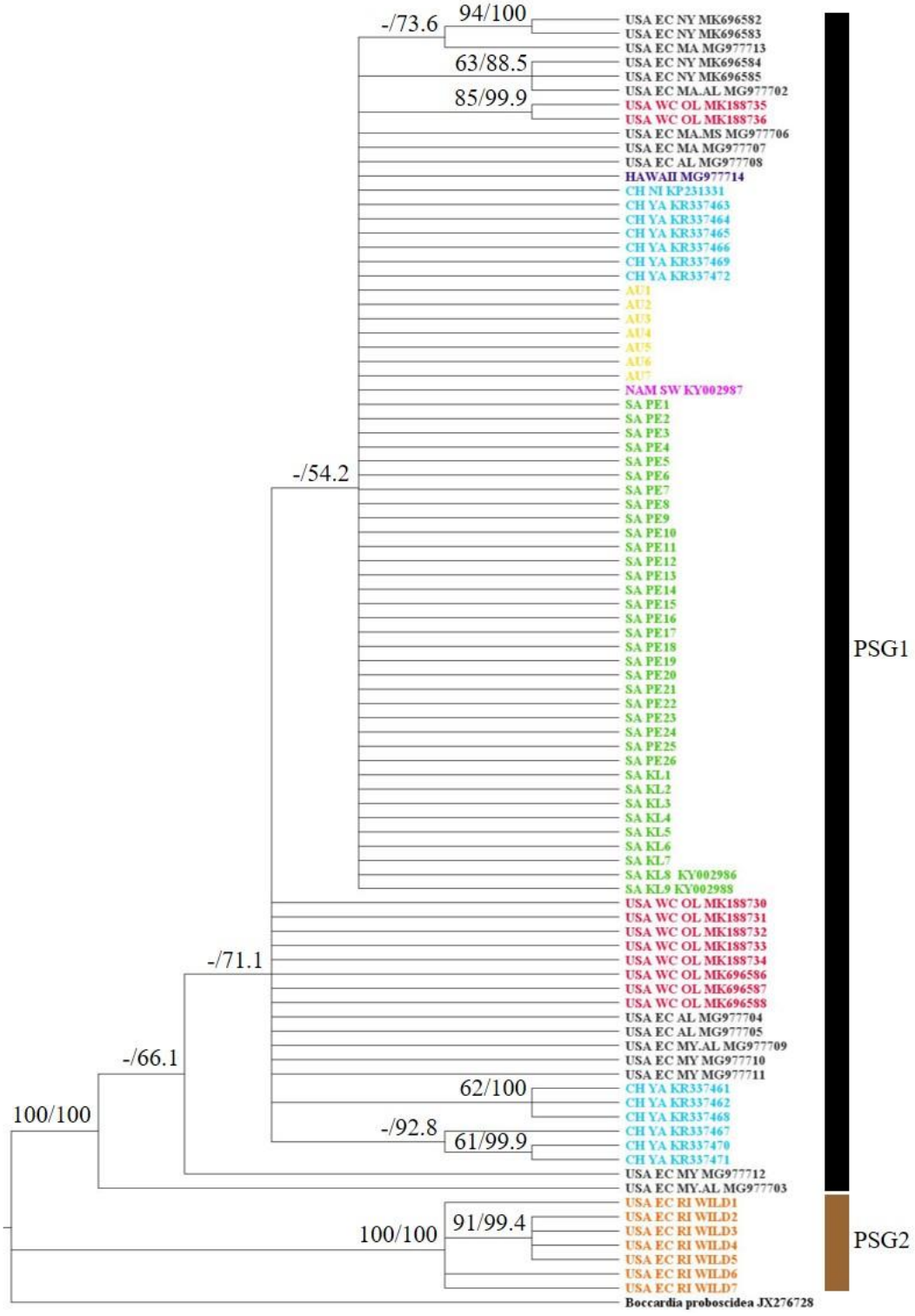


**Figure 3.1A**: Phylogenetic tree based on COI alignment of *Polydora websteri* and *Polydora* cf. *websteri*, with *Boccardia proboscidea* serving as the outgroup; maximum likelihood bootstrap (left) support > 50 % (based on 1000 bootstrap replicates) and Bayesian (right) support based on 50 % majority-rule consensus tree are indicated at the respective nodes; results from species delimitation model (ABGD) is presented to the right of the tree; species delimitation analyses grouped species into putative species groups (PSG); PSG1: Australia (AU), China (CH) in Ningbo (NI) and Yangxi (YA), Hawaii (HAW), Namibia (NAM) in Swakopmund (SW), South Africa (SA) in Kleinzee (KL) and Port Elizabeth (PE), United States of America (USA) on the east coast (EC) in Alabama (AL), Maine (MA), Maryland (MY), Massachusetts (MS) and New York (NY), and the USA on the west coast (WC) in Oakland (OL); PSG2: USA on the EC in Rhode Island (RI) collected from wild molluscs (WILD); sequences obtained from GenBank are followed by their accession numbers

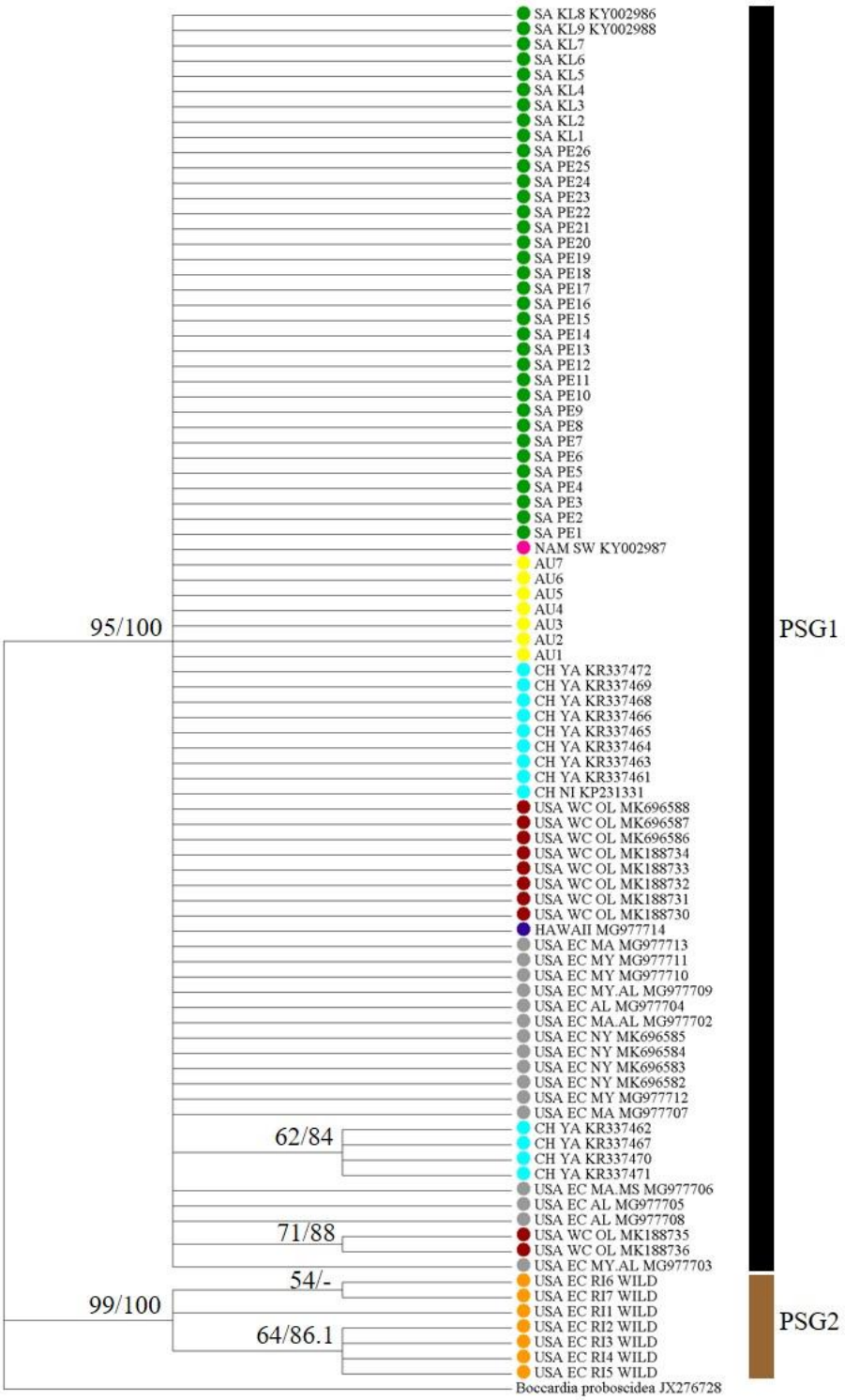


**Figure 3.1B:** Phylogenetic tree based on COI (trimmed to 277 bp) alignment of *Polydora websteri* and *Polydora* cf. *websteri*, with *Boccardia proboscidea* serving as the outgroup; maximum likelihood bootstrap (left) support > 50 % (based on 1000 bootstrap replicates) and Bayesian (right) support based on 50 % majority-rule consensus tree are indicated at the respective nodes; results from species delimitation model (ABGD) is presented to the right of the tree; species delimitation analyses grouped species into putative species groups (PSG); PSG1 (black): Australia (AU, yellow), China (CH, blue) in Ningbo (N) and Yangxi (YA), Hawaii (HAW, purple), Namibia (NAM, pink) in Swakopmund (SW), South Africa (SA, green) in Kleinzee (KL) and Port Elizabeth (PE), United States of America (USA) on the east coast (EC, grey) in Alabama (AL), Maine (MA), Maryland (MY), Massachusetts (MS) and New York (NY), and the USA on the west coast (WC, red) in Oakland (OL); PSG2 (brown): USA on the EC in Rhode Island (RI) collected from wild molluscs (WILD, orange); sequences obtained from GenBank are followed by their accession numbers

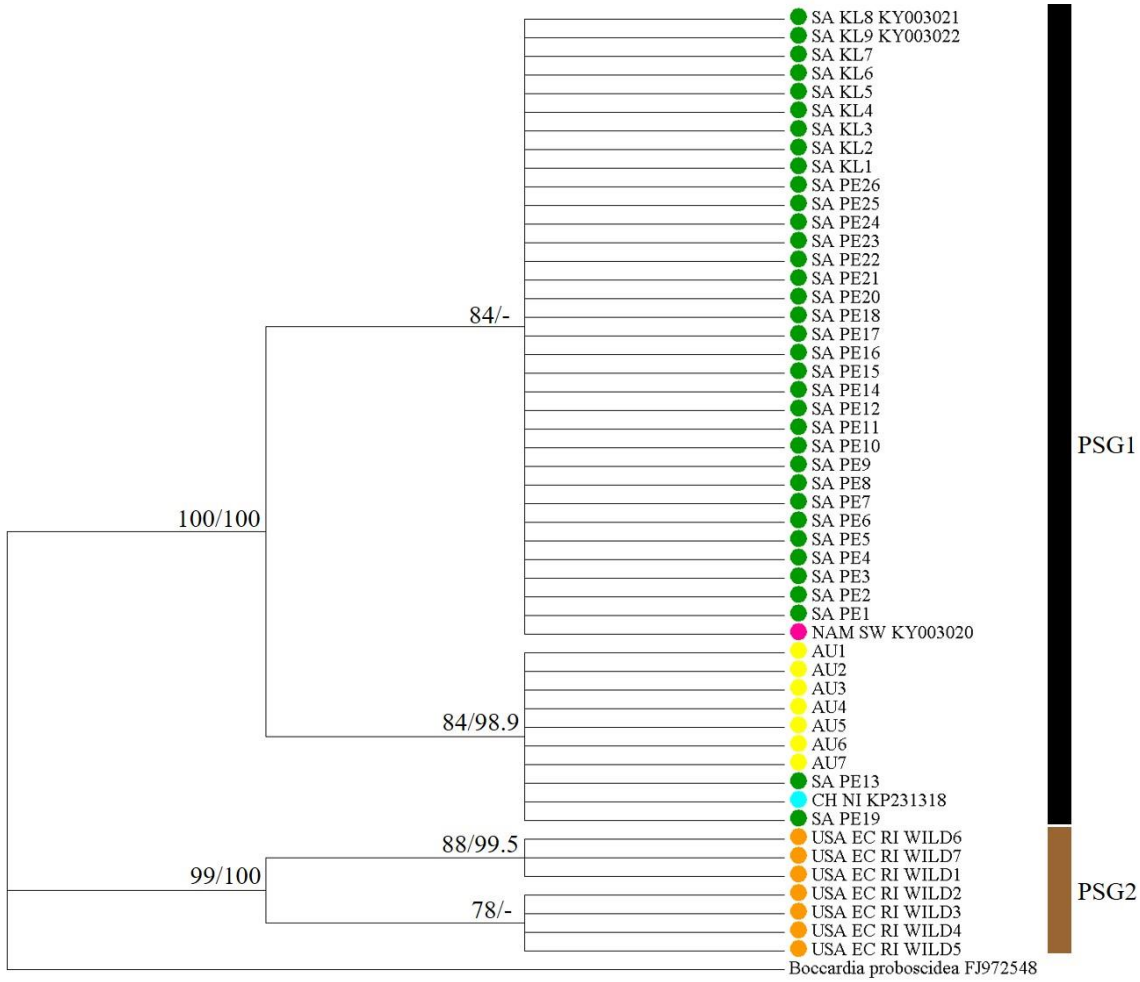


**Figure 3.2:** Phylogenetic tree based on Cyt *b* alignment of *Polydora websteri* and *Polydora* cf. *websteri*, with *Boccardia proboscidea* serving as the outgroup; maximum likelihood bootstrap (left) support > 50 % (based on 1000 bootstrap replicates) and Bayesian (right) support based on 50 % majority-rule consensus tree are indicated at the respective nodes; results from species delimitation model (ABGD) is presented to the right of the tree; species delimitation analyses grouped species into putative species groups (PSG); PSG1 (black): Australia (AU, yellow) , China (CH, blue) in Ningbo (Ni), Namibia (NAM, pink) in Swakopmund (SW), South Africa (SA, green) in Kleinzee (KL) and Port Elizabeth (PE); PSG2 (brown): USA on the EC in Rhode Island (RI) collected from wild molluscs (WILD, orange); sequences obtained from GenBank are followed by their accession numbers

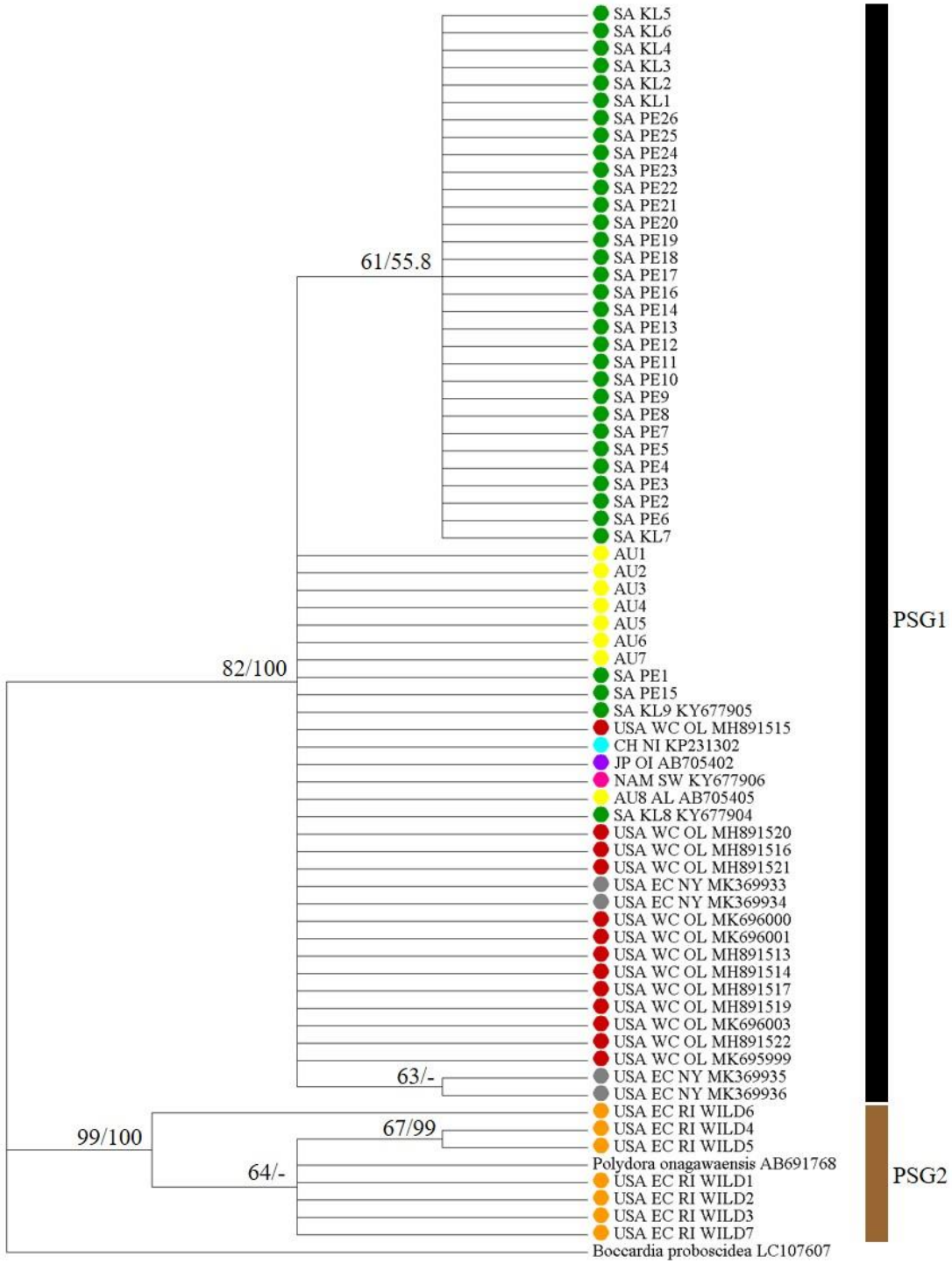


**Figure 3.3A:** Phylogenetic tree based on 18S rRNA alignment of *Polydora websteri* and *Polydora* cf. *websteri*, with *Boccardia proboscidea* serving as the outgroup; maximum likelihood bootstrap (left) support > 50 % (based on 1000 bootstrap replicates) and Bayesian (right) support based on 50 % majority-rule consensus tree are indicated at the respective nodes; results from species delimitation model (ABGD) is presented to the right of the tree; species delimitation analyses grouped species into putative species groups (PSG); PSG1 (black): Australia (AU, yellow), China (CH, blue) in Ningbo (Ni) , Japan (JP), Namibia (NAM, pink) in Swakopmund (SW), South Africa (SA, green) in Kleinzee (KL) and Port Elizabeth (PE), United States of America (USA) on the east coast (EC, grey) in New York (NY), and the USA on the west coast (WC, red) in Oakland (OL); PSG2 (brown): USA on the EC in Rhode Island (RI) collected from wild molluscs (WILD, orange) and *Polydora onagawaensis*; sequences obtained from GenBank are followed by their accession numbers

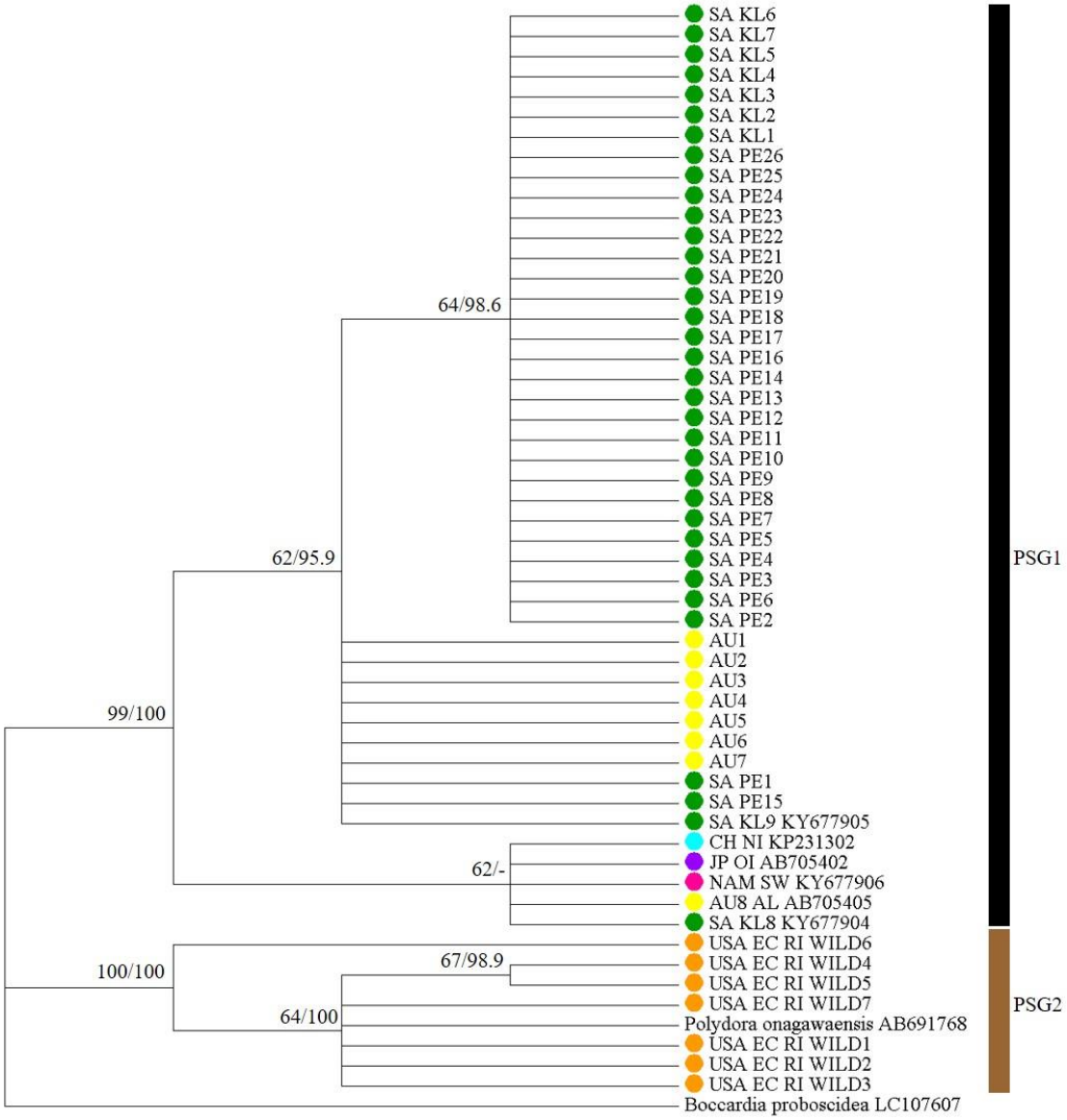


**Figure 3.3B:** Phylogenetic tree based on 18S rRNA (trimmed to 1703 bp) alignment of *Polydora websteri* and *Polydora* cf. *websteri*, with *Boccardia proboscidea* serving as the outgroup; maximum likelihood bootstrap (left) support > 50 % (based on 1000 bootstrap replicates) and Bayesian (right) support based on 50 % majority-rule consensus tree are indicated at the respective nodes; results from species delimitation model (ABGD) is presented to the right of the tree; species delimitation analyses grouped species into putative species groups (PSG); PSG1 (black): Australia (AU, yellow), China (CH, blue) in Ningbo (Ni) , Japan (JP, purple), Namibia (NAM, pink) in Swakopmund (SW), South Africa (SA, green) in Kleinzee (KL) and Port Elizabeth (PE); PSG2 (brown): USA on the EC in Rhode Island (RI) collected from wild molluscs (WILD, orange) and *Polydora onagawaensis*; sequences obtained from GenBank are followed by their accession numbers

### 3.1.4. Intraspecific and interspecific distances

Intraspecific and interspecific distances for COI, Cyt *b* and 18S rRNA are summarised in Table 3.2. The intraspecific distances among specimens identified as *Polydora websteri* and *P.* cf. *websteri* were ≤ 0.5 % for COI, ≤ 0.3 % for Cyt *b* and ≤ 0.1 % for 18S rRNA in all geographic regions. Interspecific distances between samples of *P. websteri* and *P.* cf. *websteri* from all geographic regions for COI and Cyt *b* were less than 0.6 % except for those from USA (EC RI WILD). Interspecific distances between samples of *P. websteri* and *P.* cf. *websteri* from all

geographic regions for 18S rRNA were less than 0.2 % except for those from USA (EC RI WILD). The interspecific distances of *P. websteri* and *P.* cf. *websteri* from all geographic regions, relative to those from the USA (EC RI WILD), was similar to the interspecific distance of these taxa relative to the outgroup taxa, *Boccardia proboscidea* and *P. onagawaensis*.

Interspecific distances for COI and Cyt *b* were 18.4 to 29.7 % between *P. websteri* USA (EC RI WILD) and *B. proboscidea* and *P. websteri* (including *P.* cf. *websteri*) from all other regions, i.e. one or two orders of magnitude higher than the intraspecific distances within each of these taxa. Similarly, the interspecific distances for 18S rRNA of 1.4 to 2.8 % was one or two orders of magnitude higher than the intraspecific distances for USA (EC RI WILD) and *B. proboscidea*, relative to each other and all other regions, except for *P. onagawaensis*. The interspecific distance between USA (EC RI WILD) and *P. onagawaensis* was 0 %, similar to the intraspecific distance among *P. websteri* and *P.* cf. *websteri* from all the other regions (≤ 0.5 %), and two orders of magnitude lower than their interspecific distance of 1.4 to 1.6 % between specimens from all other regions.

**Table 3.2:** Intraspecific and interspecific distances of COI, Cyt *b* and 18S rRNA between regions, using Kimura two-parameter model; intraspecific distances along the diagonal in italics with ± standard error estimates; interspecific distances below the diagonal with standard error estimates above the diagonal; putatively *Polydora websteri* and *Polydora* cf. *websteri* from South Africa (SA), Namibia (NAM), Australia (AU), China (CH), Hawaii, United States of America (USA), west coast (WC), east coast (EC), Rhode Island (RI) collected from wild molluscs (WILD), Japan (JP), *Polydora onagawaensis* (*P. ona*), *Boccardia proboscidea* (*B. pro*)

| Marker | Geographic Region | Number of Sequences | AU | CH | HAWAII | JP | NAM | SA | USA (EC) | USA (WC) | USA (EC RI WILD) | *P. ona* | *B. pro* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| COI | AU | 7 | *0.000±0.000* | 0.001 | 0.001 | – | 0.000 | 0.000 | 0.001 | 0.001 | 0.022 | – | 0.027 |
| | CH | 13 | 0.003 | *0.005±0.002* | 0.002 | – | 0.001 | 0.001 | 0.001 | 0.001 | 0.022 | – | 0.026 |
| | HAWAII | 1 | 0.001 | 0.005 | – | – | 0.002 | 0.002 | 0.002 | 0.002 | 0.022 | – | 0.027 |
| | NAM | 1 | 0.000 | 0.003 | 0.002 | – | – | 0.000 | 0.003 | 0.003 | 0.025 | – | 0.026 |
| | SA | 35 | 0.000 | 0.003 | 0.002 | – | 0.000 | *0.000±0.000* | 0.003 | 0.002 | 0.022 | – | 0.026 |
| | USA (EC) | 16 | 0.003 | 0.005 | 0.005 | – | 0.001 | 0.001 | *0.005±0.001* | 0.001 | 0.021 | – | 0.026 |
| | USA (WC) | 10 | 0.002 | 0.004 | 0.004 | – | 0.002 | 0.001 | 0.004 | *0.002±0.001* | 0.021 | – | 0.026 |
| | USA (EC RI WILD) | 7 | 0.207 | 0.210 | 0.209 | – | 0.212 | 0.209 | 0.207 | 0.207 | *0.003±0.002* | | 0.029 |
| | *B. pro* | 1 | 0.245 | 0.245 | 0.247 | – | 0.244 | 0.245 | 0.243 | 0.241 | 0.272 | – | – |
| Cyt *b* | AU | 7 | *0.000±0.000* | 0.000 | – | – | 0.004 | 0.004 | – | – | 0.024 | – | 0.037 |
| | CH | 1 | 0.000 | – | – | – | 0.004 | 0.004 | – | – | 0.025 | – | 0.038 |
| | NAM | 1 | 0.005 | 0.006 | – | – | – | 0.000 | – | – | 0.190 | – | 0.036 |
| | SA | 35 | 0.005 | 0.006 | – | – | 0.000 | *0.001±0.000* | – | – | 0.190 | – | 0.036 |
| | USA (EC RI WILD) | 7 | 0.190 | 0.184 | – | – | 0.024 | 0.024 | – | – | *0.003±0.002* | – | 0.035 |
| | *B. pro* | 1 | 0.297 | 0.291 | – | – | 0.292 | 0.292 | – | – | 0.291 | – | – |
| 18S rRNA | AU | 8 | *0.000±0.000* | 0.001 | – | 0.001 | 0.001 | 0.000 | 0.001 | 0.001 | 0.005 | 0.005 | 0.007 |
| | CH | 1 | 0.002 | – | – | 0.000 | 0.000 | 0.002 | 0.000 | 0.000 | 0.005 | 0.005 | 0.007 |
| | JP | 1 | 0.002 | 0.000 | – | – | 0.000 | 0.002 | 0.000 | 0.000 | 0.005 | 0.005 | 0.007 |
| | NAM | 1 | 0.002 | 0.000 | – | 0.000 | – | 0.002 | 0.000 | 0.000 | 0.014 | 0.005 | 0.007 |
| | SA | 35 | 0.000 | 0.002 | – | 0.002 | 0.002 | *0.000±0.000* | 0.002 | 0.002 | 0.015 | 0.005 | 0.007 |
| | USA (EC) | 4 | 0.002 | 0.000 | – | 0.000 | 0.000 | 0.002 | *0.001±0.001* | 0.000 | 0.005 | 0.005 | 0.007 |
| | USA (WC) | 13 | 0.001 | 0.000 | – | 0.000 | 0.000 | 0.001 | 0.000 | *0.000±0.000* | 0.005 | 0.005 | 0.007 |
| | USA (EC RI WILD) | 7 | 0.015 | 0.014 | – | 0.014 | 0.005 | 0.005 | 0.015 | 0.014 | *0.000±0.000* | 0.000 | 0.007 |
| | *P. ona* | 1 | 0.015 | 0.014 | – | 0.014 | 0.014 | 0.016 | 0.015 | 0.014 | 0.000 | – | 0.007 |
| | *B. pro* | 1 | 0.028 | 0.026 | – | 0.026 | 0.026 | 0.028 | 0.027 | 0.026 | 0.027 | 0.026 | – |

### 3.1.5. Haplotype network and diversity

Nine COI haplotypes from seven regions were retrieved for PSG1 with 0.268±0.064 haplotype diversity and a low nucleotide diversity of 0.001±0.000 (Figure 3.4). Within PSG1, Hap_4 was the most common, containing sequences from most regions, and was the most abundant. Eight singleton haplotypes surrounded Hap_4, of which six were from USA (EC). This corresponds to USA (EC) having the highest haplotype diversity of 0.967±0.001 (Table 3.3), relative to all the regions. All sequences from SA, NAM, HAWAII and AU were confined to one haplotype (Hap_4) suggesting a high level of genetic homogeneity. The haplogroup representing PSG1 was separated by 45 mutation differences from the PSG2 haplogroup, which retrieved three haplotypes from USA (EC RI WILD).

Two Cyt *b* haplotypes from four regions were retrieved for PSG1 with 0.359±0.070 haplotype diversity and a low nucleotide diversity of 0.002±0.000 (Figure 3.5). The most common haplotype, Hap_1 consisted of sequences from AU, CH and SA, and was separated by two mutational differences from Hap_2. The most dominant haplotype, Hap_2, consisted of sequences from SA and NAM. The haplogroup representing PSG1 was separated by 49 mutation differences from the PSG2 haplogroup. Two haplotypes from USA (EC RI WILD) were retrieved for PSG2. USA (EC RI WILD) had the highest haplotype diversity of 0.571±0.119 with a low nucleotide diversity of 0.003±0.001 (Table 3.3).

Three 18S rRNA haplotypes from seven regions were retrieved for PSG1 with 0.063±0.0042 haplotype diversity and a low nucleotide diversity of 0.001±0.000 (Figure 3.6). Amongst the haplogroups within PSG1, Hap_2, was the most common and abundant. The haplogroup representing PSG1 was separated by eight mutation differences from the PSG2 haplogroups. The PSG2 represented by Hap_1, had a high haplotype diversity of 0.607±0.164 and a low nucleotide diversity of 0.000±0.000 (Table 3.3).

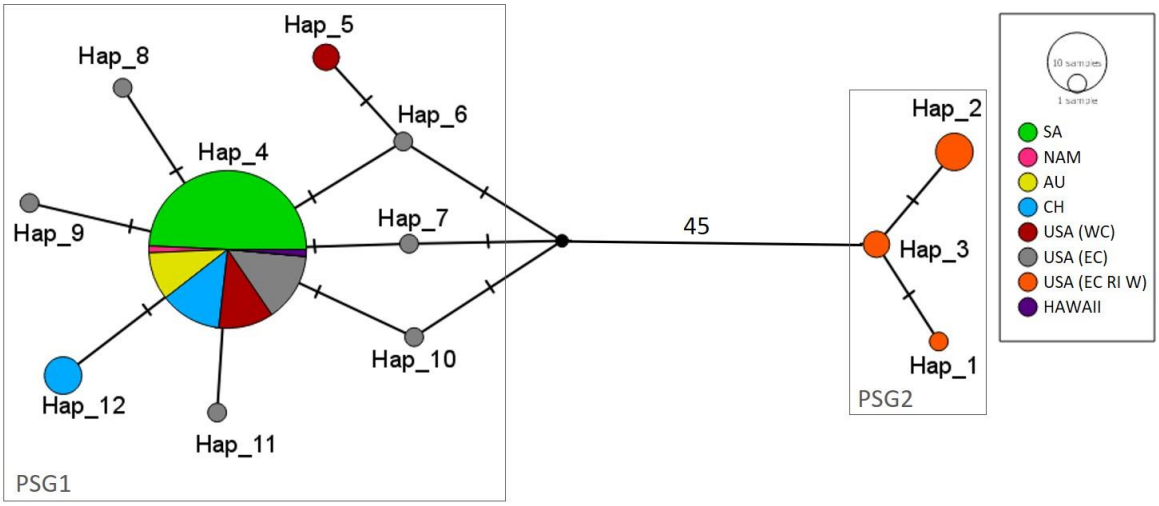


**Figure 3.4:** TCS haplotype network of *Polydora websteri* and *Polydora* cf. *websteri* for COI; each region corresponds to a colour and circle sizes are proportional to number of sequences in each haplotype (Hap); lines perpendicular to the connecting lines indicate mutation differences and black dots indicate missing haplotypes; PSG1 and PSG2 grouped by grey squares; South Africa (SA), Namibia (NAM), Australia (AU), China (CH), United States of America (USA), west coast (WC), east coast (EC), collected from wild molluscs in Rhode Island (RI W) and Hawaii

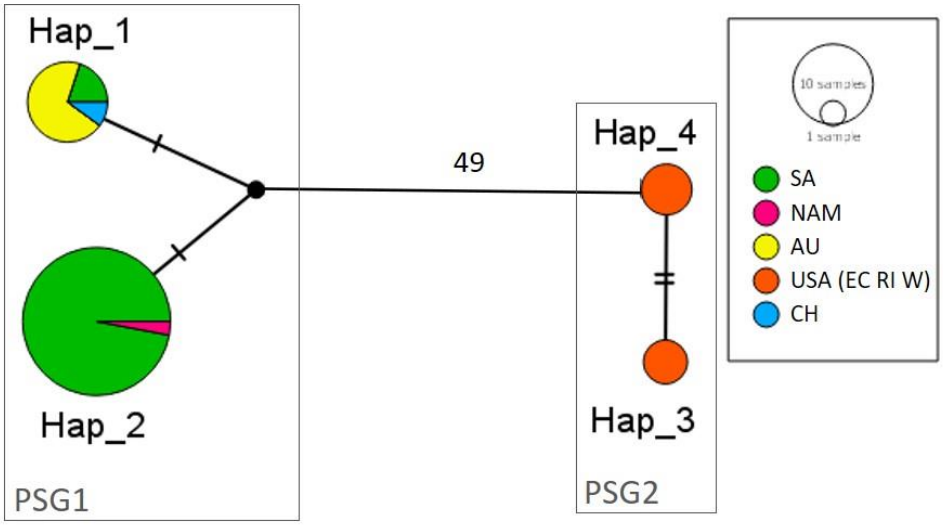


**Figure 3.5:** TCS haplotype network of *Polydora websteri* and *Polydora* cf. *websteri* for Cyt *b*; each region corresponds to a colour and circle sizes are proportional to number of sequences in each haplotype (Hap); lines perpendicular to the connecting lines indicate mutation differences and black dots indicate missing haplotypes; PSG1 and PSG2 grouped by grey squares; South Africa (SA), Namibia (NAM), Australia (AU), China (CH) and east coast (EC) of the United States of America (USA) collected from wild molluscs in Rhode Island (RI W)

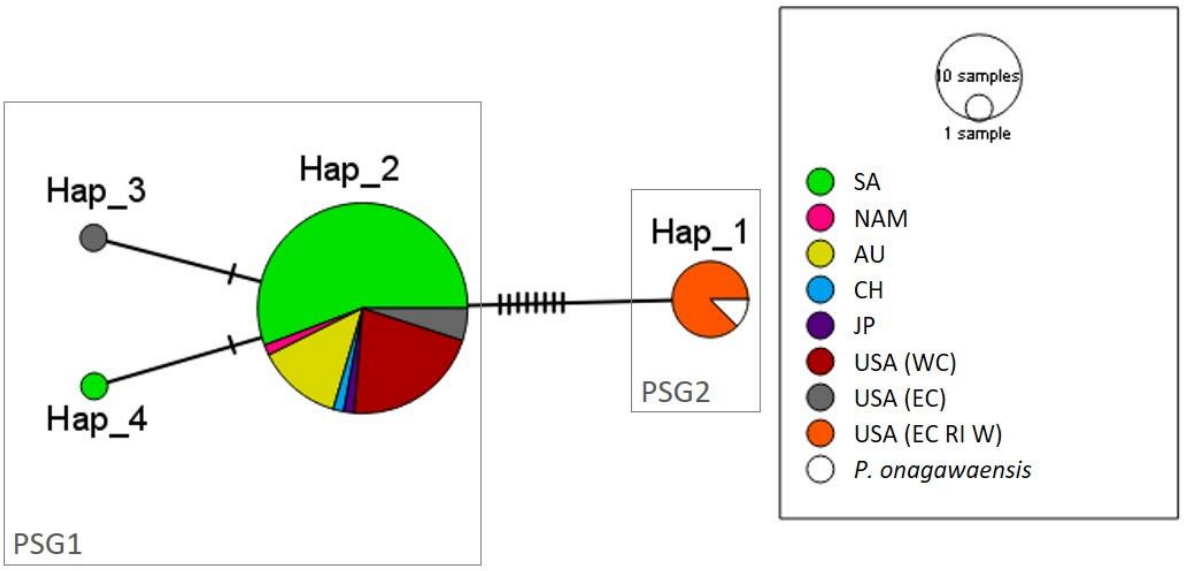


**Figure 3.6:** TCS haplotype network of *Polydora websteri* and *Polydora* cf. *websteri* for 18S rRNA, each region corresponds to a colour and circle sizes are proportional to number of sequences in each haplotype (Hap); lines perpendicular to the connecting lines indicate mutation differences and black dots indicate missing haplotypes; PSG1 and PSG2 grouped by grey squares; South Africa (SA), Namibia (NAM), Australia (AU), China (CH), Japan (JP), United States of America (USA), west coast (WC), east coast (EC), collected from wild molluscs in Rhode Island (RI W) and *Polydora onagawaensis*

**Table 3.3:** Number of sequences, number of haplotypes, haplotype diversity (Hd), nucleotide diversity (λ) and ± standard deviations (SD) of *Polydora websteri* and *Polydora* cf. *websteri* in each region for COI, Cyt *b* and 18S rRNA; South Africa (SA), Namibia (NAM), Australia (AU), China (CH), Hawaii, Japan (JP), United States of America (USA), west coast (WC), east coast (EC), Rhode Island (RI), collected from wild molluscs (WILD)

| Geographic Region | Number of Sequences | Number of Haplotypes | Hd | ± SD | λ | ± SD |
|---|---|---|---|---|---|---|
| **COI** | | | | | | |
| AU | 7 | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| CH | 13 | 9 | 0.910 | 0.068 | 0.004 | 0.001 |
| HAWAII | 1 | 1 | – | – | – | – |
| NAM | 1 | 1 | – | – | – | – |
| SA | 35 | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| USA (EC) | 16 | 13 | 0.967 | 0.001 | 0.006 | 0.001 |
| USA (WC) | 10 | 2 | 0.356 | 0.159 | 0.002 | 0.001 |
| USA (EC RI WILD) | 7 | 3 | 0.667 | 0.160 | 0.003 | 0.001 |
| **Cyt *b*** | | | | | | |
| AU | 7 | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| CH | 1 | 1 | – | – | – | – |
| NAM | 1 | 1 | – | – | – | – |
| SA | 35 | 2 | 0.111 | 0.070 | 0.001 | 0.000 |
| USA (EC RI WILD) | 7 | 2 | 0.571 | 0.119 | 0.003 | 0.001 |
| **18S rRNA** | | | | | | |
| AU | 8 | 2 | 0.250 | 0.180 | 0.000 | 0.000 |
| CH | 1 | 1 | – | – | – | – |
| JP | 1 | 1 | – | – | – | – |
| NAM | 1 | 1 | – | – | – | – |
| SA | 35 | 4 | 0.264 | 0.094 | 0.000 | 0.001 |
| USA (EC) | 4 | 2 | 0.500 | 0.265 | 0.001 | 0.000 |
| USA (WC) | 13 | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| USA (EC RI WILD) | 7 | 3 | 0.667 | 0.160 | 0.001 | 0.000 |
| USA (EC RI WILD) + *P. onagawaensis* (JP) | 8 | 3 | 0.607 | 0.164 | 0.000 | 0.000 |

### 3.2. Morphological

South African specimens match the morphological description of the lectotype of *P. websteri* (Radashevsky, 1999), and descriptions of the species from Australia, China, Japan, New Zealand and the west and east coast of the USA, but not the specimens of '*P. websteri*' collected from wild mollusc in Rhode Island, USA. To justify the scientific name selected for the morphological description below, this study therefore concludes that specimens from South Africa are *Polydora websteri* Hartman in Loosanoff and Engle (1943), and that the specimens collected from Rhode Island had been misidentified.

#### 3.2.1. Systematics

Family SPIONIDAE Grube, 1850

Subfamily SPIONINAE Söderström, 1920

Genus *Polydora* Bosc, 1802

Species *Polydora websteri* Hartman in Loosanoff and Engle 1943

(Figures 3.7 – 3.13)

*Polydora websteri*: Hartman in Loosanoff & Engle, 1943: 70 – 72, Figure 1

*Polydora websteri*: Blake, 1969: 814 – 815, Figure 2; Blake, 1971: 6 – 8, Figure 3; Foster, 1971: 26; Blake & Kudenov, 1978: 258 – 259, Figure 43 k – n; Handley & Bergquist, 1997: 191 – 205; Radashevsky & Williams, 1998: 212 – 216; Radashevsky, 1999; 107 – 113, Figure 1; Sato-Okoshi, 1999: 832 – 834, Figure 2 B; Surugiu, 2005: 67; Bonifácio, 2009; Read, 2010: 9 – 11, Figures 1 H – J, 2B, 2D, 2 F and 4 D – G; Surugiu, 2012: 50 – 53, Figure 3; Sato-Okoshi & Abe, 2013: 1280 – 1281, Figure 2; Ye et al., 2017; Rice et al., 2018

*Polydora* cf. *ciliata*: Simon, 2011

*Polydora haswelli*: Sato-Okoshi et al., 2008: 495, Figure 4 F and G

*Polydora* cf. *websteri*: Williams, 2015; Simon, 2015; Simon & Sato-Okoshi, 2015; Williams et al., 2017

#### 3.2.2. Material examined

Non-type material: South Africa: Eastern Cape: Nelson Mandela Bay (Knysna Oyster Company): 33°50′0″ S, 25°50′0″, 20 specimens, complete, 2 specimens, incomplete, coll. C.A. Simon, August 2017, from cultured *Crassostrea gigas* Thunberg, 1793.

### 3.2.3. Comparative material examined

Type material: Lectotype – LACM-AHF POLY 1628, United States: Connecticut: Long Island Sound, mouth of Milford River, coll. J. B. Engle, Milford Wildlife Laboratory, 04 January 1943, from vesicles on empty oyster shells, housed in the Los Angeles County Museum of Natural History.

United States: USA: Rhode Island: Narrow River: 41°31′8.7″ N, 71°26′43.77″ W, 2 fragmented specimens, coll. J. D. Williams, 1996 from wild molluscs.

*Polydora onagawaensis* Teramoto, Sato-Okoshi, Abe, Nishitani and Endo, 2013: Japan: Rishiri Island: 45° 20’ 04” N, 141° 22’ 20” E, 12 incomplete specimens, coll. H. Sato-Okoshi on 31 July 2019.

### 3.2.4. Description of *Polydora websteri* from South Africa

Complete specimens 4.7 to 22 mm long and 0.32 to 0.95 mm wide at chaetiger 5 (n = 22), for up to 39 to 123 chaetigers from South Africa (n = 20). Prostomium anteriorly bilobed or weakly bilobed; caruncle extending to mid chaetiger 2 or end chaetiger 3 (Figures 3.7 A – C and 3.8 B); eyes usually absent, but up to 4 arranged in trapezoid when present; occipital antennae absent. Body pigmentation absent; palps with distinct black continuous lines along feeding groves (Figure 3.9 A and 3.10 A).

Notochaetae absent on chaetiger 1, notopodial lobe small. Winged capillary notochaetae with postchaetal lamellae on chaetigers 2 to 4 and 6. Capillary notochaetae with postchaetal lamellae on posterior chaetigers reducing in size posteriorly. Winged neurochaetae with postchaetal lamellae increasing in size from chaetigers 1 to 4 and 6 (Figure 3.8 B). Neurochaetae replaced by bidentate hooded hooks in vertical row on chaetiger 7 (Figures 3.8 B and 3.11 B); up to 7 hooded hooks per fascicle, increasing up to 11 in middle chaetigers, decreasing to 1 or 2 on posterior chaetigers. Hooded hooks without companion chaetae; main fang at < 45° to apical tooth and right angle to shaft; with constriction on shaft (Figure 3.12 D).

Chaetiger 5 modified, approximately twice as large as chaetigers 4 and 6; with superior and inferior winged chaetae, shorter than capillary chaetae on the preceding chaetigers (Figures 3.10 B and C). Thick falcate spines on chaetiger 5 with prominent flange on concave side of spine, no tooth; up to 7 spines in slightly curved row (Figures 3.8 A, B, E and 3.12 A); spines alternating with pennoned companion chaetae, trips occasionally frayed (Figure 3.12 A).

Branchiae present from chaetiger 7 onwards (Figures 3.7 A – C and 3.8 A, B, E), covering approximately 50 % of chaetigers, longest on chaetigers 11 to 25 (Figures 3.7 F – H).

Pygidium cup to disc-shaped, with dorsal notch leading to anus, 1.5 ± 0.31 times wider than 5th last chaetiger (Figures 3.7 F – H and 3.11 G).

### 3.2.5. Staining pattern

Staining of prostomium varying from few irregular stained cells to stained cells forming two bars (faint or distinct) fading into dispersed stained cells towards anterior. Staining of caruncle varying from no staining pattern to few stained cells. Dorsal staining of peristomium varying from few irregular stained cells to clearly dispersed stained cells that may be concentrated along ridge of prostomium. Chaetiger 1 to 4 varying from no stain to irregularly stained cells or forming patches of stain on both sides of caruncle, fading toward latero-ventral (Figures 3.7, 3.8 and Table 3.4). Chaetiger 5 with fewest stained cells of anterior chaetigers, varying from no staining pattern to few irregular stained cells (Figure 3.7 and Table 3.4). On chaetigers 6 to 20th from the last chaetiger, stain varying from faint to distinct, patches of stained cells forming on both sides of centre intensifying towards posterior; 8th to 2nd from the last chaetiger varying from no staining pattern to stained cells forming faint or distinct patches towards posterior on both sides of centre; last chaetiger without staining pattern (Figures 3.7, 3.8 and Table 3.4). Except for chaetiger 1 to 4, no lateral staining pattern (Figure 3.8 and Table 3.4).

Ventral staining of peristomium and chaetiger 1 to 5 varying from no staining pattern to irregular stained cells. Chaetiger 6 to 14 stained cells forming a distinct band across the anterior part of chaetiger. Chaetiger 15 to 18 ranging from no staining pattern to stained cells forming a distinct band across the anterior part of chaetiger. Chaetiger 19 to last chaetiger without staining pattern (Figure 3.11 and Table 3.4).

Pygidium with distinct stained cells covering entire surface, except along edge around anus and outermost edge of disc (Figures 3.7 F – H and 3.11 G). Palps without staining pattern or continuous lines of stained cells adjacent to black continuous pigmentation lines (Figure 3.9 A). Branchiae without staining pattern (Figures 3. 7 and 3.8).

### 3.2.6. Distribution

*P. websteri* has been collected in Namibia, in South Africa on the west coast in Paternoster (Williams, 2015) and Kleinzee and on the east coast in Nelson Mandela Bay, Port Elizabeth (this study). Genetic and morphological data have confirmed the presence of *P. websteri* in Australia (Sato-Okoshi & Abe, 2013), China (Sato-Okoshi et al., 2013; Ye et al., 2017), Japan (Sato-Okoshi & Abe, 2013), Hawaii (Rice et al., 2018), Washington State on the west coast of the USA (Martinelli et al., 2020), on the east coast of the USA in Maine, Massachusetts, Maryland (Rice et al., 2018) and New York (Martinelli et al., 2020), and on the south eastern coast of the USA in Alabama (Rice et al., 2018). Reports that included only detailed

morphological descriptions indicate that *P. websteri* occurs in the Gulf of California in Mexico (Blake, 1969, 1971; Foster, 1971), New Zealand (Read, 2010), Romania and Ukraine (Surugiu, 2005, 2012). It is uncertain whether the latter morphological reports refer to the same species, however, the morphological report from Brazil (Bonifácio, 2009) may represent the 'true' *P. websteri*. The detailed morphological description of *P.* cf. *websteri* from Brazil (Barros et al., 2017) closely matches the reports of *P. websteri* above and probably represents the 'true' *P. webster*. Reports of *P. websteri* from Argentina (Diez et al., 2011), British Columbia and New Brunswick in Canada (Bergman et al., 1982; Bower et al., 1992; Clements et al., 2017), Chile (Basilio et al., 1995), Ecuador, Peru (Blake, 1983), Galapagos Islands (Keppel et al., 2019), Red Sea (Abd-Elnaby, 2019), South Island in New Zealand (Handley, 1995), Tasmania in Australia (Nell, 2001), and *P.* cf. *websteri* in Venezuela (Díaz-Díaz & Liñero-Arana, 2009) are not accompanied by detailed morphological data, making these records more difficult to confirm.

### 3.2.7. Ecology

In South Africa, *P. websteri* is currently only found boring into shells of cultured oysters, *C. gigas* (Simon & Sato-Okoshi, 2015; Williams, 2015; Williams et al., 2017). *P. websteri* is a pest of commercial molluscs in most locations where it has been reported (Simon & Sato-Okoshi, 2015), and also abundant in intertidal and shallow waters (Blake & Evans, 1973). *Polydora websteri* is not host specific and creates U-shaped burrows that induces the formation of mud-blisters by molluscs such as *Argopecten irradians* Lamarck, 1819 (Lauckner, 1983), *Crassostrea* cf. *brasiliana* Lamarck, 1819 (Bonifácio, 2009; Barros et al., 2017), *C. gigas* (Read, 2010), *Crassostrea hongkongensis* Lam & Morton, 2003 (Ye et al., 2017), *Crassostrea rhizophorae* Guilding, 1828 (Bonifácio, 2009; Barros et al., 2017), *Crassostrea virginica* Gmelin, 1791 (Loosanoff & Engle, 1943; Martinelli et al., 2020), *Crepidula fornicata* Linnaeus, 1758 (Blake, 1971), *Euspira heros* (Say, 1822) (Blake, 1971), *Littorina littorea* Linnaeus, 1758 (Blake, 1971), *Mytilus edulis* Linnaeus, 1758 (Blake & Evans, 1973), *Mytilus galloprovincialis* Lamarck, 1819 (Surugiu, 2005, 2012), *Nucella lapillus* (Linnaeus, 1758) (Blake, 1971), *Ostrea angasi* Sowerby, 1871 (Nell, 2001), *Pinctada fucata* Gould, 1850 (Simon & Sato-Okoshi, 2015), *Pinctada imbricata* Röding, 1798 (Díaz-Díaz & Liñero-Arana, 2003), *Placopecten magellanicus* Gmelin, 1791 (Blake, 1969; Blake, 1971), *Patinopecten yessoensis* Jay, 1857 (Bower et al., 1992), *Saccostrea cucullata* Born, 1778 (Skeel, 1979) and *Saccostrea glomerata* Gould, 1850 (as *Saccostrea commercialis* Sato-Okoshi et al., 2008), and also been found in limestone (Surugiu, 2005, 2012).

### 3.2.8. Remarks

Specimens of *P. websteri* from South Africa conform to the morphology of lectotype (Radashevsky, 1999) (Figure 3.13) and descriptions of conspecifics found globally (Loosanoff

& Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Kudenov, 1978; Handley & Bergquist, 1997;Sato-Okoshi, 1999; Surugiu, 2005; Sato-Okoshi et al., 2008; Bonifácio, 2009; Read, 2010; Surugiu, 2012; Sato-Okoshi & Abe, 2013; Barros et al., 2017; Ye et al., 2017; Rice et al., 2018). Some variation was present for the branchiae, pigmentation patterns and modified chaetae on chaetiger 5 but are within the ranges reported for the species (Appendix A, Table A1) or may be the result of wear and tear, and preservation.

Branchiae for South African specimens covered on average 50 % of chaetigers, slightly fewer than other descriptions where branchiae covered 60 – 80 % of the body length (Loosanoff & Engle, 1943; Blake & Kudenov, 1978; Radashevsky, 1999; Surugiu, 2005, 2012; Sato-Okoshi & Abe, 2013; Ye et al., 2017). Continuous black lines along the feeding groove on the palps was observed for most South African specimens (Figure 3.9). Pigmentation was absent from the palps of some paratype material (Radashevsky, 1999), but this may be a consequence of pigmentation fading in preserved specimens (Read, 2010). Similar pigmentation fading can also be seen on the palps for some South African (this study) and USA (Rice et al., 2018) preserved specimens, where sections of the black line are lighter, making them seem non-continuous if not carefully analysed (Figure 3.10 A). Sato-Okoshi & Abe (2013) found that while some live specimens from Japan had continuous black lines on the palps, some had discontinuous black pigmentation. It is uncertain whether this is intraspecific variation or a result of fading due to age of the worm, as certain parts of the palp pigmentation appeared faded.

South African specimens have a flange on the falcate spines with mostly pennoned companion chaetae on chaetiger 5 (Figure 3.12 A). Wear and orientation of the falcate spines on chaetiger 5 gives the appearance of a tooth and/or a sheath instead of only a flange as in South African specimens and most other descriptions (Appendix A, Table A1). Caution is also necessary when viewing the pennoned companion chaetae on chaetiger 5 because age and wear may render some to appear frayed or hastate (Read, 2010), as seen for South African specimens and other descriptions (Appendix A, Table A1).

South African specimens differ morphologically from specimens collected from wild molluscs in Rhode Island, USA in terms of the shape of modified spines on chaetiger 5, size of the pygidium, pigmentation and methyl-green staining patterns. However, the specimens collected in Rhode Island are morphologically similar to *P. onagawaensis* from Japan (see *P. onagawaensis* full description in Appendix C, Figures C1 and C2), in terms of the shape of modified spines, pygidium, body and palp pigmentation and methyl-green staining patterns.

South African specimens differ from Rhode Island specimens by having a flange instead of a tooth on the spines of chaetiger 5. Specimens from Rhode Island are similar to specimens from Japan by having a distinct tooth on the spines of chaetiger 5 (Appendix C, Figure C2 A) (Sato-Okoshi et al., 2013; Teramoto et al., 2013), whereas South African specimens and the lectotype of *P. websteri* have only a flange (Figures 3.11 A, B and in Radashevsky (1999), Figure 1 F). Another difference is that the pygidium of South African specimens is wider than the 5$^{th}$ last chaetiger, whereas the pygidium is narrower than the 5$^{th}$ last chaetiger for Rhode Island specimens (Appendix C, Figure C1).

South African specimens have continuous black lines along the feeding grooves on the palps (Figures 3.9 A and 3.10 A), whereas in specimens from Rhode Island and Japan, brown or black shading may be discontinous or absent (Figures 3.9 B and 3.10 B, C). However, caution is necessary as pigmentation patterns are not a reliable distinguishing feature to diffirentiate between species, as these patterns depend on age, fading due to light exposure and preservation materials (Read, 2010).

Methyl-green staining patterns differ (Table 3.4), mainly towards the dorsal posterior. In South African specimens and the *P*. *websteri* lectotype there is one bar on each chaetiger on both sides of the centre (Figures 3.7 and 3.13) compared to specimens from Rhode Island which have two bars on each chaetiger on both sides of the centre (Appendix C, Figure C1). Staining patterns also differ ventrally in the mid anterior. South African specimens and *P. websteri* lectotype have stained cells forming a distinct band across the anterior part of the chaetiger (Figure 3.12) compared to specimens from Rhode Island that have no ventral mid anterior staining pattern (Appendix C, Figure C1 B, E).

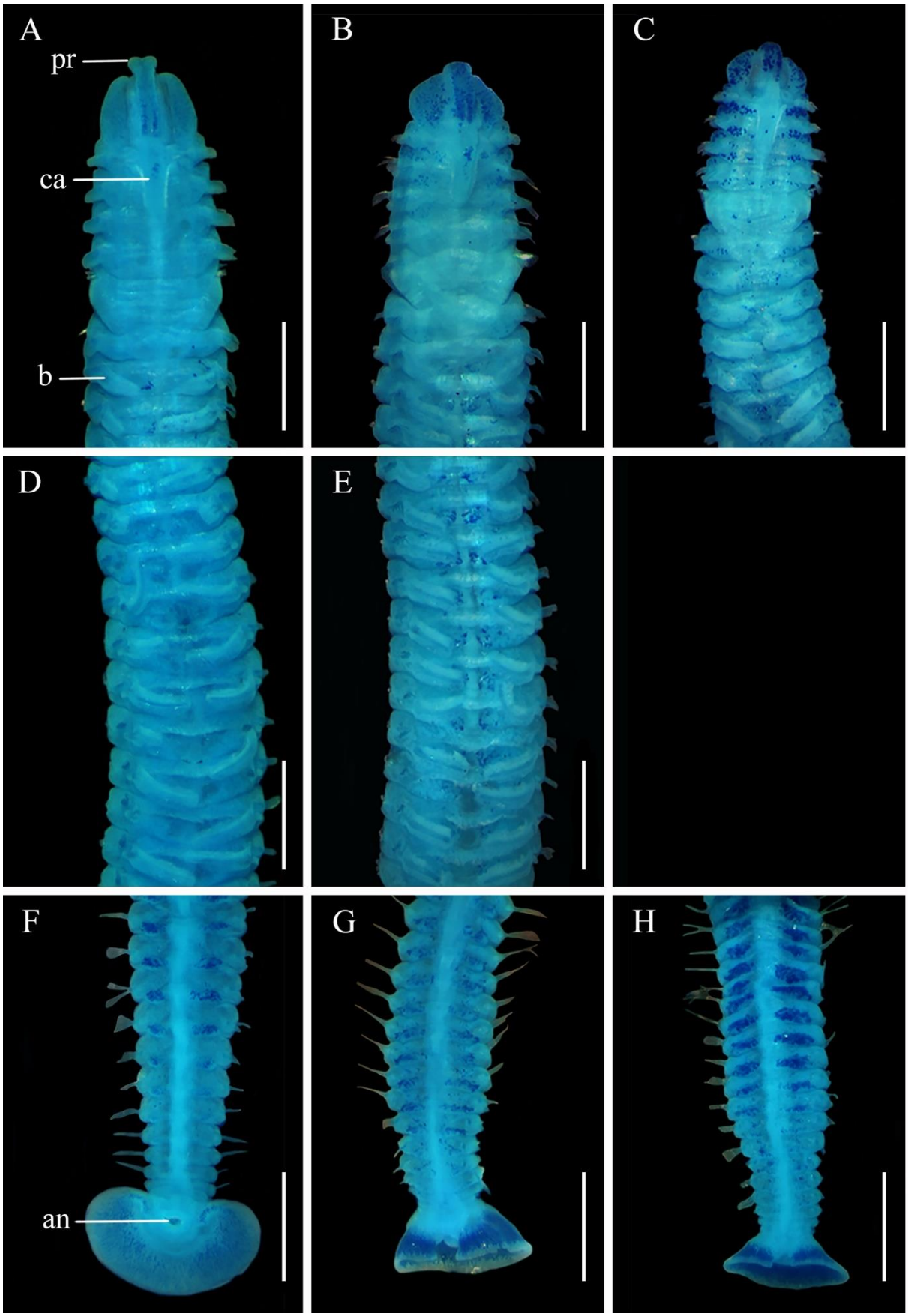


**Figure 3.7:** Dorsal view of *Polydora websteri* from South Africa, (A – C) view of anterior without palps showing prostomium (pr), caruncle (ca) and the branchia (b); (D, E) mid-body; (F – H) posterior view showing the anus (an); (A, D, F) SAMC-A089084; (B, E, G) SAMC-A089085; (C) SAMC-A089086; (H) SAMC-A089087; scale bars: 0.5 mm

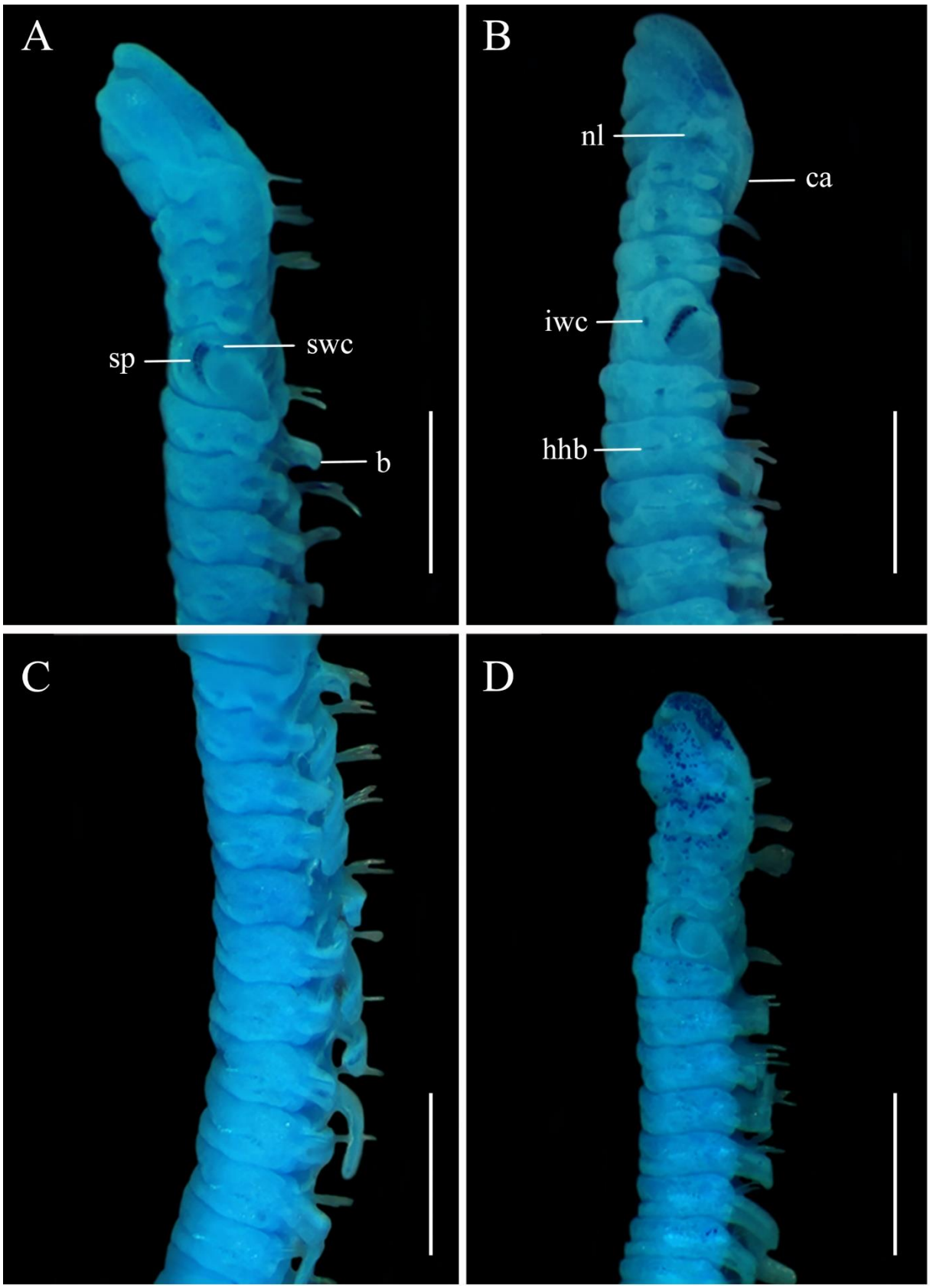


**Figure 3.8:** Lateral view of *Polydora websteri* from South Africa, (A, B, D) view of anterior without palps showing the caruncle (ca), neuropodial lobe (nl), row of spines (sp), superior winged chaetae (swc), inferior winged chaetae (iwc), where the branchiae begin (b), where the hooded hooks begin (hhb); (C) mid-body; (A, C) SAMC-A089084; (B) SAMC-A089085; (D) SAMC-A089086; scale bars: 0.5 mm

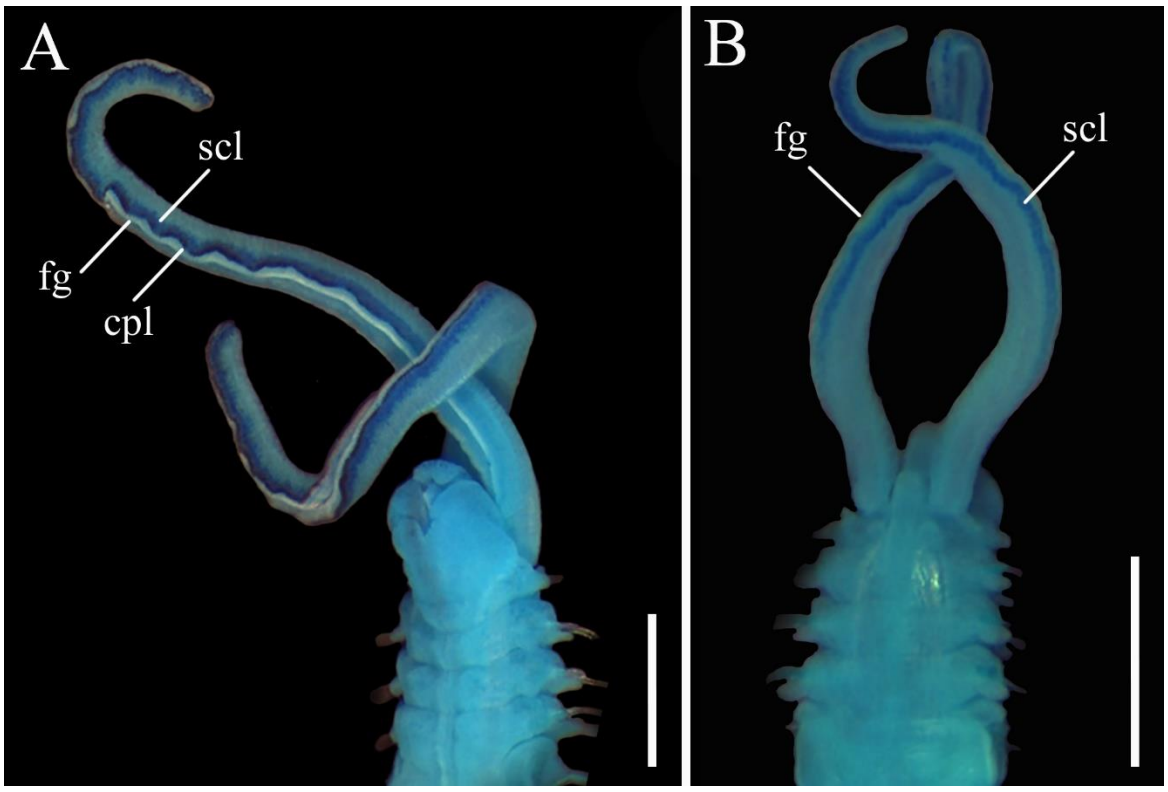


**Figure 3.9:** Anterior view of palps (A) ventral view of *Polydora websteri* from South Africa showing the continuous black pigmentation line (cpl), feeding groove (fg), stained cells line (scl); (B) dorsal, Rhode Island, USA specimen collected from wild molluscs; scale bars: 0.5 mm

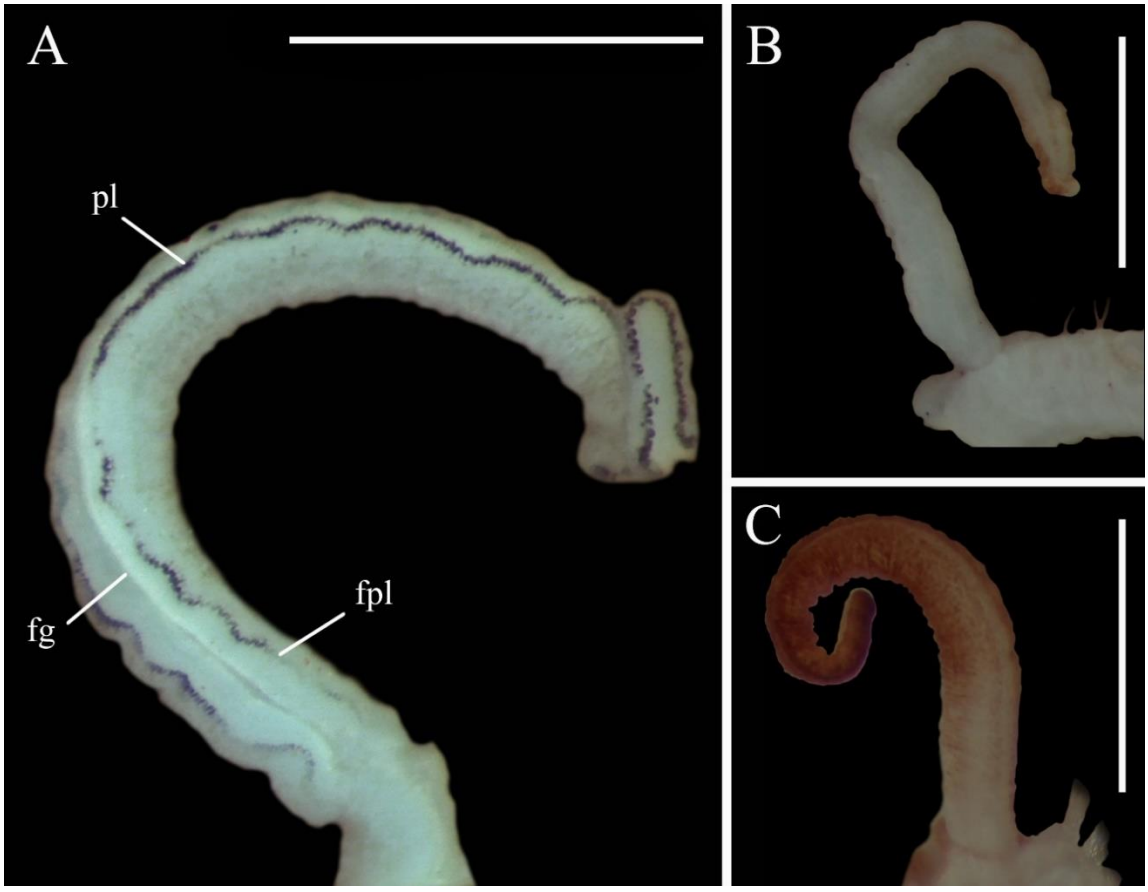


**Figure 3.10:** Anterior view of palps (A) *Polydora. websteri* from South Africa showing the black pigmentation line (pl), faded black pigmentation line (fpl), feeding groove (fg); (B) lateral view of specimen from Rhode Island, USA collected from wild molluscs; (C) dorsal view of specimen from Rhode Island, USA collected from wild molluscs; scale bars: 0.5 mm

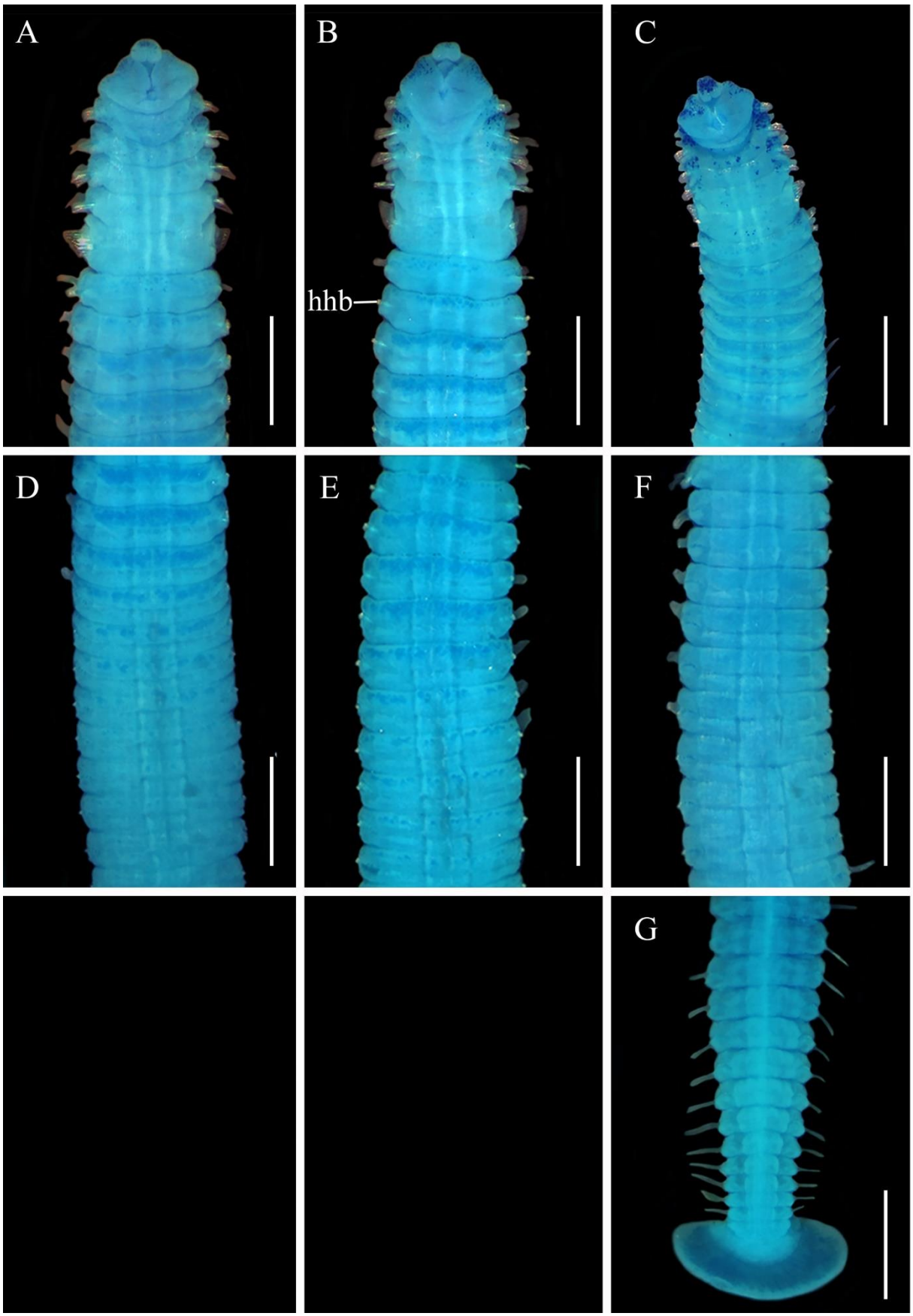


**Figure 3.11:** Ventral view of *Polydora websteri* from South Africa, (A – C) view of anterior without palps showing where the hooded hooks begin (hhb); (D – E) mid-body; (G) posterior; (A, D) SAMC-A089085; (B, E) SAMC-A089088; (C) SAMC-A089086; (F, G) SAMC-A089084; scale bars: 0.5 mm

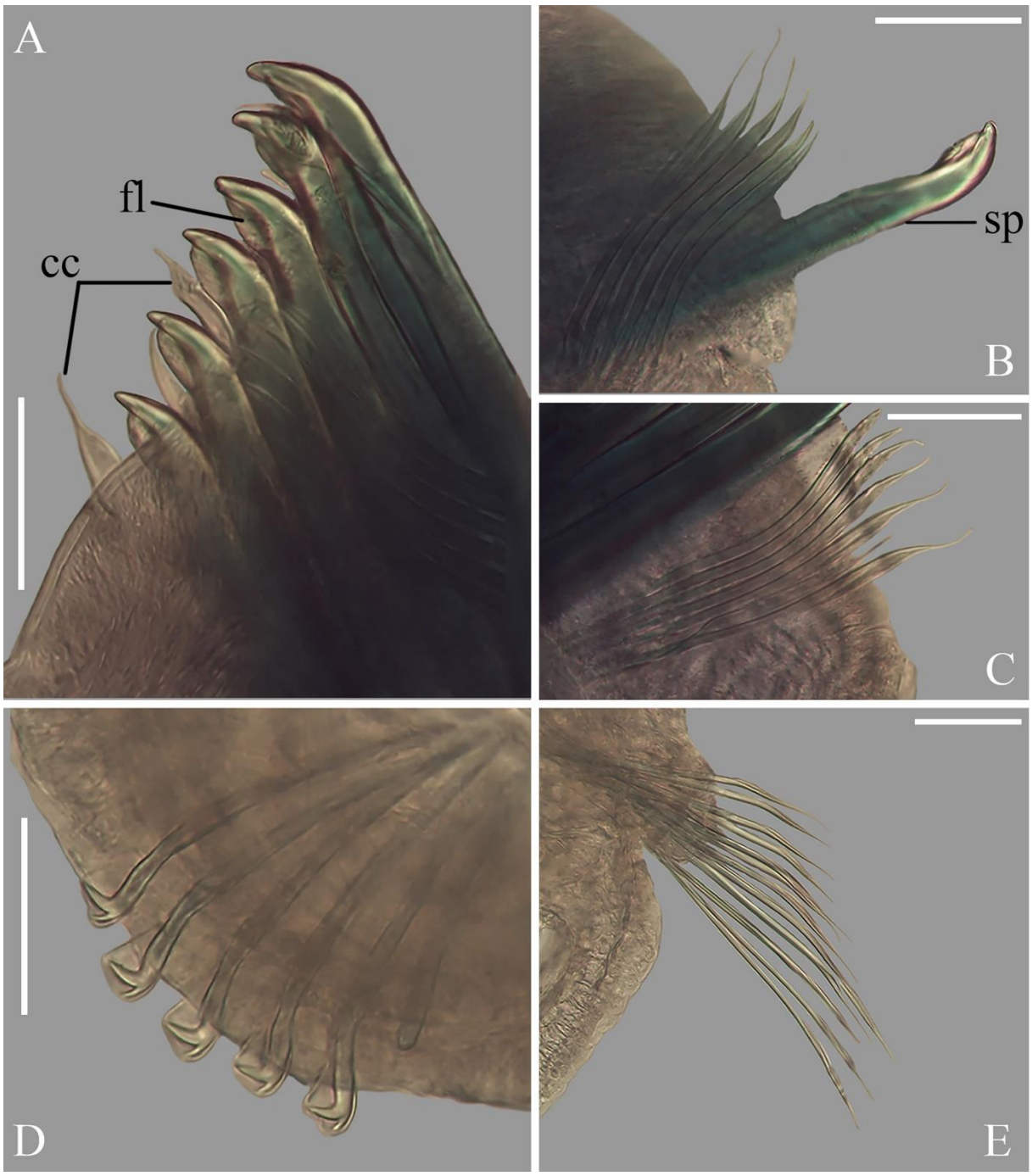


**Figure 3.12:** Chaetal structures of *Polydora websteri* from South Africa (A) falcate spines (sp) on chaetiger 5 with flange (fl) on the concave side, pennoned companion chaetae (cc); (B) spine (sp) with superior winged chaetae on chaetiger 5; (C) inferior winged chaetae on chaetiger 5; (D) hooded hooks on neuropodium of posterior chaetigers; (E) capillary notochaetae on posterior chaetigers; scale bars: 0.05 mm

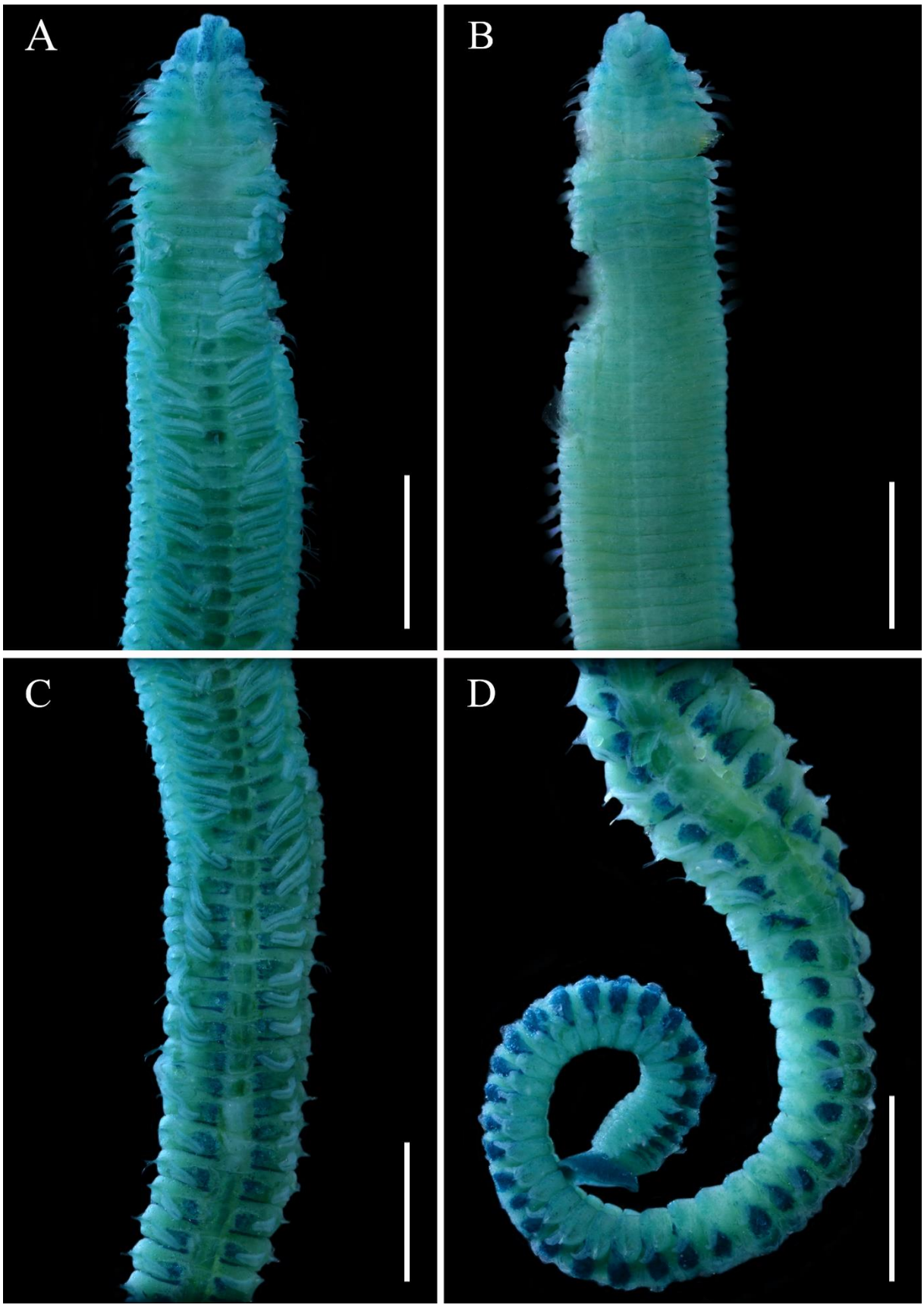


**Figure 3.13:** Lectotype of *Polydora websteri* (LACM-AHF POLY 1628) collected from oyster shells and deposited in the Los Angeles County Museum of Natural History (Radashevsky, 1999). Dorsal view of *Polydora websteri* stained with methyl-green. (A) dorsal anterior; (B) ventral anterior; (C) dorsal mid-body; (D) posterior; scale bars: 1 mm

**Table 3.4:** Methyl-green staining patterns of *Polydora websteri* from South Africa and specimens from Rhode Island, USA, bullet points represent variations in staining patterns of individual specimens; no staining pattern (NSP)

| | ***Polydora websteri* from South Africa** | | **Specimens from Rhode Island, east coast of USA** | |
|---|---|---|---|---|
| **Location** | **Dorsal** | **Ventral** | **Dorsal** | **Ventral** |
| Palps | • NSP<br>Continuous line next to the black continuous pigmentation lines | | • Continuous line along the feeding grove | |
| Prostomium | • Few irregular stained cells<br>• Stained cells forming 2 lateral bars (faint or distinct) fading into dispersed stained cells towards the anterior | • NSP | • Few irregular stained cells | • Few irregular stained cells |
| Peristomium | • Few irregular stained cells<br>• Clearly dispersed stained cells that may be concentrated along the ridge of the prostomium | • NSP<br>• Few irregular stained cells | • Few irregular stained cells | • Few irregular stained cells |
| Caruncle | • NSP<br>• 1 or 2 individual stained cells<br>• Few stained cells forming 1 or a few small patches | • NSP | • NSP | • NSP |
| Chaetiger 1 – 4 | • NSP<br>• Few irregular stained cells<br>• Stained cells (faint or distinct) forming patches on both sides of caruncle, fading toward latero-ventral | • NSP<br>• Few irregular stained cells | • NSP | • NSP |
| Chaetiger 5 | • NSP<br>• Few irregular stained cells | • NSP<br>• Few irregular stained cells | • NSP | • NSP |
| Chaetiger 6 & 7 | • NSP<br>• Few irregular stained cells<br>• Few stained cells forming patches on both sides of the centre | • Stained cells forming a distinct band across the anterior part of the chaetiger | • NSP<br>• 1 or 2 patches of stained cells | • NSP |
| Chaetiger 8 – 14 | • Few irregular stained cells<br>• Few stained cells forming patches on both sides of the centre | • Stained cells forming a distinct band across the anterior part of the chaetiger | • 2 stacked bars on either side of the central line | • NSP |
| Chaetiger 15 – 18 | • Stained cells forming patches on both sides of the centre | • NSP<br>• Stained cells forming a distinct band across the anterior part of the chaetiger | • 2 stacked bars on either side of the central line | • NSP |
| Chaetiger 19 – 20th last chaetiger | • Stained cells forming patches (faint or distinct) on both sides of the centre | • NSP | • 2 stacked bars on either side of the central line | • NSP |
| 20th – 9th last chaetiger | • Stained cells forming patches (faint or distinct) on both sides of the centre | • NSP | • 2 stacked bars on either side of the central line<br>• Few stained cells on both sides of the centre | • NSP |
| 8 & 3rd last chaetiger | • NSP<br>• Stained cells forming patches (faint or distinct) on both sides of the centre | • NSP | • 2 stacked bars on either side of the central line<br>• Few stained cells on both sides of the centre | • NSP |
| 2nd last chaetiger | • NSP | • NSP | • NSP | • NSP |

| | | | | |
|---|---|---|---|---|
| | • Stained cells forming patches (faint) on both sides of the centre | | • Few stained cells on both sides of the centre | |
| Last chaetiger | • NSP | • NSP | • NSP | • NSP |
| Pygidium | • Distinct stained cells covering the entire surface, except along the edge around the anus & the outermost edge of the pygidium | | • Distinct stained cells covering the entire surface, except along the edge around the anus & the outermost edge of the pygidium | |

# CHAPTER FOUR
# DISCUSSION

## 4.1. Clarification of *Polydora websteri* taxonomy

*Polydora websteri* from South Africa is monophyletic with *P. websteri* from Australia, China and Japan; but not with *P. websteri* from wild molluscs in the USA. South African specimens match the morphological description and staining patterns of the *P. websteri* lectotype (Radashevsky, 1999), and descriptions of the species from Australia, New Zealand, China, Japan and the east, west and gulf coasts of the USA, North America (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Blake & Kudenov, 1978; Radashevsky, 1999; Sato-Okoshi, 1999; Sato-Okoshi et al., 2008; Read, 2010; Sato-Okoshi & Abe, 2013; Ye et al., 2017; Rice et al., 2018; Martinelli et al., 2020). Specimens collected from wild molluscs near the type locality in Rhode Island, and therefore presumed to be the true *P. websteri*, clearly differed morphologically from the lectotype of *P. websteri* (Radashevsky, 1999), and morphologically and genetically from specimens collected in Australia, China, Japan, South Africa and the USA, North America. Instead, these samples were morphologically similar to *Polydora onagawaensis* from Japan, while genetic data confirmed that they were reciprocally monophyletic. Thus, concluding that specimens collected in Australia, China, Japan and South Africa are *P. websteri*, while those collected from wild molluscs in Rhode Island, thought to represent the 'true' *P. websteri* had in fact been misidentified.

The results support the preliminary study by Williams (2015) in terms of retrieving two species groups that correspond with specimens from Australia, China, Japan and South Africa clustering within the *P. websteri* clade, and specimens from Rhode Island clustering together with *P. onagawaensis* from Japan. The confusion between *P. websteri* and *P. onagawaensis* in the USA reinforces the difficulty in identifying pest polydorids using only morphology and highlights the problems which can result from such misidentifications. The *P. onagawaensis* specimens examined in this study were collected in Rhode Island more than a decade before *P. onagawaensis* was described in Japan, which may support the conclusion by Teramoto et al. (2013) that the species may not be indigenous to Japan. Confirming the identity of *P. websteri* in Australia, China, Japan, South Africa and the east, west and gulf coasts of the USA, shows that it is the second most widespread polydorid pest of aquaculture known, after *Polydora hoplura* (Sato-Okoshi et al., 2017). The distribution may be even wider if the identity of *P. websteri* from South America and Europe is confirmed; this may help to better understand the global route of invasion and consequently assist with preventing or at least minimising further spread.

### 4.1.1. Source population of *Polydora websteri* into South Africa

Haplotype sharing and lack of genetic differentiation among populations of *P. websteri* from geographically distant locations confirms anthropogenic transportation of the species into South Africa. Furthermore, this haplotype sharing of *P. websteri* populations from different countries has undoubtedly resulted from the repeated movement of molluscs (Rice et al., 2018), making it difficult to identify the source of the population of South Africa.

The high haplotype sharing and low genetic divergences of *P. websteri* specimens from Australia, China, Japan, Hawaii, South Africa and the USA suggests that all populations globally arose from the same source populations, either directly or indirectly via already invaded populations that serve as steppingstones (Figure 4.1). From among the countries with which *P. websteri* specimens in South Africa share haplotypes, South Africa has only imported oyster spat from Namibia and the USA (Haupt et al., 2010a, 2012; Williams et al., 2016), which suggests that these countries may have been sources of the local populations of this species. However, oyster farmers in South Africa continue to import oyster spat from Chile, while importation from Europe has stopped (Haupt et al., 2012). Although the presence of *P. websteri* in these regions have not been confirmed, it may have been transported there via ship ballast water, as fouling on hulls and/or infested mariculture imports from North America, where it went undetected while serving as a 'ghost population' (Hirsch et al., 2019) and source of *P. websteri* into South Africa. Additional sampling in Chile and Europe could clarify this.

*Polydora websteri* has always been accepted to be indigenous along the east coast of North America (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Radashevsky, 1999; Read, 2010). As such, it is therefore not surprising that of the populations included in the genetic analyses in this study, those from the east coast of the USA had the highest haplotype and nucleotide diversity for the COI dataset (Loosanoff & Engle, 1943; Blake, 1969, 1971; Foster, 1971; Radashevsky, 1999; Read, 2010). Recently Rice et al. (2018) showed that the genetic diversity was greater among Chinese than the USA populations and suggested *P. websteri* originated from Asia and was introduced to the USA waters via the importation of *Crassostrea gigas* from Japan. However, according to Rius et al. (2015), the introduced range often displays genetic diversity signals that are similar or higher than that of the indigenous range. *P. websteri* populations from China had high haplotype, but low nucleotide diversity, indicating a bottleneck or founding event (Lowe et al., 2004), which shows that Chinese populations may have been recently introduced. Additionally, the higher genetic diversity indices in China compared to the USA showed by Rice et al. (2018) are most likely due to either (1) larvae coming from multiple diverse source populations from the indigenous range (USA) or (2) introductions come from a few highly diverse source populations (USA) (Rius et al., 2015). Alternatively, the same argument highlighted above can be said if *P. websteri* was

indigenous in China, as farm populations could receive larvae from either (1) multiple, diverse wild source populations in China or (2) a few highly divergent wild populations in China. However, sampling localities in China are too limited to infer source population and invasion routes and therefore can only be investigated with more extensive sampling from both the USA and Asia. Furthermore, the indigenous range is characterised by genetically structured populations, while the introduced range consists of diverse but highly homogenous populations (Simon-Bouhet et al., 2006; Rius et al., 2012). These patterns were observed in the phylogenetic trees and the diversity indices of this study and suggest that *P. websteri* was introduced to China and is indigenous to the USA. However, we cannot really be sure, as *P. websteri* populations from China and the east coast of the USA were sampled from farmed molluscs only and wild populations were not available to be examined and therefore should be added in future studies to resolve this problem.

The doubt regarding the indigenous range of this polydorid species is not unique and has also arisen regarding two other important pests of mariculture, *P. hoplura* and *Polydora neocaeca* (Radashevsky & Migotto, 2017; Malan et al., 2020). Malan et al. (2020) noted the importance of identifying the indigenous range of pest polydorids as it is vital for understanding and managing their spread. Therefore, it was recommended that future investigations must include samples from their full distribution range, from both wild and cultured molluscs, to not only clarify the species' indigenous range, but also the possible invasion history. Therefore, determining the indigenous range of *P. websteri* should not be based on genetic data from specimens collected only on mollusc farms. Additional sampling of both wild and farmed populations from the USA and Asia (Rice et al., 2018), from additional locations in Asia (including China and Japan), and using both mtDNA and nuDNA markers is recommended for future studies to determine the indigenous range of *P. websteri*.

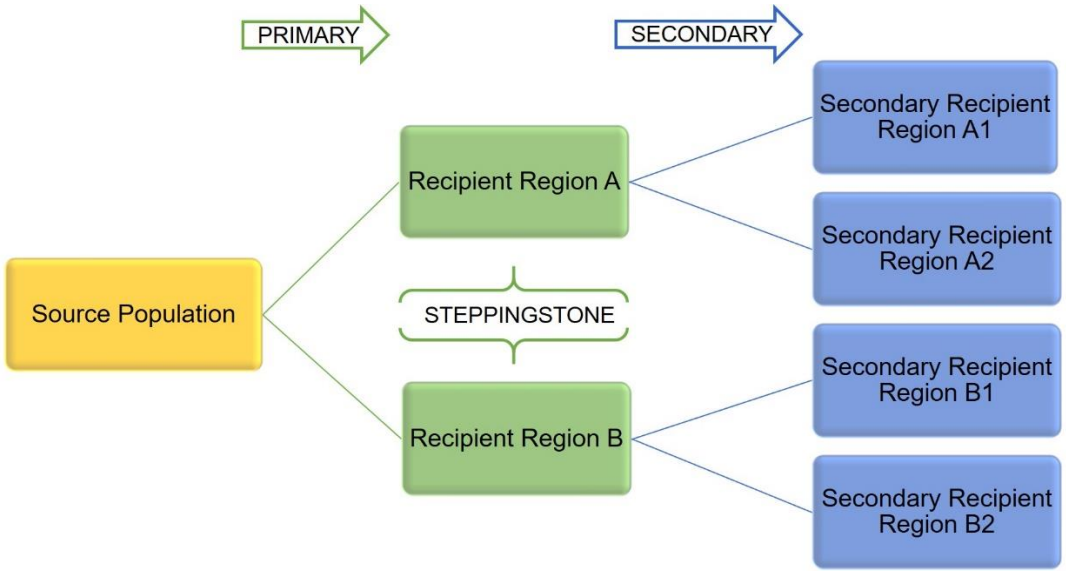


**Figure 4.1:** Illustrating the hypothetical movement of non-indigenous polydorids from a common source population directly (primary) to multiple recipient regions, resulting in high levels of haplotype sharing, followed by the recipient regions serving as steppingstones for the movement to secondary recipient regions, resulting in a secondary movement from the source population, subsequent high levels of haplotype sharing

### 4.1.2. Invasion potential of *Polydora websteri*

The proposed unified framework for biological invasions in Blackburn et al. (2011) divides the invasion process of non-indigenous species (NIS) into four stages; transport, introduction, establishment and spread. However, this framework needs to be modified when interpreting accidental introductions and spread of NIS that are associated with the intentional movement of host species within the mariculture context (Figure 4.2 and Table 4.1). Once a species has been transported to a new geographical location and passes on to the next stages in the framework, the invasion potential of that species increases, until it becomes invasive (Figure 4.2).

Among the four non-indigenous polydorid pest species infesting farmed molluscs in South Africa, *P. hoplura* and *Boccardia proboscidea* can both be considered invasive (Robinson et al., 2016). Understanding the manner in which they were introduced and spread can provide insight into how the recently introduced *P. websteri* and *P. neocaeca* arrived here, may be spread, become invasive, and how these processes may be halted (David et al., 2016). Therefore, these species will be discussed within the context of the modified framework (Figure 4.2).

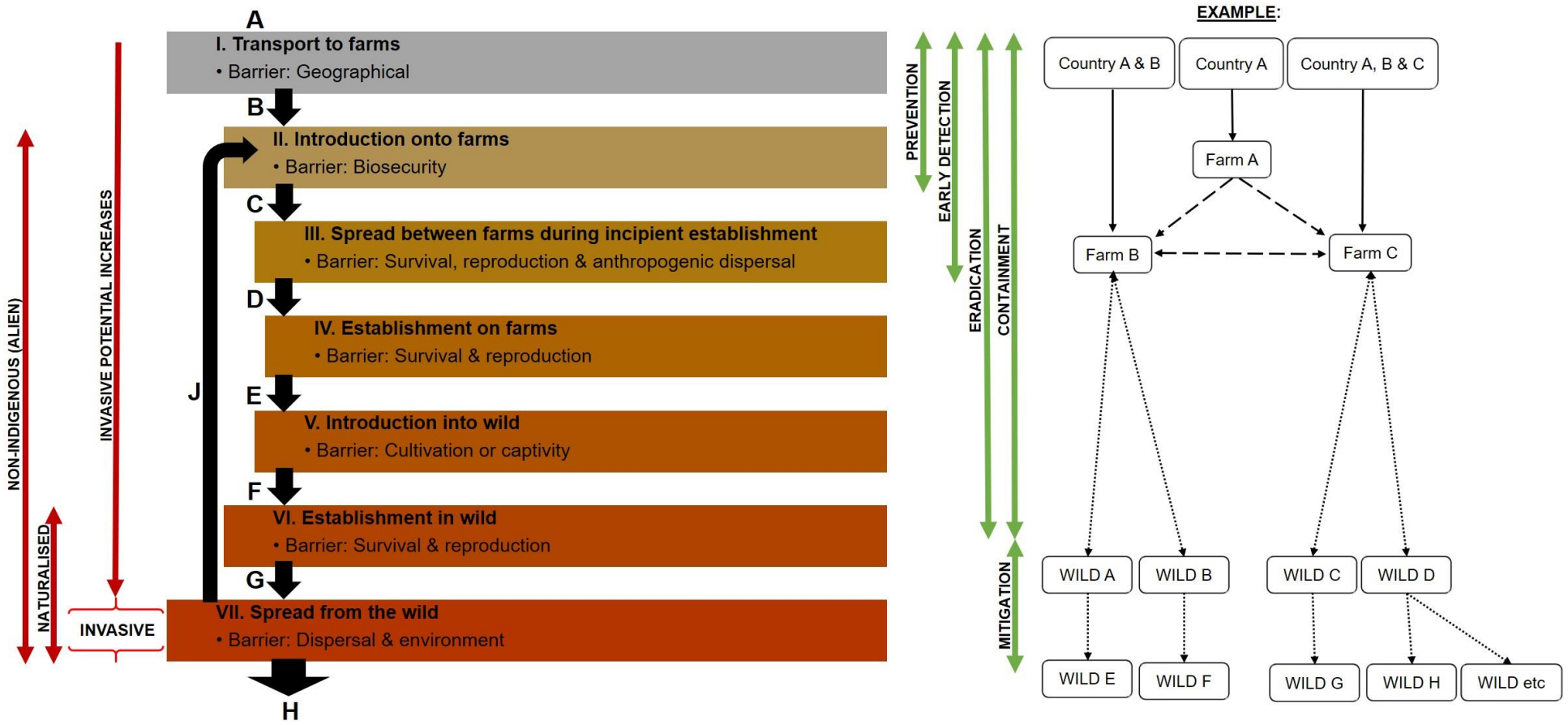


**Figure 4.2:** Framework for biological invasions (Blackburn et al., 2011) modified for invasions of marine species facilitated by movement of cultured hosts. The invasion process can be divided into stages, within each stage there are barriers that need to be overcome for a species or population to pass onto the next stage, the terminology (red arrows) depends on where in the invasion process the species have reached, and that different management interventions (green arrows) apply at different stages. The black arrows describe the movement of species along the invasion framework with respect to the barriers, and the letters associated with the arrows relate to the categorisation of species with respect to the invasion pathway given in Table 4.1 (below). The example represents different pathways (Williams et al., 2016) which may contribute to the dispersal and population structure, Farm A represents a nursery and Farm B and C represent grow-out operations, arrows represent the direction of movement via transport onto a farm with cultured hosts (represented by solid lines), anthropogenic spread bewteen farms with cultured hosts (represented by dashed lines) and spread via natural dispersal (represented by dotted lines)

**Table 4.1:** Categorisation scheme for invasive species proposed by Blackburn et al. (2011) and an interpretation of this for marine invasions via accidental introduction through the intentional mariculture introductions; the information required in order to place species within the unified framework

| | Category | Interpretation for marine systems |
|---|---|---|
| FARM | A | Not introduced; no evidence that the species has been translocated from its indigenous range or that it has arrived in a novel range |
| ↓ | B | Individuals transported beyond limits of indigenous range |
| ↓ | C | NIS unintentional introduction to mariculture facilities with minimal or no biosecurity protocols; unable to survive and form self-sustaining populations |
| ↓ | D | NIS surviving and spread unintentionally between mariculture facilities on the intentionally spread hosts, thus anthropogenic dispersal from original source of introduction; no reproduction or reproduction occurring; population not self-sustaining |
| ↓ | E | NIS farmed populations survive, reproduction occurring and form self-sustaining populations |
| WILD | F | Individuals from the NIS farmed populations released from mariculture facilities and introduced into the wild, possibly at multiple locations; these wild NIS are unable to survive, reproduce and form self-sustaining populations |
| ↓ | G | Naturalised populations survive, reproduce and form self-sustaining populations in the wild, i.e. outside of captivity or culture |
| ↓ | H | Invasive species support self-sustaining populations with individuals demonstrating natural dispersal (via larvae) from the wild source population. It can be difficult to tell the difference between numerous sites of primary introduction vs secondary spread. |
| ↓ | J | Invasive wild populations spread via natural dispersal (larvae) from wild to mariculture facilities, re-infesting mariculture stock |

#### 4.1.2.1. Transport and introduction onto mariculture farms

In the unified framework for biological invasions (Blackburn et al., 2011), transport of a species beyond the limits of its indigenous range (overcoming geographical barriers) is the first stage of invasion (Figure 4.2 I, B and Table 4.1 B). Anthropogenic vectors that have probably played a role in transporting *P. hoplura*, *B. proboscidea* and *P. neocaeca* to South Africa include infested mariculture imports, ship ballast water and hull fouling (Simon et al., 2006, 2009, Haupt et al., 2010b; David et al., 2016; Williams et al., 2016; Malan et al., 2020).

*Polydora neocaeca* was probably transported into the wild and onto an oyster farm via different anthropogenic transport vectors during different events. This NIS was first recorded in 2011 on wild scallops (*Pecten sulcicostatus* Sowerby II, 1842) in False Bay, close to a shipping port but far from mollusc farms, and may therefore have been transported through ship ballast water and hull fouling (e.g. transportation of *P. hoplura* to South Africa (Williams et al., 2016)). Later in 2017, *P. neocaeca* was recorded on cultured *C. gigas* in Port Elizabeth on the east coast of South Africa, more than 700 km away from False Bay. The occurrence of *P. neocaeca* in Port Elizabeth probably resulted from the transport of infested oyster spat from Oranjemund, Namibia, as the oysters originated from there (Malan et al., 2020). By contrast, *P. websteri* has not been found in the wild, and therefore it is unlikely that it could have been transported via ship ballast water or hull fouling to South Africa (Williams et al., 2016). It is more likely that the transport of *P. websteri* resulted from the movement of infested molluscs for mariculture, as this has been shown before (Bailey-Brock & Ringwood, 1982; Rice et al., 2018). The genetic signal for *P. websteri* and *P. neocaeca* is most similar to *B. proboscidea* in South Africa (David et al., 2016; Malan et al., 2020), which therefore suggests that they were and are transported via infested oyster imports.

Once a pest such as *P. websteri* has been transported via mariculture stock to a new geographical location, it may be unintentionally introduced as a NIS, onto mariculture facilities with limited or no biosecurity barriers (Figure 4.2 II), an additional barrier in the modified framework (Figure 4.2 C and Table 4.1 C).

#### 4.1.2.2. Spread between and establishment on mariculture farms

Under the standard framework, a NIS is recognised as ‘established’ when individuals survive in the introduced location, reproducing and forming self-sustaining populations before they escape from the point of establishment and spread via natural dispersal (Blackburn et al., 2011; Robinson et al., 2016). The modified framework identifies the incipient establishment stage when NIS survive and possibly reproduce, but do not form self-sustaining populations (Figure 4.2 III, D and Table 4.1 D). However, an introduced pest of mariculture can spread via anthropogenic means (e.g. stock exchange (Haupt et al., 2012)) between farms, before

forming a self-sustaining population on the farm and escaping. This is a likely explanation for the spread of *B. proboscidea*, which was first recorded on a mollusc farm on the south coast of South Africa in 2004, and further recorded on four mollusc farms on the west and six on the south coast by 2009, while no specimens were detected in the wild at that time (Simon et al., 2009, 2010; Boonzaaier et al., 2014). By 2013, this NIS was recorded at seven wild sites on the west and south-west coast of South Africa, although only one site was at some distance from abalone farms (David et al., 2016). This later detection in the wild may be due to a lag in establishment outside farms or due to sampling, as Simon et al. (2010) extracted worms from molluscs collected close to abalone farms, however, *B. proboscidea* was later recorded inhabiting sediment close to the abalone farms (David, 2015). Therefore, it is uncertain whether this NIS first arrived in the wild and remained undetected in sediment, followed by the spread onto the mollusc farms, or first arrived onto the mollusc farms and then spread to the wild. Either way, once *B. proboscidea* was present on the mollusc farms, the intraregional movement of infested molluscs for mariculture, allowed the NIS to spread to additional farms (Simon et al., 2009).

Similar to *B. proboscidea*, *P. websteri* has been spread between mollusc farms in South Africa via intraregional movement of infested stock (this study). Both NIS show extremely low genetic diversity even though they occur on farms 800 km or more apart. *P. websteri* has not been detected in the wild yet but was recoded on multiple farms in South Africa. This shows *P. websteri* has spread during its incipient establishment stage on the farms and may establish at multiple locations, without having naturally spread from the point of introduction. However, early detection is still possible during the incipient establishment stage before it has a chance to become a self-sustaining established population on the farms (Figure 4.2 III).

If a NIS is introduced directly into the wild, as may occur if it was introduced via ballast water or hull fouling, it must first become established before it can start spreading naturally in the wild and/or onto a farm (e.g. *P. hoplura* (David et al., 2016; Williams et al., 2016)). *P. hoplura* was first recorded on wild molluscs in 1947 in South Africa before mollusc farming began in South Africa (Haupt et al., 2010a; Williams et al., 2016). Since then, *P. hoplura* spread extensively in the wild, which was further facilitated by multiple introductions (Williams et al., 2016). Therefore, it is possible that different farms were infested independently, but the worms were further spread via the intraregional movement of infested molluscs between mariculture farms (David et al., 2016; Williams et al., 2016). As such, *P. hoplura* is the most widely known non-indigenous, invasive shell-boring pest of mariculture in South Africa (Williams et al., 2016). By contrast, *P. neocaeca* is the most recently detected non-indigenous shell-boring pest species. As this NIS was probably a combined introduction into the wild and onto a farm via different vectors, it must first establish itself in the wild, before it can spread via natural

dispersal. At the same time, the occurrence on the farm means that it may spread between farms in South Africa during its incipient establishment stage just as *P. websteri, B. proboscidea* and *P. hoplura* were spread.

In the modified framework, NIS established on farms (Figure 4.2 IV, E and Table 4.1 E), unlike the establishment stage in the unified framework (Blackburn et al., 2011), are not considered 'naturalised' as they do not have self-sustaining populations outside of captivity or culture (Robinson et al., 2016). *P. neocaeca* and *P. websteri* do not have self-sustaining populations outside of captivity or culture but have the potential to become established on multiple mollusc farms in South Africa, in the same way as *P. hoplura* and *B. proboscidea* have. The implications of NIS establishing on mollusc farms was seen in Hawaii when following the introduction, *P. websteri* rapidly became established on the farms to such an extent that it contributed to the collapse of the aquaculture industry (Bailey-Brock & Ringwood, 1982).

#### 4.1.2.3. Introduction, establishment and spread in the wild

Once a NIS has successfully established itself on mariculture farms and is not contained or eradicated (i.e. appropriately managed), it may escape via movement of larvae (Simon, 2015; Simon & Sato-Okoshi, 2015) and become established in the wild close to multiple mariculture farms (Figure 4.2 V, F and Table 4.1 F). *P. websteri* from South Africa was observed to be poecilogonous, thus has the ability to be introduced to the wild via movement of planktotrophic larvae, much like *B. proboscidea* (Simon, 2015; Simon & Sato-Okoshi, 2015; David et al., 2016).

The modified framework recognises NIS populations established in the wild but has not naturally spread (via larvae) from the point of introduction in the wild as a 'naturalised' species (Figure 4.2 VI, G and Table 4.1 G). Once *P. websteri* reaches the establishment stage in the wild, eradication is still possible under some circumstances (Figure 4.2), but may be difficult, especially if different host species are infested or the worm becomes widely established (Culver & Kuris, 2000).

The final stage in the modified framework is spread via natural dispersal of the wild invasive populations (Figure 4.2 VII, H and Table 4.1 H). David et al. (2016) showed that establishment and natural dispersal of polydorids are mainly facilitated by larval development mode, similar to that demonstrated for *P. websteri* (see also Simon & Sato-Okoshi (2015)). Additionally, *P. websteri* has survived in multiple oceans and currents, as shown by its worldwide distribution (Figure 1.1), thus emphasizing its high potential to become established and spread to various wild sites along the southern African coastline (see also *B. proboscidea* (David, 2015)). This means that if *P. websteri* becomes established in the wild it will be able to disperse and become

invasive (Simon, 2015; Simon & Sato-Okoshi, 2015). Furthermore, farmed molluscs would be vulnerable to re-infestation from wild populations (e.g. *P. hoplura* (David et al., 2016; Williams et al., 2016)) (Figure 4.2 VII, J and Table 4.1 J). However, *P. websteri* in South Africa has not yet been detected in the wild, thus it is vital to eradicate this non-indigenous pest before it spreads to the wild (Figure 4.2 and Table 4.1), and therefore, appropriate management strategies are important. These will be discussed later.

## 4.2. Management and sequence library of shell-boring polydorid pests

### 4.2.1. Importance of management in South Africa

The increasing pressure on marine biodiversity due to invasive species in South Africa increases the need to appropriately manage these NIS and ultimately prevent further invasions (Mead et al., 2011; Robinson et al., 2020). The impacts on recipient ecosystems vary according to species and the ecosystems into which they are introduced (Blackburn et al., 2014). *P. websteri* has been known to cause negative impacts to molluscs, both farmed and wild stock (e.g. Lunz, 1941; Hopkins, 1958; Turner & Hanks, 1959; Bailey-Brock & Ringwood, 1982; Bergman et al., 1982; Read, 2010; Rice et al., 2018; Martinelli et al., 2020), resulting in significant changes to environmental and socio-economic parameters (discussed in subsection 1.2.1).

The impacts of *P. websteri* and other shell-boring polydorid species on the environment and economy in South Africa increases the need for appropriate management. The main legislative platform that guides the management of NIS in South Africa is the *National Environmental Management: Biodiversity Act*, No. 10 of 2004 (NEM:BA) within the framework of the *National Environmental Management Act*, No. 107 of 1998. Chapter 5 of the NEM:BA describes steps to prevent unauthorised introduction and spread of NIS, including their management and control, to mitigate harmful environmental impacts (NEM:BA, No. 10 of 2004). Principles under section 2 (3) of NEMA (No. 107 of 1998) requires developments, including mariculture operations, to be socially, environmentally and economically sustainable. This includes the accountability and responsibility principle in section 2 (4p), which mentions that the cost of remediating the negative impact must be paid for by those responsible for harming the environment (NEMA, No. 107 of 1998). Section 2 (4) of NEMA (No. 107 of 1998) requires the sustainable developments to apply a risk aversion and precautionary approach under uncertain conditions. Uncertain conditions apply to the mollusc culture industry during the importation of spat or the intraregional movement of stock, since several studies (Simon et al., 2009; Haupt et al., 2010b, 2012; Williams et al., 2016; Malan et al., 2020; current study) have now presented evidence to link the intraregional spread of these non-indigenous polydorids to the mollusc culture industry in South Africa. Implementing stricter biosecurity protocols to prevent NIS being introduced onto and spread between farms is an example of applying the

risk aversion and precautionary approach. Additionally, the duty of care principle must be adopted for the mollusc culture industry to act with due care to avoid negative impacts on coastal resources and environments (NEMA, No. 107 of 1998). The mollusc culture industry could implement this principle by identifying and regularly monitoring polydorid species present on their farms. This way the industry acts with due care, since through early identification of a non-indigenous polydorid pest species, they can eradicate the species or implement management strategies before it spreads, thus avoiding negative impacts on the industry, coastal resources and environments. The mollusc culture industry pays attention to potential contagious viruses that may be transmitted via the oysters and thus fulfilling expectations by NEM:BA (No. 10 of 2004) and principles stipulated in NEMA (No. 107 of 1998). This study recommends that the mollusc culture industry shows the same level of precaution and duty of care towards polydorid pests.

Accurate identification of pest species is the first step of management, as different management strategies apply if the species is indigenous or non-indigenous, irrespective of whether it is new to science or not. If the pest species is indigenous, mariculture stocks are in constant danger of re-infestation from wild populations and farmers need to manage their stock accordingly, thus it is easier to manage NIS compared to indigenous pest species. By contrast, if a non-indigenous pest has been detected early on the first farm responsible for the importation, and still restricted to that farm, it could be more easily controlled or possibly eradicated through the culling of infested stock (Simon & Sato-Okoshi, 2015). This will prevent the spread and establishment on multiple farms (Figure 4.2 III). However, invasive species are often only identified once they become problematic. If these non-indigenous shell-boring polydorid pests become invasive in the wild, consequent eradication attempts on mollusc farms will be futile as they would be at constant risk for re-infestation from the wild populations (Figure 4.2 J), as is the case for *P. hoplura* in South Africa (Williams et al., 2016). It is, therefore, vital that these pests are detected early by timeous and accurate identification to prevent, or control further spread because once a NIS progresses towards the invasive stage, opportunities for eradication and containment decrease significantly.

### 4.2.2. Facilitation of management in South Africa

Management strategies for polydorids can be divided into (1) preventing further introductions and (2) containing and monitoring non-indigenous shell-boring polydorid pests already in South Africa.

Preventing, by reducing the risk of introductions of non-indigenous polydorid pests from being transported into South Africa is far more cost effective than the future potential costs of impacts and management (IUCN, 2018). A precautionary approach should be taken when importing

molluscs for mariculture, given the uncertainty of the identity and the impacts on biodiversity of the NIS (NEMA, Act 107 of 1998). Furthermore, it is important to prevent repeated introductions of non-indigenous polydorid pests already in South Africa. For example, additional introductions of *P. websteri* and *P. neocaeca* into South Africa from different source populations may lead to increased genetic diversity, which is likely to influence the invasiveness in this species (e.g. *P. hoplura* (Williams et al., 2016)), and minimise effectiveness of management strategies.

Cost effectiveness of prevention or rapid response to remove new species greatly outweighs that of ongoing management (IUCN, 2018). Culver & Kuris (2000) successfully eradicated an established non-indigenous sabellid polychaete pest in California, *Terebrasabella heterouncinata* Fitzhugh & Rouse, 1999, by removing a susceptible host species. They suggested that the eradication success resulted from early detection while the pest was still spatially restricted, followed by a rapid response that minimised the chance of spread. However, early detection cannot occur if the identity of the pest species is undetermined, thus regular and frequent monitoring is the key to early detection of new NIS. This also shows that containing further intraregional spread of non-indigenous polydorid pests between mollusc farms within South Africa is important. However, because the oyster culture industry relies on the movement of stock (Haupt et al., 2010a), containment is difficult, therefore culture stocks should be carefully monitored.

If proper precautionary measures and monitory checks for pest species are not thorough, it is possible that *P. websteri* (and *P. neocaeca*) may spread in the wild and re-infest nearby farmed molluscs, causing recurring problems as demonstrated for *P. hoplura* (Williams et al., 2016). Therefore, early detection through identification while the NIS is still spatially restricted, is important. However, South African mollusc farmers usually identify all shell-boring polydorids as '*Polydora* species' (pers. obs.), even though the polydorid group comprises nine genera. Furthermore, mollusc farmers usually do not consider the identities of individual species; rather they concentrate on the shell-boring activity which directly affects the health and eventually yield of mollusc stocks (Royer et al., 2006). This is problematic, because overlooking the fact that a '*Polydora* species' may be a NIS would affect management, as it is easier to manage an identified newly arrived NIS than an indigenous or established NIS, that may re-infest cultured mollusc stock. Identification of polydorid pest species on mollusc farms can coincide with weekly and monthly monitoring requirements of biotoxins and microbiological organisms, for which they may shuck the mollusc before testing the flesh (South Africa, Department of Forestry and Fisheries, 2016). Since these shells are discarded, no additional molluscs would need to be sacrificed to identify any polydorids present. However, it is time consuming removing whole individual worms and identifying them, especially if the farmers do not have

updated descriptions on shell-boring pest polydorids and access to the literature. A faster approach is to identify polydorids using DNA barcoding, or meta-barcoding (Borrell et al., 2017; Williams et al., 2017); collecting samples for DNA meta-barcoding would be the least time consuming as polydorids would not have to be removed from shells individually. However, with meta-barcoding no reference samples are retained for individual species, should a new species be found.

### 4.2.3. Sequence library

To facilitate rapid identification while avoiding misidentification and confusion, detailed descriptions are essential for polydorid identifications (Read, 2010). However, accurate species identification is hampered if the taxonomy of the pest species is unresolved, or if several species are morphologically similar to each other. For example, *P. websteri* is morphologically similar to *Polydora ciliata* and has probably been mistaken for it on numerous occasions (Blake, 1971). To overcome these difficulties in morphological identification, modern species identifications have combined morphology with genetics (e.g. Sato-Okoshi et al., 2017; Ye et al., 2017), as was done in this study. This is further facilitated by the development of a library of sequences for easy identification using molecular data only (Williams et al., 2017). With a reliable library of sequences for pest species, specimens can be identified much faster and more accurately, even when taxonomic expertise is lacking (Stoeckle & Hebert, 2008). However, the success of such a library depends on the correct identification of the species linked to the sequences that are available.

Of the 21 identified shell-infesting polydorid pest species that are associated with cultured or commercially harvested molluscs and the available genetic data on GenBank have increased from three species in 2010 (Walker, 2011) to 13 species (GenBank, 2020). Additionally, 17 of these pest species are NIS in part of their distributions. This provides additional impetus for expanding on the number of species for which sequences are available, including the number of sequences for the same species but from different locations within their distribution.

### 4.2.4. Morphological versus molecular identification in the context of mariculture

Using only molecular data for easy identification during routine testing may be too expensive (da Silva & Willows-Munro, 2016), especially for farmers in South Africa. However, the African Centre of DNA Barcoding provides relatively inexpensive DNA barcoding services in South Africa (ACDB, 2020). Additionally, based on the experience in generating sequences and examining the morphology of polydorid worms in this study, it is more time consuming (± 8 hours) for a trained person to genetically identify a known species compared to identifying the species morphologically (± 1 hour). Thus, in the long run it may be cheaper for farmers to learn to morphologically identify the polydorids themselves. As such, an updated taxonomic key to

identify shell-boring pest polydorids in South Africa will aid the farmers in morphological identification. Then, if they cannot identify a species or are uncertain, they can have the specimen identified molecularly. However, Read (2010) showed that similar morphology such as between *P. websteri* and *P. neocaeca* makes identification difficult, especially regarding pigmentation on palps and if the material quality is poor. Preservation methods and light exposure may also alter the pigmentation patterns (Read, 2010; Walker, 2011), making pigmentation patterns an unreliable method for species identifications. This variation in palp and body pigmentation, and wear and tear on chaetae make identification difficult when using only morphological data.

Using morphology alone may also result in overlooking cryptic invasions, which delays the detection of an invasive NIS and hampers timeous management strategies. Cryptic invasions can be the introduction and spread of non-indigenous lineages within the indigenous range of the species or the unnoticed invasion of NIS due to the misidentification as an indigenous or another invasive species (Morais & Reichard, 2018). Malan et al. (2020) suggested that *P. haswelli* and *P. neocaeca* reflect cryptic invasions. *P. neocaeca* was described as indigenous to the east coast of the USA (Williams & Radashevsky, 1999), but it is reciprocally monophyletic with *P. haswelli* (Malan et al., 2020),a species described in Australia and occurring widely on cultured molluscs in Australasia (Blake & Kudenov, 1978; Read, 2010). Thus, the native range of *P. neocaeca* is unknown and it may really be indigenous to Australia, Asia or an as yet unknown region (Malan et al., 2020). Similarly, *P. hoplura*, was first described from Italy but mistaken for a new species and described as indigenous *P. uncinata* in Japan (Radashevsky & Migotto, 2017; Sato-Okoshi et al., 2017). Using molecular data would have enabled quick identification of the species and avoided decades of confusion relating to each species. Therefore, if no sequences are available for a newly detected invading species, it could lead to cryptic invasions, diminishing the understanding of the full invasion history of a species.

### 4.3. Overview of shell-boring polydorid pests in South Africa

Simon & Sato-Okoshi (2015) found that in most countries where molluscs are farmed, the molluscs are infested by a mixture of indigenous and non-indigenous pests, with the number of non-indigenous pests in a country generally increasing with the number of species farmed in that country. In South Africa the number of non-indigenous pests is disproportionately high compared to the number of mollusc species farmed. Since Day (1967) reported *P. hoplura*, *Boccardia pseudonatrix, Dipolydora capensis* (Day, 1955) and *Dipolydora* cf. *armata*, the number of shell-boring polydorid pests on farmed molluscs in South Africa have increased to five, with the edition of *B. proboscidea* (Simon et al. 2006) and further to seven when *P. websteri* was recorded in 2009 (as *P.* cf. *ciliata* (Simon, 2011)) and *P. neocaeca* in 2011

(Malan et al., 2020). Of these, only *B. pseudonatrix* and nominal species *D. capensis* are indigenous to South Africa (Day, 1967), whereas *D.* cf. *armata* in South Africa should be considered an unresolved cosmopolitan (*sensu* Darling & Carlton, 2018) until its identity is confirmed. It could therefore be argued that there would be significantly fewer problems related with shell-boring polydorids on South African mollusc farms if there are fewer NIS.

To date, taxonomic information regarding the shell-boring species found in South Africa are available in multiple publications (Day 1967; Simon et al. 2006, 2010; Simon 2011; Malan et al. 2020) but molluscs farmers may have limited access to research literature. Here we collate all this information into a key which we hope will facilitate rapid identification by mollusc farmers. This should assist farmers in monitoring the shell-boring polydorid pest species as a first step towards identifying species that may be new to farms but already introduced into the country, and even to recognise new NIS, that are not included in this key.

### 4.3.1. Taxonomic key to shell-boring polydorids pests in South Africa, including indigenous species (IS), non-indigenous species (NIS), unresolved cosmopolitan species (?) and those not known to cause severe shells damage (*)

1. Branchiae from chaetiger 2 onwards, absent from chaetiger 5……..….2
- Branchiae from chaetiger 6–12 onwards, notopodial lobes may be present on chaetiger 5……..……..…..…..…….3
2. 1 type of modified spines on chaetiger 5……..…….……. ……..……...*Boccardiella* (No known shell-borers in South Africa)
- 2 types of modified spines on chaetiger 5 (i) simple, falcate hook (ii) expanded end usually bearing cusps or bristles…….……..…... *Boccardia*, 5
3. Chaetiger 5 moderately modified: neuropodial lobes present, 2 types of modified spines usually arranged in U or J- shape (i) simple, falcate hook (ii) usually pennoned (bent)……....*Pseudopolydora* (No known shell-borers in South Africa)
- Chaetiger 5 greatly modified: notopodial lobes absent, 1 type of modified spines usually arranged in curved row with companion chaetae…..……...……..4
4. Notochaetae absent from chaetiger 1; ~90° between shaft and main tooth, ~ 45° between teeth of hooded hooks that have distinct constriction on shaft.....…*Polydora*, 6
- Notochaetae on chaetiger 1; >90° between shaft and main tooth, < 45° between teeth of hooded hooks without constriction on shaft……...……...…*Dipolydora*, 7
5. Up to 45 mm long and 2 mm wide at chaetiger 5 for 150 chaetigers; modified spines on chaetiger 5 (i) bristle-topped (ii) simple falcate; promonent prostomium rounded or weakly incised; dark pigmentation along edge of prostomium, dorsal side of peristomium

and anterior chaetigers (most intense on 1 and 2); pygidium fleshy plug-like disc with dorsal notch, may be divided into four lobes……..……..……....*B. proboscidea* (NIS)

- Up to 20 mm long for 83 chaetigers; modified spines on chaetiger 5: (i) swollen tip, raised central cone and raised outer ridge ii) simple falcate; prostomium strongly bilobed; luminescent spots on dorsal anterior when alive; brown pigmentation on anterior part of prostomium and caruncle; darker palps with white bars; mid-dorsal ridge from chaetiger 5 to mid-chaetiger 8; pygidium reduced, forming pair of flattened cushions……..……
……...*B. pseudonatrix* (IS)

6. Up to 40 mm long and 2 mm wide at chaetiger 5 for 180 chaetigers; modified spines on chaetiger 5 falcate with flange; prostomium notched; occipital antenna present only in large specimens; dark pigmentation along prostomium, peristomium and sometimes pygidium; palps often with black bars; posterior notopodia with 1–2 heavy recurved spines and capillary chaetae; pygidium cup-shaped with dorsal notch….*P. hoplura* (NIS)

- Up to 30 mm long and 1 mm wide at chaetiger 5 for 100 chaetigers; modified spines on chaetiger 5 falcate with prominent flange; prostomium rounded to bilobed; occipital antenna absent; without dark body pigmentation; palps with contiuous black line on feeding groove edge, sometimes faded looking like discontinuous line; posterior notopodia with capillary chaetae only; pygidium cup to disc-shaped, with dorsal notch, distinctly wider than 5th last chaetiger……..……..…….*P. websteri* (NIS)
- Up to 32 mm long and 1 mm wide at chaetiger 5 for 97 chaetigers; modified spines on chaetiger 5 falcate with small flange; prostomium bilobed; occipital antenna absent; variable black pigmentation dorsally on prostomium along caruncle, patches on peristomium, and chaetigers 1–5, may form black line along mid-venter of chaetigers 2–5; palp crossed by 2–11 black bands from base to distal end, pigmentation sometimes absent; posterior notopodia with capillary chaetae; pygidium flaring disc-shaped with dorsal notch……..……....*P. neocaeca* (NIS)

7. Up to 20 mm long for 100 chaetigers; modified spines on chaetiger 5 simple hooks; prostomium rounded; posterior capillary notochaetae accompanied with 2–3 pointed spines; hooded hooks present from chaetiger 7; posterior hooks unidentate, without hoods; pygidium small with 4 lobes……..…….*D. capensis* (IS, *)

- Up to 5 mm long for 24–25 chaetigers; modified spines on chaetiger 5 falcate with collar; prostomium bilobed; posterior notochaetae with retractable stout spines, form funnel when everted and cone when retracted; hooded hooks present from chaetiger 7; 7 pairs of short branchia starting at chaetiger 7; pygidium small cup-shaped with dorsal notch……..……..…….*D.* cf. *armata* (?, *)

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions

The first alternate hypothesis was supported as specimens identified as *Polydora* cf. *websteri* in South Africa are reciprocally monophyletic with *P. websteri* from Australia, China and Japan; and not with supposed conspecifics collected near the type locality in the USA. Furthermore, specimens from South Africa, Australia, China, Japan, and *P. websteri* from Hawaii, Washington State on the west coast of the USA, on the east coast of the USA in Maine, Massachusetts, Maryland and New York, and on the south eastern coast of the USA in Alabama (Rice et al., 2018; Martinelli et al., 2020) formed a monophyletic group. Supplementary to these regions, South African specimens also matched morphological descriptions of those from New Zealand and the gulf coast of the USA, but not the specimens collected from Rhode Island, collected near the type locality in the USA. Instead the latter were morphologically similar to *P. onagawaensis* from Japan.

This study has therefore, completed the first step of pest management of confirming the presence by clarifying the taxonomy of a new non-indigenous species (NIS), *P. websteri*, on oyster farms in South Africa. Additionally, the results have shown that this NIS arrived in South Africa via the transport of infested oysters and has been spread between mariculture farms through the intraregional movement of infested oyster stock. The discussion has highlighted the importance of eradication while the non-indigenous pest species is still spatially restricted. Thus, to aid future management in identifying shell-boring polydorid pests this study has contributed to the sequence library and has developed an updated cohesive taxonomic key that can be used to identify worms by mariculture practitioners.

## 5.2. Recommendations

This study recommends that future studies confirm the identity of *P. websteri* collected in South America and Europe to help better understand the global route of invasion and consequently assist with preventing or at least minimising further additional transport onto mariculture farms, not only in South Africa but worldwide. Cost effectiveness of prevention greatly outweighs that of ongoing management. As such, the mollusc culture industry in South Africa should act with due care and adopt a precautionary approach regarding the importation of molluscs for mariculture, as the molluscs may be infested with non-indigenous shell-boring polydorid pests. Therefore, this study recommends that the industry in South Africa should implement a monitoring plan to facilitate the rapid identification of these shell-boring polydorid pests, by using a combination of the taxonomic key provided in this study and genetic identification methods.

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# APPENDIX A

**Table A1.1:** Descriptions of *Polydora websteri*

| Reference | Loosanoff & Engle (1943) | Blake (1969) | Foster (1971) | Blake (1971) | Blake & Kudenov (1978) | Radashevsky (1999) | Sato-Okoshi (1999) | Surugiu (2005) |
|---|---|---|---|---|---|---|---|---|
| Country | USA: Connecticut | Canada: Newfoundland to USA: Gulf coast, Mexico: Baja California | Canada: Newfoundland to USA: Gulf coast, Mexico: Baja California | Canada: Newfoundland to USA: Gulf coast, Mexico: Baja California | Australia | USA: Connecticut | Japan | Romania |
| Collected from wild (W) or cultured molluscs (C) | W | W, ? | W, ? | W, ? | ? | W | C, W | ? |
| Length (mm) | 20 | 20 | 8.66 | 20 | 10 | 15 | 15 | 20 |
| Width (mm) | ? | ? | ? | ? | 0.5 | 1 at chaetiger 7 | 0.7 at chaetiger 5 | ? |
| Number of Chaetigers | 105 | ? | 47 | 100 | 100 | 104 | 100 | 100 |
| Caruncle extends to chaetiger | End of 3 | 3 | Between 1 to 4 | End of 2 | Mid of 3 | End of 2 | End of 2 | Between 2 & 3 |
| Shape of anterior margin of prostomium | Bilobed | Rounded to weakly incised | Bilobed | Strongly to weakly incised | Weakly bilobed | Bilobed | Rounded/ weakly incised | Weakly incised |
| Occipital antennae | ? | ? | Absent | ? | Absent | Absent | ? | ? |
| Shape of spines on chaetiger 5 | Heavy, falcate, chitinous sheath around one side | Heavy, falcate, lateral flange/sheath | Falcate, lateral flange/sheath | Heavy, falcate, lateral flange/sheath | Falcate, lateral flange | Falcate, lateral flange | Falcate, lateral flange | Falcate, lateral flange |
| Shape of companion chaetae on chaetiger 5 | Pennoned, terminate in acute point | Pennoned | Smooth/frayed distal sheath | Pennoned | Bilimbate | Pennoned | Pennoned, slender with distal wing | Lanceolate |
| Shape of neuropodial hooded hooks | 2 well developed teeth | Bidentate, constriction at shaft | Bidentate, constriction at shaft | Bidentate, in a series, constriction at shaft | Bidentate, constriction at shaft | Vertical row, bidentate, constriction at shaft | Bidentate, constriction at shaft | Vertical row, bidentate, constriction at shaft |
| Angle of tooth/fang | Major tooth at right angle to shaft | ? | ? | Main fang at right angle to shaft | Wide angle between teeth, main fang at right angle to shaft | ? | Main fang at right angle to shaft | Main fang at right angle to shaft |
| Shape of Pygidium | Disc-shaped | Cup-shaped | Flaring disc-shaped | Cup-shaped | Disc-shaped | Cup-shaped | Flaring/ disc-shaped | Cup-shaped |
| Pygidium with dorsal notch | Present | Present | Present | Present | Present | Present | Present | Present |

**Table A1.2:** Descriptions of *Polydora websteri* and *P.* cf. *websteri* (*)

| Reference | Sato-Okoshi et al. (2008) | Bonifácio (2009)* | Read (2010) | Surugiu (2012) | Sato-Okoshi & Abe (2013) | Barros et al. (2017) | Ye et al. (2017) | Rice et al. (2018) | Martinelli et al. (2020) |
|---|---|---|---|---|---|---|---|---|---|
| Country | Australia | Brazil | Australia, New Zealand | Romania (?), Ukraine (C) | Australia (C), Japan (C, W) | Brazil | China | East and south eastern coast of USA, Hawaii | East and west coast of USA |
| Collected from wild (W) or cultured molluscs (C) | C | ? | C, ? | C, ? | C, W | ? | C | C | C |
| Length (mm) | 15 | 14 | 24 | 20 | 18 | 18.4 | 15 | 20 | ? |
| Width (mm) | ? | 0.69 | 1.2 | 1.1 at chaetiger 5 | 1 at chaetiger 5 | 1.2 at chaetiger 5 | 1 at Chaetiger 5 | ? | 1 at chaetiger 7 |
| Number of Chaetigers | ? | 94 | 110 | 116 | 120 | 136 | 100 | 100 | 60 < 100 < |
| Caruncle extends to chaetiger | Mid of 3 | Mid of 2 | Mid of 3 | End of 2 | End of 2/3 | Mid 3 | End of 3/ Mid 4 | 3 to 4 | End 2 |
| Shape of anterior margin of prostomium | Widely bilobed | Bilobed | Weakly bilobed/notched | Weakly incised | Strongly to weakly bilobed | Bilobed | Weakly bilobed/incised | Weakly notched, bilobed | Bilobed |
| Occipital antennae | ? | Absent | Absent | Absent | ? | Absent | Absent | Absent | Absent |
| Shape of spines on chaetiger 5 | Falcate, with lateral tooth | Falcate, lateral flange | Falcate, subterminal lateral flange | Falcate, distally curved, subterminal flange | Falcate, lateral tooth/sheath | Falcate, subterminal lateral flange | Heavy, falcate, lateral flange/sheath on concave side/flange absent | Falcate, later flange | Major falcate, lateral flange |
| Shape of companion chaetae on chaetiger 5 | Pennoned | Bilimbate | Hastate with flat broad blades, frayed tips | Pennoned, slender, more ventral closely adjoining spines | Pennoned, slender | Hastate, flat broad blades, frayed tips | Pennoned | Hastate, interspersed with spines | Pennoned, sometimes exhibiting frayed tips |
| Shape of neuropodial hooded hooks | ? | Bidentate, constricted at shaft | Bifid, shaft constricted (manubrium) | Vertical row, constriction, manubrium at shaft | Bidentate, constriction at shaft | Bidentate | Bidentate, constriction at shaft | ? | Bidentate, constriction at shaft |
| Angle of tooth/fang | ? | ? | Right angles to shaft with wide angle to apical tooth | Main fang at right/acute angle to shaft, wide acute angle to small apical tooth | Main fang at right angle to shaft, acute angle with apical tooth | ? | ? | ? | Approximately right angle between main fang & shaft |
| Shape of Pygidium | Flaring disc-shaped | Disc-shaped | Flat shallow disc-shaped | Cup-shaped | Flaring- to disc-shaped | Disc-shaped | Disc-/cup-shaped | Flared, disc-shaped | Cup-shaped |
| Pygidium with dorsal notch | Present | Present | Present | Present | Present | Present | Present | ? | Present |

# APPENDIX B

**Table B1:** PCR primers, cycling conditions, template DNA and molecular biology grade water volume included in the PCR reaction volume. South African (SA), Australian (AU) and United States of America (USA) specimen samples

| Marker | Code | Direction | Sequence (5'- 3') | Cycle Conditions<br>Williams et al. (2017) | Expected fragment size (base pairs)<br>Williams et al. (2017) | PCR Reaction Volume Includes: | |
|---|---|---|---|---|---|---|---|
| | | | | | | Template DNA | Molecular Biology Grade Water |
| COI | Dorid_COI.3F<br>Dorid_COI.1R<br>Williams et al. (2017) | Forward<br>Reverse | AAGGWATACCTACAGAAAARATACC<br>CTGTGAATAGRGGRAATCAGTTTAT | 1 cycle: 95 °C for 4 minutes;<br>30 cycles: 95 °C for 30 seconds, 50 °C (SA & AU)/ 45 °C (USA) for 30 seconds, 72 °C for 1 minute; 1 cycle: 72 °C for 5 minutes | 684 bp | SA: 3 µl<br>AU: 4 µl<br>USA: 4 µl | SA: 7.5 µl<br>AU: 6.5 µl<br>USA: 6.5 µl |
| Cyt *b* | Cytb424F<br>Boore & Brown (2000)<br>Cytb-bp-876<br>Oyarzun et al. (2011) | Forward<br><br>Reverse | GGWTAYGTWYTWCCWTGRGGWCARAT<br><br>RAAWARRAAGTATCAYTCAGG | 1 cycle: 94 °C for 4 minutes;<br>35 cycles: 94 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 30 seconds; 1 cycle: 72 °C for 7 minutes | 410 bp | SA: 1 µl<br>AU: 3 µl<br>USA: 3 µl | SA: 9.5 µl<br>AU: 7.5 µl<br>USA: 7.5 µl |
| 18S rRNA | 18S-1F1<br>18S-1R632<br>18S-2F576<br>18S-2R1209<br>18S-3F1129<br>18S-R1772<br>Nishitani et al. (2012) | Forward<br>Reverse<br>Forward<br>Reverse<br>Forward<br>Reverse | AACCTGGTTGATYCTGCCAG<br>ACTACGAGCTTTTTAACYGCARC<br>GGTAATTCCAGCTCYAATRG<br>AAGTTTYCCCGTGTTGARTC<br>GCTGAAACTTAAAGRAATTGACGGA<br>TCACCTACGGAAACCTTGTTACG | 1 cycle: 94 °C for 4 minutes,<br>35 cycles: 94 °C for 30 seconds, 54 °C for 30 seconds, 72 °C for 30 seconds; 1 cycle: 72 °C for 10 minutes | 1780 bp | SA: 1 µl<br>AU: 2 µl<br>USA: 2 µl | SA: 9.5 µl<br>AU: 8.5 µl<br>USA: 8.5 µl |

**Table B2:** Sequences of *Polydora websteri*, *Polydora* cf. *websteri* and outgroup taxa used that were obtained from GenBank (2020); east coast (EC), west coast (WC)

| Marker | Species | Accession number | Location | Reference | Primers (F: Forward/R: Reverse) | Number of Base pairs | Primer Reference |
|---|---|---|---|---|---|---|---|
| COI | *P.* cf. *websteri* | KY002987 | Swakopmund, Namibia | Williams et al. (2017) | Dorid_COI.3F/ Dorid_COI.1R | 491 bp | Williams et al. (2017) |
| | *P.* cf. *websteri* | KY002986, KY002988 | Kleinzee, South Africa | Williams et al. (2017) | | 491 bp | Williams et al. (2017) |
| | *P. websteri* | KP231331 | Ningbo, China | Ye & Wang (2015)[1] | | 939 bp | Ye et al. (2017) |
| | *P. websteri* | KR337461 – KR337472 | Yangxi County, Guangdong Province, China | Ye et al. (2017) | X1-FF2/X1-R6; X1-F2/X1-R2 | 853 bp | Ye et al. (2017) |
| | *P. websteri* | MG977704, MG977705, MG977708 | Alabama, EC, USA | Rice et al. (2018) | PwCO1LP-For/ PwCO1LP-Rev | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977702, MG977703 | Alabama & Maine, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977709 | Alabama & Maryland, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977713 | Maine, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977706 | Maine & Massachusetts, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977710 – MG977712 | Maryland, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977707 | Massachusetts, EC, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MG977714 | Hawaii, USA | Rice et al. (2018) | | 794 bp | Rice et al. (2018) |
| | *P. websteri* | MK696582 – MK696585 | New York, EC, USA | Martinelli et al. (2020) | Dorid_COI.3F/ Dorid_COI.1R | 617–658 bp | Williams et al. (2017) |
| | *P. websteri* | MK188730 – MK188736, MK696586 – MK696588 | Oakland, WC, USA | Martinelli et al. (2020) | | 574–654 bp | Williams et al. (2017) |
| | *Boccardia proboscidea* | JX276728 | Canada | Paterson & Gibson (2012)[2] | | 595 bp | |
| Cyt *b* | *P.* cf. *websteri* | KY003020 | Swakopmund, Namibia | Williams et al. (2017) | Cytb424F/ Cytb-bp-876R | 367 bp | Boore & Brown (2000); Oyarzun et al. (2011) |
| | *P.* cf. *websteri* | KY003021, KY003022 | Kleinzee, South Africa | Williams et al. (2017) | | 367 bp | Boore & Brown (2000); Oyarzun et al. (2011) |
| | *P. websteri* | KP231318 | Ningbo, China | Ye & Wang (2015)[1] | | 547 bp | Boore & Brown (2000) |
| | *B. proboscidea* | FJ972548 | Alamitos Bay, California & False Bay Harbour, Washington, USA | Simon et al. (2009) | | 309 bp | Boore & Brown (2000) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18S rRNA | *P*. cf. *websteri* | KY677906 | Swakopmund, Namibia | Williams et al. (2017) | 18S-1F1/18S-1R632; 18S-2F576/18S-2R1209; 18S-3F1129/18S-R1772 | 1716 bp | Nishitani et al. (2012) |
| | *P*. cf. *websteri* | KY677904, KY677905 | Kleinzee, South Africa | Williams et al. (2017) | | 1716 bp | Nishitani et al. (2012) |
| | *P. websteri* | AB705405 | Albany, Australia | Sato-Okoshi & Abe (2013) | | 1771 bp | Nishitani et al. (2012) |
| | *P. websteri* | KP231302 | Ningbo, China | Ye & Wang (2015)[1] | | 1709 bp | Nishitani et al. (2012) |
| | *P. websteri* | AB705402 | Nakatsu, Oita, Japan | Sato-Okoshi & Abe (2013) | | 1771 bp | Nishitani et al. (2012) |
| | *P. websteri* | MK369933 – MK369936 | New York, EC, USA | Martinelli et al. (2020) | 18S-1F1/18S-1R632 | 569–625 bp | Nishitani et al. (2012) |
| | *P. websteri* | MH891513 – MH891517, MH891519 – MH891522, MK695999 – MK696003 | Oakland, WC, USA | Martinelli et al. (2020) | | 614–643 bp | Nishitani et al. (2012) |
| | *Polydora onagawaensis* | AB691768 | North east, Japan | Teramoto et al. (2013) | 18S-1F1/18S-1R632; 18S-2F576/18S-2R1209; 18S-3F1129/18S-R1772 | 1771 bp | Nishitani et al. (2012) |
| | *B. proboscidea* | LC107607 | Sasuhama, Japan | Abe et al. (2016) | | 1768 bp | Nishitani et al. (2012) |

[1]Ye, L. T. & Wang, J. Y. *Patinopecten yessoensis* infested by *Polydora brevipalpa*. Unpublished.

[2]Paterson, I. G. & Gibson, G. D. Adelphophagy and variable larval development in *Boccardia acus* (Annelida: Spionidae): Evidence for a case of poecilogony. Unpublished.

**Table B3:** Best-fit model of evolution for maximum likelihood phylogenetic trees for each target region; trimmed data set with no missing data (T)

| Marker | Best-Fit Model of Evolution for Maximum Likelihood Analysis |
|---|---|
| COI | Tamura 3-parameter + Gamma Distributed with Invariant Sites |
| COI (T) | Tamura 3-parameter + Gamma Distributed with Invariant Sites |
| Cyt *b* | Tamura 3-parameter + Gamma Distributed |
| 18S rRNA | Kimura 2-parameter |
| 18S rRNA (T) | Kimura 2-parameter |

**Table B4:** The minimum intraspecific pairwise distance (Pmin) and the maximum intraspecific pairwise distance (Pmax) values were calculated using Kimura 2-parameter model for species delimitation; trimmed data set with no missing data (T)

| Marker | Pmax | Pmin |
|---|---|---|
| COI | 0.197 | 0.005 |
| COI (T) | 0.207 | 0.005 |
| Cyt *b* | 0.190 | 0.005 |
| Cyt *b* (T) | 0.180 | 0.005 |
| 18S rRNA | 0.015 | 0.001 |
| 18S rRNA (T) | 0.015 | 0.001 |

# APPENDIX C

Family SPIONIDAE Grube, 1850

Subfamily SPIONINAE Söderström, 1920

Genus *Polydora* Bosc, 1802

*Polydora onagawaensis* Teramoto, Sato-Okoshi, Abe, Nishitani and Endo, 2013

(Figures B1, B2, 3.9 B and 3.10 B, C)

*Polydora* sp.: Sato-Okoshi, 1999: 836.

*Polydora onagawaensis*: Sato-Okoshi et al., 2013: 406 – 407, Figure 2; Teramoto et al., 2013: 31 – 45, Figures 2 – 3

*Polydora websteri*: Williams 2015: 3 – 57

**Description**

Complete specimens 5.0 and 9.7 mm long and 0.45 and 0.52 mm wide at chaetiger 5, for up to 63 and 75 chaetigers from USA (n = 2); between 5.7 to 10.2 mm long and 0.35 to 0.70 mm wide at chaetiger 5, for up to 62 to 77 chaetigers from Japan (n = 12). Prostomium anteriorly rounded or weakly bilobed; caruncle extending to end chaetiger 2 or 3 (Figure C1 A); up to four eyes arranged in trapezoid; occipital antennae absent. Body pigmentation brown or black shading may be on prostomium, peristomium, body, palps (Figure 3.10 B, C) and pygidium or absent.

Notochaetae absent on chaetiger 1, only small notopodial lobe (Figure C1 A). Winged capillary notochaetae with postchaetal lamellae on chaetigers 2 to 4 and 6. Capillary notochaetae with postchaetal lamellae on posterior chaetigers reducing in size posteriorly. Winged neurochaetae with postchaetal lamellae increasing in size from chaetigers 1 to 4 and 6. Neurochaetae replaced by bidentate hooded hooks in vertical row on chaetiger 7 (Figures C1 B, C); up to 7 hooded hooks per fascicle, increasing up to 9 in middle chaetigers, decreasing to 1 or 2 on posterior chaetigers. Hooded hooks without companion chaetae; with main fang at < 45° to apical tooth and right angle to shaft; with constriction on shaft (Figures C2 B, C).

Chaetiger 5 modified, approximately twice as large as chaetiger 4 and 6; with superior (Figure C2 A) and inferior winged chaetae, shorter than capillary chaetae on the preceding chaetigers. Thick falcate spines on chaetiger 5 with a tooth on the concave side of the spine; 5 to 6 spines in slightly curved row (Figure C1 C); spines alternating with pennoned companion chaetae, occasionally exhibiting frayed tips (Figure C2 A).

Branchiae present from chaetiger 7 onwards (Figure C1 C), for 30 to 60 % of chaetigers, longest on chaetigers 10 to 16 (Figure C1 G).

Pygidium small teacup-shaped, with dorsal notch leading to anus; 5th last chaetiger 1.36 ± 0.15 times wider than pygidium (Figures C1 G, H).

**Staining pattern**

Staining patterns shown on Figures C1, 3.9 B and Table 3.4. Few irregular stained cells on dorsal and ventral of prostomium and peristomium. No dorsal and ventral staining pattern on caruncle and chaetiger 1 to 5. Chaetiger 6 to last chaetiger without ventral staining pattern. Chaetiger 6 and 7 dorsal staining patterns ranging from none to 2 patches of stained cells. Chaetiger 8 to 3rd last dorsal staining pattern of 2 bars on both sides of centre of chaetiger; 2nd last chaetiger with few stained cells on dorsal on both sides of centre of chaetiger; last chaetiger without staining pattern. Pygidium with distinct stained cells covering entire surface, except along edge around anus and outermost edge of teacup (Figure C1 G, H). Palps continuous line of stained cells adjacent to feeding groove (Figure 3.9 B). Branchiae without staining pattern (Figure C1).

**Distribution**

Narrow River, Rhode Island, USA (this study), Japan and China (Sato-Okoshi et al., 2013; Teramoto et al., 2013).

**Ecology**

Forms U-shaped, unbranching burrows in mollusc shells and may be a pest of commercial molluscs (Teramoto et al., 2013). Reported in China infesting wild and cultured *Crassotrea gigas*, *Chlamys farreri* Müller, 1776, *Haliotis discus hannai* Reeve, 1846; wild *Omphalius rusticus* Gmelin, 1791 and cultured *Patinopecten yessoensis* (Sato-Okoshi et al., 2013). Reported in Japan infesting wild *C. gigas*, *O. rusticus* and cultured *P. yessoensis* (Teramoto et al., 2013). In shells of wild molluscs off Narrow River, Rhode Island in USA (this study).

**Remarks**

The specimens from USA, purportedly *P. websteri*, and *P. onagawaensis* from Japan examined in this study resemble *P. onagawaensis* described from Japan and China (Sato-Okoshi, 1999; Sato-Okoshi et al., 2013; Teramoto et al., 2013) in noto- and neurochaetal structure and shape of prostomium. Winged superior and inferior chaetae, pennoned shaped companion chaetae and the presence of falcate spines with a tooth on chaetiger 5 all resemble *P. onagawaensis* (Sato-Okoshi et al., 2013; Teramoto et al., 2013).

The caruncle extended to between the end of chaetiger 2 to the end of chaetiger 3 for samples examined, but can extend to chaetiger 4 as in previous descriptions (Sato-Okoshi, 1999; Teramoto et al., 2013; Sato-Okoshi et al., 2013). The length of *P. onagawaensis* samples from these previous descriptions was longer than samples examined for this study and thus, the extension of the caruncle may be related to the length (Williams & Radashevsky, 1999).

Teramoto et al. (2013) noted a tooth or flange on the spines, Sato-Okoshi et al. (2013) noted a tooth and sheath and Sato-Okoshi (1999) noted only a flange on the spines of chaetiger 5. However, a consequence of wear and orientation may result in the falcate spines on chaetiger 5 appearing to have a flange or sheath (Read, 2010).

High variation in palp and body pigmentation patterns were evident in previous descriptions (Sato-Okoshi, 1999; Sato-Okoshi et al., 2013; Teramoto et al., 2013), however, as discussed above, pigmentation patterns are unreliable for species identification and should not be used as a distinguishing feature to differentiate between *P. websteri* and *P. onagawaensis.*

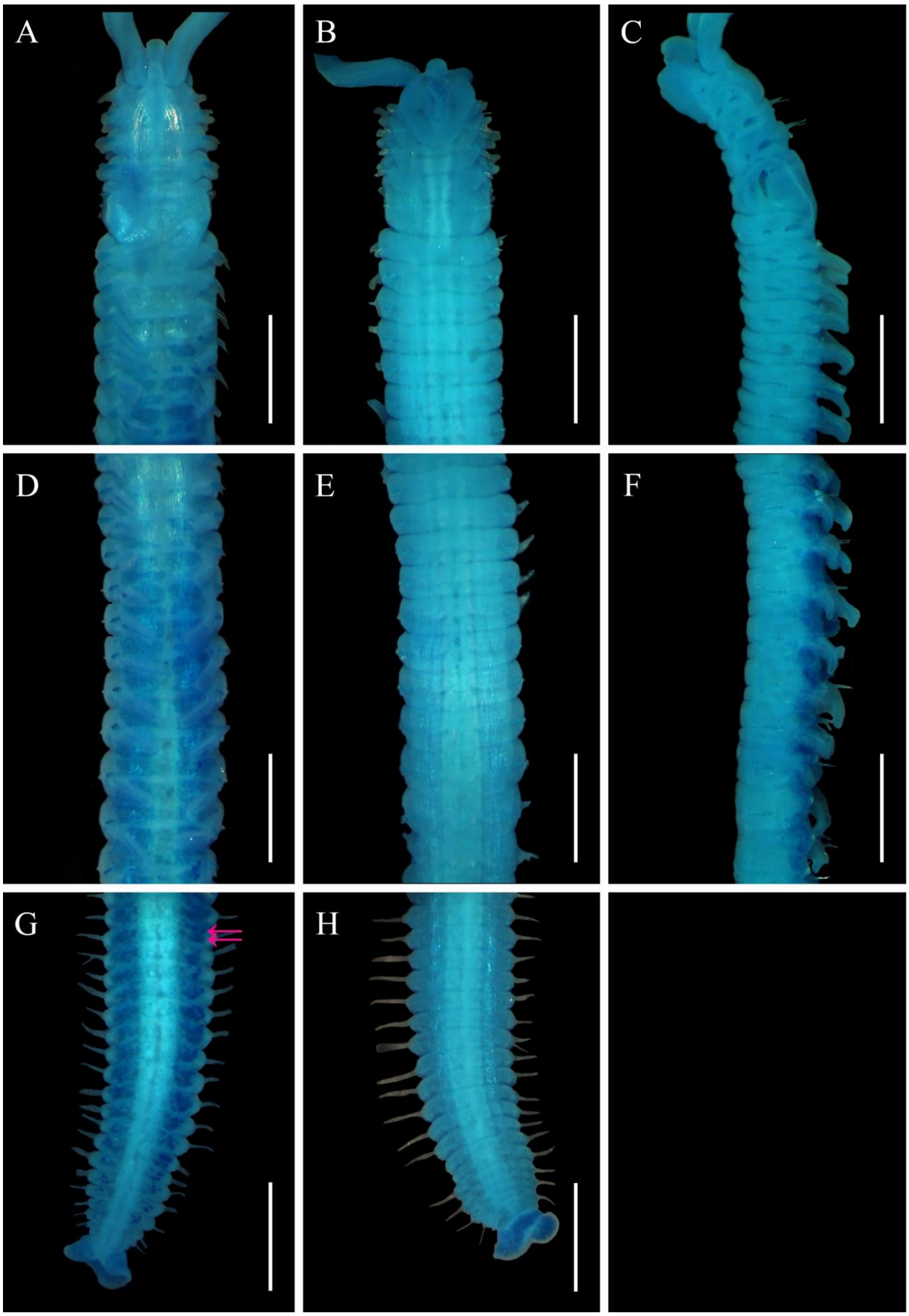


**Figure C1:** Specimen from Rhode Island, USA; (A) dorsal anterior; (B) ventral anterior; (C) lateral anterior; (D) dorsal mid-body; € ventral mid-body; (F) lateral mid-body; (G) dorsal posterior; (H) ventral posterior; arrows indicate bars on chaetiger; scale bar: 0.5 mm

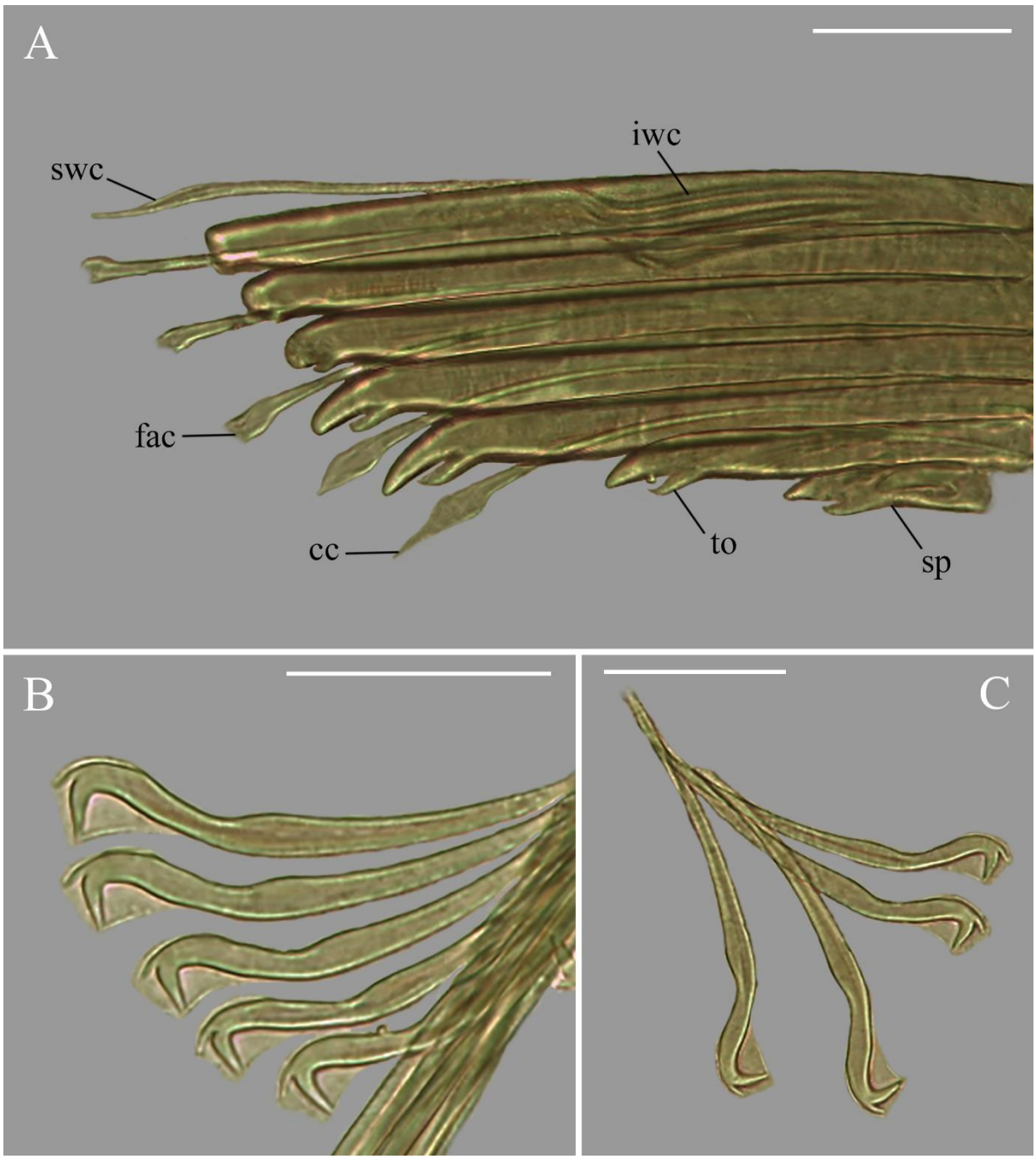


**Figure C2:** Chaetal structures from Rhode Island, USA specimen; (A) falcate spines (sp) with a tooth (to) on the concave side, alternating pennoned companion chaetae (cc), frayed companion chaetae (fac), superior (swc) and inferior winged chaetae (iwc) on chaetiger 5; (B, C) hooded hooks on neuropodium. Scale bar represents 0.05 mm