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## Molecular analysis and new records of the invasive polychaete *Boccardia proboscidea* (Annelida: Spionidae)

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### Abstract

The spionid polychaete *Boccardia proboscidea* Hartman, 1940 is a tube-dweller and shell/stone-borer widely occurring in temperate waters across the world and considered invasive in many areas. It was originally described from California, USA, and later reported from Pacific Canada, the Asian Pacific, Australia, New Zealand, Argentina, South Africa, and northern Europe. The Bayesian inference analysis of sequence data of three gene fragments (836 bp in total) of the mitochondrial *16S* rDNA, nuclear *28S* rDNA, and *Histone 3* has shown that individuals from the Pacific coasts of Canada and the USA, Argentina, Australia, South Africa, the United Kingdom, and Mediterranean France were genetically very similar (maximal average *p*-distance value, 0.49%, was between *16S* rDNA sequences). We consider these individuals to be conspecific and report the earliest records of *B. proboscidea* from the UK and a possible first Mediterranean record in the Gulf of Lion. The high *16S* haplotype diversity of *B. proboscidea* detected in the north-eastern Pacific suggests a native distribution for the species in the northern Pacific and subsequent introductions through human activities to other parts of the world. The histories of these introductions are reviewed and the hypotheses about times and places of introductions are updated.

**Keywords:** Polychaete; Distribution; Non Indigenous Species; West Mediterranean; France.

## Introduction

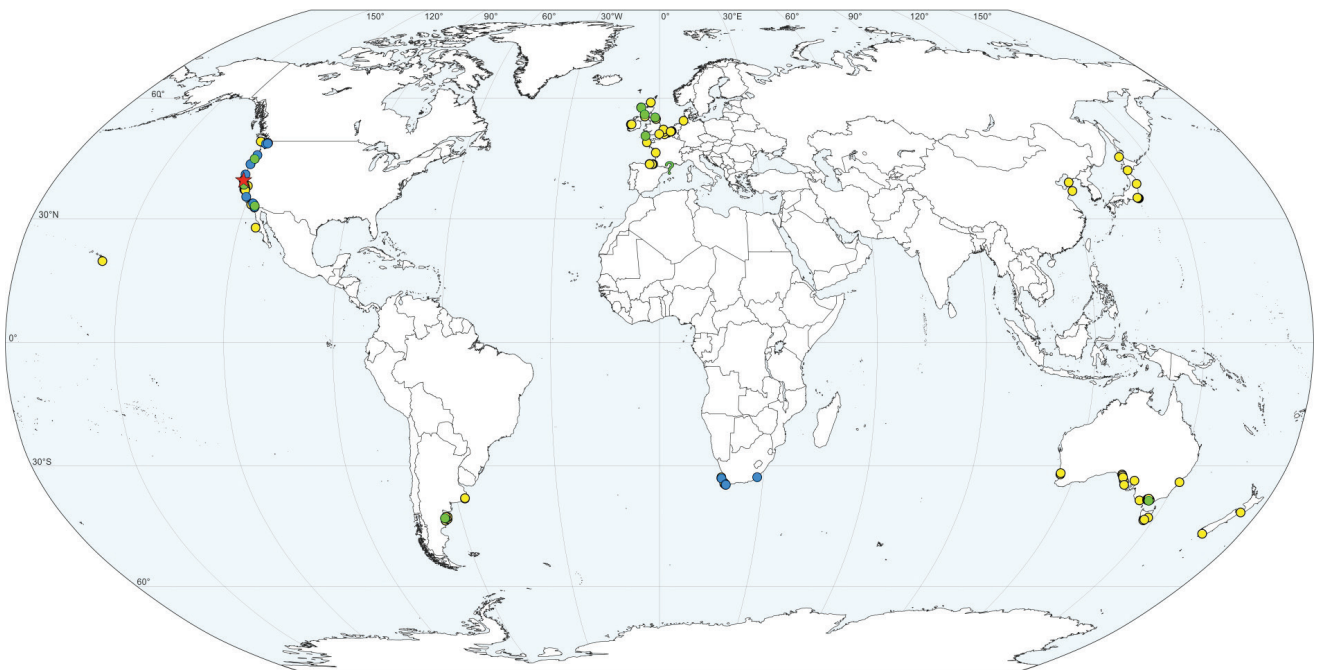
*Boccardia proboscidea* Hartman, 1940 was originally described from northern California (see Radashevsky & Harris, 2010; Fauchald *et al.*, 2011; Figs 1, 2A) and later widely reported from the Pacific coast of North America, from British Columbia, Canada, south to southern California, USA (Hartman, 1940, 1941, 1944, 1954; Berkeley & Berkeley, 1950, 1952; Hartman & Reish, 1950; Woodwick, 1963, 1977; Reish, 1971; Light, 1977, 1978; Hobson & Banse, 1981; Dorsey *et al.*, 1983; Oyarzun *et al.*, 2011), and also in Japan (Imajima & Hartman, 1964; Sato-Okoshi, 2000; Abe *et al.*, 2019a, b), Korea (Paik, 1975, 1989), and China (Yang & Sun, 1988; Sun, 1994; Zhou *et al.*, 2010). It is considered as an introduced species in Australia, Tasmania and New Zealand (Pollard & Hutchings, 1990; Jones, 1991; Hewitt *et al.*, 2004; Read, 2004; Sliwa *et al.*, 2009), Hawaii (Bailey-Brock, 2000), South Africa (Simon & Booth, 2007; Simon *et al.*, 2009, 2010a; Mead *et al.*, 2011), Europe (Martínez *et al.*, 2006; Hatton & Pierce, 2013; Kerckhof & Faasse, 2014; López & Richter, 2017; Spilmont *et al.*, 2018), and Argentina (Diez *et al.*, 2011; Jaubet *et al.*, 2011; Radashevsky, 2011; Jaubet *et al.*, 2013, 2015, 2018).

Simon *et al.* (2009) reported the first sequence data of two mitochondrial genes (*16S* rDNA and *cytochrome b*, *Cyt b*) for *B. proboscidea* and showed a genetic similarity between worms from South Africa and the Pacific coast of North America. Oyarzun *et al.* (2011) reviewed the records of the species and sampled in search of worms along the Pacific coast of North and Central America. They found *B. proboscidea* from Vancouver Island, British Columbia, Canada, south to La Jolla, California, USA, and showed that this Pacific North American population

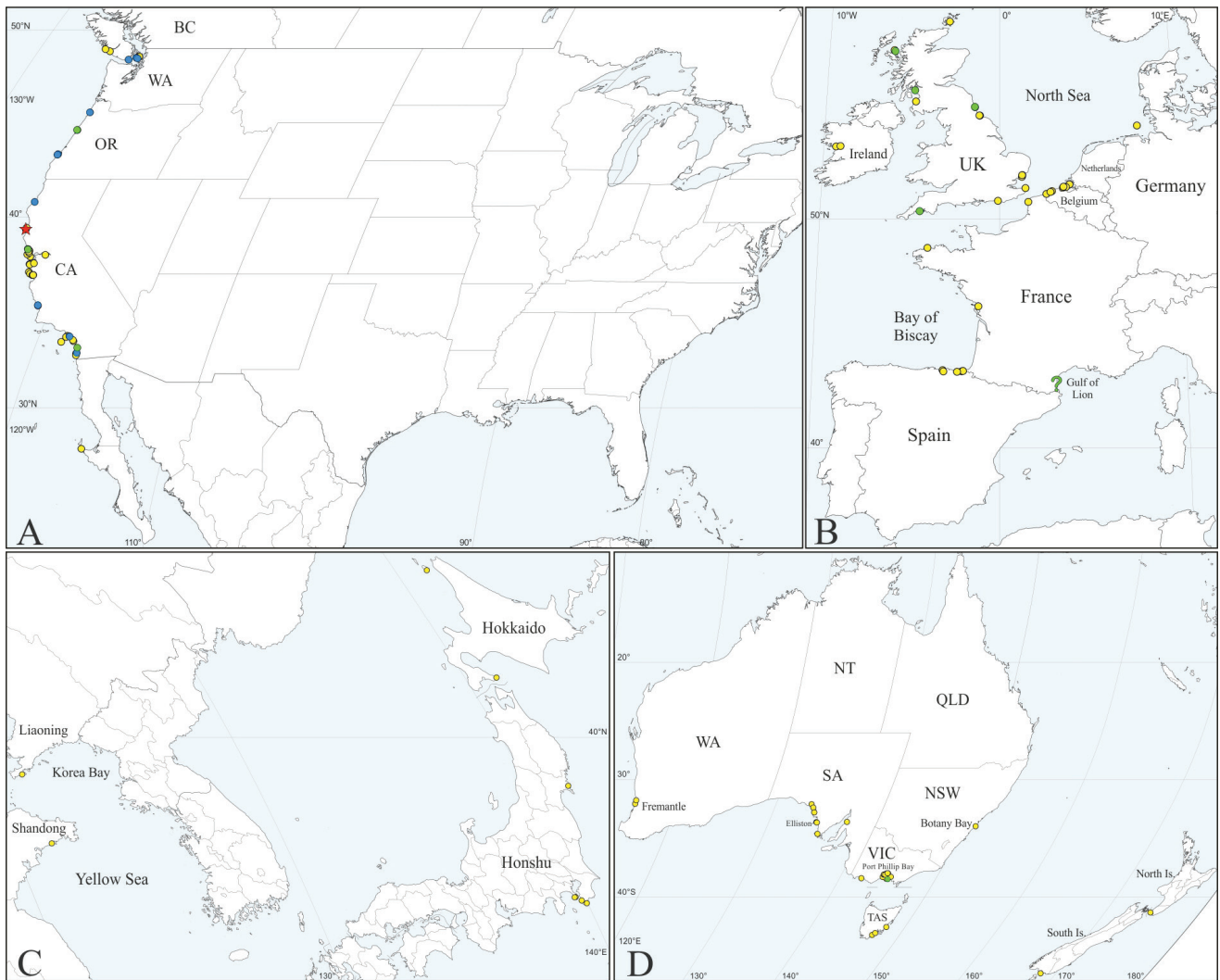
was structured geographically, by the well-documented biogeographic break near Point Conception, California.

The reproduction and larval development of *B. proboscidea* have been described from California and Washington, USA, and British Columbia, Canada (Hartman, 1940, 1941; Woodwick, 1977; Gibson, 1997; Gibson *et al.*, 1999; Smith & Gibson, 1999; Gibson & Smith, 2004; Oyarzun & Strathmann, 2011; Gibson & Carver, 2013; Oyarzun & Brante, 2014), Australia (Blake & Kudenov, 1981), South Africa (David & Simon, 2014), and Argentina (Jaubet *et al.*, 2015). The species exhibits development that varies both within a single brood and among broods produced by different females. Some females produce many small larvae that have a two week pelagic period before metamorphosis. Other females produce broods containing both planktotrophic larvae and nurse-egg-ingesting (adelphophagic) offspring that complete all or most of their development inside the capsule. Molecular analysis (RAPD-PCR) showed that a significant proportion of genetic variance is attributable to geographic origin, and not to developmental type (Gibson *et al.*, 1999), while experimental studies suggested that individual larval fates are determined very early in development and that once their fate is determined, hatching size and intracapsular survival are affected by maternal food provisioning and sibling interaction (Oyarzun & Brante, 2014). David & Simon (2014) assessed various aspects of the larval development of *B. proboscidea* to determine the potential of the species to become established and extend its range along the South African coast.

We collected new and re-examined museum specimens of *B. proboscidea* collected worldwide. Herein, we review previous reports and provide new records, confirm the conspecificity of the distant populations by



**Fig. 1:** Map showing world-wide records of *Boccardia proboscidea*. Red star – type locality of *Boccardia proboscidea* Hartman, 1940: Caspar, Mendocino County, California, USA. Green circles and a question mark – specimens sequenced in the present study (see Table 1). Blue circles – specimens from South Africa, Pacific Canada, and the USA sequenced by Simon *et al.* (2009, 2019), and Pacific USA sequenced by Oyarzun *et al.* (2011). Yellow circles – adults identified based on the morphology only (see Table S1).



**Fig. 2:** Maps showing world-wide records of *Boccardia proboscidea*. A, Pacific coast of North America. B, Europe. C, Pacific coast of Asia. D, Australia and New Zealand. Symbols are the same as on Figure 1.

means of molecular analysis, and discuss the native distribution and possible vectors of introduction for the species around the globe.

## Material and Methods

### Material

Collections were made or reviewed from intertidal and shallow water habitats around the UK, Ireland, Argentina, Australia, China, France, Germany, Pacific Canada and the USA (Figs 1, 2). Sediments collected for this study were washed in the field on a 500  $\mu\text{m}$  mesh sieve and *Boccardia* worms retained in the residue were removed and examined alive under light microscopes in the laboratory. We also collected a range of potential substrata including gastropods, bivalves, barnacles, mud- and siltstones, etc. manually in the intertidal zone and in shallow water using SCUBA equipment. Polychaetes were removed after cracking infested shells and stones with a hammer and pliers. For molecular analysis, specimens were preserved in 95% ethanol. Voucher specimens were fixed in 10% formalin solution, rinsed in

fresh water and then transferred to 70% ethanol. After examination, formalin preserved specimens were deposited in the polychaete collections of the Museum of the Institute of Marine Biology (MIMB), Vladivostok, Russia; Natural History Museum (NHMUK), London, UK; Orkney Islands Council (OIC), Kirkwall, Orkney, Scotland, UK; Senckenberg Museum (SMF), Frankfurt am Main, Germany; Museums Victoria (MV), Melbourne, Australia; Natural History Museum of Los Angeles County (LACM-AHF), Los Angeles, CA, USA, and the United States National Museum of Natural History, Smithsonian Institution (USNM), Washington, D.C., USA. We additionally examined Spionidae specimens deposited in the polychaete collections of the above mentioned museums and also in the Australian Museum (AM), Sydney, Australia; the California Academy of Sciences, Department of Invertebrate Zoology (CASIZ), San Francisco, CA, USA; Universidad Autónoma de Nuevo León (UANL), Nuevo León, México; Museo de Zoología de la Universidad de Costa Rica (MZUCR), San José, Costa Rica, and the Canadian Museum of Nature (CMNA), Ottawa, Canada. Information about samples used for molecular analysis is given in Table 1. Complete information about newly collected material and museum samples examined during



**Table 1.** Sampling location data, museum registration numbers and GenBank accession numbers of sequences obtained in the present study (plain text) and earlier provided by Radashevsky *et al.* (2014) (in bold). *Boccardia* individuals boring in stones or mollusc shells are noted; tube-dwelling individuals are given without notes.

Species	Location	Coordinates	Date	Voucher Habitat	GenBank accession number*		
					16S	28S	Histone 3
1. <i>Dipolydora quadrilobata</i>	Kandalaksha Bay, White Sea, Russia	66.5513° N, 33.123° E	21 Sep 2011	MIMB 36675	MH493041-45	MH493012-16	MH511506-10
2. <i>Boccardia perata</i>	Sea of Japan, Russia	42.8925° N, 132.735° E	19 Oct 2011	MIMB 33071	MH493046-47	MH493017-18	MH511511-12
3. <i>B. perata</i>	Sea of Japan, Russia	42.8925° N, 132.735° E	15 Jul 2013	MIMB 33072	MH493048-49	MH493019-20	MH511513-14
4. <i>Boccardia proboscidea</i>	Puerto Madryn, Golfo Nuevo, Chubut, Argentina	42.7835° S; 65.0085° W	31 Dec 2009	MIMB 33643	<b>KJ546337-41</b> MH493022-23	<b>KJ546209-13</b> MH492993-94	<b>KJ546195-97</b> MH511488-89
5. <i>B. proboscidea</i>	Cerro Avanzado, Golfo Nuevo, Chubut, Argentina	42.83° S, 64.8848° W	11 Aug 2015	MIMB 36629, boring in mudstone	MH493033	MH493004	MH511498
6. <i>B. proboscidea</i>	Puerto Madryn, Golfo Nuevo, Chubut, Argentina	42.76316° S, 65.0336° W	11 Aug 2015	MIMB 36630	MH493034	MH493005	MH511499
7. <i>B. proboscidea</i>	Flinders, Victoria, Australia	38.47527° S, 145.02524° E	24 Sep 2013	MIMB 36628; MV F202860	MH493029	MH493000-01	MH511493-94
8. <i>B. proboscidea</i>	? Leucate, Gulf of Lion, Mediterranean, France	42.8778° N, 3.0229° E	26 Jun 2014	used in molecular analysis	MH493031-32	MH493002-03	MH511497
9. <i>B. proboscidea</i>	Cullercoats Bay, England, UK	55.0335° N, 1.4288° W	17 Nov 2008	MIMB 33677	MH493021	MH492992	MH511487
10. <i>B. proboscidea</i>	Looe, England, UK	50.35134° N, 4.452° W	21 Mar 2011	MIMB 33678	MH493038-39	MH493009-10	MH511503-04
11. <i>B. proboscidea</i>	Isle of Skye, Hebrides, Scotland, UK	57.58643° N, 6.14722° W	6 Mar 2013	MIMB 33065	MH493030	—	MH511495-96
12. <i>B. proboscidea</i>	White Bay, Great Cumbrae Is., Scotland, UK	55.791° N, 4.9091° W	12 Oct 2016	MIMB 33681, boring in shells of live limpets <i>Patella vulgata</i> Linnaeus, 1758	MH493035-37	MH493006-08	MH511500-02
13. <i>B. proboscidea</i>	Bodega Bay, California, USA	38.30445° N, 123.05681° W	4 Aug 2012	MIMB 28078	MH493027-28	MH492998-99	MH511492
14. <i>B. proboscidea</i>	Bodega Bay, California, USA	38.3157° N, 123.0545° W	4 Aug 2012	MIMB 28080	<b>KJ546323-27</b>	<b>KJ546204-08</b>	<b>KJ546181-85</b>
15. <i>B. proboscidea</i>	Carlsbad, Los Angeles Co., California, USA	33.1416° N, 117.3382° W	11 Sep 2017	MIMB 36627, boring in shells of live Pacific oysters <i>Crassostrea gigas</i>	MH493040	MH493011	MH511505
16. <i>B. proboscidea</i>	Yaquina Bay, Oregon, USA	44.62367° N, 124.059° W	27 Jul 2012	MIMB 36625, boring in mudstone	MH493024-26	MH492995-97	MH511490-91

\* Two last digits are shown for the second and other numbers in a successive series.

this study is given in Table S1. A complete list of the museums and other collections (and their acronyms) holding the examined or reported samples is given in Table S2. Table S1 also includes material reported by Petch (1995), Sato-Okoshi & Okoshi (1997), Sato-Okoshi (2000), Sato-Okoshi *et al.* (2008), Zorita *et al.* (2013), David & Simon (2014), Kerckhof & Faasse (2014), Kind & Kuhlenskamp (2017), Spilmont *et al.* (2018), Zettler *et al.* (2018), and Abe *et al.* (2019a) for which no museum deposits were noted, and the material reported by Read (2004), Martínez *et al.* (2006), and Hatton & Pierce (2013) which was deposited by the authors upon our request. It also includes reliable records of *B. proboscidea* from Spain, Belgium, the Netherlands and the United Kingdom kept in private collections or not preserved after examination by other authors. To link some sequences used in the molecular analysis in the present study with the corresponding data, unique numbers from the first author's database (VIR) are given to samples in the Table S1 (at the end of each record). These numbers without letters precede specific names on the phylogenetic tree (Fig. 5).

It was not always the custom in old studies to provide the coordinates of sampling sites and coordinates from some commercial surveys could not be traced; for those samples (including most reports by Olga Hartman, Marian Pettibone, Edith and Cyril Berkeley, and the CASIZ deposits), the coordinates were collected from the Google Earth map according to the original descriptions of the locations. Worldwide sampling locations of *B. proboscidea* noted in Table S1 are plotted on maps using the QGIS 3.6.1 software (Figs 1, 2).

Two worms were found boring in a shell of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) served live in a restaurant in Banyuls-sur-Mer, France, in June 2014. The shell was examined in the laboratory but, because it was treated with lemon juice, the worms were in inappropriate shape for specific morphological identification. However, two kinds of heavy spines characteristic for *Boccardia* spp. were observed in the worms in chaetiger 5. The worms were fixed in ethanol for further molecular examination. According to the restaurant manager, the oysters were from Leucate Lagoon, the main aquaculture facility in the region. We visited Leucate Lagoon on the next day (26 June 2014) and again in November 2018, examined tens of live oysters collected by fishermen, but no *B. proboscidea* worms were found. The sequences obtained from the specimens are noted in Tables 1, 1S and on the maps (Figs 1, 2B) therefore refer to Leucate Lagoon with question mark.

We examined specimens from the Pacific coast of Mexico (UANL 379, 2257) and Costa Rica (MZUCR 280-02) reported as *B. proboscidea* by de León-González *et al.* (1993) and Sibaja-Cordero & Echeverría-Sáenz (2015), respectively. We also collected and sequenced *Boccardia perata* (Chlebovitsch, 1959) and *Dipolydora quadrilobata* (Jacobi, 1883) (see Table 1) to evaluate genetic divergences between *Boccardia* species and between remote conspecific populations of *B. proboscidea*.

## DNA extraction, amplification, and sequencing

We used the DNA Wizard Genomic DNA Purification Kit and the ReliaPrep gDNA Tissue Miniprep System (Promega Corporation, Madison, WI, USA) for DNA extraction and purification, with the standard protocol for animal tissue. Polymerase chain reaction (PCR) amplification of D1 region of nuclear 28S rDNA and *Histone 3*, and mitochondrial 16S rDNA gene fragments was accomplished with the primers and conditions described by Radashevsky *et al.* (2014, 2016). Purified PCR products were bidirectionally sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using the Big-Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers as for PCR. The consensus sequence of each gene region of each specimen was assembled from the two complementary sequences using SeqScape v 2.5 (Applied Biosystems). GenBank accession numbers of the obtained sequences are given in Table 1.

## Data analysis

We aligned DNA sequences using the MAFFT v7.2 software with the default settings (automatically chosen algorithms) (Katoh *et al.*, 2002; Katoh & Standley, 2013). Ambiguous positions and gaps were excluded from subsequent analysis using GBlocks (Castresana, 2000). The nucleotide datasets were combined using the supermatrix approach. Most individual concatenated sequences obtained in the present study (33 of 41) comprised fragments of three genes. The uncorrected values of sequence divergence (pairwise distances,  $p$ ) both within and between groups were calculated in MEGA 5.1.

We used MrBayes 3.2.6 via the CIPRES web portal (Miller *et al.*, 2010) for the Bayesian analysis of 5,000,000 generations, four parallel chains and sample frequencies set to 500, in two separate runs. Based on the convergence of likelihood scores, 25% sampled trees were discarded as burn-in. Analysis of the combined data set was partitioned and the models of substitution were determined using Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada & Crandall, 1998): HKY+I was used for 16S, HKY for 28S, and TrN+G for *Histone 3*.

In the analysis, we also included sequences of *B. proboscidea* obtained in our earlier study (Radashevsky *et al.*, 2014), 15 haplotypes of 16S rDNA of *B. proboscidea* from South Africa (FJ434486, AB973944), Pacific Canada (FJ434487–89) and the USA (FJ972541–47) obtained by Simon *et al.* (2009, 2019), and from the Pacific USA (JN600628–34) obtained by Oyarzun *et al.* (2011). The phylogenetic tree was rooted using the sequences of *Dipolydora quadrilobata* according to a preliminary phylogenetic analysis of molecular data for spionid polychaetes, where *D. quadrilobata* appeared sister to *Boccardia* (Radashevsky *et al.*, unpublished).

The haplotype network was constructed based on a statistical parsimony approach using TCS (Clement *et al.*, 2000).

## Results

### Spionidae Grube, 1850

#### *Boccardia proboscidea* Hartman, 1940

*Boccardia proboscidea* Hartman, 1940: 382–387, fig. 1; 1941: 299–304, pl. 46, figs 22–28, pl. 47, figs 30–37; 1944: 259. Hartman & Reish, 1950: 27. Woodwick, 1963: 133–137, figs 2–4; 1977: 348–352, figs 1–12 (larval morphology). Imajima & Hartman, 1964: 279–280, pl. 36, figs a–f. Reish, 1971: 1464. Paik, 1975: 11, pl. 8, fig. 70; 1989: 457–458, fig. 168. Blake & Kudenov, 1978: 238, fig. 33 a–c; 1981: 176–177. Light, 1978: 147–149, textfig. 148. Kudenov, 1979: 163. Dorsey, 1982: 48–53. Hutchings & Turvey, 1984: 14–15. Yang & Sun, 1988: 212, fig. 95 A–F. Sun, 1994: 357. Petch, 1995: 26–28. Gibson, 1997: 215–220, figs 1–3. Sato-Okoshi & Okoshi, 1997: 487. Gibson *et al.*, 1999: 746–749, figs 3–6. Smith & Gibson, 1999: 179–184, figs 1–4. Bailey-Brock, 2000: 27–29, fig. 1. Sato-Okoshi, 2000: 447–448. Leonart, 2001: 24, figs 45, 46, 48; 2002: 30, fig. 3.17. Gibson & Smith, 2004: 136–145, figs 1–3. Handlinger *et al.*, 2004: 39. Read, 2004: *Boccardia proboscidea*. Zippel, 2004: 12. Martínez *et al.*, 2006: 59–62, figs 4, 5. Blake & Ruff, 2007: 371, 375. Simon & Booth, 2007: 502–507. Sato-Okoshi *et al.*, 2008: 498. Simon *et al.*, 2009: 18–24; 2010a: 594–596, figs 4, 5; 2010b: 52–59, figs 4, 7. Haupt *et al.*, 2010: 56. Zhou *et al.*, 2010: 3, fig. 1 A–F. Jaubet *et al.*, 2011: 190–195, fig. 2; 2013: 217–220, figs 3, 4; 2015: 613–617, figs 2–5 (adult & larval morphology); 2018: 355–361. Oyarzun *et al.*, 2011: 489–500. Oyarzun & Strathmann, 2011: 83–88, figs 2, 3. Garaffo *et al.*, 2012: 274–277, fig. 2. Gibson & Carver, 2013: 13–19, figs 1–6 (larval morphology). Hatton & Pearce, 2013: 2–3, figs 2 &

3. David & Simon, 2014: 24–29. Kerckhof & Faasse, 2014: 3–4, figs 4–6. Oyarzun & Brante, 2014: 246–351, fig. 1. Elías *et al.*, 2015: 532–535. Wijnhoven *et al.* 2017: 25. Spilmont *et al.*, 2018: 1111–1118, fig. 2 b, c. Abe *et al.*, 2019a: 7–9, fig. 6b; 2019b, 19–20, fig. 4C. Kakkonen *et al.*, 2019: 57.

*Boccardia* sp.: Simon *et al.*, 2006: 168–171, fig. 1a.

*Polydora (Boccardia) proboscidea*: Berkeley & Berkeley, 1950: 51–52; 1952: 17–18, figs 26, 27. Hobson & Banse, 1981: 38, fig. 5i, j. Hartmann-Schröder, 1982: 85–86.

*Polydora californica* Treadwell, 1914: 203–204. California. LACM-AHF POLY 638 (type). Radashevsky & Harris, 2010: 203–207. Fauchald *et al.*, 2011: 134–136. *Fide* ICZN, 2012: 232–234.

Not *Boccardia proboscidea*: Carrasco, 1974: 186–187, figs 1–4; 1976: 8–11, figs 2, 8A, 13 A–C. *Fide* Blake & Kudenov, 1978.

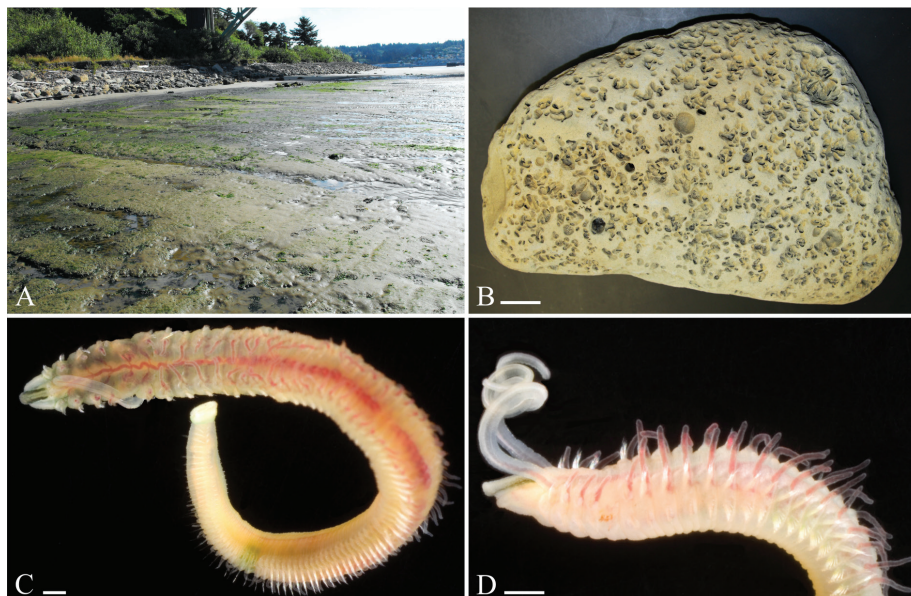
Not *Boccardia proboscidea*: Fauchald, 1977: 47. *Fide* Petch, 1995.

Not *Boccardia proboscidea*: de León-González *et al.*, 1993: 879. Salazar-Vallejo & Londoño-Mesa, 2004: 59. Soto & Paterson, 2010: 76. Sibaja-Cordero & Echeverría-Sáenz, 2015: 69, fig. 5J, K.

Not *Polydora (Boccardia) proboscidea*: Hartmann-Schröder, 1989: 43.

#### Adult morphology

Up to 45 mm long and 2 mm wide for 150 chaetigers. Individuals with more than 50 chaetigers with dark green pigment (appearing almost black in formaldehyde-fixed specimens), diffused on anterior lateral sides of prostomium, on dorsal side of peristomium and anterior chaetigers (most intense on chaetigers 1 and 2) (Figs 3C, D, 4H, I).



**Fig. 3:** Habitat and adult morphology of *Boccardia proboscidea*. A, flat intertidal zone comprised of mudstone densely populated by worms. B, piece of mudstone heavily perforated by worms. C, complete individual. D, anterior end, left lateral view. Scale bars: A – 1 cm; C, D – 500  $\mu$ m. A, B – Newport, near Yaquina Bay bridge, Oregon, USA (MIMB 36625). C, D – Griffiths Island, Victoria, Australia (MV F242601). Photographs C & D by Leon Altoff, Marine Research Group of the Field Naturalists Club of Victoria.





**Fig. 4:** Habitat, reproduction and morphology of *Boccardia proboscidea* from Helgoland Island, North Sea, Germany. A, B, intertidal soft red sandstone heavily perforated by worms. C, female in a burrow with recently laid egg capsules with numerous small eggs. D, advanced egg capsule (attached by two thin stalks to female's burrow) with one small mainly planktotrophic larva, three large entirely lecithotrophic larvae, and a few nurse eggs yet to be eaten by large lecithotrophic larvae. E–G, adult chaetae. E, bristle-topped spine (entire and distal end) from notopodium of chaetiger 5. F, simple falcate spine from notopodium of chaetiger 5. G, bidentate hooded hooks from midbody neuropodium. H, complete individual. I, anterior end, left lateral view. Scale bars: A, B – 1 cm; C – 1 mm; D – 200  $\mu$ m; H – 500  $\mu$ m; I – 300  $\mu$ m. Photographs by Britta Kind and Ralph Kuhlenkamp.

Prostomium anteriorly rounded to blunt, with frontal margin entire to incised; posteriorly extending to end of chaetiger 3 as a low caruncle. Occipital antenna absent. Three pairs of black eyes usually present.

Chaetiger 1 with short capillaries and postchaetal lamellae in both rami. Posterior notopodia with capillaries only.

Chaetiger 5 larger than chaetigers 4 and 6 (Figs 3C, 4I), with two kinds of heavy spines in notopodia and up to six short ventral capillaries; dorsal superior capillaries and postchaetal lamellae absent. Heavy spines comprise up to 7 (usually 4) bristle-topped spines (Fig. 4E) and 6 (usually 3) simple falcate spines (Fig. 4F); typically one less falcate spine than the number of bristle-topped spines.

Hooks in neuropodia from chaetiger 7, up to 13 in a series, accompanied by 1–5 inferior capillaries invariably in chaetigers 7–9, and 1–3 alternating capillaries in 3–10 posterior chaetigers. Hooks bidentate; shaft slightly curved, without constriction (Fig. 4G).

Branchiae on chaetigers 2–4 and from chaetiger 6 throughout most of body. Small individuals, with fewer than 25–30 chaetigers, with branchiae from chaetiger 7 to chaetigers 9–10.

Pygidium fleshy, with one pair of dorsal lobes and one pair of slightly bigger ventral lobes, white due to numerous glandular cells with striated content. Both lateral and midventral clefts separating dorsal and ventral lobes usually well developed in small individuals but fused in big worms, thus whole pygidium appearing as a fleshy pad (Fig. 4H).

#### *Habitat*

Adults usually occur intertidally, inhabiting muddy or sandy tubes in mud flats or other soft sediments, on exposed rocky shores and pier pilings between shells of various mytilids and barnacles, or boring into clay, shale, sandstone, mudstone or volcanic rock (Figs 3A, B, 4A,

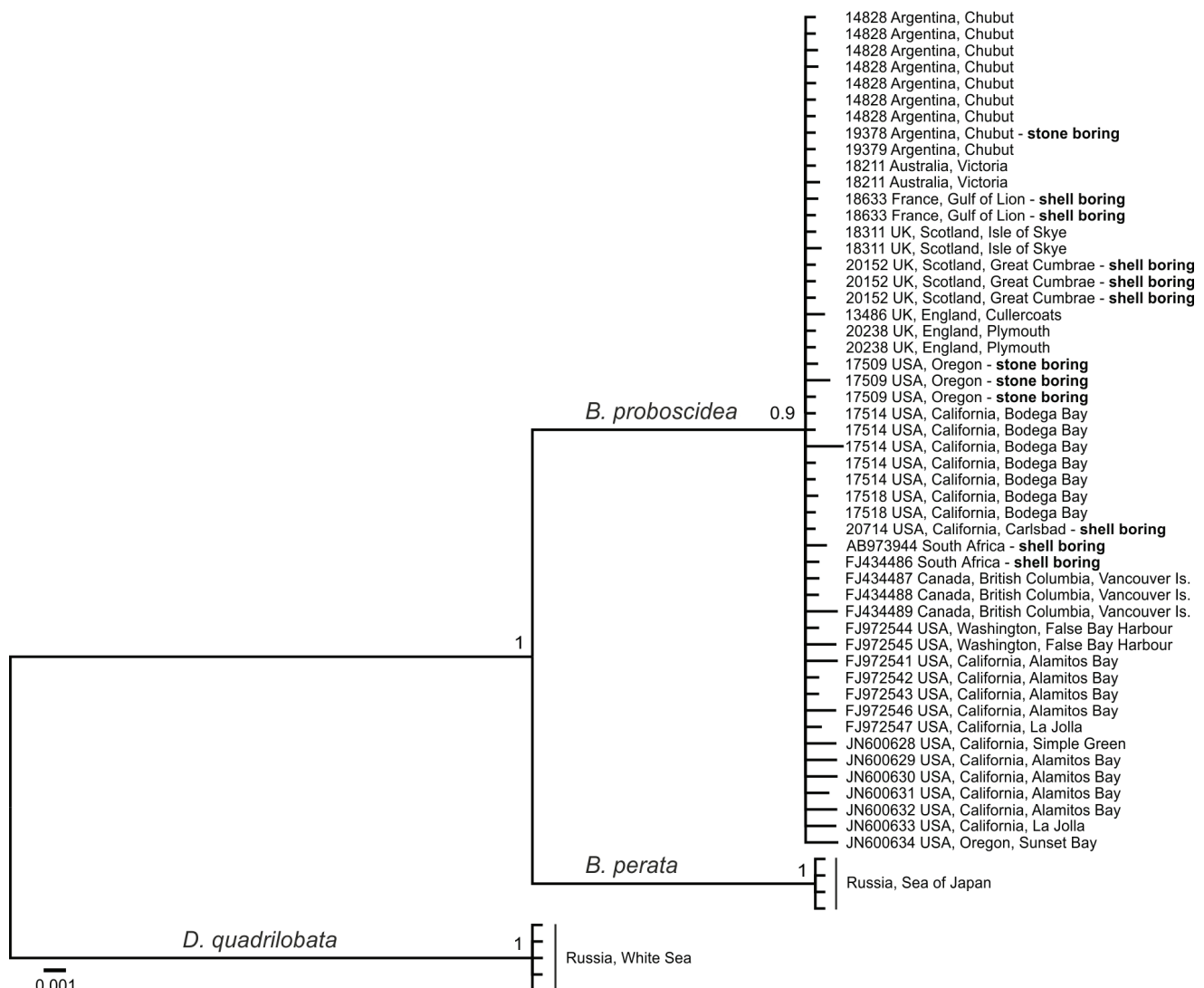


B). High density colonies often form around sewage outfalls. In organically impacted sites around Mar del Plata's sewage discharge in Argentina, *B. proboscidea* built a reef-like structure with density up to 1,500,000 individuals per one square meter (Jaubet *et al.*, 2011, 2015). Occasionally, worms were reported associated with coralline algae (Petch, 1995; David & Simon, 2014) and sponge (Abe *et al.* 2019a), and possibly bored into these hosts. Occurrence of worms in abalone and oyster shells was often explained as a secondary occupation, inhabiting burrows and blisters originally made by *Polydora* or *Dipolydora* spp. Original perforation of mollusc shells by *B. proboscidea* was never clearly demonstrated. During this study, we found worms in burrows in oyster, mussel and gastropod shells in France, California and Scotland. At least in two cases, other boring polydorins were absent in the shells. We interpret these observations as evidence that *B. proboscidea* has the ability to bore into mollusc

shells in a same way as *Polydora* or *Dipolydora* spp. However, it is unclear why this ability is rarely realized. The present study confirmed the conspecificity of stone-boring, shell-boring and tube-dwelling individuals and found no consistent intraspecific genetic difference between them.

#### Molecular analysis

The combined alignment, with gaps excluded, comprised in total 836 bp, including 307 bp (99%) for *16S* rDNA, 284 bp (99%) for *28S* rDNA, and 245 bp (100%) for *Histone 3*. They contained 99 (11.8%) variable sites, 93 (11%) of which were informative. The general Bayesian analysis showed that *B. proboscidea* individuals from distant populations were genetically similar and formed a well-supported (PP = 0.9) clade (Fig. 5). The maximum



**Fig. 5:** Majority rule consensus tree of the Bayesian inference analysis of the combined *16S* (307 bp), *28S* (284 bp), and *Histone 3* (245 bp) sequences (836 bp in total) of *Boccardia* rooted with sequences of *Dipolydora quadrilobata*. Posterior probabilities are shown on the branches. Species names are followed by the names of the collecting locations (in parentheses). The *16S* sequences of specimens from South Africa (FJ434486), Pacific Canada (FJ434487–89) and USA (FJ972541–47) were provided by Simon *et al.* (2009), and those from Pacific USA (JN600628–34) by Oyarzun *et al.* (2011). The *28S* sequence of a specimen from South Africa (AB973944) was provided by Simon *et al.* (2019). The numbers without letters preceding specific names are unique numbers from the VIR database linking the individuals on the tree with the sampling data in the Table S1.

uncorrected *p*-distance value within this clade was 1.95% (average 0.49%) for *16S* rDNA sequences, whereas sequences of *28S* rDNA and *Histone 3* were invariable. The average *p*-distances between individuals of *B. proboscidea* and *B. perata* were 0.35% for *28S* rDNA, 3.7% for *Histone 3* and maximal 8.9% for *16S* rDNA.

The haplotype network analysis was based on 159 sequences of *16S* rDNA, 129 of which were previously obtained by Simon *et al.* (2009, 2019) and Oyarzun *et al.* (2011). The 307 bp aligned sequences comprised 14 variable positions and 5 parsimony-informative sites. The analysis recovered a single network comprising seventeen haplotypes (Fig. 6). Thirty sequences obtained in the present study comprised five haplotypes different from each other by one or two base substitutions. Three of these haplotypes were previously reported by Oyarzun *et al.* (2011) and two new haplotypes, P (MH493026) and O (KJ546325), each with a single representative, were found in Pacific North America. Haplotypes K and A were most common, found in 45% and 33% of individuals, respectively. Haplotype K was found in individuals occurring along Pacific North America (Canada and USA) and in Europe (France and UK), whereas haplotype A was found in individuals occurring worldwide, from the most northern to the most southern locations. Individuals from the southern hemisphere (Africa, Argentina, Australia) had only haplotype A. Haplotypes B, M, P, and R were present only in individuals from Washington and Oregon. The remaining haplotypes, including haplotype D observed in 9% of individuals, were present only in individuals from California.

## Discussion

The molecular analysis showed that individuals of *B. proboscidea* from France, North America, Argentina, South Africa, Australia and the UK were genetically very similar to each other (Fig. 5). The maximum *p*-distanc-

es (*16S* *p*=1.95%) between individuals were similar to distances between sympatric individuals of other spionids examined in our earlier studies (Radashevsky *et al.*, 2014, 2016). Identical mitochondrial *16S* rDNA haplotypes were found in individuals from geographically distant regions, indicating their close relationships. The results of our molecular analysis confirm the conclusions about the conspecificity of distant populations of *B. proboscidea* inferred by Petch (1995) based on examination of adult morphology. Petch (1995) provided a set of diagnostic morphological features for the species, which we have updated in the present study.

The present analysis also supports the conspecificity of individuals living in different habitats: in sandy and silty tubes in soft sediments and boring into the shells of various mollusks and into different kinds of stone. Such habitat plasticity was previously suggested for some other spionid polychaetes but was only shown by molecular analysis for *Dipolydora carunculata* (Radashevsky, 1993) (Radashevsky & Pankova, 2013).

As with many other marine species introduced to non-native localities, the native distribution of *B. proboscidea* had been uncertain. Without any consideration and apparently based on the type locality only, the species was assumed to be native to California and introduced from British Columbia to Baja California and all other locations (Jaubet *et al.*, 2018), or native to the Pacific coast of North America and Japan (Spilmont *et al.*, 2018). Remarkably, *B. proboscidea* has never been reported from the Atlantic coasts of North America and, until recently, had not been found in South American or European waters. The occurrence of all 17 haplotypes of *16S* partition recognized in *B. proboscidea* from Pacific North America strongly supports a native distribution of the species in this region (Fig. 6). Indeed, when only considering California, although the abundance of haplotypes K, A, and D remains greater than the other haplotypes, the proportions are more balanced, suggesting a well established population with balanced diversity. On the contrary, the occur-



**Fig. 6:** Haplotype network of the *16S* rDNA gene in *Boccardia proboscidea*, calculated in TCS. Haplotypes are labeled after Oyarzun *et al.* (2011), indicated by colored circles and their frequency is indicated by the size of the circles. Multiple colors indicate haplotypes shared by more than one sampling locality, with sections scaled by frequency. Sequences of individuals from Witty's Lagoon, Vancouver Island, Canada (reported by Simon *et al.*, 2009), and False Bay and Cattle Point, Washington, USA (reported by Simon *et al.*, 2009, and Oyarzun *et al.*, 2011) are combined and shown in the network as a sample from Washington, USA.

rence of single haplotypes in Europe (haplotype K) and in South Africa, Australia and South America (haplotype A) suggests a recent foundation event for each. The exclusive occurrence of one or the other of these haplotypes suggests at least two distinct routes of colonization from the original distribution zone (North Pacific): one for European locations, and one for the Southern Hemisphere.

Haplotype analysis was not the primary objective of this study and we used the set of genes originally chosen for phylogenetic studies of Spionidae (see Radashevsky *et al.*, 2016). The 16S rDNA has widely been proven to be highly suitable for molecular identification at the species level for spionids (Radashevsky *et al.*, 2014, 2016; Simon *et al.*, 2019). However, the more variable mitochondrial *cytochrome b* (*cyt b*) was shown to be more suitable for intraspecific studies (Simon *et al.*, 2009; Williams *et al.*, 2017). For example, Simon *et al.* (2009) detected seven haplotypes of *cyt b* in *B. proboscidea* in South Africa, where only one 16S haplotype had been found. Future analysis of *cyt b* may therefore reveal more details in the invasion history of the species around the world.

The genetic diversity of *B. proboscidea* in the Asian Pacific, including Japan, Korea and China, has never been studied so far; thus there are no data to hypothesize about the origin of worms in this region. The species might have been distributed in the past all along the northern Pacific without interruption and then, due to changes in the geology and climate of the region, survived in temperate waters only, resulting in disjunct distributions on the American and Asian coasts. It is also plausible that the species evolved on the Pacific coast of North America and later was introduced to other regions. Support for the former hypothesis can be seen in a common occurrence of the species in Chinese coastal waters (Yang & Sun, 1988; Zhou *et al.*, 2010). Support for the latter hypothesis, however, can be seen in the absence of the species in the low boreal waters of the Asian coast of Russia, both around the islands (Sakhalin and Kuril Islands) and along the mainland. Polychaetes with disjunct amphipacific distributions usually occur in this region (Uschakov, 1971). The two hypotheses should be further evaluated in a molecular analysis of individuals from Pacific Asia.

*Boccardia proboscidea* has been reported from the Pacific coast of Panama by Fauchald (1977). Petch (1995) re-examined specimens from Panama (USNM 66369); we have done likewise, and found them different from *B. proboscidea* from temperate waters. The species has also been reported from the Pacific coast of Mexico (de León-González *et al.*, 1993) and Costa Rica (Sibaja-Cordero & Echeverría-Sáenz, 2015). We re-examined the Mexican (UANL 379, 2257) and Costa Rican (MZUCR 280–02) specimens, and also found them different from *B. proboscidea*. At the same time, we found *B. proboscidea* from Punta Eugenia, Baja California Sur, Mexico (LACM-AHF Poly 11203) identified by Olga Hartman but never reported in the literature. Thus, on the Pacific coast of North America, the species has been recorded from Port Albion, British Columbia, Canada (48.9513° N) south to Punta Eugenia, Baja California Sur, Mexico (27.8487° N) (Fig. 1B).

Outside of the North Pacific, *B. proboscidea* was first reported from Australia by Blake & Kudenov (1978) who collected numerous adults in Port Phillip Bay, Victoria (Fig. 2D), in December 1976, and adults with egg capsules in January–March 1977. Soon after that, Kudenov (1979) and Dorsey (1982) reported dense intertidal, intensely reproducing settlements of the species near sewage outfalls in Port Phillip Bay. Based on these reports, *B. proboscidea* was suggested to be introduced to Australia with ballast waters discharged in Port Phillip Bay, which hosts the largest seaport in Australia (Pollard & Hutchings, 1990; Jones, 1991; Hewitt *et al.*, 2004). However, *B. proboscidea* was collected in Elliston, South Australia in 1979 (Hutchings & Turvey, 1984) only three years after the first records from Port Phillip Bay. Elliston is more than 1,000 km west of Port Phillip Bay and is also at least 200 km west of the nearest major ports in Spencer Gulf (Fig. 2D). Therefore, either there were multiple introductions of *B. proboscidea* to southern Australia, or *B. proboscidea* was introduced much earlier and dispersed locally but remained undetected until the first major environmental surveys occurred in the 1970s.

In South America, *B. proboscidea* was first reported from Concepción, Chile, by Carrasco (1974, 1976). Blake & Kudenov (1978: 238), however, suggested that “his descriptions match those of *B. tricuspa* (Hartman), and his specimens should probably be referred to the latter species.” Soto & Paterson (2010) reported *B. proboscidea* collected from the Región de Aisén, southern Chile, in 2002. However, we re-identified their specimens as *Boccardia claparedei* (Kinberg, 1866) based on the photos provided by the authors on our request (Soto *in litt.*, 27 Nov 2018). *Boccardia proboscidea* is therefore considered to be currently absent from Chilean waters. The species is well established, however, in Argentina. It was first reported as forming large biogenic reefs around the sewage outfall of Mar del Plata, Buenos Aires Province (Jaubet *et al.*, 2011, 2013, 2015; Sanchez *et al.*, 2011; Garaffo *et al.*, 2012; Elías *et al.*, 2015), and in relatively pristine areas around Puerto Madryn, northern Patagonia, Chubut Province (Diez *et al.*, 2011; Radashevsky, 2011) (Fig. 1A). Later it was found to be widely distributed along the Argentinean coast (Becherucci *et al.*, 2016, 2018; Garaffo *et al.*, 2016; Jaubet *et al.*, 2018). Jaubet *et al.* (2018) suggested that the species might have been introduced into Argentina from Australia with shipments of Australian bauxite to the aluminum plant in Puerto Madryn that was installed in 1974. Two of the five major bauxite mines in Australia are in the northern part of the continent, where *B. proboscidea* has never been found. Three other mines are in Western Australia, close to Fremantle (Fig. 2D), the largest and busiest international cargo port in the state, with bauxite as one of the major export commodities (<https://www.fremantleports.com.au>). *Boccardia proboscidea* was present in Fremantle in 1975 (Hartmann-Schröder, 1982). It is not possible to determine at present whether the species was first introduced to Port Phillip Bay (Victoria) or to Fremantle (Western Australia). It might have been dispersed along the southern coast of Australia by means of coastal traffic



and water currents and then transported from Australia to Puerto Madryn, Argentina.

In Europe, *B. proboscidea* was first discovered in 1996 on the rocky intertidal near a sewage outfall at San Sebastián, Bay of Biscay, Spain (Martínez *et al.*, 2006) (Fig. 2B). Martínez (*in litt.*, 21 Nov 2018) provided additional records of the species based on specimens collected in northern Spain during environmental monitoring programs and Studies for Public Administrations after 1997 (see Table S1). In 1999, *B. proboscidea* was collected near Roscoff, Brittany, France (USNM 186423), and in 2014 it was found on an intertidal rocky reef near Wimereux, Hauts-de-France, and also near La Rochelle, French coast of the Bay of Biscay (Spilmont *et al.*, 2018). As early as 2000 and then in 2011, worms were found on groynes along the Belgian coast and among the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) in the south-western Dutch delta (Kerckhof & Faasse, 2014). In the British Isles, *B. proboscidea* was first collected in August 1998 on Horsey Island, Essex, south-eastern England (MIMB 33675). In 2000, worms were found at Shotley, Suffolk, near the ports of Felixstowe, Suffolk, and Harwich, Essex (recorded at generic level in an unpublished report, full details in the present study), and also in Galway Bay on the west coast of Ireland (Brendan O'Connor, *in litt.*, 5 Dec 2018). In 2002, they were found in the Nervión estuary, southeastern Bay of Biscay, Spain (Zorita *et al.*, 2013). In 2004 and 2005, *B. proboscidea* was found in the Tees Estuary, north-eastern England; in 2006, it was found in the Clyde Sea, Scotland (Myles O'Reilly, *in litt.* 5 Dec 2018), and in 2008 in King Edwards Bay, Tynemouth, and Cullercoats Bay, north-eastern England (MIMB 33676), and on Helgoland Island, North Sea, Germany (present study, identified on a photograph of broken intertidal sandstone on 29 July 2008). In 2016, the population on Helgoland Island was well established, dense and actively reproducing (Kind & Kühlenkamp, 2017; Zettler *et al.*, 2018; Fig. 4 A–D). In 2011, the species was found on rocky intertidal shores in Looe, Cornwall, near Plymouth (MIMB 33678), and in 2016 it was present in Kent (MIMB 33679) and Sussex (Robin Shrubsole, *in litt.*, 3 Dec 2016), all of which are along the English Channel. In 2011 and 2013, worms were found intertidally on the Isle of Skye, north-west Scotland (Hatton & Pierce, 2013), and in 2016 we collected them in Great Cumbrae Island, western Scotland (MIMB 33681). Discoveries of *B. proboscidea* in different parts of northern Europe within a short period of time (late 1990s – early 2000s) might have resulted from either several introductions of the species or the dispersal of once-introduced larvae over the region due to intense coastal traffic and water currents. It is, however, also possible that the species had been present but unrecorded in previous decades, as it is easily missed by many routine survey methods (its preference for intertidal firm or hard substrata excludes it from most routine grab and core samples). Our discovery of *B. proboscidea* in an oyster in a restaurant in Banyuls-sur-Mer represents an alarm bell towards the possible occurrence of the species in Mediterranean environments and raises concerns for Leucate

Lagoon, a major aquaculture facility in Mediterranean France. However, our failure to find more worms in oysters cultured in the lagoon shows that additional survey work would be required to establish a sound Mediterranean record.

In South Africa, *B. proboscidea* was first discovered in 2004, infesting the abalone *Haliotis midae* Linnaeus, 1758 cultured in land-based tanks on a farm in Hermanus, on the south-west coast of the country (Simon *et al.*, 2006, as *Boccardia* sp.; Simon & Booth, 2007) (Fig. 1). Due to anthropogenic movement of abalone, the worms were spread over onshore farms on the west and east coasts of the country (Simon *et al.*, 2009, 2010; Boonzaaier *et al.*, 2014). Recently they were found in low densities at seven wild sites on the west and south-west coasts, indicating that establishment of *B. proboscidea* in the wild has begun (Simon, 2015; David *et al.*, 2016).

Due to aquaculture, *B. proboscidea* was also introduced to an oyster farm at Keahole, Hawai'i, with a shipment of *Ostrea edulis* Linnaeus, 1758. Imported oysters were heavily infested with adult worms whose burrows contained egg capsules with late-stage larvae (Bailey-Brock, 2000; MIMB 33682). The oysters were maintained in raceways with the seawater coming from depths of 15 to 300 m, which was ideal for aquaculture. Wild individuals of *B. proboscidea* have not been recorded to date and the survival of the species in tropical Hawaiian waters seems to be hardly possible. Although the adelphophagic larvae of *B. proboscidea* have their highest survival rates at 21°C, temperatures above 24°C were found to be lethal for pre-hatched planktotrophic larvae (David & Simon, 2014). Because of this adaptation, confirmed records of wild *B. proboscidea* are limited mainly to temperate waters.

Review of previous reports and our new records of *B. proboscidea* show a wide spread of the species in temperate waters across the globe (Figs 1, 2). *Boccardia proboscidea* has reproductive characteristics that favor establishment and spread (*e.g.*, poecilogonous development, early maturation, parthenogenesis and continuous brood production throughout the year). Adults and larvae of this species are tolerant of variation in salinity, survive high organic pollution and are adapted to live in diverse habitats. They are very competitive and are able to modify benthic communities, suppressing and eliminating native species in newly occupied areas (Jaubet *et al.*, 2013, 2018; Elías *et al.*, 2015). The spread of the species may threaten biodiversity and raises concern in countries to which it has been introduced.

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## Supplementary material

**Table S1.** Sampling location data and museum registration numbers of newly collected material of *Boccardia proboscidea*; museum samples examined during this study; material reported by Petch (1995), Sato-Okoshi & Okoshi (1997), Sato-Okoshi (2000), Sato-Okoshi *et al.* (2008), Zorita *et al.* (2013), David & Simon (2014), Kerckhof & Faasse (2014), Kind & Kuhlenkamp (2017), Spilmont *et al.* (2018), Zettler *et al.* (2018), and Abe *et al.* (2019a) for which no museum deposits were noted; material reported by Read (2004), Martínez *et al.* (2006), and Hatton & Pierce (2013) which was deposited by the authors upon our request; reliable records from Spain, Belgium, the Netherlands and the United Kingdom kept in private collections or not preserved after examination by other authors.

**Table S2.** List of the museums and collections (and their acronyms) holding the examined or reported samples of *Boccardia proboscidea*.

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