

## Evidence for the validity of *Protatlanta sculpta* (Gastropoda: Pterotracheoidea)

Deborah Wall-Palmer<sup>1,2,6</sup>, Alice K. Burridge<sup>2,3</sup>, Katja T.C.A. Peijnenburg<sup>2,3</sup>, Arie Janssen<sup>2</sup>, Erica Goetze<sup>4</sup>, Richard Kirby<sup>5</sup>, Malcolm B. Hart<sup>1</sup>, Christopher W. Smart<sup>1</sup>

<sup>1</sup>*School of Geography, Earth and Environmental Sciences, Plymouth University, Drake Circus, Plymouth, PL4 8AA, United Kingdom*

<sup>2</sup>*Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, the Netherlands*

<sup>3</sup>*Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, P.O. Box 94248, 1090 GE Amsterdam, the Netherlands*

<sup>4</sup>*Department of Oceanography, University of Hawai'i at Mānoa, 1000 Pope Road, Honolulu, HI 96822, USA*

<sup>5</sup>*Marine Biological Association, Citadel Hill, Plymouth, PL1 2PB, United Kingdom*

<sup>6</sup>*E-mail: deborah.wall-palmer@plymouth.ac.uk*

**Key words:** Atlantic Ocean, biogeography, DNA barcoding, morphometrics, *Protatlanta*, shelled heteropod

### Abstract

The genus *Protatlanta* is thought to be monotypic and is part of the Atlantidae, a family of shelled heteropods. These microscopic planktonic gastropods are poorly known, although research on their ecology is now increasing in response to concerns about the effects of ocean acidification on calcareous plankton. A correctly implemented taxonomy of the Atlantidae is fundamental to this progressing field of research and it requires much attention, particularly using integrated molecular and morphological techniques. Here we use DNA barcoding, shell morphology and biogeography to show that the genus *Protatlanta* includes at least two valid species in the Atlantic Ocean. *Protatlanta souleyeti* and *Protatlanta sculpta* were found to be separate species, with different shell morphology and separated by a K2P genetic distance of 19% sequence divergence at the Cytochrome Oxidase 1 gene. This evidence supports the revival of the species name *P. sculpta*, which was described by Issel in 1911, but has not been recognised as a valid species since 1915.

### Contents

Introduction .....	423
<i>Taxonomic history of the genus Protatlanta</i> .....	424
Material and methods .....	425
<i>Morphometric analysis</i> .....	425
<i>DNA barcoding</i> .....	426
Results and discussion .....	427
<i>Shell morphology</i> .....	427
<i>DNA barcoding</i> .....	430
<i>Biogeography</i> .....	431
Conclusions .....	432
Acknowledgements .....	432
References .....	433
Appendix .....	435

### Introduction

The genus *Protatlanta* Tesch, 1908 is one of three shelled heteropod genera within the family Atlantidae. All members of the Atlantidae are microscopic (<12 mm), holoplanktonic gastropods that have a foot modified for swimming, a long proboscis, large, complex eyes and flattened shells with keels. Defining characteristics of the three genera within the Atlantidae are largely based on shell morphology and shell composition (see Seapy, 1990; Seapy, 2011 for species key). The most diverse genus, *Atlanta* Lesueur, 1817, contains 19 species (Seapy, 2011) and is characterised by an aragonite shell and keel. The genus *Protatlanta* is characterised by an aragonite shell with a fine, transparent, conchiolin keel and the genus *Oxygyrus* Benson, 1835 is characterised by an aragonite juvenile shell, enclosed within adult whorls of conchiolin and a conchiolin keel. Both *Protatlanta* and *Oxygyrus* are thought to be monotypic genera, although several extinct species of *Protatlanta* have been described (Gabb, 1873; Janssen, 2007; Janssen, 2012a, b).

Due to their small size and difficulty to sample and identify, we know very little about the Atlantidae in general (Wall-Palmer *et al.*, 2016) and even less about the genus *Protatlanta*. Specimen collections and published records show that protatlantids are widespread in the oceans, occurring globally in tropical, subtropical and temperate regions (Fig. 1). Protatlantids can also make up a high proportion of Atlantidae specimens caught in plankton samples, for example, protatlantids made up a total of 29% of Atlantidae specimens caught

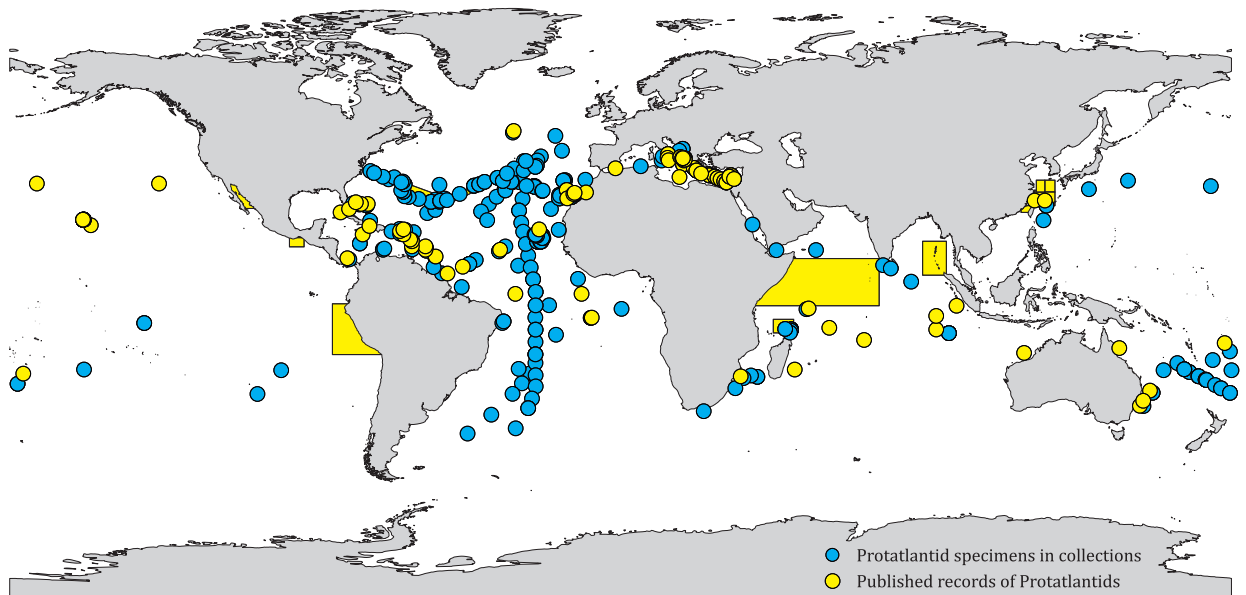


Fig. 1. Biogeography of the genus *Protatlanta*. Published data (yellow) compiled by Wall-Palmer *et al.* (2016). Specimens in collections (blue) are from surface sediment and plankton samples stored at the Naturalis Biodiversity Center (Leiden), Natural History Museum (London), Zoological Museum (Copenhagen), VU (Amsterdam) and the Plymouth Marine Laboratory (Plymouth).

in all plankton tows during the Atlantic Meridional Transect cruise in 2014 (AMT24, Burrige *et al.* in press). There is currently a lot of interest in planktonic gastropods as potential bioindicators of ocean changes, in particular, ocean acidification. However, thus far, the focus of these studies has been on the shelled pteropods (*e.g.* Bednaršek *et al.*, 2012). Similarities between the thin aragonite shell and surface ocean habitat of the shelled pteropods and the shelled heteropods, means that the Atlantidae are also likely to be vulnerable to ocean acidification and could act as valuable bioindicators.

Despite their wide distribution, relatively high abundance within the planktonic gastropod community and likely threat from global changes, we still know very little about the protatlantids. It is becoming increasingly important to investigate these planktonic organisms, to resolve their species boundaries and to determine their distributions and ecology as a baseline for future research of the effects of ocean changes.

#### *Taxonomic history of the genus Protatlanta*

The first extant species of *Protatlanta* was introduced by Gray (1850), described from a specimen collected

in the Atlantic Ocean. Gray (1850) identified this specimen as *Atlanta lamanoni* (a homonym of *Steira lamanoni* Eschscholtz 1825), after a species dubiously illustrated by Lamanon (1797) that is now considered to be a synonym of *Atlanta peronii* (Janssen, 2012c). Smith (1888) realised that Gray's specimen was not *A. lamanoni*, but a new species and, in the description of specimens from the Challenger expedition, named this new species *Atlanta souleyeti*. In 1908, Tesch recognised that the conchiolin keel of this species required the introduction of a new intermediate genus between *Atlanta* and *Oxygyrus*, thus establishing the genus *Protatlanta* containing one species, *Protatlanta souleyeti*. *Protatlanta souleyeti* is a small species with an aragonite shell of up to 2 mm in diameter and a conchiolin keel. It is characterised by a rounded, slightly elevated shell spire that has no ornamentation (Smith, 1888; Seapy, 2011).

Issel (1911, 1915) described a further species and a variety of *Protatlanta*, *Protatlanta sculpta* Issel, 1911 from offshore of the Cape Verde Islands, Atlantic Ocean and *Protatlanta sculpta* var. *mediterranea* Issel, 1915 from offshore of Messina, Mediterranean Sea. Issel (1911) described the main features of *P. sculpta* as having a smaller juvenile shell than *P. souleyeti*, with brown colouration. The spire is de-

scribed as being ornamented with irregular, wavy and frequently interrupted spiral lines, becoming just two lines in the final whorl of the juvenile shell. The final, adult whorl bears no ornamentation. Issel (1911) also suggested that the keel of *P. sculpta* inserts closer to the aperture than in *P. souleyeti*. Despite the clear morphological features that characterise *P. sculpta*, the species name *P. sculpta* has not been used since 1915 (Issel, 1915), having been assumed a synonym of *P. souleyeti* since this time. A number of authors have noted this ‘second form’ of *Protatlanta*, particularly in the Atlantic Ocean (Batten and Dumont, 1976; Richter and Seapy, 1999; Seapy 2011). Fossil specimens have even been described from the Pliocene of the Philippines (Janssen, 2007), although the name *P. sculpta* was not used.

*Protatlanta sculpta* var. *mediterranea* is described as having generally the same shape and ornamentation as *P. sculpta* from the Atlantic Ocean, but lacking the brown colouration. Unlike *P. sculpta*, this variety was recognised as a valid taxon until 1970 (Di Geronimo, 1970). We did not find any specimens matching this description from the Mediterranean Sea or elsewhere during this study. Therefore, we are unable to determine whether this is a valid variety of *P. sculpta*.

Two extinct species of *Protatlanta* with shell ornamentation have been described, both from the Miocene fossil record. *Protatlanta rotundata* (Gabb, 1873) was described from the Miocene of the Dominican Republic and *Protatlanta kbiraensis* Janssen, 2012b was described from the Langhian (15.97–13.65 Ma, Middle Miocene) of Malta and Italy. Descriptions and illustrations of specimens by Janssen (2007, 2012a) suggest that these species may have had spiral lines on the juvenile shell.

Here we investigate whether the morphological characteristics described by Issel (1911), and identified in specimens from recent plankton sampling, indicate a second species of *Protatlanta*. A combination of DNA barcoding of the COI gene, morphometrics and biogeography of specimens from the Atlantic Ocean are used to address the original description of *P. sculpta* to determine whether this species name should be revived. We follow an integrated taxonomic approach, combining molecular and morphological evidence (e.g. McManus and Katz, 2009; Padial *et al.*, 2010), and consider a species to be different when it is genetically distinct and shows corresponding differences in morphological characters.

## Material and methods

Specimens of *Protatlanta* used for this study were sourced from a number of collections. Fresh specimens for molecular and morphological analysis were collected from the Atlantic Ocean during the Atlantic Meridional Transects AMT24 (N=404, late September to October 2014) and AMT20 (N=68, October to November 2010). Oblique tows were carried out using a CalBOBL bongo net with an aperture diameter of 0.71 m for AMT24 and a WP2 bongo net with an aperture diameter of 0.57 for AMT20. Both nets had a mesh size of 200 µm. For sample collection during AMT24, a flowmeter (General Oceanics 2030RC) was mounted at the mouth of one of the nets to measure the volume of water sampled for quantitative analysis (Burridge *et al.*, in press). Specimens from AMT24 were fixed and preserved in 96% ethanol and stored at -20°C prior to DNA barcoding. Specimens from AMT20 were fixed and stored in 96% ethanol and stored at room temperature. Storage at room temperature is not optimal for the preservation of DNA, therefore, specimens from AMT20 were not used for DNA barcoding. Station information and sampling parameters for all specimens are given in Table S1.

A total of seven specimens from the recent fossil record (0–10 ka) were used for morphometric analysis. Fossil specimens were collected from a marine sediment core from site CAR-MON 2, offshore of Montserrat, Lesser Antilles (Le Friant *et al.*, 2008; Wall-Palmer *et al.*, 2014). For the biogeography, additional specimens were examined from the Challenger collection and the National Institute of Oceanography Discovery collection (N=66, surface sediment and plankton samples Natural History Museum, London), the ACE-ASIA, KH-11-10, VANC10MV and CANCAP Expeditions collections (N=100, plankton samples and surface sediment, Naturalis Biodiversity Center, Leiden), the Dana and Thor collections (N=337, plankton samples, Zoological Museum, Copenhagen) and the APNAP-I-1986 and G0-Snellius-II collections (N=21, plankton samples, Vrije Universiteit, VU, Amsterdam). Collection information is given in Table S1. All biogeographical data was plotted using the software QGIS (QGIS Development Team, 2016).

### Morphometric analysis

Morphometric analysis was performed on 19 adult specimens from AMT20 and AMT24 and from the recent fossil record of site CAR-MON 2 (Le Friant *et al.*,

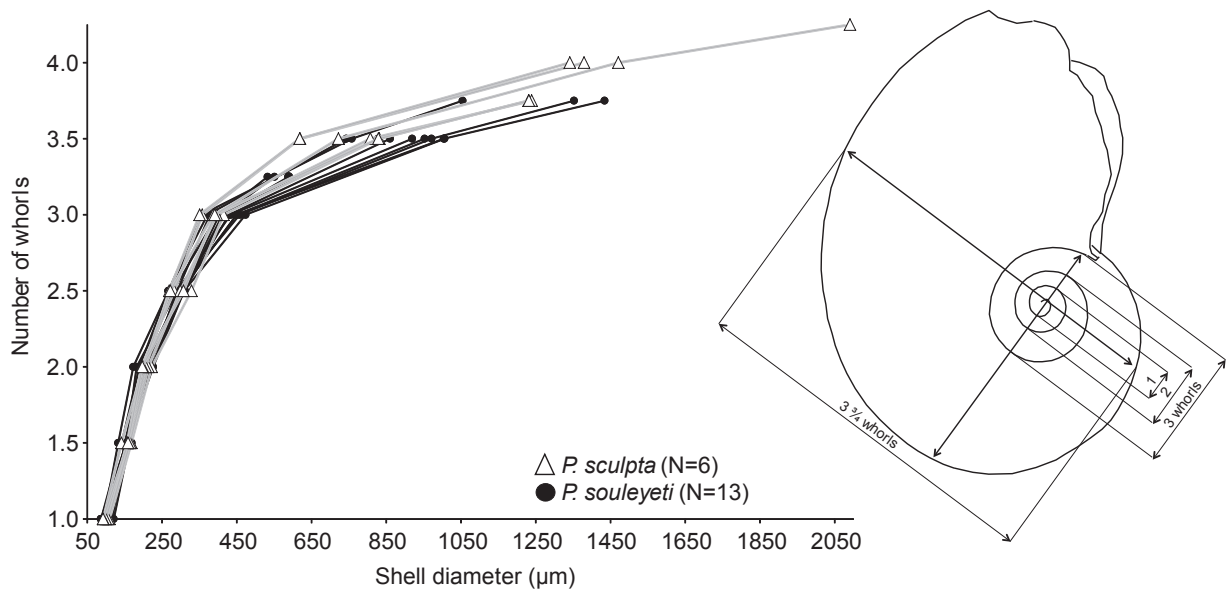


Fig. 2. Method of whorl counting of *Protatlanta* shells, following the method of whorl numbering by Seapy (1990) and morphometric shell measurements for *P. souleyeti* (black circles, black lines) and *P. sculpta* (white triangles, grey lines) shells. For specimens analysed, see supplementary Table S1. Specimen used for method schematic is *P. sculpta* from Figure 3k.

2008; Wall-Palmer *et al.*, 2014). Images of the apical side of each shell were obtained using light microscopy and scanning electron microscopy. The ImageJ software FIJI (Schindelin *et al.*, 2012) was used to measure the shell diameter perpendicular to the nucleus of the protoconch (Fig. 2) at half whorl intervals throughout each shell.

#### DNA barcoding

A random sample of 18 undamaged adult (N=7) and juvenile (N=11) specimens from the Atlantic Ocean were selected from AMT24 samples for DNA barcoding. A mixture of ornamented and non-ornamented specimens of varying colour (brown and white) were analysed. All specimens were imaged prior to analysis using a Zeiss automated z-stage light microscope. DNA extraction was carried out on whole specimens, using the NucleoMag 96 Tissue kit by Macherey-Nagel on a Thermo Scientific KingFisher Flex magnetic bead extraction robot, with a final elution volume of 75 µl. The standard CO1 barcoding fragment (Hebert *et al.*, 2003) was amplified using primers jgLCO1490 and jgHCO2198 (Geller *et al.*, 2013). Primers were tailed with M13F and M13R for sequencing (Messing, 1983). PCR reactions contained 17.75 µl mQ, 2.5 µl 10x PCR

buffer CL, 0.5 µl 25mM MgCl<sub>2</sub>, 0.5 µl 100mM BSA, 1.0 µl 10 mM of each primer, 0.5 µl 2.5 mM dNTPs and 0.25 µl 5U Qiagen Taq, with 1.0 µl of template DNA, which was diluted 10 or 100 times for some samples. PCR was performed using an initial denaturation step of 180 s at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 50°C and 40 s at 72°C, and finishing with a final extension of 300 s at 72°C and pause at 12°C. Sequencing was carried out by Macrogen, Europe.

Sequences were examined and edited in MEGA 6 (Tamura *et al.*, 2013), aligned using Mafft v7 (<http://mafft.cbrc.jp/alignment/server/>) and are available from GenBank (Table 1). Additional GenBank CO1 sequences from heteropods identified as *Atlanta inclinata*, *Oxygyrus inflatus*, *Firoloida desmarestia*, *Pterotrachea hippocampus* and *Pterotrachea coronata* (Jennings *et al.*, 2010) were used in the analysis to represent the families and genera most closely related to the protatlantids. CO1 sequences of the gastropods *Crassitoniella flammea*, *Pisinna albizona*, *Bembicium nanum*, *Echinolittorina riisei*, *Tectonatica sagraiana*, *Natica vittata* and *Ataxocerithium* sp. from GenBank (Williams and Reid, 2004; Colgan *et al.*, 2003, 2007; Huelsken *et al.*, 2008; Ayre *et al.*, 2009) were used as outgroups. These all belong to Hypsogastropoda

Table 1. Station, location and GenBank accession codes for specimens used for DNA barcoding.

	Institute accession number	Juvenile or adult?	Station	Latitude	Longitude	Genbank accession code
<i>Protatlanta souleyeti</i>	Psou_AMT24_06_01	A	AMT24_06	31.30°N	27.73°W	KU841493
	Psou_AMT24_06_02	J	AMT24_06	31.30°N	27.73°W	KU841494
	Psou_AMT24_06_03	J	AMT24_06	31.30°N	27.73°W	KU841495
	Psou_AMT24_06_04	A	AMT24_06	31.30°N	27.73°W	KU841496
	Psou_AMT24_06_05	J	AMT24_06	31.30°N	27.73°W	KU841497
	Psou_AMT24_18_01	J	AMT24_18	11.95°S	27.02°W	KU841500
	Psou_AMT24_19_01	A	AMT24_19	14.66°S	25.07°W	KU841501
	Psou_AMT24_19_02	A	AMT24_19	14.66°S	25.07°W	KU841502
	Psou_AMT24_23_01	A	AMT24_23	27.76°S	25.01°W	KU841506
Psou_AMT24_25A_01	A	AMT24_25A	34.18°S	27.21°W	KU841507	
<i>Protatlanta sculpta</i>	Pscu_AMT24_09_01	J	AMT24_09	20.45°N	29.27°W	KU841485
	Pscu_AMT24_09_02	J	AMT24_09	20.45°N	29.27°W	KU841486
	Pscu_AMT24_09_03	J	AMT24_09	20.45°N	29.27°W	KU841487
	Pscu_AMT24_10_01	J	AMT24_10	18.80°N	31.42°W	KU841488
	Pscu_AMT24_16_01	J	AMT24_16	3.89°S	25.03°W	KU841489
	Pscu_AMT24_16_02	J	AMT24_16	3.89°S	25.03°W	KU841490
	Pscu_AMT24_16_03	J	AMT24_16	3.89°S	25.03°W	KU841491
	Pscu_AMT24_25_01	A	AMT24_25	34.18°S	27.22°W	KU841492

(within Caenogastropoda), supposedly closely related to the heteropods, as suggested by Jennings *et al.* (2010). A maximum-likelihood tree was constructed based on these data using nucleotide sequences and a General Time Reversible model with different rates at the three codon positions (+CP) model with 1000 bootstraps in RAxML (Stamatakis, 2014). Kimura-2-parameter (K2P) genetic distances were calculated between and within species belonging to the family Atlantidae using MEGA 6 (Tamura *et al.*, 2013).

## Results and discussion

### Shell morphology

Two clear groups of *Protatlanta* were identified: ornamented specimens that are comparable to *P. sculpta*, as described by Issel (1911) and non-ornamented specimens that follow the description of *P. souleyeti*. The differences in shell ornamentation are most obvious in juvenile specimens (Fig. 3a-d). Non-ornamented specimens have completely smooth shells with no ornamentation (Fig. 3a-b, i-j), whereas ornamented specimens have several spiral lines (Fig. 3c-d). The main spiral lines of ornamented specimens begin on the apical side of the shell at the second whorl as irregular,

interrupted spiral lines and become more regular lines in the third whorl (Fig. 3c-d). A third, main spiral line is present and visible in apertural view in juvenile shells, but it is covered by subsequent whorls, so that it is not visible in the adult form (Fig. 3d, 3l). This ornamentation is clearly visible with light microscopy, however, scanning electron microscopy reveals additional, finer details, including lines of pores and multiple finer spiral lines in the transition from juvenile to adult whorl (Fig. 3c-d). The final, adult whorl of ornamented specimens does not have any surface ornamentation (Fig. 3h, 3k) and is identical to that of non-ornamented specimens. Here, we present the results of this study as comparisons between these two, potentially different species.

The morphometric analysis of 19 adult protatlantid shells reveals that both ornamented (N=6) and non-ornamented (N=13) specimens show a similar pattern of shell diameter per whorl, with greater variation in diameter with increasing whorl number for both species (Fig. 2). Adult ornamented specimens ( $3 \frac{3}{4}$  to  $4 \frac{1}{4}$  whorls) were often  $\frac{1}{4}$  to  $\frac{3}{4}$  of a whorl larger than non-ornamented specimens ( $3 \frac{1}{2}$  whorls). However, two larger non-ornamented specimens were found with  $3 \frac{3}{4}$  whorls. In general, ornamented shells begin to inflate rapidly at 3 whorls whereas non-ornamented shells begin to inflate rapidly at  $2 \frac{1}{2}$  to 3 whorls (Table 2).

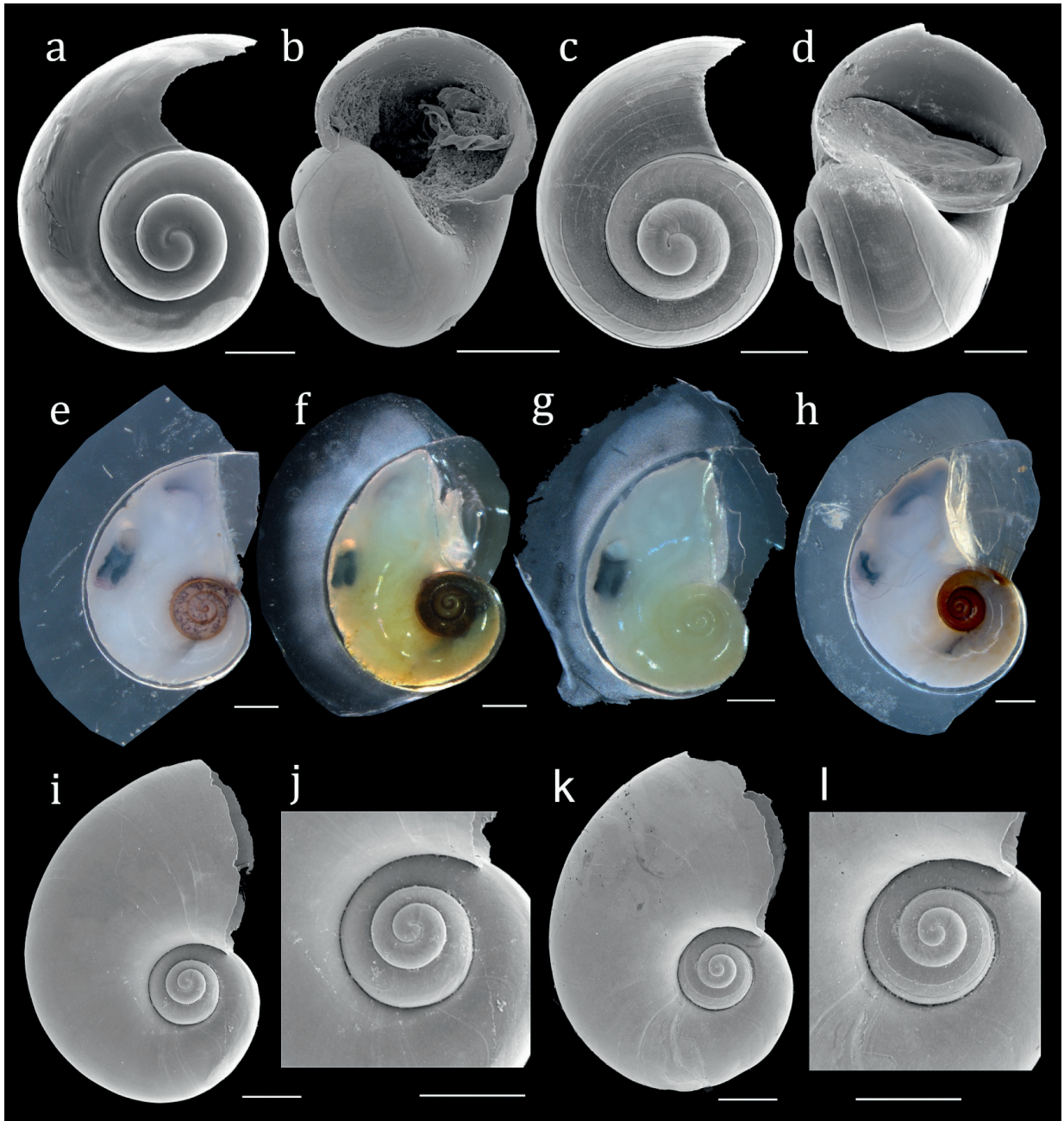


Fig. 3. SEM and light microscope images demonstrating the variation in shell colouration and ornamentation in *Protatlanta*, a-b) *P. souleyeti* juvenile, c-d) *P. sculpta* juvenile, e-g) *P. souleyeti* adult, h) *P. sculpta* adult, i-j) *P. souleyeti* adult, k-l) *P. sculpta* adult. Scale bars are 100  $\mu\text{m}$  for e-l, 200  $\mu\text{m}$  for a-d.

Based on these data, we suggest that shell diameter per whorl cannot be used as a diagnostic feature to separate the two groups of *Protatlanta*. These data also show that, contrary to Issel's (1911) description, specimens with ornamentation do not have a smaller

juvenile shell than specimens without ornamentation. In the Atlantic Ocean, specimens of both groups are, in fact, almost identical in size and proportions. It is also apparent that specimen colour cannot be used as a feature to characterise the two groups (Table 2). In

*P. souleyeti*

*P. sculpta*



Table 2. Comparison summary of *P. souleyeti* and *P. sculpta*.

2½ - 3 whorls in spire	3 - 3¼ whorls in spire
3½ - 3¾ whorls in adult shell	3¾ - 4¼ whorls in adult shell
Spire diameter 350 µm	Spire diameter 350 µm
Adult shell diameter <2 mm	Adult shell diameter <2 mm
No shell ornamentation	Spire surface ornamented with three spiral lines. Adult whorl no ornamentation.
Soft tissues within the spire vary in colour from white to dark brown.	The soft tissues and shell of the spire are brown.
Global distribution. Preference for oligotrophic gyres in Atlantic Ocean.	Distribution probably restricted to Atlantic Ocean. Preference for regions outside of oligotrophic gyres.
Latitude range 47°N to 40°S	Latitude range 41°N to 34°S

Table 3. Average K2P pairwise genetic distance of Cytochrome Oxidase 1 sequences for *P. souleyeti*, *P. sculpta* and representatives of the genera *Atlanta* and *Oxygyrus*.

	<i>P. souleyeti</i>	<i>P. sculpta</i>	<i>A. inclinata</i>	<i>O. inflatus</i>
<i>P. souleyeti</i> (N=10)	0.00			
<i>P. sculpta</i> (N=8)	0.19	0.00		
<i>A. inclinata</i> (N=2)	0.20	0.20	0.00	
<i>O. inflatus</i> (N=2)	0.24	0.24	0.24	0.00

agreement with the original description of *P. sculpta* by Issel (1911), ornamented specimens were found to always have a brown colouration of the shell in the first 2 ½ to 3 whorls (Fig. 3). This colouration is sometimes very faint in juvenile specimens, being restricted to the

whorl sutures, but becomes darker in adult specimens, emphasised by brown-coloured soft tissues. Non-ornamented specimens have a colourless shell, but the colour of soft tissues can be seen, ranging from white to mottled yellow-brown or dark brown (Fig. 3). This

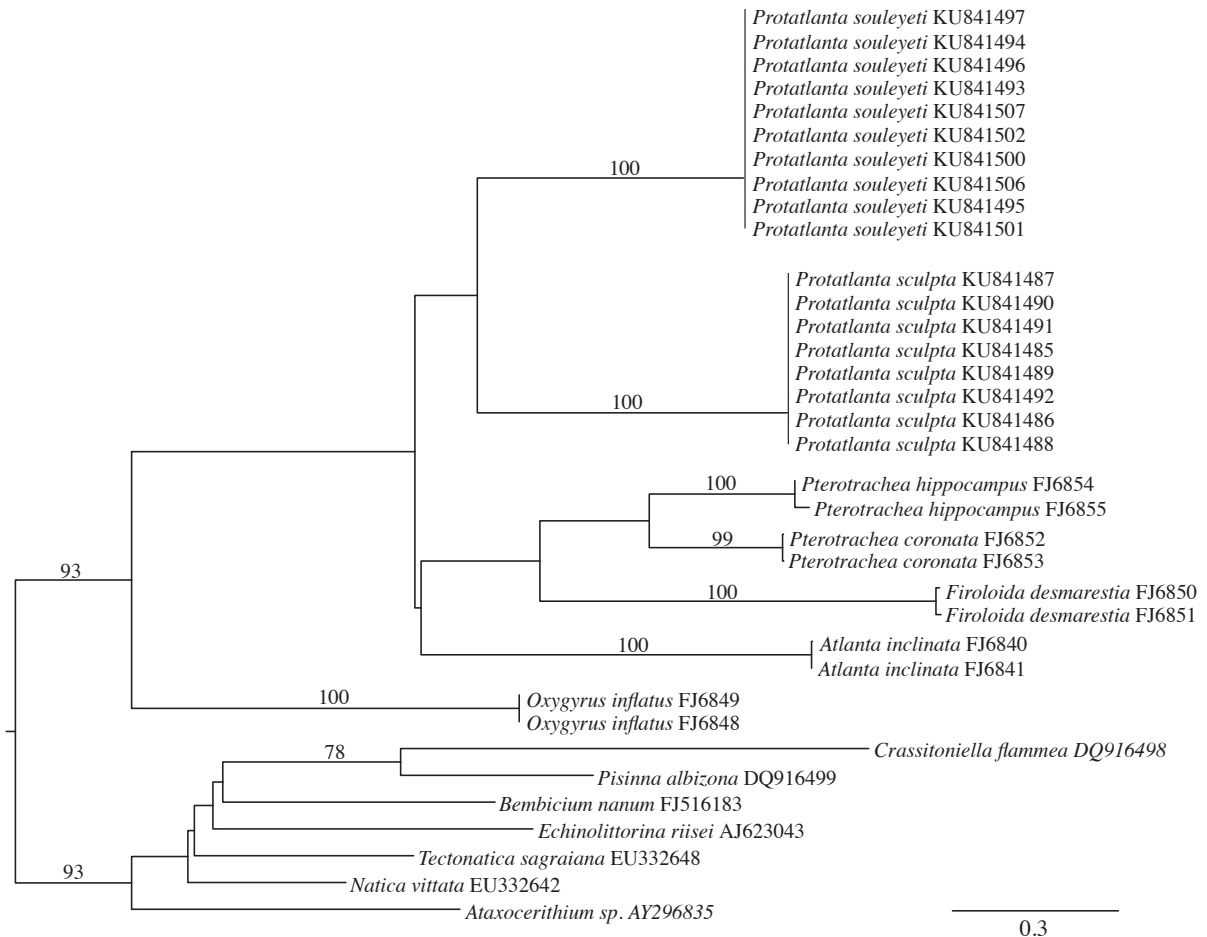


Fig. 4. Maximum-likelihood tree showing relationships based on Cytochrome Oxidase 1 DNA sequences of *Protatlanta* and other heteropod genera, including several Hypsogastropoda outgroups (see text for more details). Branch lengths are proportional to the amount of inferred change, indicated by the scale bar. Only bootstrap supports (1000 replicates) above 70% are displayed. GenBank accession codes are presented in Table 1. Sequences from Jennings *et al.* (2010) begin with FJ, other outgroup sequences are from GenBank.

variation in soft-tissue colour of non-ornamented specimens showed no geographical pattern.

#### DNA barcoding

DNA barcoding of 18 specimens of *Protatlanta* from the Atlantic Ocean revealed two monophyletic clades of *Protatlanta* with maximum bootstrap support (Figs 4, 5). The first clade consists of non-ornamented specimens (N=10), representing the species *P. souleyeti*. The second clade is exclusively made up of ornamented specimens (N=8). The ornamentation on these specimens is comparable to *P. sculpta*, as described by

Issel (1911) and we therefore consider this second clade to be a separate species, *P. sculpta*. The average K2P pairwise distance (Table 3) between *P. sculpta* and *P. souleyeti* is 0.19. Average K2P pairwise distances show that the family Atlantidae overall, is highly diversified based on the CO1 gene. Representatives of the other Atlantidae genera *Atlanta* and *Oxygyrus* showed an average pairwise distance of 0.20 and 0.24 from *P. souleyeti* and 0.20 and 0.24 from *P. sculpta*, respectively. This molecular analysis supports that *P. sculpta* is a valid species and that shell ornamentation is an effective way to identify *P. sculpta* from *P. souleyeti*.



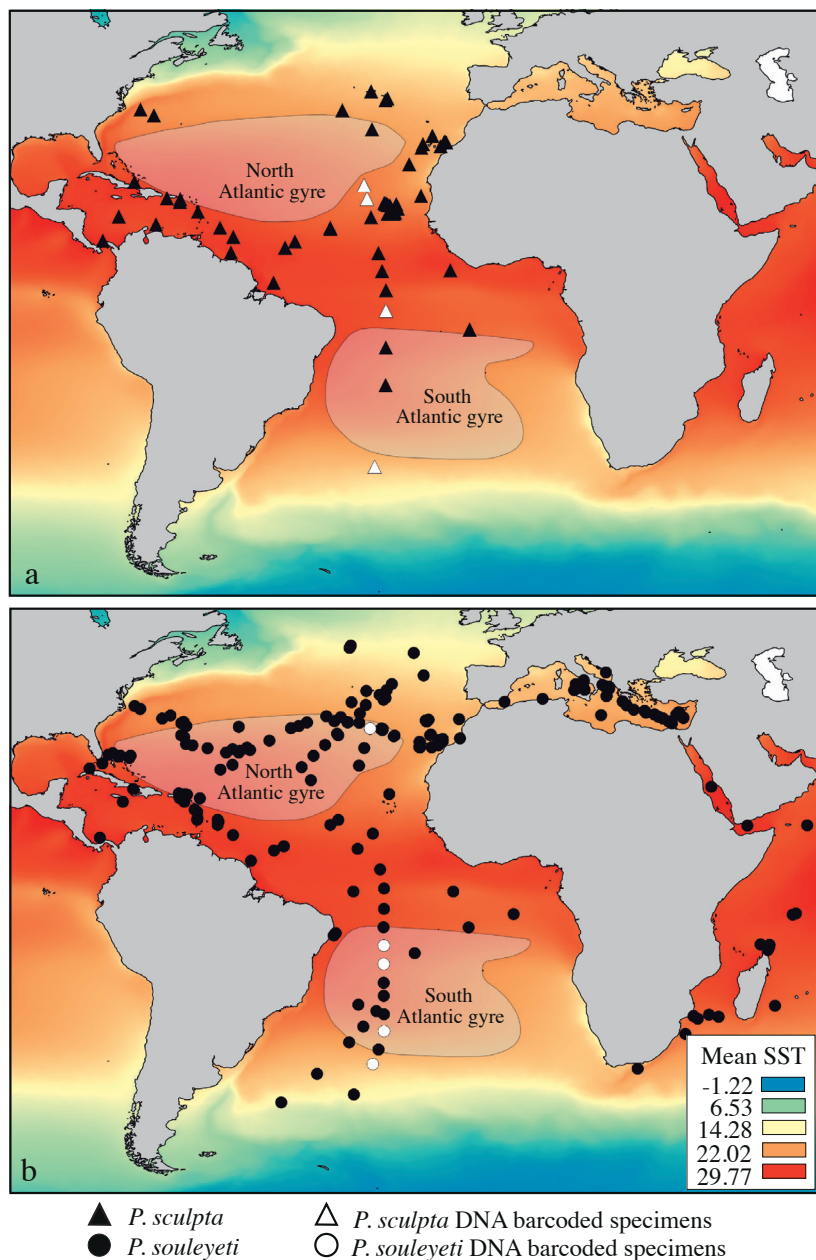


Fig. 5. *Protatlanta* biogeography in the Atlantic Ocean (a) occurrences of *P. sculpta* (in black) showing stations included in DNA barcoding (in white) and (b) occurrences of *P. souleyeti* (in black) showing stations included in DNA barcoding (in white). Average sea surface temperature from Tyberghein *et al.* (2012).

### Biogeography

All specimens of *Protatlanta* (Table S1) were assessed for shell ornamentation. Following the results of this study, specimens with shell ornamentation were identified as *P. sculpta* and non-ornamented specimens were identified as *P. souleyeti* (Fig. 5). These specimens (N=1003) indicate that *P. sculpta* is distributed throughout the Atlantic (Fig. 5a). However, quantita-

tive sampling along the meridional transect of AMT24 shows that *P. sculpta* is rarely found within the subtropical gyres, being most abundant in the equatorial upwelling region (Figs 5a, 6). Conversely, quantitative sampling shows that *P. souleyeti*, although widespread in the Atlantic Ocean, has a preference for the northern and southern oligotrophic subtropical gyres (Figs 5b, 6). This suggests that the two species have different ecological preferences, with *P. souleyeti* preferring

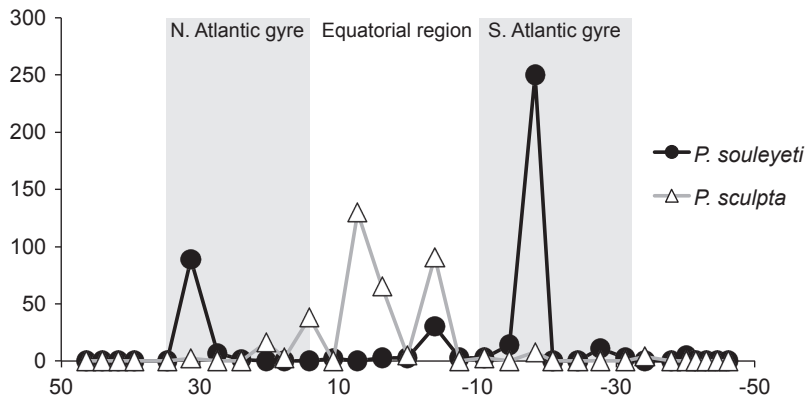


Fig. 6. Abundance (specimens per 1000 m<sup>3</sup>) of *P. souleyeti* (black circles, black lines) and *P. sculpta* (white triangles, grey lines) along the AMT24 meridional transect sampled in 2014. Specimens sorted from 25% or 50% of the quantitative zooplankton sample depending on bulk sample volumes.

oligotrophic regions and *P. sculpta* preferring more nutrient rich waters.

Samples from the Indian and Pacific oceans permitted initial investigation of the wider distribution of these two species. Specimens of *P. souleyeti* were found worldwide, however, only a single specimen of *P. sculpta* was found outside of the Atlantic Ocean. This single specimen of *P. sculpta* (ZMUC\_GAS\_782) was collected in the Marquesas Islands and a specimen of *P. souleyeti* was collected at the same site.

## Conclusions

Based on the combination of molecular, morphological and biogeographical data presented here, we conclude that there are at least two, valid, extant species of *Protatlanta* in the Atlantic Ocean, *P. souleyeti* and *P. sculpta*. DNA barcoding of the CO1 gene supports differences in shell morphology, verifying that *P. souleyeti* and *P. sculpta* are separate species. The ability to use shell ornamentation to identify between the two species of *Protatlanta* ensures that they can be identified in sea floor sediments, in plankton samples and in the fossil record, where *P. sculpta* is known from the Pliocene (Piacenzian).

Biogeographical data have revealed that *P. souleyeti* and *P. sculpta* inhabit different oceanographic provinces in the Atlantic Ocean and are, therefore, ecologically differentiated. Initial analysis of sites from the Indian and Pacific oceans shows that *P. souleyeti* is found worldwide, whereas *P. sculpta* may be restricted to the Atlantic Ocean.

Aragonite shelled holoplanktonic gastropods (theosome pteropods and Atlantidae heteropods) are con-

sidered to be threatened by, and important bioindicators of, ocean acidification. However, for many species we still do not have an adequate understanding of their species boundaries and biogeography, which is essential to recognise where to monitor for potential changes and what constitutes significant changes in species distribution. This study demonstrates that using an integrative taxonomic approach to reveal species boundaries is an important step in ensuring accurate species identification, which is crucial to understanding species distribution patterns.

## Acknowledgements

We would like to thank Andreia Salvador and Jon Ablett from the Natural History Museum (London), Jeroen Goud and Bram van der Bijl from the Naturalis Biodiversity Center (Leiden), Tom Schiøtte and Martin Vinther Sørensen from the Zoological Museum (Copenhagen), Brett Metcalfe and Gerald Ganssen from Vrije Universiteit (Amsterdam), Atushi Tsuda from the University of Tokyo and Rachel Harmer from the Plymouth Marine Laboratory (Plymouth) for allowing access to Atlantidae collections. We would also like to thank Aline Nieman, Kevin Beentjes and Frank Stokvis (Naturalis) for help with DNA barcoding of specimens and Rachel Harmer (Plymouth Marine Laboratory) for plankton collection on cruise AMT20. We would like to acknowledge the scientists and crew who took part in cruises ACE-ASIA, KH-11-10, AMT20 and AMT24, and the Atlantic Meridional Transect (AMT) programme. This study is a contribution to the international IMBER project and was supported by the UK Natural Environment Research Council National Capability funding to Plymouth Marine Laboratory and the National Oceanography Centre, Southampton. This is contribution number 299 of the AMT programme. We would like to acknowledge the Plymouth Electron Microscopy Centre for help with SEM imaging. We are extremely grateful to Rosie Oakes and four anonymous reviewers for their detailed and constructive comments on our manuscript. This research was

carried out with funding from the Leverhulme Trust (RPG-2013-363, 2014–2017, DW-P, CWS, RK) and a Martin-Fellowship from Naturalis Biodiversity Center (DW-P).

## References

- Ayre DJ, Minchinton TE, Perrin C. 2009. Does life history predict past and current connectivity for rocky intertidal invertebrates across a marine biogeographic barrier? *Molecular Ecology* 18: 1887-1903.
- Batten RL, Dumont MP. 1976. Shell ultrastructure of the Atlantidae (Heteropoda, Mesogastropoda) *Oxygyrus* and *Protatlanta*, with comments on *Atlanta inclinata*. *Bulletin of the American Museum of Natural History* 157: 267-310.
- Bednaršek N, Tarling GA, Bakker DCE, Fielding S, Jones EM, Venables HJ, Ward P, Kuzirian A, Lézé B, Feely RA, Murphy EJ. 2012. Extensive dissolution of live pteropods in the Southern Ocean. *Nature Geoscience* 5: 881-885.
- Burridge AK, Goetze E, Wall-Palmer D, Le Double S, Huisman J, Peijnenburg KTCA. In press. Epipelagic distribution of planktic gastropods along a meridional transect in the Atlantic Ocean. *Progress in Oceanography*.
- Colgan DJ, Ponder WF, Beacham E, Macaranas J. 2007. Molecular phylogenetics of Caenogastropoda (Gastropoda: Mollusca). *Molecular Phylogenetics and Evolution* 42: 717-737.
- Colgan DJ, Ponder WF, Beacham E, Macaranas JM. 2003. Molecular phylogenetic studies of Gastropoda based on six gene segments representing coding or non-coding and mitochondrial or nuclear DNA. *Molluscan Research* 23: 159-178.
- Di Geronimo I. 1970. Heteropoda e Pteropoda Thecosomata in sedimenti abisali recenti dello Ionio. *Thalassia Salentina* 4: 41-115.
- Gabb WM. 1873. On the topography and geology of Santo Domingo. *Transactions of the American Philosophical Society New Series* 15: 49-259.
- Geller J, Meyer C, Parker M, Hawk H. 2013. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources* 13: 851-861.
- Hebert PD, Cywinska A, Ball SL. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270: 313-321.
- Huelsken T, Marek C, Schreiber S, Schmidt I, Hollmann M. 2008. The Naticidae (Mollusca: Gastropoda) of Giglio Island (Tuscany, Italy): Shell characters, live animals, and a molecular analysis of egg masses. *Zootaxa* 1770: 1-40.
- Issel R. 1911. Molluschi Eteropodi raccolti dal Capitano G. Chierchia durante il viaggio di circumnavigazione della R. Nave Vettor Pisani negli anni 18182-83-84-85. *Annuario del Museo Zoologico della R. Università di Napoli, Nuova Serie* 3: 1-11.
- Issel R. 1915. Atlantidae e Carinaria. *Memorie R. Comitato Tassografico Italiano, Memoria LII*: 3-26.
- Janssen AW. 2007. Holoplanktonic Mollusca (Gastropoda: Pterotracheoidea, Janthinoidea, Thecosomata and Gymnosomata) from the Pliocene of Pangasinan (Luzon, Philippines). *Scripta Geologica* 135: 29-144.
- Janssen AW. 2012a. Early Pliocene heteropods and pteropods (Mollusca, Gastropoda) from Le Puget-sur-Argens (Var), France. *Cainozoic Research* 9: 145-187.
- Janssen AW. 2012b. Systematics and biostratigraphy of holoplanktonic Mollusca from the Oligo-Miocene of the Maltese Archipelago. *Bollettino del Museo Regionale di Scienze Naturali, Torino* 28: 197-601.
- Janssen AW. 2012c. Late Quaternary to Recent holoplanktonic Mollusca (Gastropoda) from bottom samples of the eastern Mediterranean Sea: systematics, morphology. *Bollettino Malacologico* 48: supplemento: 1-105.
- Jennings RM, Bucklin A, Ossenbrügger H, Hopcroft RR. 2010. Species diversity of planktonic gastropods (Pteropoda and Heteropoda) from six ocean regions based on DNA barcode analysis. *Deep-Sea Research* 57: 2199-2210.
- Lamanon [JHR de P de]. 1797. *Mémoire sur les cornes d'amon, et description d'une espèce trouvée entre les tropiques dans la mer du sud*. Pp. 134-139 in: Millet-Mureau, M.L.A. ed, *Voyage de la Pérouse autour du monde*. Vol. 4. Imprimerie de la République, France.
- Le Friant A, Lock EJ, Hart MB, Boudon G, Sparks RSJ, Leng MJ, Smart CW, Komorowski J-C, Deplus C, Fisher JK. 2008. Late Pleistocene tephrochronology of marine sediments adjacent to Montserrat, Lesser Antilles volcanic arc. *Journal of the Geological Society, London* 165: 279-289.
- McManus GC, Katz LA. 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? *Journal of Plankton Research* 31: 1119-1129.
- Messing J. 1983. New M13 vectors for cloning. *Methods in Enzymology* 101: 20-78.
- Newman LJ. 1990. The taxonomy, distribution and biology of *Atlanta gaudichaudi* Souleyet, 1952 (Gastropoda, Heteropoda) from the Great Barrier Reef, Australia. *American Malacological Bulletin* 8: 85-94.
- Padial JM, Miralles A, De la Riva I, Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* 7: 16.
- QGIS Development Team. 2016. QGIS Geographical Information System. Open Source Geospatial Foundation Project. <http://qgis.osgeo.org>
- Richter G, Seapy RS. 1999. Heteropoda. Pp. 621-647 in: Boltovskoy D, ed, *South Atlantic zooplankton*. Backhuys Publishers, Leiden.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9: 676-682.
- Seapy RR. 2011. Atlantidae. Available at <http://tolweb.org/Atlantidae> accessed 22nd July 2015.
- Seapy RR. 1990. The pelagic family Atlantidae (Gastropoda: Heteropoda) from Hawaiian waters: a faunistic survey. *Malacologia* 32: 107-130.
- Smith EA. 1888. Report on the Heteropoda collected by HMS Challenger during the years 1873-1876. *Challenger Reports, Zoology* 23: 1-51.
- Stamatakis A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* 30: 1312-1313.
- Tamura K, Stecher G, Peterson D, Filipiński A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.
- Tyberghein L, Verbruggen H, Pauly K, Troupin C, Mineur F, De

- Clerck O. 2012. Bio-ORACLE: a global environmental dataset for marine species distribution modelling. *Global Ecology and Biogeography* 21: 272-81.
- Tesch JJ. 1908. Systematic monograph of the Atlantidae (Heteropoda) with enumeration of the species in the Leyden museum. *Notes from the Leyden Museum* 30: 1-30.
- Wall-Palmer, D., Smart, C.W., Hart, M.B., Leng, M.L., Borghini, M., Manini, E., Aliani, S., Conversi, A. 2014. Late Pleistocene pteropods, heteropods and planktonic foraminifera from the Caribbean Sea, Mediterranean Sea and Indian Ocean. *Micropaleontology* 60: 557-578.
- Wall-Palmer D, Smart CW, Kirby R, Hart MB, Peijnenburg KTCA, Janssen AW. 2016. A review of the ecology, palaeontology and distribution of atlantid heteropods (Caenogastropoda: Pterotracheoidea: Atlantidae). *Journal of Molluscan Studies* 82: 221-234.
- Williams ST, Reid DG. 2004. Speciation and diversity on tropical rocky shores: a global phylogeny of snails of the genus *Echinolittorina*. *Evolution* 58: 2227-2251.

Received: 16 November 2015

Revised and accepted: 8 April 2016

Published online: 9 November 2016

Editor: D. Huang

## Online supplementary information

*SI*. Collection, station and storage information for all specimens analysed in this study. \* Denotes specimens used for morphometric analysis in Fig. 2.

## Appendix

### Systematic descriptions

Superfamily Pterotracheoidea Rafinesque, 1814

Family Atlantidae Rang, 1829

Genus *Protatlanta* Tesch, 1908

*Type species.* *Protatlanta souleyeti* (Smith, 1888). Originally named *Atlanta lamanoni* Gray, 1850 and subsequently renamed *Atlanta souleyeti* by E.A. Smith.

*Diagnosis.* Genus of Atlantidae with an aragonite shell and a conchiolin keel. The aperture is rounded and sub-triangular in shape without a slit in the shell periphery (as in *Atlanta*) where the keel ends.

*Description.* The shell is dextrally coiled and flattened, lenticular in shape, consisting of up to 4 ¼ whorls with a total shell diameter of up to 2 mm. The spire (central, juvenile part of the shell) is slightly elevated, with incised sutures and consists of 2 ½ to 3 ¼ whorls. The final adult whorl inflates rapidly and gives the shell an oval shape when viewed from the apical side. The shell is calcareous with a tall, extremely transparent and strongly truncated conchiolin keel.

*Remarks.* The keel of *Protatlanta* is extremely transparent and is often difficult to see in wet specimens. The keel in lost is fossil material, even in relatively recent sediments.

*Protatlanta souleyeti* (Smith, 1888)

(Fig. 5a-b, e-g, i-j)

*Material examined.* See supplementary Table S1.

*Diagnosis.* *Protatlanta* species with no shell ornamentation.

*Description.* The spire typically consists of 2 ½ to 3 whorls with a diameter of less than 450 µm. The spire has a smooth surface with no ornamentation. The shell is colourless, however the spire may vary in colour from white to dark brown due to the colour of the soft tissues (Fig. 3e-g). The complete adult shell consists of 3 ½ to 3 ¾ whorls. Eyes are type a and operculum is type a (Seapy, 2011).

*Remarks.* Juvenile specimens are particularly difficult to identify as their lack of ornamentation and dis-

tinctive features can cause confusion with juveniles of *Atlanta peronii* and *Atlanta lesueurii*. However, the aperture of juvenile *P. souleyeti* is more rounded than species in the genus *Atlanta*.

*Distribution.* Distribution in the Atlantic Ocean is mainly restricted to northern and southern oligotrophic subtropical gyre regions and equatorial waters. Specimens also found in the Pacific and Indian oceans. The overall latitudinal range is between 47°N to 40°S.

*Protatlanta sculpta* Issel, 1911

(Fig. 3c-d, h, k-l)

*Material examined.* See supplementary Table S1.

*Diagnosis.* *Protatlanta* species with shell ornamentation on the spire consisting of three irregular spiral lines.

*Description.* The spire typically consists of 3 to 3 ¼ whorls with a diameter of less than 450 µm. The spire surface is ornamented with three spiral lines, 2 on the spire and 1 along the edge of the shell, in line with the aperture. The spiral lines are present on the second and third whorls and may be partially irregular and interrupted. Scanning electron microscopy (SEM) reveals additional finer ornamentation, including lines of pores on the first whorl and numerous fine spiral lines in the third whorl. The final, adult whorl does not have any ornamentation. The spire shell and sutures has a brown colouration, even in fossil specimens. The final adult shell consists of 3 ¾ to 4 ¼ whorls. Eyes are type a and operculum is type a (Seapy, 2011).

*Remarks.* Ornamentation is similar to *Atlanta gaudichaudi* in the first whorls (Newman, 1990) and may cause confusion with juveniles. *Atlanta plana* also has similar ornamentation, however, the spire of *P. sculpta* is lower than that of *A. plana* and *A. gaudichaudi*. Juveniles of *P. sculpta* have a light brown colouration.

*Distribution.* *P. sculpta* is found throughout the Atlantic Ocean from latitudes 41°N to 34°S, but rarely within the northern and southern oligotrophic subtropical gyre regions. Only a single specimen was found outside of the Atlantic Ocean close to the Marquesas Islands in the south Pacific.

