



Orange is the new white: taxonomic revision of *Tritonia* species (Gastropoda: Nudibranchia) from the Weddell Sea and Bouvet Island

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Abstract

Among nudibranch molluscs, the family Tritoniidae gathers taxa with an uncertain phylogenetic position, such as some species of the genus *Tritonia* Cuvier, 1798. Currently, 37 valid species belong to this genus and only three of them are found in the Southern Ocean, namely *T. challengeriana* Bergh, 1884, *T. dantarti* Ballesteros & Avila, 2006, and *T. vorax* (Odhner, 1926). In this study, we shed light on the long-term discussed systematics and taxonomy of Antarctic *Tritonia* species using morpho-anatomical and molecular techniques. Samples from the Weddell Sea and Bouvet Island were dissected and prepared for scanning electron microscopy. The three molecular markers COI, 16S, and H3 were sequenced and analysed through maximum-likelihood and Bayesian methods. The phylogenetic analyses and species delimitation tests clearly distinguished two species, *T. challengeriana* widely spread in the Southern Ocean and *T. dantarti* endemic to Bouvet Island. Colouration seems to be an unreliable character to differentiate among species since molecular data revealed both species can either have orange or white colour morphotypes. This variability could be explained by pigment sequestration from the soft coral species they feed on. Morphological analyses reveal differences between Antarctic and Magellanic specimens of *T. challengeriana*. However, the relationship between *T. challengeriana* specimens from these two regions remains still unclear due to the lack of molecular data. Therefore, the validity of the *T. antarctica* Martens & Pfeffer, 1886, exclusively found in Antarctic waters requires further systematic work.

Keywords Phylogenetic analyses · Southern Ocean · Species delimitation tests · Tritoniidae taxonomy

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Introduction

Antarctic benthic fauna tend to present long life cycles, slow growth rates due to slow metabolism, and direct development, and this is particularly true for molluscs (Peck et al. 2006; Moles et al. 2017). All these common characteristics seem to be the consequence of the peculiar characteristics of the Southern Ocean (SO), e.g. low temperatures, relative stability in the frequency of physical disturbance, and pronounced seasonality (Dayton et al. 1994; Jörger et al. 2014; Chown et al. 2015) aided by the onset of the Antarctic Circumpolar Current (ACC), ca. 25 Mya (Beu et al. 1997). During the late Eocene glacial periods, shelf fauna was completely impoverished with some species migrating into shelters and deep-sea waters, these being one of the major shelters for eurybathic species during the Last Glacial Maximum (Thatje et al. 2005). Certain taxa were able to re-colonize shallow waters during interglacial periods or when iceberg scouring wrecked the benthic communities and left free space available (Thatje et al. 2005). The deeper

shelf of the Antarctic continent and the periodic destruction of benthic habitat on the shelf were hypothesized as natural evolutionary drivers towards eurybathy (i.e. the capacity of species of living at a wide depth range), a widely shared feature of the Antarctic benthic fauna (Thatje et al. 2005; Allcock and Strugnell 2012). Numerous taxa present circum-Antarctic distributions due to the action of the ACC, the main responsible for the connectivity between populations due to the clockwise dispersion of larvae and/or adults around the SO (Thatje 2012; Riesgo et al. 2015). On the other hand, the Polar Front acts as a North–South barrier for water exchange above 1000 m depth (Clarke et al. 2005). The idea of the SO being isolated by the Polar Front has been challenged during the last years, revealing species connectivity and genetic flow with the adjacent areas (e.g. South Africa and the Magellanic region; Griffiths 2010; Chown et al. 2015).

Gastropods are one of the major taxa represented in the SO, with numerous species still being discovered (e.g. Moles et al. 2018, 2019; Fassio et al. 2019; Layton et al. 2019). In the SO, nudibranchs are currently represented by less than a hundred recognized species (Moles 2016; De Broyer et al. 2019), although this species richness could increase with the application of molecular techniques. Among nudibranchs, the Dendronotida gathers several taxa with unassigned or unstable phylogenetic position (Goodhear et al. 2015). One of these taxa is the family Tritoniidae, among which the genus *Tritonia* Cuvier, 1798, appears to be the most speciose (WoRMS Editorial Board 2018). Currently, 37 valid species belong to the genus *Tritonia*, and only three of them are found in the SO, with Antarctic, Sub-Antarctic, and Magellanic distributions, namely *T. dantarti* Ballesteros & Avila, 2006, *T. vorax* (Odhner, 1926), and *T. challengeriana* Bergh, 1884, respectively. *Tritonia vorax* was firstly described from South Georgia as *Duvaucelia vorax* by Odhner (1926) and then transferred into *Tritonia* by Marcus in 1959. *Tritonia dantarti* was described in 2006 from Bouvet Island (Ballesteros and Avila 2006). *Tritonia challengeriana*, instead, was described for the first time by Bergh (1884) from the Magellan Strait. Since then, the latter species has been found in South Georgia, the Falkland Islands, Tierra del Fuego, and in several Antarctic locations (Antarctic Peninsula, Ross Sea, Scotia Arc; Wägele 1995; Schrödl 2003). In Antarctica, *T. antarctica* Pfeffer in Martens & Pfeffer, 1886, was first described by Pfeffer (1886) from South Georgia, and later ascribed to *T. challengeriana* by Odhner (1926), as *Duvaucelia*. Years later, Wägele (1995) differentiated between Magellanic specimens which were identified as *T. challengeriana* and specimens occurring south of the Antarctic convergence, regarded as *T. antarctica*. This was based on the presence of oral lips and the absence of mantle glands in *T. antarctica*. However, Schrödl (1996) mentioned that oral lips may also be present in *T. challengeriana* from the

Chilean Patagonia. Mantle glands were found in histological sections of *T. antarctica* from South Georgia although in much lower numbers than in *T. challengeriana* from the Magellan area, and this led to synonymize again *T. antarctica* with *T. challengeriana* (Schrödl 2003). According to Schrödl (2003, 2009), other described species are no longer valid and are considered synonyms of *T. challengeriana*, i.e. *Microlophus poirieri* Rochebrune & Mabille, 1889, *Candiella australis* Berg, 1898, and *Tritonia appendiculata* Eliot, 1905. Descriptions of type material were often limited to a single individual and thus, these identifications might be unreliable (Wägele 1995; Schrödl 2003, 2009; Shields 2009). Furthermore, until now, no molecular data are available for any of these species, when given the wide range of distribution that *T. challengeriana* seems to present. So, the implementation of molecular tools could prove helpful to solve this phylogenetic conundrum. We aim to combine molecular techniques, used here for the first time in this species complex, with detailed morpho-anatomical analysis to shed light into the long-term discussed systematics and taxonomy of Antarctic *Tritonia* species.

Materials and methods

Sample collection

Specimens were collected by Agassiz trawl, bottom trawl, and Rauschert dredge at the Sub-Antarctic Bouvet Island and the eastern Weddell Sea in 1998 during the ANT XV/3 (Arntz and Gutt 1999) and in 2003–2004 during the ANT XXI/2 cruises (Brey 2005) of the R/V Polarstern (Alfred Wegener Institute, Bremerhaven, Germany) (Fig. 1). In order to build the maps of Fig. 1 the R packages ggplot2, marmap, and ggsn were used. The specimens of *Tritonia* were collected at 130–789 m depth at 17 different stations (Online Resource 1). Specimens were photographed on board and preserved in either Karnovsky, 70% ethanol, or 10% formalin in seawater for morpho-anatomical analyses, or frozen and later transferred to 96% ethanol, for molecular analyses. Specimens are deposited at the Centre of Biodiversity Resources (CRBA) in the Faculty of Biology, Universitat de Barcelona.

DNA amplification and extraction

Total genomic DNA was extracted from foot tissue with the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Molecular markers included two fragments of the mitochondrial genes cytochrome *c* oxidase I (COI) and 16S *rRNA*, and the nuclear gene *histone 3* (H3). Partial sequences of the protein-encoding COI marker were amplified using the

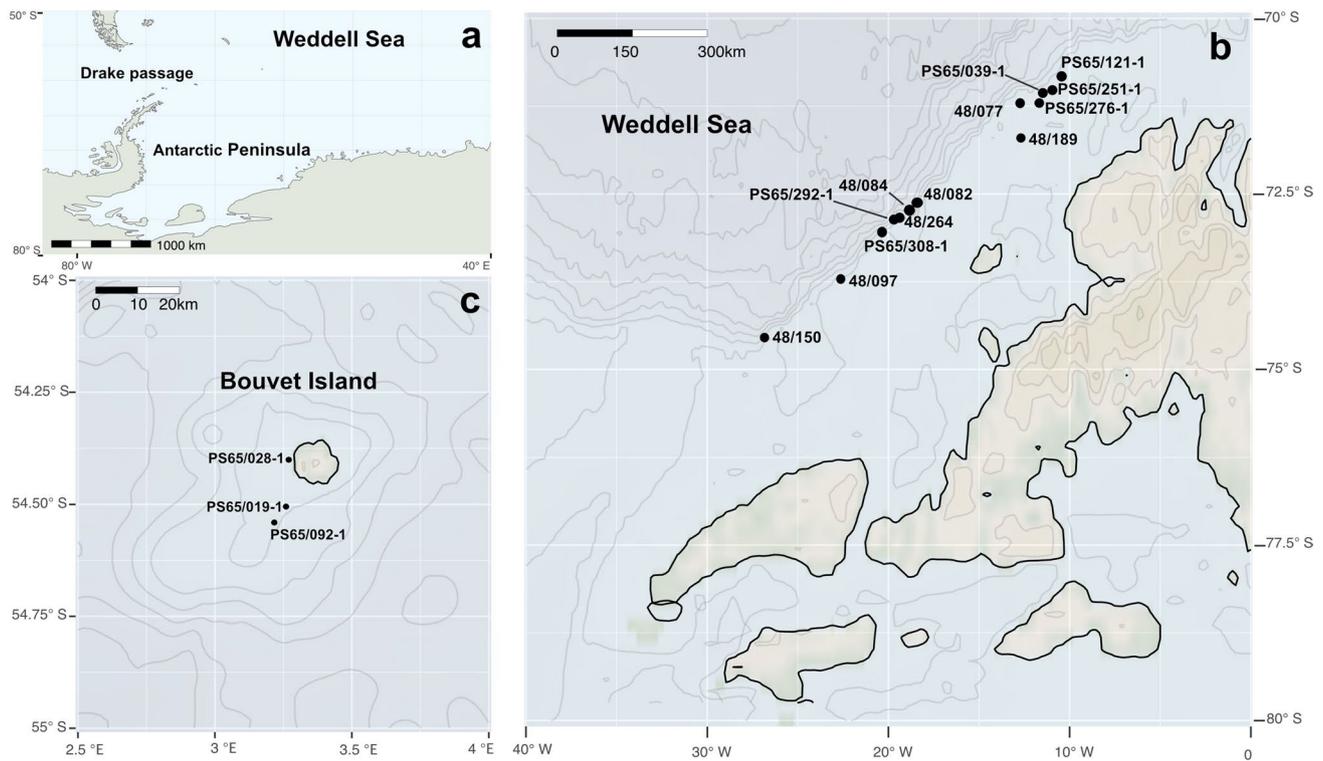


Fig. 1 Map of the Western Antarctic region showing the sampling stations. **a** General overview of the Western Antarctic region. **b** Map showing the sampling stations in the Weddell Sea. **c** Map of Bouvet Island showing sampling stations

primers LCO1490 and HCO2198 (Folmer et al. 1994), the 16S marker was amplified using 16Sar-L and 16Sbr-H (Palumbi et al. 2002), and the H3 marker was amplified with H3AD5'3' and H3BD5'3' (Colgan et al. 1998). PCR amplifications were carried out in a 10 μ L-reaction including 5.1 μ L of Sigma dH₂O, 3.3 μ L REDExtract-N-Amp PCR ReadyMix (Sigma Aldrich, St. Louis, MO, USA), 0.3 μ L of each primer, and 1 μ L of genomic DNA, following standard protocols implemented in our lab (Moles et al. 2016). The PCR for COI consisted of an initial denaturation step at 95 °C for 3 min, 39 cycles of denaturation at 94 °C for 45 s, annealing at 48–50 °C for 30 s, extension at 72 °C for 2 min, and a final extending step at 72 °C for 10 min. The PCR program for 16S involved an initial denaturing step at 94 °C for 3 min, 39 cycles of denaturation at 94 °C for 30 s, annealing at 44–52 °C for 30 s, extension at 72 °C for 2 min, and a final extending step at 72 °C for 10 min. For H3 amplifications, we used an initial denaturation step at 94 °C for 3 min, 35 amplification cycles (94 °C for 35 s, 50 °C for 1 min, and 72 °C for 1 min and 15 s), and a final extension at 72 °C for 2 min. Amplified products were sequenced at the UB Scientific and Technological Centers (CCiT-UB) on an ABI 3730XL DNA Analyzer (Applied Biosystems, CA).

Phylogenetic analyses

Chromatograms were visualized, edited, and assembled in Geneious Pro 8.1.5 (Kearse et al. 2012). To check for contamination, sequences were compared against the GenBank database using the BLAST algorithm (Basic Local Alignment Search Tool; Altschul et al. 1990). Single-gene sequences were aligned with the MUSCLE algorithm and alignments were trimmed to a position at which more than 50% of the sequences had nucleotides. Missing positions at the ends were coded as missing data. We used Gblocks 0.91b on the final trimmed alignment for identifying and excluding blocks of ambiguous data in the single, non-coding gene alignments of 16S, using both relaxed and stringent settings (Talavera and Castresana 2007). The total dataset contained 41 specimens of *Tritonia* and 17 closely related outgroup taxa (Online Resource 2). The concatenated alignment consisted of 1415 characters, including COI with 3rd codon position (ca. 601 bp), 16S unmodified (ca. 486 bp), and H3 with 3rd codon position (ca. 328 bp). The best-fit model of evolution (GTR + Γ + I; Yang 1996) was chosen using the Akaike information criterion (AIC; Posada and Buckley 2004) implemented in jModelTest 2.1.7 (Posada 2008) with the selected partition for each gene (Online Resource 3).

For each gene, a maximum-likelihood (ML) analysis was conducted, the final result was given by a concatenated alignment of all three genes. ML analyses were conducted using RAxML 8.1.2 (Stamatakis 2014), a GTR model of sequence evolution with corrections for a discrete gamma distribution and invariable sites (GTR + Γ + I; Yang 1996) was specified for each gene partition, and 500 independent searches were conducted. Nodal support was estimated through a bootstrap algorithm (500 replicates) using the GTR-CAT model (Stamatakis et al. 2008). Bayesian inference (BI) was performed on the concatenated alignment of the three genes using MrBayes 3.2.5 (Ronquist et al. 2011). Two runs were conducted in

MrBayes for 10 million generations, sampling every 2000 generations, using random starting trees. 25% of the runs were discarded as burn-in after checking for stationarity with Tracer 1.7 (Rambaut et al. 2018). Bootstrap support (BS) and posterior probabilities (PP) were thereafter mapped onto the optimal tree from the independent searches. Only nodal support values of > 70 and > 0.85 for BS and PP, respectively, were depicted in Fig. 2. The tree was rooted using the four selected Proctonotoidea species *Curnon granulosa*, *Leminda millecra*, and two species of *Dirona*, as the sister group to the rest of the Dendronotoidea species included in this study (following Goodhear et al. 2015).

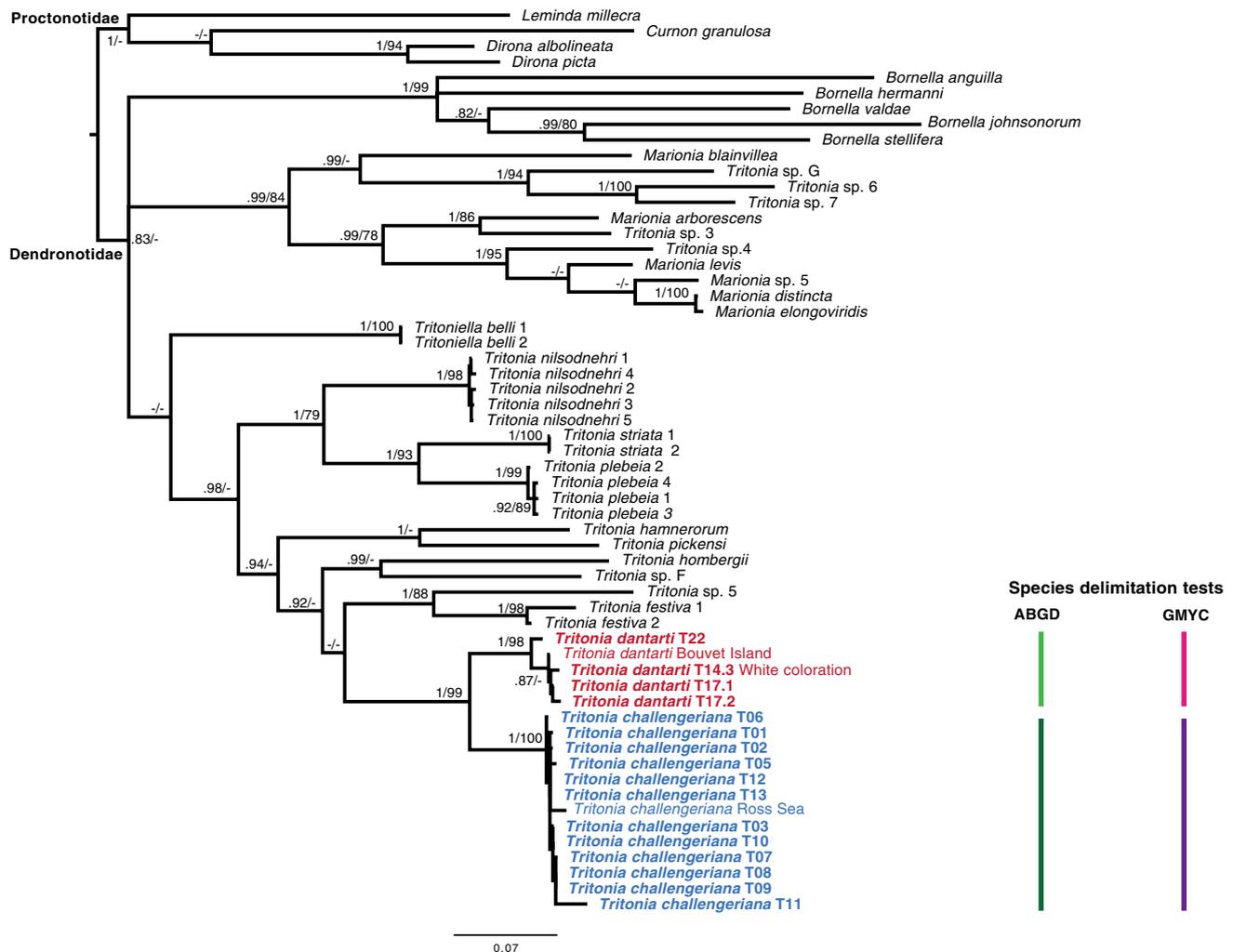


Fig. 2 Phylogenetic tree of *Tritonia* species and outgroup species considered using Bayesian inference (BI) and maximum likelihood (ML) on the combined COI, 16S, and H3 datasets. BI topology is shown in the main figure, and numbers on the nodes indicate posterior probability values (BI) and bootstrap support values (ML). Only values > 75 and > 0.85 depicted for ML and BI, respectively. Specimens in red are from Bouvet Island (Sub-Antarctic distribution) and

the specimens in blue are from the Weddell Sea (Antarctic distribution). The specimen of *T. dantarti* from GenBank placed in the red cluster was registered as *T. antarctica* (Voucher n. CASIZ171177). The results of the ABGD (green/left) and GMYC (purple/right) analyses are represented in bars, distinguishing the two SO species groups studied

Species delimitation tests

To examine the molecular distinctiveness of the different Antarctic *Tritonia* morpho-species, we used the Automatic Barcode Gap Discovery (Pulliandre et al. 2011) via the web interface (ABGD; <http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>). ABGD of the COI alignment was run using the K80 calibrated index of genetic distance with transition/transversion ratio (TS/TV) equal to 2.0. We applied default values for P_{\min} , P_{\max} , and a relative gap of 1.0. Additionally, with the same alignment, a Generalized Mixed Yule Coalescent (GMYC; Fujisawa and Barraclough 2013) analysis was performed. The ultrametric tree, necessary for the GMYC, was generated with BEAST (Suchard et al. 2018), with GTR + Γ + I substitution model, and a lognormal relaxed clock with a rate of 1.0, for 10 million generations. TreeAnnotator was used to discard 25% as burn-in. The GMYC was performed on the webserver (<https://species.h-its.org/gmyc/>) with single threshold parameters.

Morphological analyses

Photographs of whole animals were taken with a Nikon d300 Sigma 105 mm f 2.8–32. Total length (L) was measured aided by a calliper. Specimens were dissected sagittally with the aid of fine forceps under a stereomicroscope. Radula and jaws were obtained from the buccal bulb after dissolving the oral bulb's soft tissue in a 10% NaOH solution for up to four hours and later rinsed with distilled water in ultrasound baths. The reproductive system was depicted and the penial papilla extracted. This was critical point dried before mounting on stubs with carbon sticky-tabs, as for the radulae and jaws, for scanning electron microscopy (SEM). The stubs were carbon-coated and images were taken using a J-7100F Jeol scanning electron microscope at the UB Scientific and Technological Centers (CCiT-UB).

Results

Phylogenetic analyses

ML and BI analyses recovered a tree with maximum support for *T. challengeriana* specimens from both the Weddell and the Ross Seas (PP = 1, BS = 100), and for *T. dantarti* including only the Sub-Antarctic specimens from Bouvet Island (PP = 1, BS = 98; Fig. 2). One specimen attributed to *T. antarctica* (voucher number CASIZ171177) clustered with our specimens of *T. dantarti* and thus, might be considered a misidentification. The sister taxa to both SO species sequenced were the North Pacific *T. festiva*, a clade composed of the type species of the genus *T. hombergii*, and an unidentified species of *Tritonia*. The SO species clustered in

a clade with high support from the BI of different *Tritonia* species. We recovered the unidentified *Tritonia* sp. 3, *Tritonia* sp. 6, *Tritonia* sp. 7, and *Tritonia* sp. G all in a well-supported clade with all sequenced *Marionia* species. The relationships of the Antarctic monotypic *Tritoniella belli* were not found in our analyses. The relationships between *Bornella*, *Marionia*, *Tritoniella*, and *Tritonia* clades were not recovered in this study.

The ABGD analysis additionally supported the taxonomic identity of *T. challengeriana* and *T. dantarti*, with an intraspecific variation of 1.7 and 1.9% on average, respectively (Table 1). Intraspecific variation within other *Tritonia* species considered in this study ranged from 0 to 7%. The interspecific variation of *T. challengeriana* and *T. dantarti* ranged from 12 to 14% (Table 1). The GMYC analysis also recovered both species as different, as for the ABGD analysis (Online Resource 4).

Systematics

Class GASTROPODA Cuvier, 1795

Subclass HETEROBRANCHIA Burmeister, 1837

Order NUDIBRANCHIA Cuvier, 1817

Suborder CLADOBRANCHIA Willan & Morton, 1984

Family TRITONIIDAE Lamarck, 1809

Genus *Tritonia* Cuvier, 1798

Type species: *Tritonia hombergii* Cuvier, 1803

***Tritonia challengeriana* Berg, 1884**

Tritonia challengeriana Bergh, 1884: 45–47, pl. 11, figs. 16–19; Eliot 1907: 354–355; 1907: 3–5; Wägele 1995: 41–45; Schrödl 2003: 97–101

Tritonia antarctica Pfeffer in Martens & Pfeffer, 1886: 112, pl. 3, figs. 6a,b; Wägele 1995: 21–46

Microlophus poirieri Mabile & Rochebrune, 1889: 11–12, pl. 6, figs. 1a,b

Tritonia poirieri (Mabile & Rochebrune, 1889): Wägele 1995: 43

Candiella australis Bergh, 1898a: 536–539, pl. 31, figs. 17–25

Tritonia appendiculata Eliot, 1905: 526, figs. 6–10

Tritonia australis (Bergh): Dall 1909: 202

Duvaucelia challengeriana (Bergh): Odhner 1926: 35–37, pl. 1, fig. 14

Duvaucelia poirieri (Rochebrune & Mabile): Odhner 1926: 38–39

Myrella poirieri (Rochebrune & Mabile): Odhner 1963: 51–52

Marionia cucullata (Couthouy in Gould): Vicente and Arnaud 1974: 539, figs. 6, 7, pl. 3, figs. 1–3

Material examined: Out of the 37 specimens collected, 32 were frozen, two were fixed in 10% formalin in seawater, two in 70% ethanol, and one in 96% ethanol. South of Vestkapp, eastern Weddell Sea, 73°36.6'S, 22°24.7'W, 736 m depth: 1 sp., dissected and sequenced, T08, voucher CRBA-92763, L = 25 mm, barcode MN651129. Halley Bay, 74°35.8'S, 26°55.0'W, 789 m depth: 1 sp., dissected and sequenced, T10, voucher CRBA-92765, L = 22 mm, barcode MN651131. North of Kapp Norvegia, 71°07.34' S, 11°27.80'W, 146 m depth: 1 sp., dissected, T25, voucher

CRBA-92800 L = 26 mm. Drescher Inlet, 72°05.18'S, 19°38.62'W, 598 m depth: 1 sp., dissected T28, L = 42 mm (Online Resource 1).

External morphology (Fig. 3a–c): Body length 22–42 mm after preservation (Table 2). Body wider dorsally than ventrally. Colour of preserved specimens milky white, beige-brownish when viscera seen by transparency; live specimens homogeneously white to orange. Dorsal mantle surface smooth, with subepithelial white knobs, found mostly in posterior region of mantle. White pigmentation

Fig. 3 Preserved specimen of *Tritonia challengeriana* from the eastern Weddell Sea (T28; voucher CRBA-92804). **a** Dorsal view. **b** Lateral view. **c** Ventral view. **d** Scanning electron microscopy (SEM) of the radula showing the tricuspidate rachidian teeth, the first and subsequent lateral teeth (T08; voucher CRBA-92763). **e** Detail of the jaw's denticles (SEM) (T08; voucher CRBA-92763). **f** Schematic drawing of the reproductive system (T28; voucher CRBA-92804). *am* ampulla; *cgl*, capsule gland; *mgl*, mucous gland; *re*, seminal receptacle; *vd*, vas deferens; *vg* vaginal duct. **g** Detail of the penial papilla (SEM) (T28; voucher CRBA-92804)

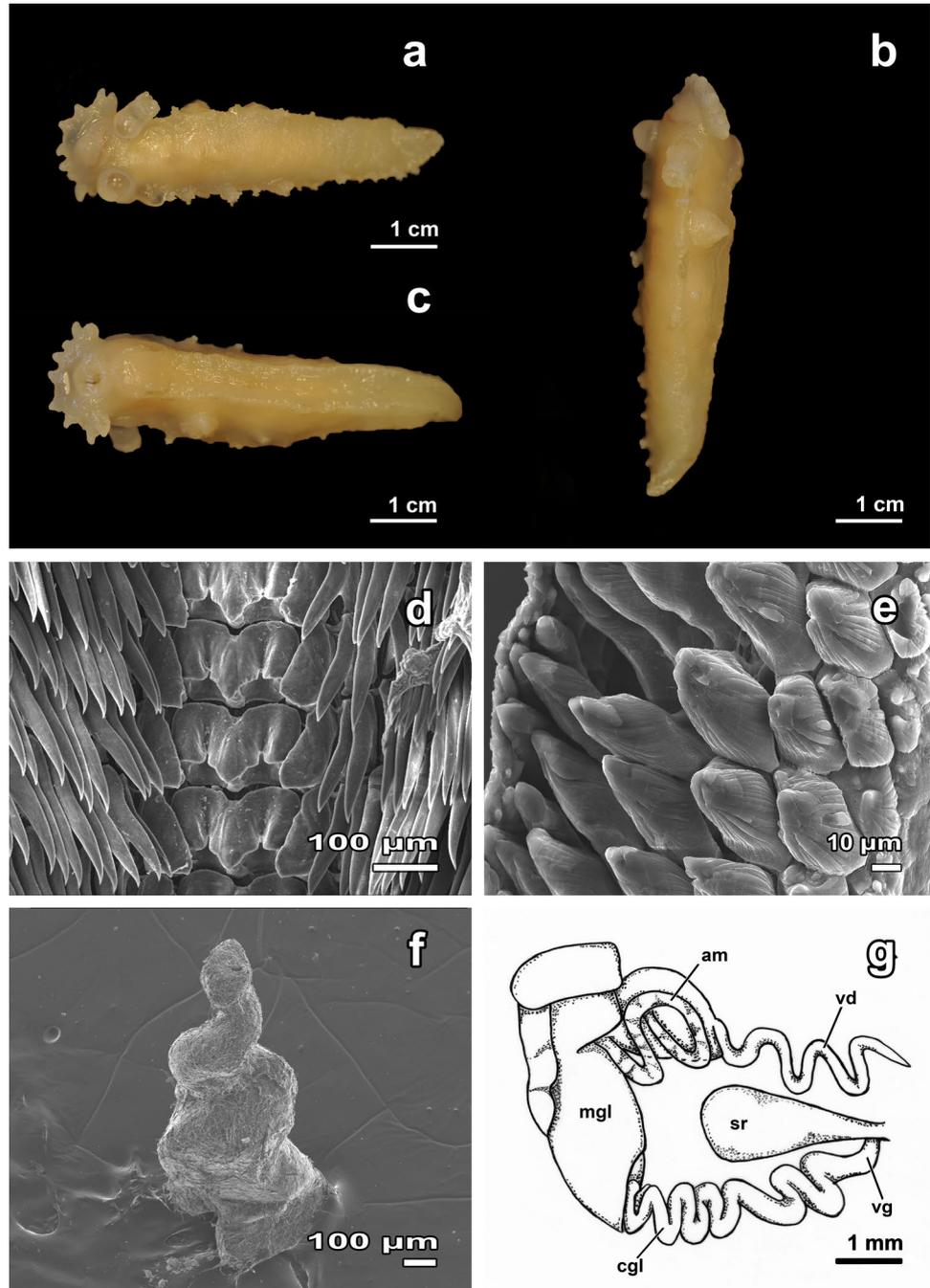


Table 1 Kimura two-parameter distances (K80) between and within groups for the putative species of *Tritonia* included in our analyses

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | Within groups |
|---|--------------------------|-----------|-----------|-----------|-----------|-----------|----------|-----------|------|---------------|
| 1 | <i>T. challengeriana</i> | | | | | | | | | 1 0–1.5 |
| 2 | <i>T. dantarti</i> | 7.9–14 | | | | | | | | 2 0–0.019 |
| 3 | <i>T. festiva</i> | 12.6–20.5 | 17.7–19 | | | | | | | 3 0–0.134 |
| 4 | <i>T. hamnerorum</i> | 11.1–18.1 | 18.4–20 | 21.1–21.3 | | | | | | 4 n.c. |
| 5 | <i>T. hombergii</i> | 12.5–22.3 | 20.5–22.5 | 18.7–19.1 | 22.7 | | | | | 5 n.c. |
| 6 | <i>T. nilsodneri</i> | 8.9–22.5 | 11.4–21.6 | 11.1–21 | 9.2–16.8 | 10–21.3 | | | | 6 0–2 |
| 7 | <i>T. pickensi</i> | 19.4–25.7 | 22.6–24.1 | 21.1–21.3 | 17.2 | 10.1–20.5 | | | | 7 n.c. |
| 8 | <i>T. plebeia</i> | 12.4–22.6 | 21.7–23.3 | 21.4–22.3 | 20.2–20.6 | 23.5–23.9 | 9.1–18.9 | 19.9–20.8 | | 8 0.1–7 |
| 9 | <i>T. striata</i> | 13.4–23.6 | 21.2–23 | 20.1–20.3 | 21.3 | 10–20.6 | 21.9 | 15.6–21.9 | 16.6 | 9 0.00 |

n.c. non-computable since there was a single sequence available

seen on notal margin, gills, and margin of rhinophoral sheath (Fig. 3a). Rhinophoral sheath broad; margin and plumes smooth. Gills ramified, dichotomous, large or small, from 6 to 19 per side, situated in parallel to each other. Oral veil prominent, bilobed or not. Five to ten velar processes present. Mouth surrounded by thick lips without distinct oral tentacles. Foot perimeter smaller than notal surface (Fig. 3c). Genital papilla found on right side of body. Anal opening placed at 1/2 of body length (Fig. 3b).

Digestive system (Fig. 3d, e): Oral lips smooth, large. Oral tube short. Oral bulb and pharynx thick. Radular formula 30–37 × (33–49)1.1.1.(33–49); transparent in colour. Rachidian teeth presenting median prominent cusp, one smaller cusp found per each side (Fig. 3d). Two different lateral teeth, with one short, broad cusp. Jaws yellowish, curved towards inside; several rows of conic denticles, curved, striated, as border denticles (Fig. 3e). Jaws 5–9 mm in length. Ratio jaw:body 0.21–0.3 in length. Oesophagus running dorsally from pharynx. Salivary glands thin, elongated, running laterally from first half of body, then ventrally following oesophagus. Stomach situated ventrally. Intestine generally striated, originating dorsally from stomach, turning right, ending in anal opening.

Reproductive system (Fig. 3f, g): Reproductive system androdialucic. Gonad large, wrinkled, covering digestive gland posteriorly. Gonoduct opening in ampulla connected by spermiduct to vas deferens. Bifurcation into vas deferens and oviduct not easily detected (Fig. 3g). Penial sheath terminal, conical, thin; penial papilla conical, slightly twisted (Fig. 3f). Seminal receptacle voluminous, with short duct; going shortly into genital opening. Granulated capsule gland at bottom of wide mucous gland, preceding short oviduct.

Ecology (Fig. 4a–c): The specimens were collected from 146 to 789 m depth. Sclerites of alcyonarian octocorals were found in the gut contents of three out of the four specimens studied.

Distribution: Argentinian Patagonia (Marcus et al. 1969), Falkland Islands (Eliot 1907), Chilean Patagonia (Bergh 1884; Schrödl 1996) to Ancud Bay (Schrödl 1996), South Georgia (Odhner 1926), Adélie Land (Vicente and Arnaud 1974), Victoria Land (Ross Sea, Schiaparelli et al. 2006), and eastern Weddell Sea (Wägele 1995; this study).

Remarks: Most synonyms of genera and species to *T. challengeriana* were based on external morphological similarities (Wägele 1995; Schrödl 2003). For instance, Rochebrune and Mabilie (1889) described *Microlophus poirieri* from Patagonia, Falkland Islands, and South Georgia, based only on their external morphology and colouration. *Microlophus poirieri* was later synonymized to *T. challengeriana* by Wägele (1995) based on their external morphology. *Marioinia cucullata* was described from Adélie Land (Vicente and Arnaud 1974) but the similarity in oral lips' shape and the low number of gills allowed Wägele (1995) to synonymize

this genus and species to *T. antarctica*. *Tritonia appendiculata* Elliot, 1905 was described from Harbour, Scotia Bay, the South Orkney Islands at 16 m depth. Its body colour was greenish-yellow, this is the only character that differs from our specimens since most of the morphological characters overlap with *T. challengeriana*. For instance, the species presents 19 gills per side and, on the dorsal surface, subepithelial knobs organized as “warts” were present (Eliot 1905). The oral veil presents twelve simple digitate processes and the lips are projected on each side of the mouth. The relation of jaw length (10 mm) to body length (51.5 mm) is 0.19 for *T. appendiculata*, while Wägele (1995) found a similar ratio for *T. challengeriana* of 0.23, as in our study. Thus, our data support Wägele’s synonymy. *Tritonia poirieri* Mabile & Rochebrune, 1891, det. Odhner 1926, was found at Fitzroy Channel, at 14 m depth. The species’ body shape resembles that of *Doris*, with the notal margin bent downward. Other than the peculiar body shape, there were not enough differences to identify *T. poirieri* as a distinct species from *T. challengeriana* (Wägele 1995).

Wägele (1995) differentiated Magellanic specimens of *T. challengeriana* from the specimens occurring south of the Polar Front, regarded as *T. antarctica* Martens & Pfeffer, 1886. The major difference was the presence of oral lips and mantle glands, exclusively found in *T. antarctica*. Later on, Schrödl (2003) described these two characters in *T. challengeriana* from Chilean Patagonia and synonymized it to *T. antarctica*. Our specimens are morphologically similar to the *T. antarctica* specimens described by Wägele (1995), with visible white knobs on the dorsal surface of the body and the presence of conspicuous oral lips. Our description of *T. challengeriana* overlaps with the measurements and descriptions from Pfeffer (1886) and Wägele (1995), thus highlighting a major similarity to *T. antarctica* than to the Magellanic *T. challengeriana*. On the other hand, *Tritonia vorax* (Odhner, 1926) is found in South Georgia (Wägele 1995), Burdwood Bank, and the Chilean Patagonia (Odhner 1926; Schrödl 1996). Living specimens present a whitish to brownish colouration, with white or opaque white reticulations on the notal surface. Preserved specimens can be whitish, yellowish or pinkish and their notum can be more

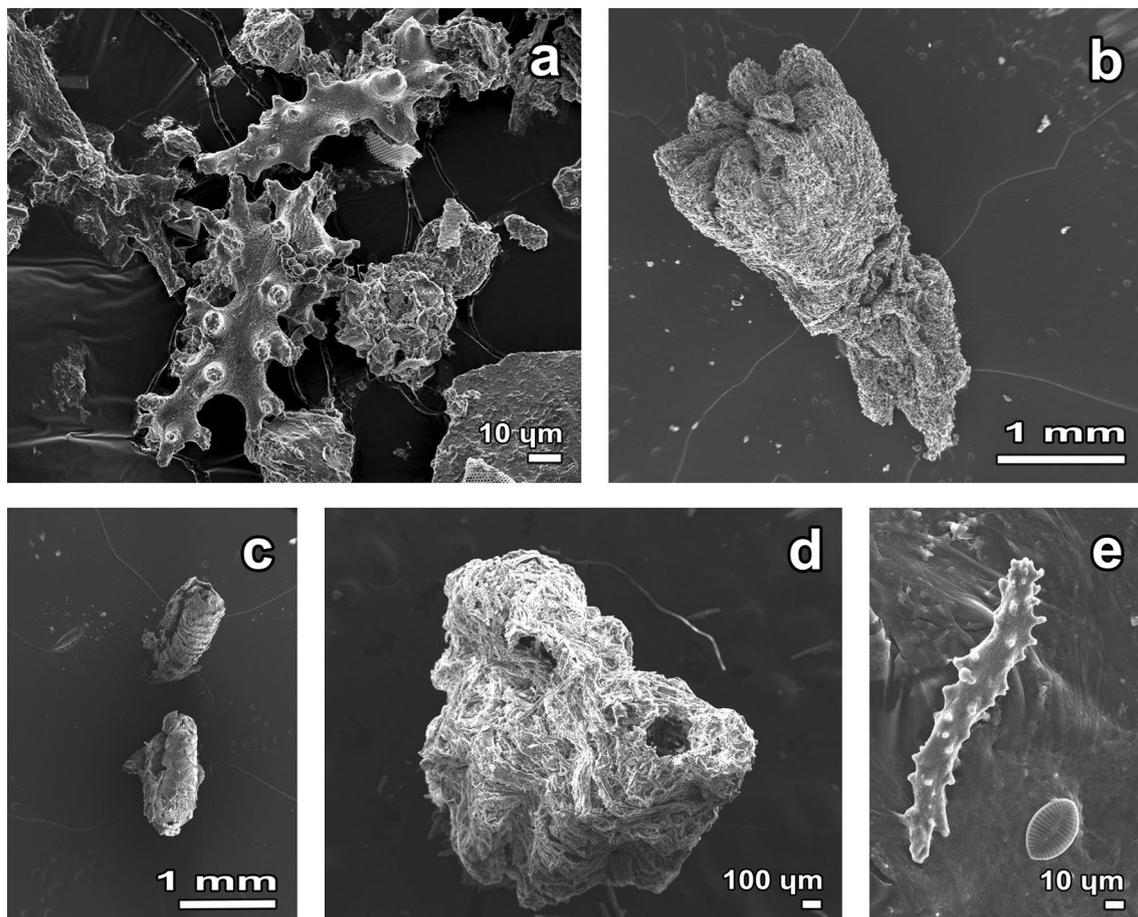


Fig. 4 Gut content found in the intestine of the examined specimens. **a, c** Octocoral structures found in *Tritonia challengeriana*. **b** Alcyonarian sclerites and diatom found in *T. challengeriana*. **d, e** Alcyonarian spicules found in *T. dantarti*

or less smooth. This species differs from *T. challengeriana* by having less number of gills, extremely large and strong jaws, which cause an elevated mediodorsal protuberance in between the rhinophores, and the lack of oral lips, with higher jaws:body length ratio than that of *T. challengeriana* (Table 2). Differences between *T. challengeriana* and *T. dantarti* are discussed in the Remarks section below.

Tritonia dantarti Ballesteros & Avila, 2006

Material examined: Thirteen specimens collected at stations PS65/028-1 and PS65/029-1 in Bouvet Island. Six specimens were fixed in 70% ethanol, four were frozen, one in 96% ethanol, and two in Karnowsky. Bouvet Island, 54°30.1'S, 3°13.97'W, 260 m depth: 1 sp., dissected and sequenced, T14.3, voucher CRBA-92771, L = 18 mm, barcode MN651134; 54°22.49'S, 3°17.58'W, 130 m depth: 1 sp., dissected, T16, voucher CRBA-92782, L = 23 mm; 54°22.49'S, 3°17.58'W, 130 m depth: 1 sp., dissected, T18.1, voucher CRBA-92787, L = 20 mm, barcode (Online Resource 1).

External morphology (Fig. 5a–c): Body short, thick; 18–23 mm length. Colour beige to milky white in preserved specimens, living specimens sometimes completely white (specimens T14.3, voucher CRBA-92771 and T14.4 voucher CRBA-92772, Fig. 6a) or bright orange on dorsal surface, with warts forming a reticulation; white laterally (specimen T15.1 voucher CRBA-92777, Fig. 6b). Dorsal mantle surface smooth with subepithelial white knobs. Notal margin unpigmented. Rhinophores with large sheath; smooth margin with emerging plumes. Single small gills or largely ramified, from 15 to 29 per side (Fig. 5a). Oral veil not prominent, bilobed or not; velar processes, short, 9 to 19 in number. Lips thick, surrounding buccal bulb, without recognizable tentacles. Foot narrower than notum (Fig. 5c). Genital papilla on right side at 1/3 of body length. Anal opening at 1/2 of body length (Fig. 5b). Length and morphometrical data reported in Table 2.

Digestive system (Fig. 5d–e): Oral lips thick, smooth. Pharynx large, compact; with a pair of curved jaws of yellowish margin. Rachidian teeth broad, monocuspid (Fig. 5d). Radular formula: 36–37 x (43–46)1.1.1.(43–46). Oesophagus running dorsally from pharynx. Jaw denticles broad, conical, striated, hooked on top, arranged in several rows (Fig. 5e). Jaws 5–6.5 mm in length. Ratio jaw:body 0.25–0.28 mm in length. Salivary glands large, isodiametric, running laterally in first body half, then ventrally under oesophagus. Stomach situated ventrally. Intestine generally striated, originating dorsally from stomach, turning to right side, ending at anal opening.

Reproductive system (Fig. 5f–g): Reproductive system situated between buccal bulb and digestive gland. Gonad brownish, warty, covering digestive gland. Genital papilla opening in a bulb, spermiduct could not be observed (Fig. 5g). Seminal receptacle wide. Penis thin, flagellated

(Fig. 5f), occasionally conical. Penial papilla with conical shape. Mucous gland well developed, situated on top of entire system; granulated capsule gland preceding short oviduct, often convoluted.

Ecology (Fig. 4d, e): Specimens of *T. dantarti* were collected in Bouvet Island at 130–134 m depth, in sea bottoms dominated by ophiuroids (*Ophionotus victoriae*), sea stars (*Porania antarctica*), holothuroids, sedentary polychaetes, hydroids, alcyonarians, different actinian species, amphipods, and pycnogonids. Gut contents showed that *T. dantarti* feeds on alcyonarians of the genus *Alcyonium*.

Distribution: Northwest and southeast of Bouvet Island.

Remarks: *Tritonia dantarti* is clearly distinguished from its counterpart *T. vorax* by the possession of oral lips, completely lacking in *T. vorax*. In *T. dantarti* the oral veil can be bilobed or not, while it is always bilobed in *T. vorax*. Moreover, *T. dantarti* presents lesser teeth rows and a monocuspidate rachidian tooth, while *T. vorax* presents a higher number of rows with a tricuspid rachidian tooth. Additionally, the jaws:body length ratio is higher in *T. vorax* (Table 2).

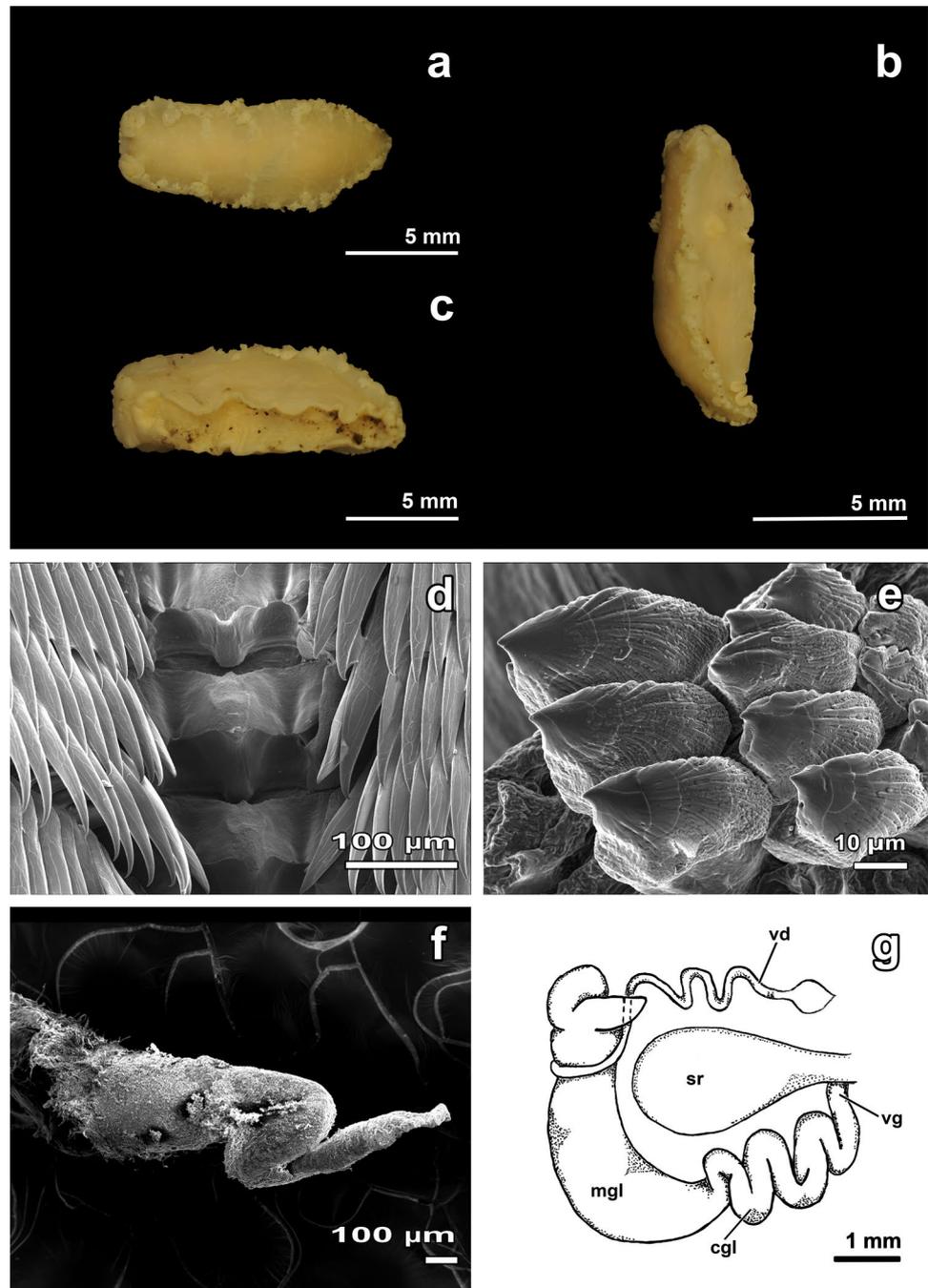
Tritonia dantarti was described by possessing a conspicuous orange colouration in the dorsum of living specimens (see Fig. 6a,c,e in Ballesteros and Avila 2006; Fig. 6b in this study). This was, in fact, the main difference from *T. challengeriana*, but here molecular evidence of both white and orange colour-morphs is given for *T. dantarti*. An additional diagnostic character is the presence of a warty reticulation in the notal surface of living specimens of *T. dantarti*, which has not been obviously observed in our preserved specimens and is completely lacking in *T. challengeriana*. Moreover, *T. challengeriana* generally presents fewer velar processes and fewer clusters of gills, but some overlap exists for both species, and a broad range of morphological differences is especially misleading in preserved specimens of both species. Our results agree with previous descriptions for both species (Wägele 1995; Schrödl 2003; Ballesteros and Avila 2006) (Table 2).

Discussion

Taxonomy and morphology of Antarctic *Tritonia* species

The specimens reviewed in this study from the high Antarctic belonged to the only current valid species *Tritonia challengeriana*, while the specimens from Bouvet Island belonged to *T. dantarti*. Phylogenetic analyses and species delimitation tests recovered these two species with strong support (Fig. 2), including the specimens of *T. challengeriana* from the Weddell Sea and the only sequenced specimen

Fig. 5 Preserved specimen of *Tritonia dantarti* from Bouvet Island (T16; voucher CRBA-92782). **a** Dorsal view. **b** Lateral view. **c** Ventral view. **d** Scanning electron microscopy (SEM) of the radula showing the rachidian teeth, the first and subsequent lateral teeth. **e** Detail of the jaw denticles (SEM). **f** Detail of the penial papilla (SEM). **g** Schematic drawing of the reproductive system. *am* ampulla, *cgl* capsule gland, *mgl* mucous gland, *re* seminal receptacle, *vd* vas deferens, *vg* vaginal duct



from the Ross Sea (PP = 1, BS = 100), and the specimens of *T. dantarti* from Bouvet Island (PP = 1, BS = 98). Morpho-anatomical analyses showed that on the dorsal body surface in living specimens of *T. dantarti* warts and reticulation are visible. Nonetheless, the bright orange colouration (Ballesteros and Avila 2006) may no longer be a valid diagnostic character, since both milky white and orange colour morphotypes from Bouvet Island were found here, as it has been described for *T. challengeriana* from both South

America and high Antarctic regions (Fig. 7). These results were supported by our molecular analyses. Besides this, no other clear diagnostic characters were found in the morpho-anatomical analysis to allow discrimination among these two species. For instance, shape and body measurements, the number of velar processes, the shape and number of gills, the radular formula, and the shape of the jaws are not quite discernible between *T. dantarti* and *T. challengeriana*. Both species overlap in the range of the aforementioned characters (Table 2), as also reported by Wägele (1995), Schrödl

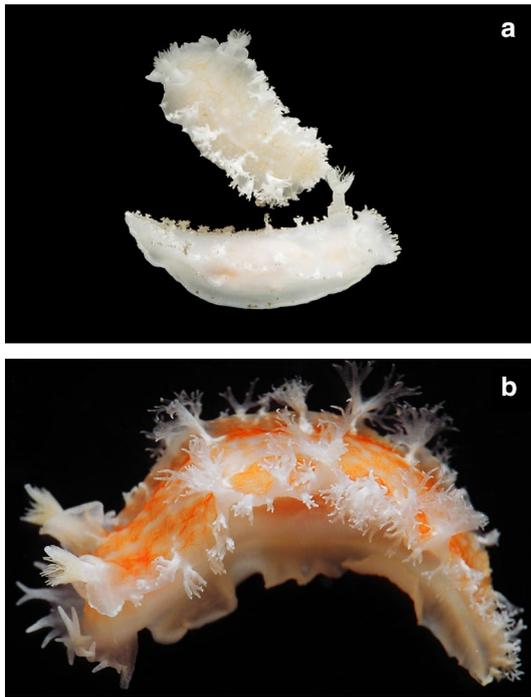


Fig. 6 Specimens of *Tritonia dantarti* from Bouvet Island (photographs by M. Ballesteros). **a** Two specimens collected at 260 m of depth from Bouvet Island displaying whitish colouration (T14.3; voucher CRBA-92771, and T14.4; voucher CRBA-92772). **b** Specimen from Bouvet Island with orange colouration, collected at 130 m depth (T15.1; voucher CRBA-92777)

(2003), and Ballesteros and Avila (2006). Nonetheless, *T. challengeriana* seems to present lesser oral tentacles and gill clusters than *T. dantarti*, but still, this might be subjected to ontogenetic development.

The validity of *T. antarctica* has been questioned in a few studies (see Remarks section of *T. challengeriana*). Wägele (1995) sustained the existence of *T. antarctica* for the presence, in Antarctic specimens, of subepithelial glands (externally visualized as knobs), which were lacking in Magellanic specimens. Later on, Schrödl (2003) suggested the contrary, showing that the glands were present on the dorsal surface of the specimens from the Magellanic area, even if sporadically and in a lower number. Our specimens seem to be similar to the *T. antarctica* specimens described by Wägele (1995). Pictures of living specimens from the Magellanic region (Fig. 7a–c) do not show visible knobs, which are easily detectable on specimens from Antarctica (Fig. 7d–f). However, the morphological analysis suggests that *T. challengeriana* and *T. antarctica* could be considered to be different species, given the evidence of the visible knobs on the dorsal surface present on Antarctic specimens. Unfortunately, we cannot confirm the validity of *T. antarctica*, since there are no molecular data available for *T. challengeriana* from the Magellanic region to date. Southern American material and

additional samples from around Antarctica could be very useful to shed light into the Southern Hemisphere *Tritonia* species systematics.

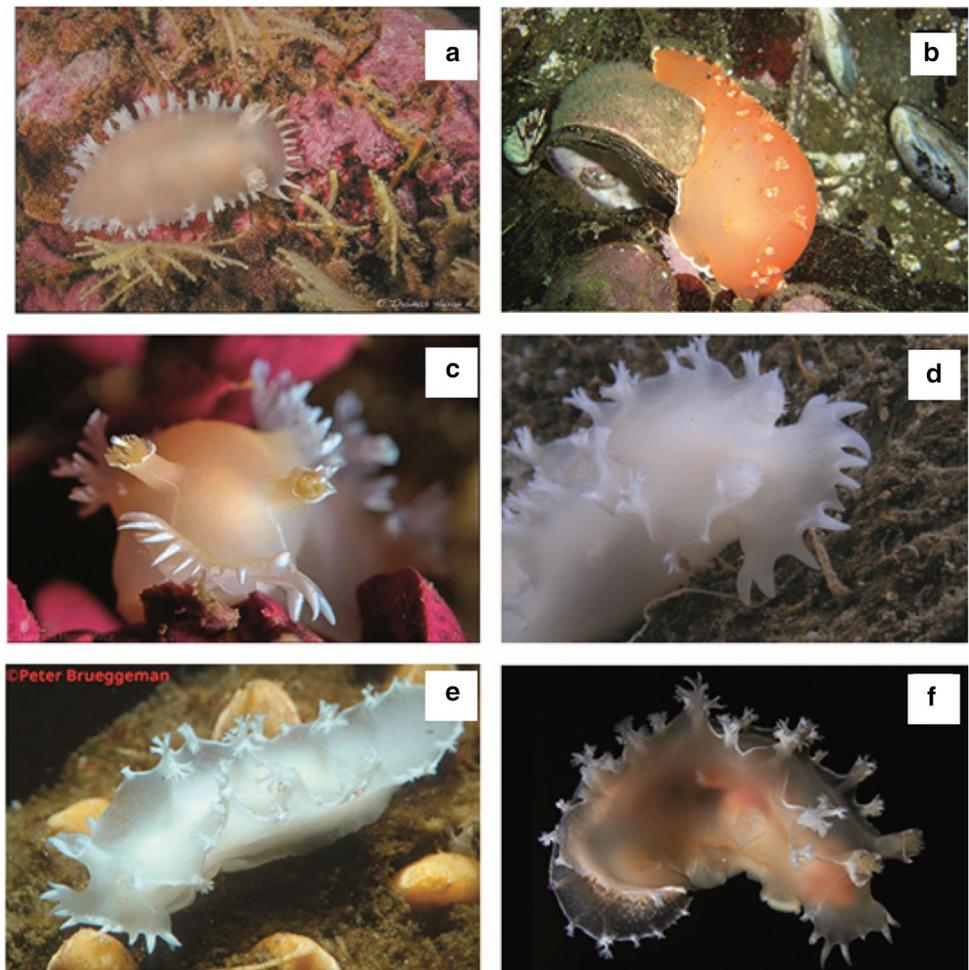
The colouration issue

Members of the family Tritoniidae feed almost exclusively on octocorals, including sea pens, alcyonarian soft corals, and gorgonians, sometimes being cryptic in shape and colouration upon them (García-Matucheski and Muniain 2011). In the SO, *Tritonia* species feed mostly on alcyonarian soft corals (Wägele 1995; Schrödl 2003; García-Matucheski and Muniain op. cit.). Here, we found soft coral sclerites in the gut contents of both *T. challengeriana* and *T. dantarti*. The *Alcyonium* species living in the SO are *A. antarcticum* Wright & Studer, 1889, *A. grandis* Casas, Ramil & van Ofwegen, 1997, *A. haddoni* Wright & Studer, 1889, *A. paucilobatum*, *A. sollasi* Wright & Studer, 1889, and *A. southgeorgensis* Casas, Ramil & van Ofwegen, 1997, and they can all present a yellow, cream or orange colouration, while they tend to be brighter in the Magellanic region (Casas et al. 1997). Through the evolution, and related to the loss of the shell, nudibranchs have developed a plethora of defensive strategies against predators (Avila et al. 2018). These defences include chemicals (natural products), which can be either de novo synthesized by the own slug or gathered from their prey (i.e. kleptochemistry). An example of kleptochemistry in Antarctica is found in *Tritoniella belli* Eliot, 1907 which obtains its defensive natural products from its prey, the anthozoan *Clavularia frankliniana* Roule, 1902 (McClintock et al. 1994). Some dietary metabolites can be brightly coloured pigments, as for some *Alcyonium* spp. natural products (Abdel-Lateff et al. 2019), and their sequestration and display in the external tissue of the slug may provide an additional mimetic defensive strategy. Although the development of a bright colouration may sometimes represent a warning mechanism (i.e. aposematism; Aguado and Marin 2007; Haber et al. 2010; Avila et al. 2018), the bright orange colouration found in both *T. challengeriana* and *T. dantarti* may not represent an aposematism mechanism, since the majority of visually guided predators, such as fishes or decapods, are not especially diversified in Antarctica (De Broyer and Danis 2011; Moles 2016). Nevertheless, antipredation assays suggest *T. challengeriana* is chemically protected against sympatric sea star predators, although the compounds have not been identified yet (Avila et al. 2018). This strategy has been proved in other Antarctic species, such as *Bathydoris hodgsoni* Eliot, 1907 and *Tritoniella belli* Eliot, 1907 (McClintock et al. 1994; Avila et al. 2000). We propose here that the colouration in *T. challengeriana* and *T. dantarti* varies locally in direct relation to diet and therefore cannot be used as a diagnostic character for these species.

Table 2 Diagnostic characters of *Tritonia challengeriana*, *T. dantarti*, and *T. vorax* from the present study and the literature

| | <i>T. challengeriana</i> | | | <i>T. dantarti</i> | | <i>T. vorax</i> | |
|-----------------------------------|----------------------------------|----------------------------------|---|--|--|---|--|
| | Weddell Sea | Antarctic Peninsula, Weddell Sea | Bouvet Island | Bouvet Island | | South Georgia | |
| Body size after preservation (mm) | 21–42 | 23–65 | 18–23 | 18–33 | | 22–60 | |
| Colouration | Beige-brownish, milky white | White, yellow, brown | Beige, milky white | Bright orange, white | | White, pink, cream | |
| Dorsal mantle | Smooth with subepithelial knobs | Warty | Smooth with subepithelial knobs | With crests | | Smooth | |
| Oral lips | Present | Present | Present | Present | | Absent | |
| Velum | Not prominent, sometimes bilobed | Sometimes bilobed | Prominent or not, bilobed | Wide, semitransparent, bilobed | | Bilobed | |
| Num. of velar processes | 5–10 | < 15 | 9–19 | < 15 | | > 15 | |
| Num. of gill clusters per side | 6–14 | 6–30 | 15–29 | 20–33 | | 20–40 | |
| Size of gill clusters | Large or small | Small or medium | Large or small | Large or small | | Tiny or small | |
| Shape of gill clusters | Ramified or dichotomous | Digitated | Ramified, highly digitated | Highly ramified | | Dichotomous | |
| Salivary glands | Thin, elongated | Long, ribbon-like | Large, isodiametric | Small, elongated | | Long, ribbon-like | |
| Rachidian tooth | Broad, prominent monocuspidate | Broad, prominent central cusp | Broad, prominent monocuspidate | Broad, monocuspidate, central cusp and two lateral rounded protuberances | | Broad, tricuspidate | |
| Radular formula | 30–37 × (33–49).1.1.1.(33–49) | 31–46 × (37–63).1.1.1.(37–63) | 36–37 × (43–46).1.1.1.(43–46) | 30–38 × (35–48).1.1.1.(35–48) | | 54–71 × (79–115).1.1.1.(79–115) | |
| Jaw/body length proportions | 0.21–0.3 | 0.25 | 0.25–0.28 | 0.27 | | > 0.4 | |
| Jaw denticles | Conical, striated, hooked | Conical, striated | Conical, striated, hooked | Conical, slightly curved | | Conical, striated | |
| Penis | Flagellated and/or conical | Digitiform to conical | Thin, flagellated and/or conical | – | | Digitiform to conical | |
| Gut contents | Alcyonarian spicules, diatoms | <i>Cephalodiscus</i> sp. | Alcyonarian spicules, diatoms, – octocoral polyps | – | | Alcyonarian species, amphipods (<i>Gammaropsis</i>), tanaidaceans | |
| Reference | Present study | Wägele (1995) and Schrödl (2003) | Present study | Ballesteros and Avila (2006) | | Wägele (1995) | |

Fig. 7 Underwater photographs of alleged *Tritonia challengeriana* from its current range of distribution. **a** Puerto Raúl Marín Balmaceda, Chile (photograph by T. Heran). **b** Comau Fjord, Chile (photograph by D. Thompson). **c** Punta Porra, Chile (photograph by T. Heran). **d** Ross Sea, Antarctica (photograph by S. Harper). **e** Ross Sea, Antarctica (photograph by P. Brueggeman). **f** Antarctic Peninsula, Antarctica (photograph by G. Giribet)



Distribution and cryptic speciation

From our molecular phylogenetic analyses, we observe that while *T. dantarti* seems to have a restricted, endemic distribution in Bouvet Island, *T. challengeriana* seems to present a disjunct (i.e. found in both the Weddell and Ross Seas) and probably circumpolar distribution. This distribution could be partially explained by the action of the ACC (Thatje 2012). When the Drake Passage was narrower, the ACC flow was particularly intense in the Antarctic region, carrying adults or egg masses attached to floating debris (i.e. rafting phenomenon) to new habitats all around Antarctica (i.e. circumpolar distribution). Through genetic drift and selection these populations could diverge sufficiently to either yield a new species by allopatric speciation (Allcock and Strugnell 2012)—which might have been the case for the restricted *T. dantarti*—or widening its geographical range, as for *T. challengeriana*. Even if the rafting phenomenon allowed a long-distance dispersal in organisms that not produce free-swimming larvae (Thatje op. cit.) during glacial cycles, the existence of shelters where the ice shelf did not cover homogeneously the shelf, acted as refugia (Thatje et al. 2005;

Fraser et al. 2014; Chown et al. 2015). There, species isolation could have led to cryptic speciation (Wilson et al. 2009). In fact, because of the existence of cryptic species, the current species richness of gastropods in the Antarctic and Sub-Antarctic regions is higher than previously thought (Linse et al. 2007). Likewise, new cephalaspidean molluscs with low character displacement have been recently described based on molecular data in the same region (Moles et al. 2017, 2019). The nudibranch *Doris kerguelenensis* (Bergh, 1884) seems to also present this trend; molecular data evidenced a complex genetic structure that suggests much diversity than a single recognized species (Wilson et al. 2009). This hypothesis is corroborated by the wide variety of natural products used against predators. Yet, due to the lack of morphological analyses, the taxonomy of *D. kerguelenensis* is still not solved (Wilson et al. 2013). Both *T. challengeriana* and *T. dantarti* are two different species, but the relationship between *T. challengeriana* specimens from the Antarctic and the Magellanic regions remains still unclear, thus the validity of *T. antarctica* requires further systematic work. Additional samples from other locations in Antarctica, Sub-Antarctic Islands, and South America are urgently

needed to shed light on the systematics of the *T. challengeriana* species group. Sampling in poorly known areas of the SO, such as the Amundsen Sea or the western Weddell Sea, and the continental shelves underneath floating ice shelves (Griffiths 2010), with the increasing application of molecular techniques and complementary molecular markers with higher resolution (e.g. EPIC markers, microsatellites, and/or genome- or transcriptome-derived SNPs; Riesgo et al. 2015; Moles et al. 2019; Moles and Giribet 2021) are required to further evaluate cryptic speciation and increasing our knowledge on the biodiversity of most invertebrate taxa in the SO.

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Author contributions CA conducted the field work taking part in the expeditions and collecting the samples. JM conceived and designed the study. MER and JM conducted the experiments and analysed the data. MER and JM wrote the manuscript. CA contributed with financial support. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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