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A new deep-sea Cirripedia of the genus *Heteralepas* from the northeastern Atlantic

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Abstract. A new species of the sessile deep-sea barnacle, *Heteralepas* (Crustacea, Cirripedia), *Heteralepas gettysburgensis* sp. nov., is described. The specimens were collected at a depth of 225 m at the Gettysburg Seamount on the Gorringe Bank, located in the Portuguese Exclusive Economic Zone, approximately 200 km off the southwestern coast of mainland Portugal. Extensive morphological and molecular (COI, 12S and 16S) analyses were carried out to separate the species from its nearest congeners with similar geographic distribution, i.e., Atlantic waters.

Keywords. Gorringe Bank, Lepadiformes, naked barnacle, seamount.

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Introduction

A hydrophone, deployed on 23 August 2015, was collected from a depth of 255 m at the Gettysburg seamount (36º34′42.46″ N, 11º35′49.02″ W), located in the Gorringe Bank in the Madeira-Tore geological complex (North-East Atlantic), on 28 August 2016. It was fully colonized by polyps of the hydrozoan *Oceania armata* Kölliker, 1853 and specimens of the new species of *Heteralepas* Pilsbry, 1907 proposed in this study.

Currently, twenty-three species of the genus *Heteralepas* are accepted at the World Register of Marine Species (Chan & Southward 2010). All species are distributed in the Atlantic, Pacific and Indo-Pacific waters. However, only six species are registered for the Atlantic, of which three are for the eastern Atlantic.

We carried out comparisons of our specimens to species with similar diagnostic characters and to species with close geographic distribution, i.e., Atlantic waters. None of the previously described species

matches, neither morphologically nor genetically, the new species *Heteralepas gettysburgensis* sp. nov. presented here.

Material and methods

Specimens were collected from a depth of 255 m at the Gettysburg Seamount (36º34′42.46″ N, 11º35′49.02″ W) in the Gorringe Bank, located in the Madeira-Tore geologic complex (Fig. 1). Fifteen specimens were morphologically analyzed, of which ten were dissected for the analysis of internal characters and five for molecular analyses.

For the morphological analysis, an optical microscope (Leica MZ 12) was used. Drawings were made using a camera lucida attached to the optical microscope. Pictures were obtained with a digital camera (SONY DFW-SX910) connected to an optical microscope (OLYMPUS SZX9), using an image analyser (Visilog, TNPC Software, v.4).

A small piece of muscle tissue from each of the five specimens was used for DNA extraction using the E.Z.N.A Mollusc DNA Kit (Omega Biotek), following the manufacturer's instructions. Three mitochondrial genetic markers (the barcode region of the cytochrome *c* oxidase subunit I (COI-5P), 12S and 16S) were amplified in a Techne TC-PLUS thermal cycler (VWR International, LLC, Radnor, USA). For the COI-5P, the Go Taq ® G2 colorless master mix (Promega) and the primer pair LoboF1 (5'-KBTCHACAAAYCAYAARGAYATHGG-3') and LoboR1

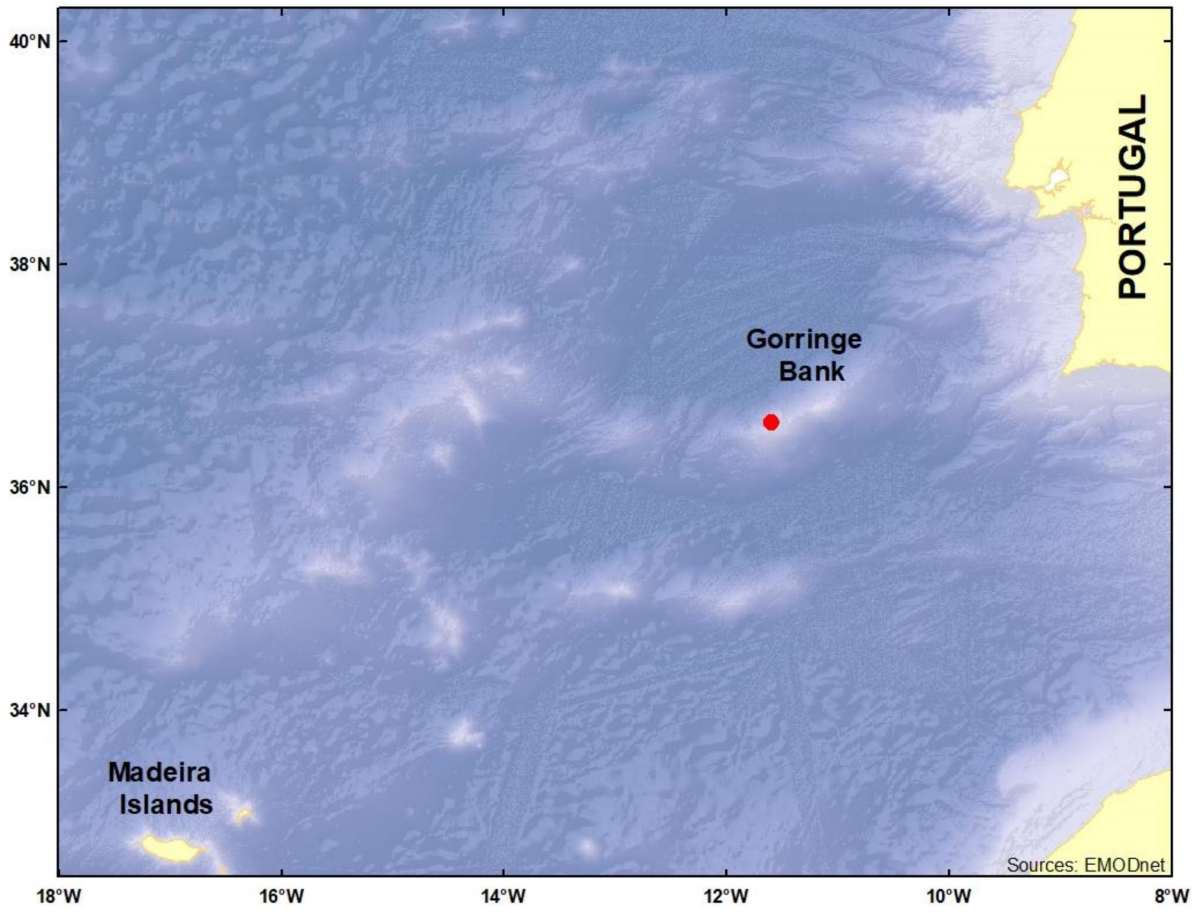

Fig. 1. Map showing the location of the sampling site (red dot).

(5'-TAAACYTCWGGRTGWCCRAARAAYCA-3') (Lobo *et al.* 2013) were used. The PCR thermal cycling conditions were as follows: 1) 94 °C (5'); 2) 5 cycles: 94 °C (30''), 45 °C (1'30''), 72 °C (1'); 3) 45 cycles: 94 °C (30''), 54 °C (1'30''), 72 °C (1'); 4) 72 °C (5'). Each reaction contained 12.5 µL 1 × Go Taq master mix plus 10 µm of each primer (1.5 µL for each primer), 4 µL of DNA template and completed with sterile milliQ-grade water to make up a total volume of 25 µL. For 12S, puRe Taq Ready-To-Go PCR beads (Amersham Biosciences) and the primer pair L13337-12S (YCTACTWTGYTACGACTTATCTC; Machida *et al.* 2002) and H13842-12S (TGTGCCAGCASCTGCGGTTAKAC; Machida *et al.* 2004) were used by performing the following thermal cycling conditions: 1) 94 °C (5'); 2) 40 cycles: 94 °C (5''), 45 °C (5''), 72 °C (10''). Each reaction contained 4 µL of DNA template, 1 µL (10 µm) of each primer and completed with sterile milliQ-grade water to make up a total volume of 25 µL. For 16S, puRe Taq Ready-To-Go PCR beads (Amersham Biosciences) and the primer pair 16L2 (TGCCTGTTTATCAAAAACAT; Schubart *et al*. 2002) and 16H2 (AGATAGAAACCAACCTGG; Schubart *et al.* 2000) were used by performing the following thermal cycling conditions: 1) 94 °C (4'); 2) 35 cycles: 95 °C (45''), 48 °C (45''), 72 °C (1'30''); 3) 72 ºC (5'). Each reaction contained 4 µL of DNA template, 1 µL (10 µm) of each primer and completed with sterile milliQ-grade water to make up a total volume of 25 µL. COI-5P amplified products were purified using the RapidTips (Diffinity Genomics); 12S and 16S amplified products were purified using ExoSap IT (Amershan Biosciences). They were subsequently sequenced bidirectionally by GATC Biotech AG (Konstanz, Germany).

Sequence trace files were individually edited and the primer sequences were removed. The obtained sequences were aligned using Clustal W in MEGA v. 7.0 (Kumar *et al.* 2016). COI-5P sequences were checked for possible stop codons, indels or unusual amino acid sequences (see Song *et al.* 2008). BOLD Identification System tool (BOLD-IDS) (Ratnasingham & Hebert 2007) and GenBank BLASTn searchs (Altschul *et al.* 1990) were used to confirm if the sequences obtained for the new species match with existing taxa in the public databases. Available DNA barcodes belonging to the same genus were included in our alignment. The neighbour joining method was carried out to calculate intra- and interspecific distances using the Kimura-2-parameter (K2P) model (Kimura 1980). A data set was created in BOLD (Data set ID-HETSPP https://doi.org/10.5883/DS-HETSPP) comprising the COI sequences generated in this study plus the available sequences of *Heteralepas* specimens. All original sequences generated in this study were submitted to GenBank and correspond to accessions numbers MF695097 – MF695101, MF695104 – MF695108 and MF695113 – MF695121, including the sequences of the hydrozoan *Oceania armata*.

Results

Taxonomic description

Infraclass Cirripedia Burmeister, 1834
Superorder Thoracica Darwin, 1854
Order Lepadiformes Buckeridge & Newman, 2006
Suborder Heteralepadomorpha Newman, 1987
Family Heteralepadidae Nilsson-Cantell, 1921
Heteralepas Pilsbry 1907

Heteralepas gettysburgensis sp. nov.
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Figs 2–6

Diagnosis

Demarcation between capitulum and peduncle clear. Capitulum wider than capitulo-peduncular junction. Capitulum slightly longer than peduncle, both with folds. Carinal margin thickened. Aperture more than ⅓ and less than ½ height of the capitulum, with crenulated lips. Lips surrounding aperture clearly set off from the surrounding capitulum by a demarcation border. Mandible with four teeth covered by fine setae, as well as its posterior side. Lower margin of the teeth with numerous pectinations: the first tooth with ten, the second, third and fourth ones with five, five and six pectinations, respectively. First maxilla with two strong acuminate teeth of unequal length on the upper angle.

Etymology

The specific epithet alludes to the location where the specimens were collected: the Gettysburg Seamount, Gorringe Bank, in the Madeira-Tore geologic complex.

Material examined

Holotype:

NORTH-EAST ATLANTIC: located approximately 200 km off the southwestern coast of Portugal, Gettysburg Seamount, Gorringe Bank, Madeira-Tore geological complex, 36º34′42.46″ N, 11º35′49.02″ W, 255 m depth, 28 Aug. 2016 (MB11-000943).

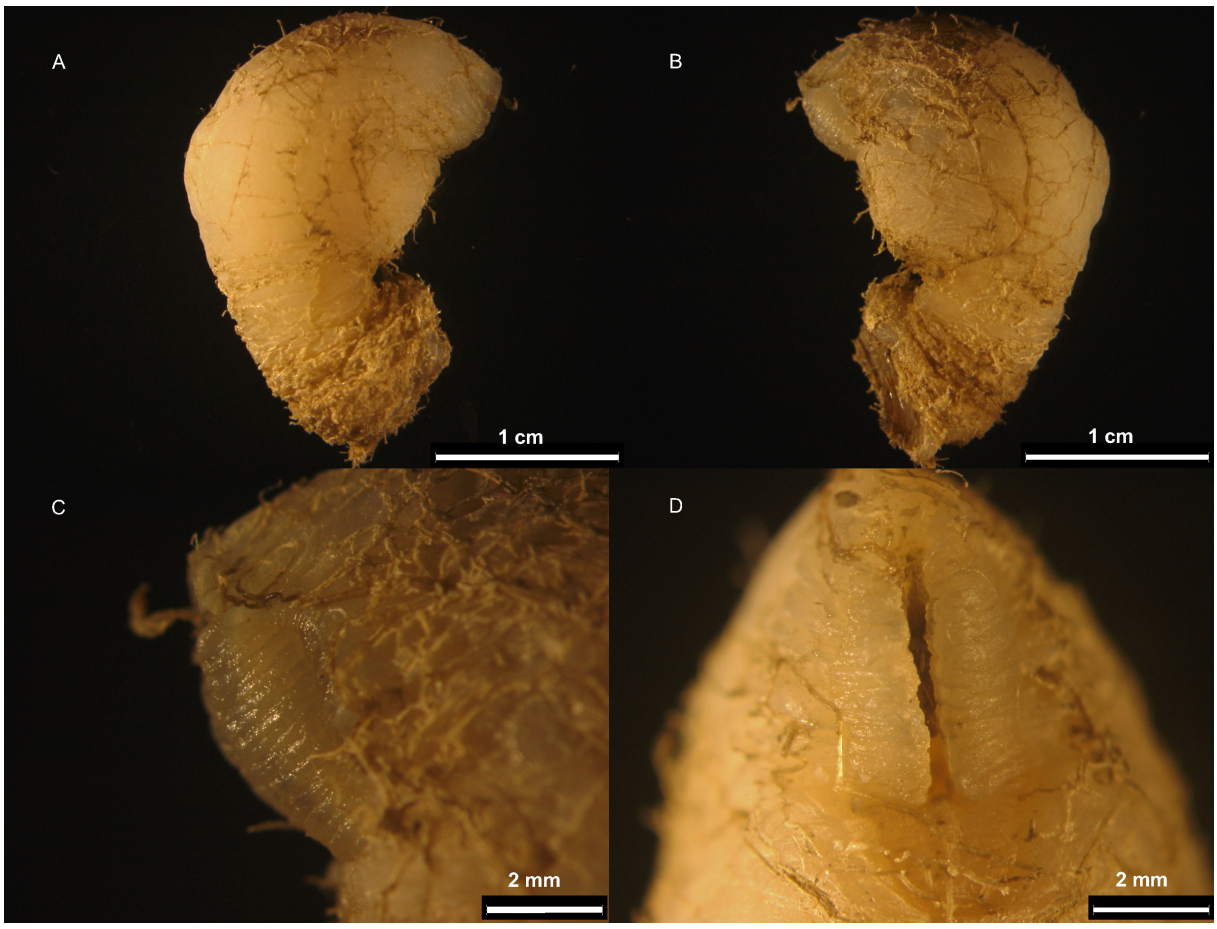

Fig. 2. External morphology of *Heteralepas gettysburgensis* sp. nov. (holotype) **A–B**. Side views showing the capitulum and peduncle. **C**. Side view of the aperture. **D**. Front view of the aperture.

Paratypes:
NORTH-EAST ATLANTIC: 14 specimens, same data as for holotype (MB11-000944 – MB11-000957).

Description

The fresh specimens were translucent yellowish, allowing observation of the cirri movement inside the capitulum. Demarcation between capitulum and peduncle clear. Capitulum wider than capitulo-

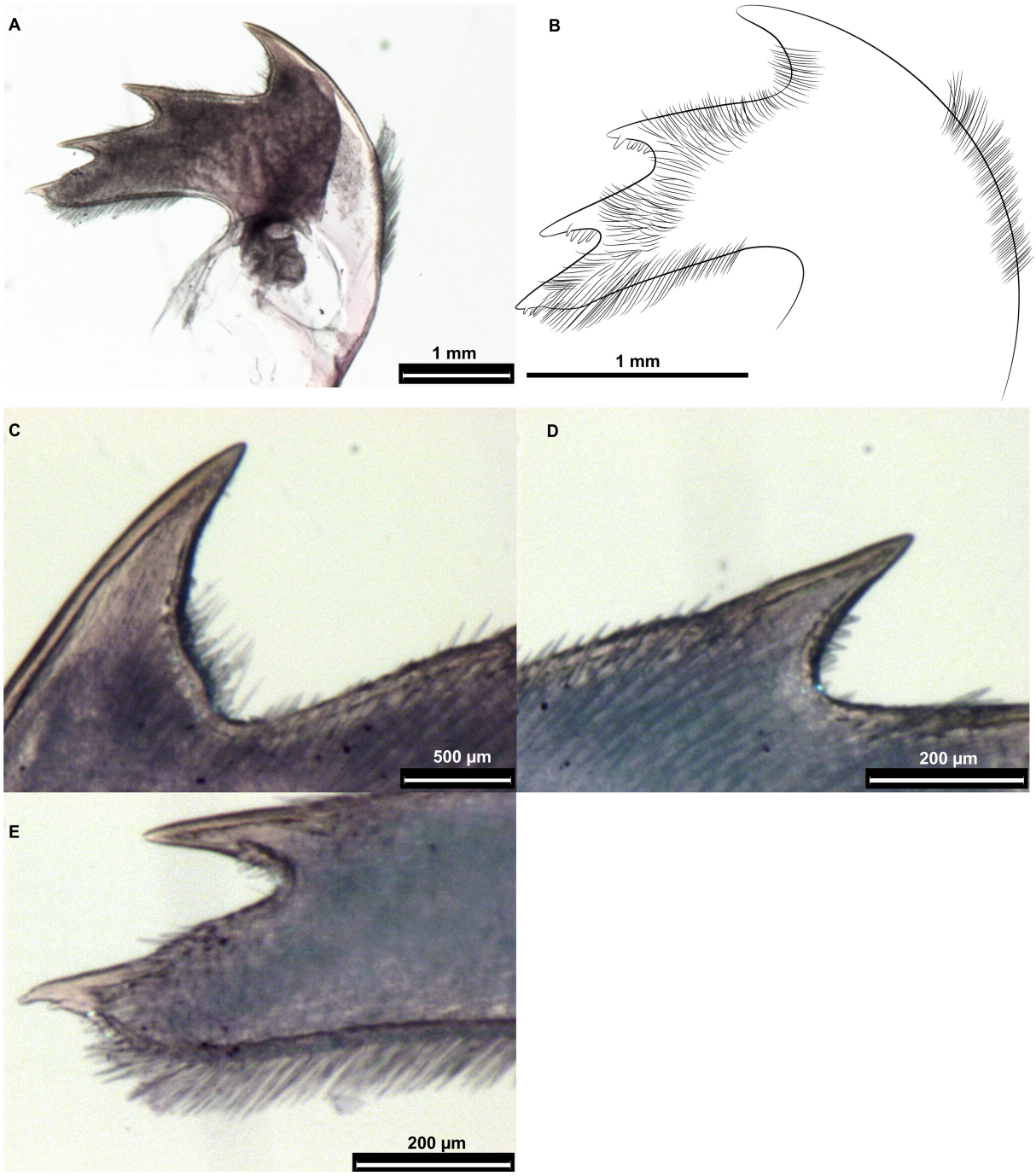

Fig. 3. Line drawings and pictures of the mouth parts of *Heteralepas gettysburgensis* sp. nov. (holotype) **A–B**. Mandible. **C**. Tooth I. **D**. Tooth II. **E**. Tooth III. **F**. Tooth IV.

peduncular junction (Fig. 2A–B). Capitulum 1.2 to 1.6 times longer than peduncle, cylindrical. Both peduncle and capitulum with folds. Aperture more than ⅓ and less than ½ of the capitulum height. Lips surrounding the aperture crenulated and clearly set off from the surrounding capitulum by a demarcation border (Fig. 2C–D). Carinal margin thickened. Mandible (Fig. 3A–B) with four teeth covered by fine setae as well as its posterior side. Lower margin of the teeth with pectinations: first tooth

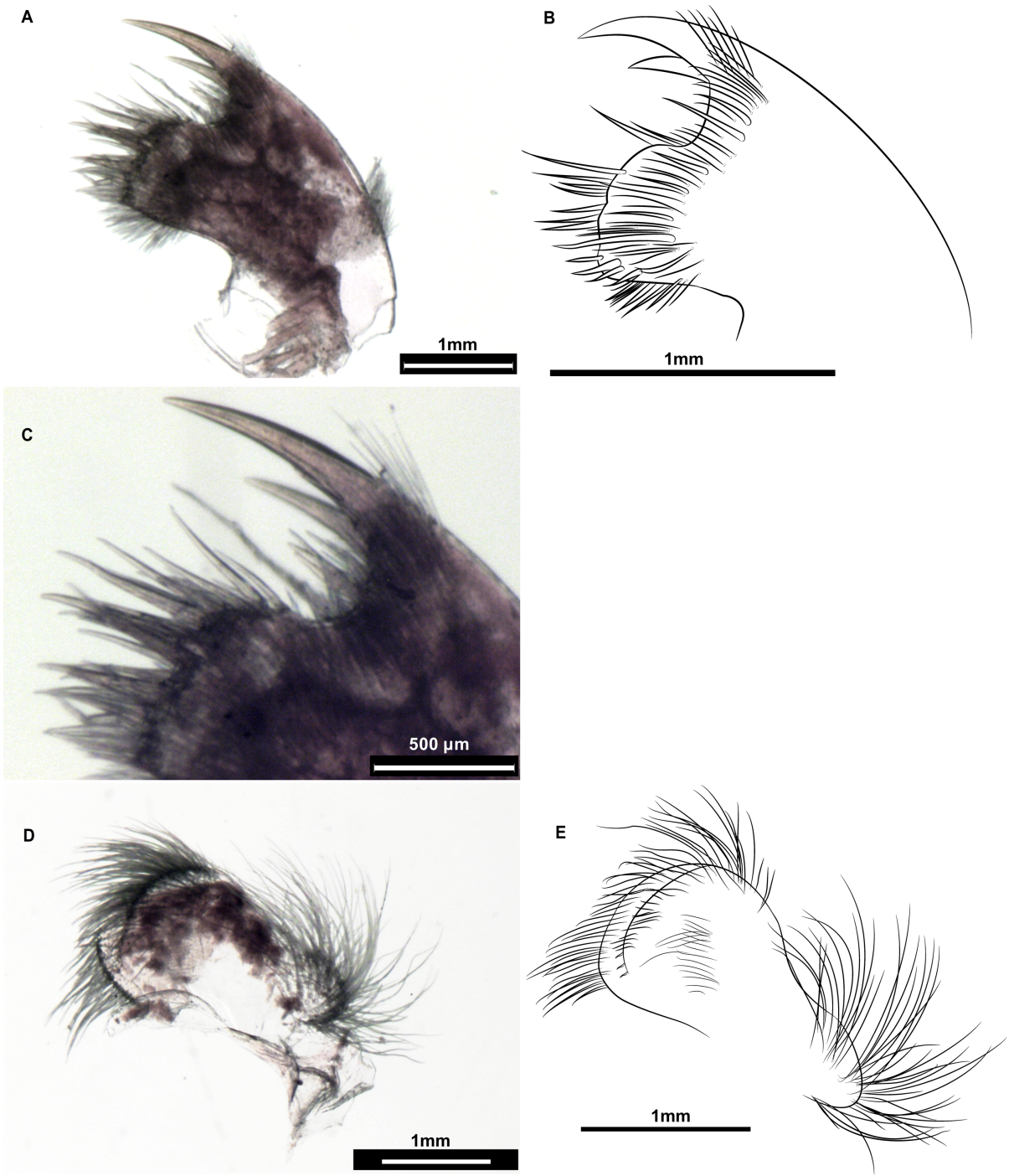

Fig. 4. Line drawings and pictures of the mouth parts of *Heteralepas gettysburgensis* sp. nov. (holotype) **A–B**. Maxilla I. **C–D**. Maxilla II.

with ten, second, third and fourth ones with five, five and six pectinations, respectively (Fig. 3C–F). Distance between first and second tooth 1.5 times that between second and third tooth. Latter distance similar to the distance between third and fourth tooth. First maxilla deeply notched, with two strong acuminate teeth of unequal length on upper angle, numerous setae and short to long spines on the cutting margin. Posterior margin with a dense clump of fine setae. (Fig. 4A–C).Second maxilla with two lobes covered with numerous long serrulate setae, those on posterior lobe longer than those on anterior lobe (Fig. 4D–E). Cirral setation lasiopod. Cirrus I separated from posterior pairs, with unequal rami. Anterior ramus slightly shorter and wider at base than posterior ramus (Fig. 5A–B). Both with numerous setae on upper and lower margins. Base of cirrus I with one filamentous appendage. Cirrus II with anterior ramus slightly shorter than posterior ramus (Fig. 6A). Anterior and posterior rami of cirri III and IV very similar (Fig. 6B–C). Cirri V and VI similar (Fig. 6D and 4C–D, respectively), with inner rami atrophied. Inner ramus of cirrus VI shorter than inner ramus of cirrus V. Caudal appendage slightly longer than pedicel of cirrus VI (Fig. 5E–F). Penis relatively long, annulated, with numerous long and short fine setae distally scattered throughout (Fig. 5G–H). Table 1 displays the measurements, ratios, mean, standard deviation and coefficient of variation concerning the morphological characters mentioned.

Discussion

All specimens were collected from the same site, at a depth of 255 m in the northeastern Atlantic. Seven species occur in the Atlantic: *H. belli* (Gruvel, 1902), *H. cantelli* Buhl-Mortensen & Newman, 2004, *H. cornuta* (Darwin, 1851), *H. lankesteri* (Gruvel, 1900), *H. luridas* Zevina, 1975, *H. microstoma* (Gruvel, 1901) and *H. segonzaci* Young, 2001, of which only three species occur in the east Atlantic (*H. cornuta*, *H. microstoma* and *H. segonzaci*). The new species differs distinctly from *H. cornuta* due to the absence of conspicuous triangular projections in the capitulum (Fig. 2A–B). The species *H. microstoma* has a peduncle much longer than the capitulum, being the opposite of our specimens, where the capitulum is slightly longer than the peduncle. The original description by Gruvel (1902) gives an account of the number of articles of cirrus I (inner and outer rami), the inner rami of cirri V and VI and the caudal appendage. All cirri have a greater number of articles than our specimens except the caudal appendage (Table 2). The peduncle of *H. segonzaci*, from a depth of 2235 m, is three times shorter than the capitulum and the aperture is more than half the length of the capitulum (Young, 2001) while our specimens have an aperture length less than half that of the capitulum, and the rami of cirri III and IV have fewer articles than *H. segonzaci*. Comparing with the other species recorded from the western Atlantic, *H. belli* has the capitulum shorter than the peduncle and the cuticle is almost smooth, with some irregular folds (Gruvel, 1902), while our specimens have the capitulum longer than the peduncle and the cuticle with numerous folds. Moreover, in *H. bellii* the aperture is half as high as the capitulum height (Gruvel, 1902) while in our species it is less than half its height. In *H. lankesteri* the capitulum is approximately as long as the peduncle (ratio length of capitulum to length of peduncle (C/P) varies from 0.6 to 1.3) (Gruvel, 1900), while in our specimens the ratio varies between 1.2 and 1.6. The cirri rami with a different number of articles and the non-pectinated mandible of *H. lankesteri* (Gruvel, 1900) also distinguish it from our species (Table 2). Finally, the small size of *H. luridas* (maximum registered size 9.5 mm), from the Caribbean, and the smaller number of articles of all cirri rami (Zevina, 1975) strongly distinguish it from our specimens (Table 2).

Regarding the species outside of the Atlantic, *H. japonica* Aurivillius, 1892 and *H. canci* Chan, Tsang & Shih, 2012 are the most similar species externally, probably due to the large morphological variability of *H. japonica*, unlike the species analyzed in this study (Table 1). However, they have crests on the carinal region of the capitulum, the rami of the cirri have fewer articles than our specimens and the ratio ‘height of aperture/height of capitulum’ is higher (Chan et al., 2009). The teeth of the mandible of *H. japonica* appear to be without pectinations or might have low pectinations on the lower margins of teeth 1–3, especially 2 and 3 (Buhl-Mortensen & Newman 2004), while the new species exhibits

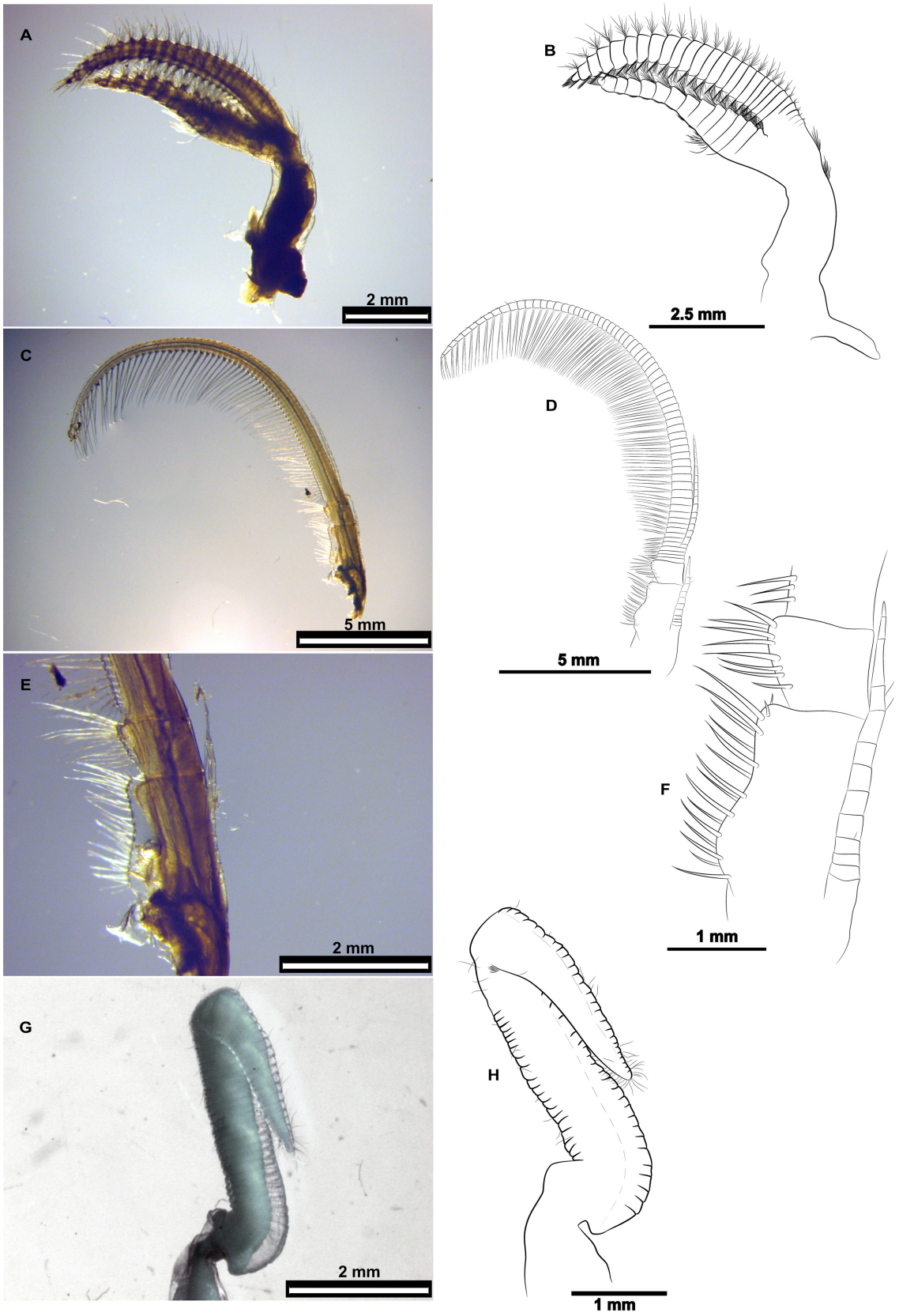

Fig. 5. Line drawings and pictures of *Heteralepas gettysburgensis* sp. nov. (holotype) **A–B**. Cirrus I. **C–D**. Cirrus VI. **E–F**. Caudal appendage. **G–H**. Penis.

numerous pectinations on the lower margin of the four teeth. Also, the maximum length of the peduncle and capitulum of *H. japonica* reported by Buhl-Mortensen & Newman (2004) is 11.6 and 3.6 cm respectively, quite distinct from the new species described in this study (1.4 and 1.6 cm, respectively). On the other hand, the sequences of the COI obtained for our specimens had divergences of 16 to 21 % with *H. japonica* and 16 to 17% with *H. canci* (Chan *et al.* 2009). The intraspecific distances obtained for our specimens were 0.3 to 0.7%. Many studies have been carried out using the mitochondrial DNA sequences of the COI-5P, which have confirmed that DNA barcodes (Hebert *et al.* 2003) are a reliable tool to discriminate species of crustaceans (Lobo *et al.* 2013, 2016), including cirripedes (Chan *et al.* 2009). Ratnasingham & Hebert (2013) suggested that 2.2% of average intraspecific distance is a reference threshold for within-species boundaries, which is quite far from the divergences found in this study. In addition, the marker 12S presented divergences approximately of 6.5% and 7.5 %, with the species *H. canci* and *H. japonica*, respectively, being within the expected values for barnacle species within the same genus (Chan *et al.* 2007, 2009). The marker 16S also showed 7 % of genetic divergence with the only specimen of *Heteralepas* (unidentified) available in public databases (Schiffer & Herbig 2016, GenBank accession KT947465), which is in accordance with the results obtained for other crustaceans (Brasher *et al*. 1992; Machado *et al.* 1993). *H. adiposa* Zevina, 1982 and *H. cygnus* Pilsbry, 1907, are similar to *H. microstoma*, but they differ from it in having numerous small calcareous knobs, four mandible teeth, and a great number of caudal segments (Zevina & Kolbasov 2000). Therefore, their diagnostic characters are also distinct from the specimens analyzed in this study. *H. mystacophora* Newman, 1964 has a smooth capitulum and the superior margins of the second and third teeth of the

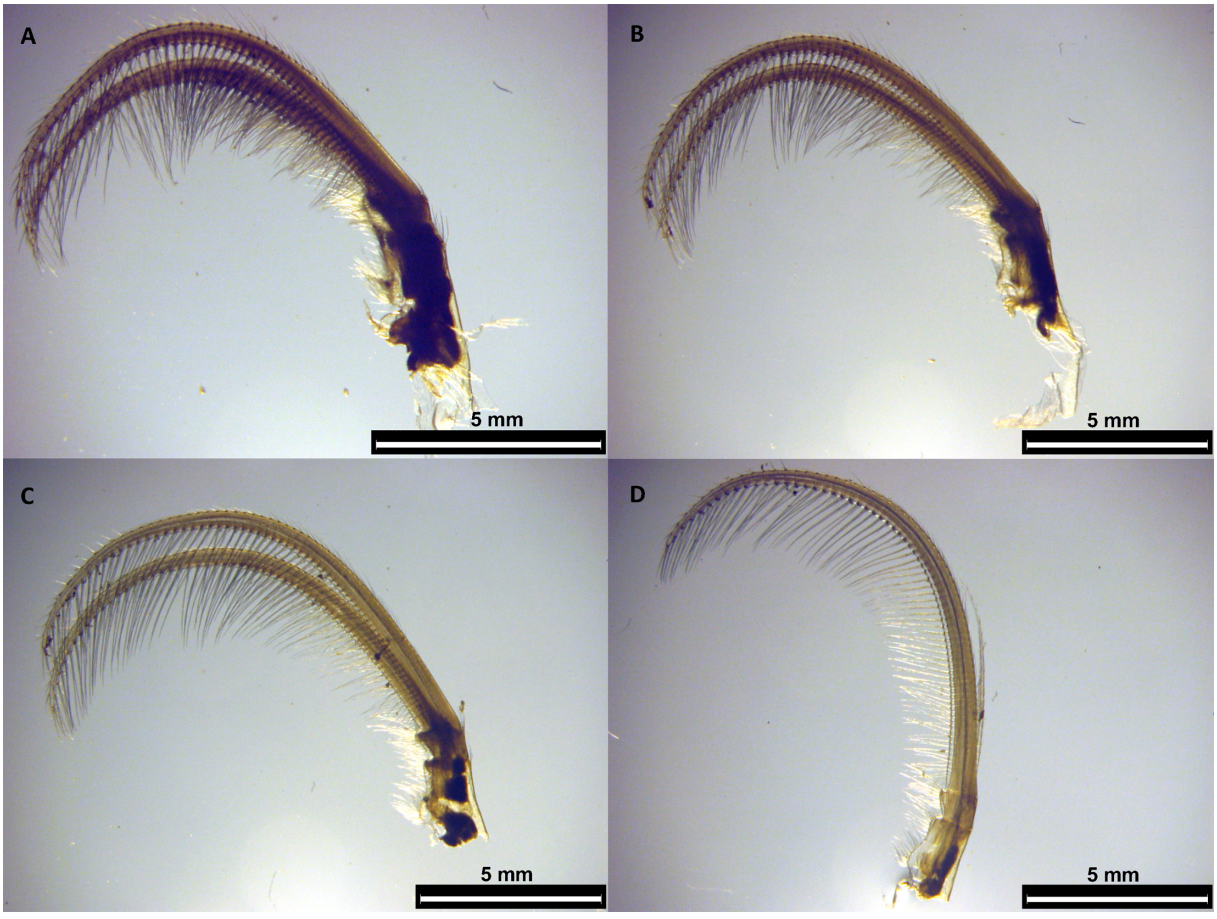

Fig. 6. Pictures of *Heteralepas gettysburgensis* sp. nov. (holotype) **A**. Cirrus II. **B** Cirrus III. **C**. Cirrus IV. **D**. Cirrus V.

Table 1. External and internal morphological variation for specimens analyzed in this study. (- lack of information.)

		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	Mean	Std dev	Coefficient of variation
Capitulum	Width	0.87	1.1	0.88	0.95	1.08	0.92	1.12	0.96	0.96	1.18	1.0	0.1	0.09
	Length	1.47	1.45	1.33	1.5	1.55	1.34	1.54	1.53	1.38	1.58	1.5	0.1	0.05
Peduncle	Width	0.76	0.75	0.64	0.72	0.84	0.82	0.81	0.82	0.81	0.93	0.8	0.1	0.07
	Length	1.1	0.91	0.97	1.03	1.28	1.13	1.08	1.19	0.95	1.36	1.1	0.1	0.10
	C/P	1.34	1.59	1.371	1.456	1.21	1.19	1.43	1.29	1.45	1.16	1.3	0.1	0.08
Aperture	Length	0.61	0.47	0.5	0.57	0.57	0.49	0.58	0.54	0.56	0.69	0.6	0.0	0.08
	A/C	0.41	0.32	0.376	0.38	0.37	0.37	0.38	0.35	0.41	0.44	0.4	0.0	0.06
Cirrus I Articles	Inner ramus	13	15	14	13	14	16	14	15	14	15	14.3	0.8	0.05
	Outer ramus	28	28	28	28	28	28	29	24	30	28	27.9	0.8	0.03
Cirrus II Articles	Inner ramus	51	50	46	52	51	53	51	48	53	51	50.6	1.6	0.03
	Outer ramus	66	58	59	61	59	63	64	58	64	59	61.1	2.5	0.04
Cirrus III Articles	Inner ramus	65	67	61	65	69	67	66	62	68	61	65.1	2.3	0.04
	Outer ramus	66	67	62	71	72	70	65	66	71	62	67.2	3.0	0.05
Cirrus IV Articles	Inner ramus	67	67	61	70	73	69	71	65	75	64	68.2	3.4	0.05
	Outer ramus	71	68	69	73	75	71	71	66	75	74	71.3	2.4	0.03
Cirrus V Articles	Inner ramus	69	70	64	72	73	-	73	71	78	67	70.8	2.9	0.04
	Outer ramus	22	19	17	22	27	19	26	21	19	20	21.2	2.4	0.12
Cirrus VI Articles	Inner ramus	70	69	60	71	76	70	73	64	78	67	69.8	3.8	0.06
	Outer ramus	20	18	17	17	17	17	21	20	18	19	18.4	1.3	0.07
	Caudal appendage	13	13	13	13	16	13	16	13	11	8	12.9	1.4	0.11

mandible support several widely spaced spinules (Zullo & Newman, 1964), while the species described here presents a wrinkled capitulum and has spinules in the inferior margin in the four teeth of the mandible. The capitulum of *H. nicobarica* Annandale, 1909 is indistinctly separated from the peduncle, the aperture height is ¼ of the capitulum height and the peduncle is similar to or longer than the capitulum (Annandale, 1909). Contrarily, the new species presents a clear demarcation between capitulum and peduncle, the aperture height is > ⅓ and < ½ of the capitulum height and the peduncle is shorter than the capitulum (see Table 1). In *H. rex* (Pilsbry, 1907), the capitulum is almost as long as the peduncle, and lips are slightly crenulated or irregularly warty and do not protrude in adults (Pilsbry, 1907). In our specimens the capitulum is slightly longer than the peduncle and the lips are clearly crenulated and protruding. In *H. utinomii* Newman, 1960, the length of the peduncle is one third of that of the capitulum (Newman, 1960), which distinguishes it from our specimens whose peduncle is only slightly shorter than the capitulum.

Table 2. Comparison of diagnostic characters between the new species *H. gettysburgensis* sp. nov. and *H. belli* (Gruvel, 1902), *H. canci* Chan, Tsang & Shih, 2012, *H. cantelli* Buhl-Mortensen & Newman, 2004, *H. japonica* Aurivillius, 1892, *H. lankesteri* (Gruvel, 1900), *H. microstoma* (Gruvel, 1901) and *H. segonzaci* Young, 2001. The table was based on Buhl-Mortensen & Newman (2004). C/P = Ratio of length of capitulum (C) to length of peduncle (P); A/C = Ratio of height of aperture (A) to height of capitulum (C). Characters coincident with the new species are underlined.

Heteralepas species		*belli*	*canci*	*cantelli*	*japonica*	*lankesteri*	*luridas*	*microstoma*	*segonzaci*	*gettysburgensis* sp. nov.
Demarcation between capitulum & peduncle		Clear	Varying	Weak		Clear		Weak		Clear
Width of capitulum to capitulo–peduncular junction		Wider	Varying	Slightly wider carinal side		Wider		Slightly wider		Wider
Capitulum	Width	2.5		2	0.6–2.3 (1.5)	1.2–2.0		2.3		0.9–1.2 (1)
	Length	1.6		3	0.9–3.6 (2.0)	1.7–2.2		1.7		1.3–1.6 (1.5)
Peduncle	Width	0.95		1.2	0.5–1.9 (0.9)	0.9–1.3		0.9		0.6–0.9 (0.8)
	Length	3.2		3	0.5–11.6 (3.8)	1.3–3.8		2.4		0.9–1.4 (1.1)
	C/P	0.5		1.0	0.2–1.8 (0.5)	0.6–1.3		0.7	3	1.2–1.6 (1.4)
Carinal margin thickened		Yes		No	Varying	Yes		Yes	Yes	Yes
Aperture	Length									0.5–0.7 (0.6)
	A/C	1/2		1/2	1/2	1/3		1/2?	>1/2	> 1/3 < 1/2
	Flaring from side	Yes		Partly	No	Yes		No?		Yes
	Crenulated	No		Slightly		Conspicuously				Yes
	Lower margin demarcated	No		No		Yes				Yes
	Tubular	?Yes		No	No	?Yes		Yes		Yes
Cirrus I Articles	Inner ramus		6	18	10–12	13	13	17	14–15	13–16
	Outer ramus		12	24	16–18	21	22	32	27–28	24–30
Cirrus II Articles	Inner ramus		24	42		50	35		50–54	46–53
	Outer ramus		26	51		55	40		64–66	58–66
Cirrus III Articles	Inner ramus		47	56		50	40		72–75	61–69
	Outer ramus		46	57		55	41		73–79	62–72
Cirrus IV Articles	Inner ramus		47	56		50	41		76–79	61–71
	Outer ramus		47	58		55	41		75–79	66–75
Cirrus V Articles	Inner ramus	27	19	25	13–29	19	15	29	3+–29	19–27
	Outer ramus		50	60	36–69	74–92	57		77–78	64–78
Cirrus VI Articles	Inner ramus	27	18	22	13–27	16	14	26	22–23	17–21
	Outer ramus		49	60	37–68	89	54		75–81	64–78
	Caudal appendage	15	6	13	4–12	10		15	13–15	8–16
Mouth parts	Mandible pectination	Yes		Yes	Yes	No		Yes		Yes

Final Remarks

Specimens of *Heteralepas* are not encountered often since they live in deep-sea environments. The combination of the morphological and molecular data, reveals that the new species *Heteralepas gettysburgensis* sp. nov. is distinct from all the species described so far for this genus. It is recorded from the type locality at a depth of 255 m in the Gettysburg Seamount of the Gorringe Bank only, located in the Portuguese Exclusive Economic Zone, approximately 200 km off the southwestern coast of mainland Portugal. Due to its outstanding biodiversity, including several priority species and habitats, the Portuguese Institute for Nature Conservation and Forests, proposed in 2015 to declare the Gorringe Bank as a Site of Community Importance (SCI) under the Natura 2000 network. The present work not only contributes to improve the knowledge of the benthic biodiversity in this deep-sea environment, but also provides both a morphological description and genetic data (three mitochondrial markers: COI-5P, 12S and 16S), which will be a relevant contribution to help clarifying potential issues regarding the taxonomy of the genus *Heteralepas*.

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