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A new *Okenia* Menke, 1830 from the Azores Islands, Portugal

(Mollusca, Nudibranchia, Goniodorididae)

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A new species of nudibranch mollusc of the genus *Okenia* Menke, 1830 is described from Pico Island, in the archipelago of the Azores (Portugal). This genus has a worldwide distribution but there were no published records of *Okenia* species from this area. *Okenia picoensis* spec. nov. shows intraspecific variation in its colour pattern. It may present white colouration with the top of their appendices yellow, or bright yellow with the top of its appendices orange. The description is complemented with molecular data obtained from the mitochondrial genes cytochrome c oxidase and 16S rRNA. The new species is compared with other *Okenia* regarding morphological characters as well as genetic distances and geographic distribution. A preliminary phylogenetic tree based on COI sequences including all *Okenia* available at the moment is also included.

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Introduction

The genus *Okenia* Menke, 1830 is a controversial taxon of nudibranchs belonging to the family Goniodorididae. Gosliner (2004) published the first phylogenetic study based on morphological characters. He concluded that *Okenia* was a monophyletic taxon only when *Hopkinsia*, *Hopkinsiella* and *Sakishimaia* were considered as synonyms of *Okenia*. Ten years later, Pola et al. (2014) presented the first molecular data with genetic distances between some species of *Okenia*. However, as the authors stated the study was incomplete since there were many missing species due to the lack of fresh, well-preserved material for molecular studies. To date, the genus *Okenia* is comprised of 52 species, of which eight have been described since 2005 (Bouchet & Gofas 2016). Surprisingly, there are no published records of *Okenia* species found in the archipelago of the Azores (Portugal).

In recent years, many studies have dealt with the description of new species morphologically identical but reproductively isolated cryptic species (Knowlton 1993), or with species morphologically recognizable only after other methods have unveiled their existence, and then known as pseudocryptic (Knowlton 2000). Within heterobranch gastropods we can find many examples (Jörger & Schrödl 2013, Carmona et al. 2014a, Kienberger et al. 2016, Lindsay & Valdés 2016, among many others), also within the genus *Okenia* (Pola et al. 2014). However, many heterobranchs show also different grades of intraspecific

variation, and this is an interesting matter of study as well (Pola et al. 2008, Carmona et al. 2014b).

In the present study, we describe a new species of *Okenia* from the Island of Pico (Azores Archipelago) that shows intraspecific variation in its colour pattern. The description is complemented with partial sequences obtained from the mitochondrial genes cytochrome c oxidase (COI) and 16S rRNA. The new species is compared with other *Okenia* regarding morphological characters, genetic distances and geographical distribution. We also obtained, for the first time, molecular sequences of these two genes and Histone 3 (H3) for the species *Okenia vena* Rudman, 2004 and *Okenia aspersa* (Alder & Hancock, 1845) as well as for an undescribed species from the Mediterranean Sea. A preliminary phylogenetic tree based on COI sequences including all the *Okenia* species available is also included. Following the study by Pola et al. (2014), we do not attempt to present a complete molecular phylogeny of the genus *Okenia*, but by adding information we try to clarify the phylogenetic relationships within this genus that is in need of a deep morphological and molecular revision (Gosliner 2004, Pola et al. 2014, Pola 2015).

Material and methods

Source of specimens. Between June and November of 2013, nine specimens of *Okenia* sp. were collected by SCUBA diving to depths of between 8 and 30 meters off the rocky shore of Pico Island, in the Archipelago of Azores (Portugal). All the specimens were collected, photographed and preserved in 96 % ethanol by Justin Hart. The material examined is deposited in the Museu Nacional de História Natural e da Ciência of Lisbon (MB), the Museo Nacional de Ciencias Naturales of Madrid (MNCN) and the Zoologische Staatssammlung München in Munich (ZSM).

Morphological examination. The external morphology was examined using photographs of the living animals as well as laboratory observations. The internal organs were examined by removing them from the animal through a dorsal incision and drawn under a Nikon SMZ-1500 dissecting microscope with a camera lucida attachment. Special attention was paid to the morphology of the radula and the reproductive system. The buccal mass of each specimen dissected was removed and dissolved in 10 % sodium hydroxide to remove surrounding tissue. Labial cuticle and radula were rinsed in water. The labial cuticles and penises were dried by critical point under an Emmitech K850 and these structures and radulae were mounted and sputter coated for examination under a Hitachi S3000N scanning electron microscope (SEM) at the "Servicio Interdepartamental de Investigación" (SIDI), Universidad Autónoma de Madrid.

Molecular analysis

Taxon sampling. Initial sampling for molecular analysis included six specimens of *Okenia* sp. from Pico Island, two specimens of *Okenia vena* from Australia, one specimen of *Okenia aspersa* from France and one species of *Okenia* sp. A from Italy. We successfully obtained eight sequences for COI, six for 16S and three for the Histone H3 (none H3 for *Okenia* sp. from Pico) (Table 1). In addition, the same 22 taxa used by Pola et al. (2014) were added from GenBank, including nine species of *Okenia*, 12 of other nudibranchs and the pleurobranchid *Berthella martensi* (Pilsbry, 1896) as outgroup. *Okenia hiroi* (Baba, 1938) recently added by Jung et al. (2014) is also included (KF648920).

DNA extraction, amplification and sequencing. DNA extractions and PCR amplifications were performed at the Universidad de Cádiz (UCA), Spain. DNA was extracted from foot tissue of specimens preserved with 96 % ethanol, and performed using the DNeasy Blood and Tissue Kit Qiagen at UCA following the manufacturer's instructions. Partial sequences of COI, 16S and H3 were amplified by polymerase chain reaction (PCR)

Table 1. Vouchers, localities and Genbank accession numbers of the new specimens of *Okenia* included for molecular analyses in this study.

Species	Voucher	Locality	COI	16S	H3
Okenia aspersa	MNCN15.05/70410	France, Cape Ferret	KY661374	KY661368	KY661382
Okenia picoensis spec. nov.	MB28-004386	Azores, Pico Island	KY661375	–	–
Okenia picoensis spec. nov.	MB28-004389	Azores, Pico Island	–	–	–
Okenia picoensis spec. nov.	ZSM Mol 20170110	Azores, Pico Island	–	KY661369	–
Okenia picoensis spec. nov.	MNCN15.05/60181	Azores, Pico Island	KY661376	KY661370	–
Okenia picoensis spec. nov	MNCN15.05/70406	Azores, Pico Island	KY661377	–	–
Okenia picoensis spec. nov.	MB28-004387	Azores, Pico Island	KY661378	–	–
Okenia sp. A	MNCN15.05/70411	Italy, Sabaudia Lake	KY661379	KY661371	–
Okenia vena	MNCN15.05/70408	Australia, Nelson Bay	KY661380	KY661372	KY661383
Okenia vena	MNCN15.05/70409	Australia, Nelson Bay	KY661381	KY6613673	KY661384

using LCO1490 and HCO2198 universal primers for COI (Folmer et al. 1994), 16S ar-L and 16S br-H for 16S (Palumbi et al. 1991) and H3AD5'3' and H3BD5'3' for H3 (Colgan et al. 1998). The master mix for the PCR was prepared in the following order: nuclease-free water up to 25 ml volume reaction, 2.5 ml of Qiagen buffer, 2.5 ml of dNTP (2 mM), 5 ml of 'Q-solution' (Qiagen), 1.5–3.5 mM magnesium chloride, 1 ml of each forward and reverse primer (10 mM), 0.25 ml of DNA polymerase (250 units) and 2–3 ml of DNA. COI amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39–40 cycles of 30–45 s at 94 °C, 30–45 s at 46 °C (annealing temperature) and 1–2 min at 72 °C with a final extension of 5 min at 72 °C. 16S amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39 cycles of 39–45 s at 94 °C, 30–50 s at 45–51.5 °C (annealing temperature), 2 min at 72 °C, with a final extension of 5–10 min at 72 °C. H3 amplification was performed with an initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45–60 s at 94–95 °C, 45 s at 50 °C (annealing temperature), 2 min at 72 °C, with a final extension of 10 min at 72 °C. Successful PCR products obtained at UCA were purified and sequenced by Macrogen, Inc. All new sequences obtained were deposited in GenBank.

Sequence alignment and analysis. Since we could not get any H3 sequences for the *Okenia* sp. specimens from Pico and there are not 16S sequences available on GenBank for other *Okenia* species, only COI sequences were assembled, edited and aligned using Genious 6.1.6 (Drummond et al. 2009). Protein-coding sequences were translated into amino acids for confirmation of alignment. The alignment was checked by eye using MacClade 4.06 (Maddison & Maddison 2005). Pairwise uncorrected *p*-distance values between species were calculated using PAUP*4.0b 10.0 (Swofford 2002).

Model selection and phylogenetic analyses. The GTR+I+G evolutionary model was selected using MrModelTest 2.3 (Nylander et al. 2004) under the Akaike information criterion (Akaike 1974). Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses were conducted for COI with *Berthella martensi* as outgroup. BI analysis was performed using the software package MrBayes version 3.1.2b (Ronquist & Huelsenbeck 2003) for ten million generations with two independent runs and sampling frequency of 1000. The model implemented was that estimated with MrModeltest 2.3. ML analysis was performed using the software package RAxML v7.04 (Stamatakis et al. 2008). To determine the nodal support in ML a 50 000 bootstrap analysis was implemented. MP analysis was performed by heuristic searches under TBR branch swapping and 100 random replicates using PAUP* 4.0b10 (Swofford 2002). All characters were left unweighted and gaps were treated as missing characters (Ogden & Rosenberg 2007). We used nonparametric bootstrapping (1000 pseudo replicates) in the MP analysis to assess nodal support (Felsenstein & Kishino 1993). Only nodes supported by bootstraps values ≥75 (Hillis & Bull 1993) and posterior probabilities ≥0.96 were considered statistically significant (Alfaro et al. 2003). The trees obtained were shown in FigTree v.1.3.1 (Morariu et al. 2008) and edited in Adobe Photoshop CC 2014.

Results

Nudibranchia Cuvier, 1817
Euctenidiacea Tardy, 1970
Family Goniodorididae H. Adams & A. Adams, 1854

Genus *Okenia* Menke, 1830

Type species: *Okenia elegans* (Leuckart, 1828), by monotypy.

For a detailed synonymy and diagnosis of the genus see Rudman (2004).

Okenia picoensis spec. nov.

Figs 1–3

Etymology. The specific epithet, a Latin adjective, refers to the island where the new species was found.

Distribution. This species is known only from Pico Island, Azores Archipelago (Portugal) (present study).

Type material. Holotype: Azores, Pico Island, Porto Calhau, near shore, 8 m depth, 5 mm (alive), 3 mm (preserved), collected by J. Hart, 25 Jun. 2013 (Photo_6, "yellow"; COI; SEM: radula, penis) (MB28-004386). – Paratypes: Azores, Pico Island, Porto Calhau, 12 m, 3 mm (alive), 1.5 mm (preserved), collected by J. Hart, 26 Nov. 2013 (Photo_1, "white"; COI; SEM: radula) (MB28-004387). Azores, Pico Island, Porto Calhau. 8 m, 4 mm (alive), 2 mm (preserved), collected by J. Hart, 30 Nov. 2013 (Photo_3, "white"; COI y 16S; SEM: radula, penis, mantle). (MNCN15.05/60181). Azores, Pico Island, Porto Calhau. 8 m, 3 mm (alive), 2 mm (preserved), collected by J. Hart, 19 Nov. 2013 (Photo_4, "yellow"; 16S) (ZSM Mol 20170110). Azores, Pico Island, Santo Mateus, 16 m, 4 mm (alive), 2.5 mm (preserved), collected by J. Hart, 25 Nov. 2013 (Photo_5, "yellow"; SEM: radula, spicules, mantle) (MB28-004388).

Additional material. Azores, Pico Island, Porto Calhau. 8 m, 4 mm (alive), 2 mm (preserved), collected by J. Hart, 30 Nov. 2013 (Photo_2, "white") (ZSM Mol 20170111). Azores, Pico Island, Santo Mateus, 16 m, 3 mm (alive), 2 mm (preserved), collected by J. Hart, 25 Nov. 2013 (Photo_5, "yellow"; COI; SEM: penis, mantle) (MNCN15.05/70406). Azores, Pico Island, near shore, 8 m, 3 mm (alive), 2 mm (preserved), collected by J. Hart, 25 Jun. 2013 (Photo_7, "yellow"; SEM: radula) (MNCN15.05/70407). Azores, Pico Island, Sao Caetano, 30 m, 2.5 mm (alive), 1.5 mm (preserved), collected by J. Hart, 2 Nov. 2013 (Photo_8, "yellow"; SEM: radula, penis) (MB28-004389).

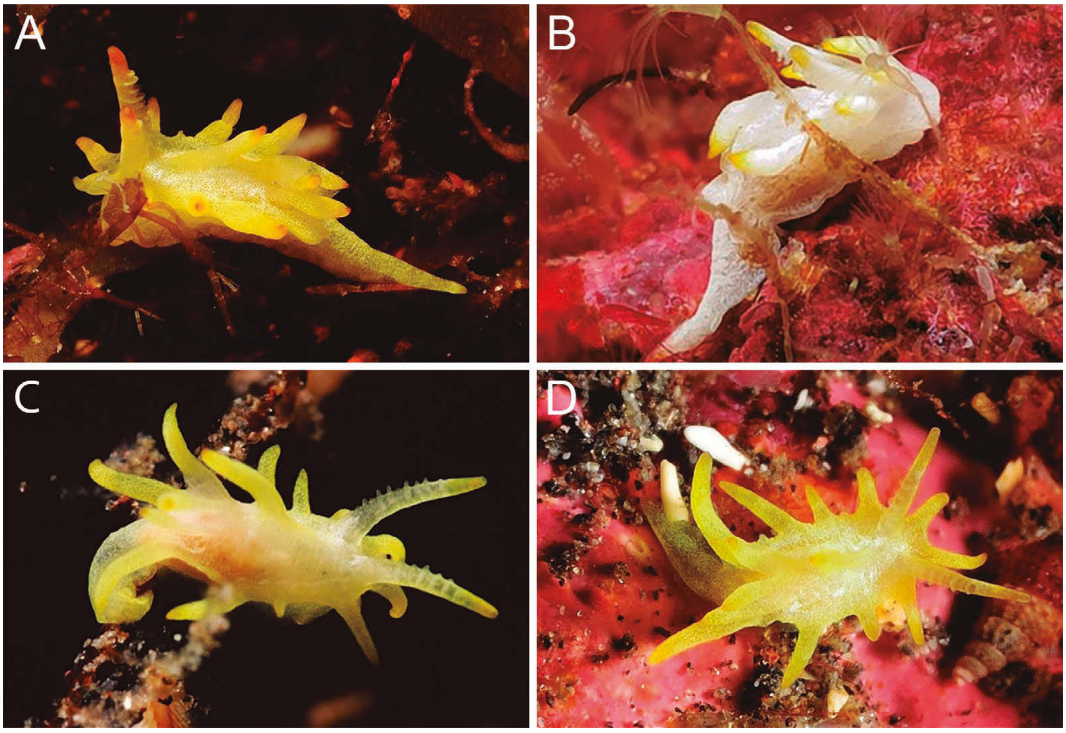

Fig. 1. *Okenia picoensis* spec. nov. Photographs of the living animals from Pico Island. **A.** Holotype, MB28-004386 (5 mm). **B.** Paratype, MNCN15.05/60181 (4 mm). **C.** MB28-004389 (2.5 mm). **D.** MNCN15.05/70406 (3 mm). Photographs by Justin Hart.

Description

External morphology (Fig. 1). Living animals up to 5 mm in length. Body high and elongate. Mantle covered by long spicules of different sizes and with bulges along them. There is a well-developed notal border with five lateral papillae, symmetrically distributed on each side of body. Two anteriormost papillae situated in front of rhinophores, two behind gill and remaining three on each side between rhinophores and gill. Papillae elongate, narrow and cylindrical, increasing in length and width from anterior to posterior papillae. A single medial papilla present mid-dorsally anterior to gill. It arises from a mid-dorsal ridge, which extends from rhinophores to beginning of papilla. Rhinophores elongated and slender bearing between 7 and 9 lamellae at the dorsal and lateral portion, but not at anterior part. Tips of rhinophores lack any lamellae. Gill comprised by 4 unipinnate branches arranged in an arch around anus; their shape and length similar to those of papillae. Two anterior branches share same stalk. Foot long and slender. A thick muscle ring surrounding mouth. Two oral tentacles relatively short at anterior part, on both sides of mouth. Reproductive opening on right side of body.

Colour pattern (Fig. 1). Species showing intraspecific colour variation. Some specimens bright yellow with ends of rhinophores, gill branches and tail coloured in orange (Fig. 1A). Some specimens white but with same ends coloured in yellow (Fig. 1B). There are also specimens with yellowish transparent ground colouration that may or may not have tips of these structures orange or yellow coloured (Fig. 1C–D). In all cases specimens entirely covered by bright spicules.

Foregut anatomy (Figs. 2A, 3). Buccal bulb (Fig. 2A) thick and muscular. Buccal pump large expanding dorsally and posteriorly. Radular sac short descending ventrally. Thin oesophagus inserts into buccal bulb behind buccal pump. Nervous system surrounding this union. Rounded salivary gland present on either side of buccal bulb at point where oesophagus enters mass. Labial cuticle surrounding lips and expanding inside buccal pump; with signs of individual jaw elements (Fig. 3A–B). Radular formula of all dissected specimens 19× 1.1.0.1.1 (Fig. 3C). Inner lateral teeth have a pointed

cusp with a masticatory margin bearing between 20 and 30 denticles (Fig. 3D–E). Denticles at either end of row shorter. Outer teeth small, with broad quadrangular base and large hook cusp (Fig. 3F).

Reproductive system (Fig. 2B). Located at anterior third of body. Thin and elongate hermaphroditic duct begins at ovotestis, inside digestive-hermaphrodite gland. It expands into a small oval ampulla. A short postampullar duct runs inside female gland mass and splits into a thin short oviduct and large and tubular prostatic portion of vas deferens. Distal end of prostatic part narrows into relatively short and thin ejaculatory duct that terminates in penis lacking penial spines. Vagina shorter than vas deferens but similar in width. It connects to a large spherical bursa copulatrix. A short duct emerges from vagina before entering bursa copulatrix and leads to a large and elongate seminal receptacle. Bursa copulatrix and seminal receptacle more or less similar in size. Uterine duct runs from base of seminal receptacle and enters female gland mass.

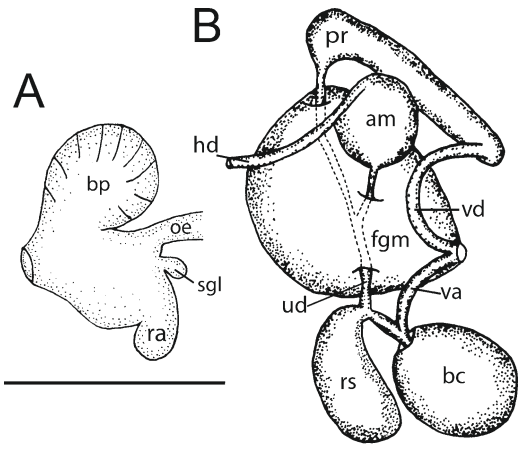

Fig. 2. *Okenia picoensis* spec. nov. **A.** Buccal bulb. **B.** Reproductive system. Abbreviations: **am**, ampulla; **bc**, bursa copulatrix; **bp**, buccal pump; **fgm**, female gland mass; **hd**, hermaphroditic duct; **oe**, oesophagus; **pr**, prostate; **ra**, radular sac; **rs**, seminal receptacle; **sgl**, salivary gland; **ud**, uterine duct; **va**, vagina; **vd**, vas deferens. Scale bars: 0,5 mm.

Molecular results

Amplifications were not successful for some genes (Table 1). Therefore, we only aligned the COI sequences of 658 bp. The phylogenetic tree based on COI sequences including all *Okenia* available at the moment is shown in Figure 4. It shows that the monophyly of the genus (IB = 1, ML = 97, MP = 100) is highly supported. It also confirms that all colour patterns found in *Okenia picoensis* spec. nov. are intraspecific variation (IB = 1, ML = 97, MP = 100). Nevertheless, the relationship between the included species within this genus is not resolved. Three clades of sister species were well supported, clustering *Okenia rosacea* (MacFarland, 1905) and *Okenia hiroi* (IB = 1, ML = 96, MP = 75), *Okenia amoenula* (Bergh, 1907) and *Okenia aspersa* (IB = 0.99, ML = 83, MP = 75) and *Okenia felis* Gosliner, 2010 and *Okenia picoensis* spec. nov. (IB = 1, ML = 94, MP = 51). The COI genetic distance between *O. felis* and *Okenia picoensis* spec. nov. is 16.8 %. Table 2 depicts the minimum COI gene pairwise uncorrected *p*-distances amongst sister species of *Okenia* in Figure 4.

Discussion

Okenia picoensis spec. nov. is the first *Okenia* species recorded from Azores Archipelago (Portugal). Morphologically, there are not many others *Okenia* species with similar features to *Okenia picoensis* spec. nov. in the Atlantic Ocean (see Table 2 in Valdés & Ortea 1995). Regarding its colouration and external morphology, only two Atlantic taxa resemble *Okenia picoensis* spec. nov.. Valdés et al. (2006) shows a picture labelled as *Okenia* sp. 1 (page 126) taken by Jeff Hamann in Flamingo Bay, Virgin Island (Bahamas). From the picture, *Okenia* sp. 1 shares the same number and distribution of lateral and middorsal papillae. However, most of the specimens in Hamann's collection dried out and the specimen of *Okenia* sp. 1 pictured in Valdés et al. (2006) is not available for study (Ángel Valdés personal communication). Another *Okenia* species from the Atlantic Ocean worth to compare with is *Okenia miramarae* Ortea and Espinosa, 2000, which was described based on two specimens collected in Miramar, La Habana (Cuba). The reproductive system was not described for this species. We tried to examine the type material of this species deposited in "Instituto

Table 2. Minimum COI gene pairwise uncorrected *p*-distances amongst sister species of *Okenia* in Figure 4.

Species	COI genetic distances (%)
Between specimens of *Okenia picoensis* spec. nov.	0–1.5
Okenia rosacea vs. *Okenia hiroi*	12.6
Okenia amoenula vs. *Okenia aspersa*	12.6
Okenia felis vs. *Okenia picoensis* spec. nov.	16.8
Okenia sp. A vs. *Okenia picoensis* spec. nov.	17

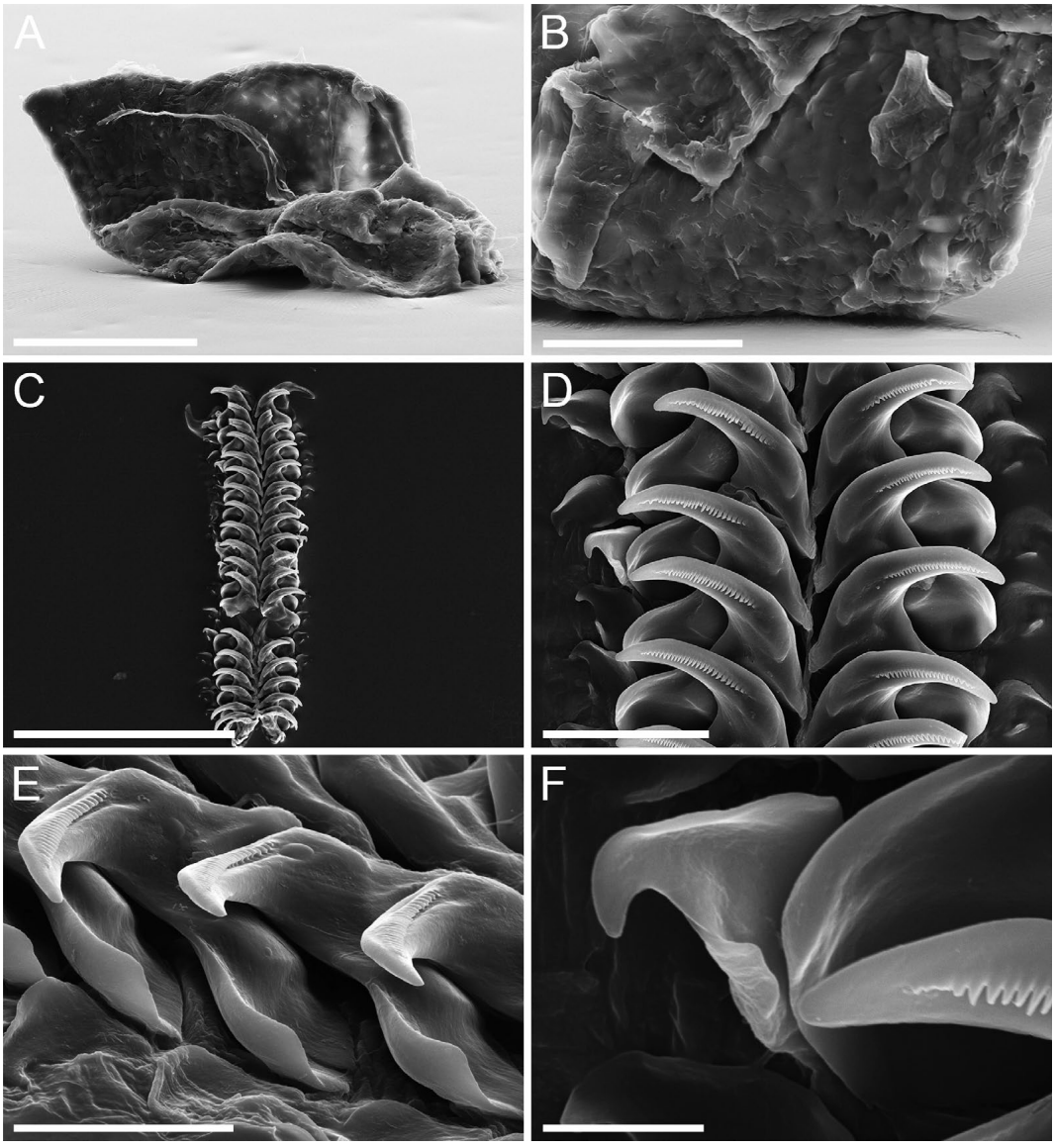

Fig. 3. *Okenia picoensis* spec. nov. Scanning electron micrographs (Holotype, MB28-004386). **A.** Labial cuticle. **B.** Detail of the cuticle. **C–D.** Radula. **E.** Outer laterals. **F.** Inner laterals. Scale bars: A, 100 µm; B, 30 µm; C, 300 µm; D, 50 µm; E, 10 µm; F, 30 µm.

de Oceanología" (La Habana) for comparison with *Okenia picoensis* spec. nov., but we did not get any response for months from that institution. Thus, since we found enough anatomical differences between both species, we compare them using the available information in the original description (Ortea & Espinosa 2000) (Table 3). First at all, *Okenia miramarae* was described as having a snow-white background colour with the tips of the lamellae, rhinophores and gill coloured in light orange. In addition, *O. miramarae* presents a purplish pink stain behind the gill. In our material, we can find specimens having bright yellow background with the tips of the rhinophores, the gill branches and the tail coloured in orange or snow-white specimens with the same ends coloured in yellow but not the pattern described

for *O. miramarae*. Moreover, none of our specimens show the purplish pink stain behind the gill. The most distinctive feature of *Okenia miramarae* is the extraordinarily large lateral papillae on each side of the gill. The original description of this species states that these latero-gill appendages exceed the length of the tail when the animal moves. Although these papillae are long in our samples they are never as long as described for *O. miramarae*. Other differences are that the rhinophores of *O. picoensis* spec. nov. have lamellae and not tubercles in the upper half as do has *O. miramarae*. Also, all the specimens of *O. picoensis* have 19 rows of radular teeth whereas there are 25 rows in *O. miramarae.* This difference in number is important since the number of teeth seems to be constant in *Okenia* species (Pola et al. 2014; Pola 2015) and Ortea & Espinosa (2000) used the number of rows of teeth to help distinguish between *O. impexa* and *O. cupella*. *Okenia impexa* Marcus, 1957 and *Okenia cupella* (Voger & Schultz, 1970) are considered to be amphi-atlantic species (Valdés & Ortea 1995) but no recent studies have been carried out to prove this assumption. Species occurring on both eastern and western coastlines have been widely accepted and regarded as moderately common in opisthobranchs (Carmona et al 2011). García & Bertsch (2009) recognized 134 species occurring on both sides of the Atlantic Ocean, approximately 12.6 % of the total opistobranch diversity in this realm. However, modern molecular tools are proving that many of them are not amphi-Atlantic species but examples of cryptic species complex (e. g., Carmona et al. 2011, 2014a,b,c; Padula et al. 2014).

Thus, until more specimens of *Okenia miramarae* from the type locality become available to study, we are strongly convinced that *Okenia picoensis* is a new species for the genus.

Recently, Moro et al. (2016) reported three specimens of *O. miramarae* from Taliarte, Gran Canaria (Canary Islands) based on two photographs of two specimens and the similarity of their external morphological features with those of the original description. However, the authors did not collect nor examine any of these specimens. The specimens depicted by Moro et al. (2016) (p. 21, pl. 7) are very similar to some specimens of *O. picoensis* spec. nov. (Fig. 1B). We suspect that the specimens found in the Canary Islands are likely *Okenia picoensis* spec. nov., but further anatomical and molecular studies of new specimens from Canary Islands are needed to clarify this matter.

Based on morphological similarity *Okenia picoensis* spec. nov. can also be compared with *Okenia japonica* Baba, 1949, known from Japan, Hong Kong and Ryukyu Islands (Gosliner 2004), *Okenia felis* Gosliner, 2010, known from California (Gosliner 2010) and *Okenia cochimi* Gosliner & Bertsch, 2004, known from Mexico (Baja California Bay) (Gosliner & Bertsch 2004). All of them present a mid-dorsal papilla in front of the gill (Gosliner 2004, 2010, Gosliner & Bertsch 2004). This papilla in *O. felis* and *O. picoensis* spec. nov. arises from a mid-dorsal edge (Gosliner 2010). They also share the distribution of their lateral papillae but not the number of them. *Okenia picoensis* spec. nov. and *O. cochimi* have five lateral papillae (Gosliner & Bertsch 2004) while

Table 3. Comparative table between *Okenia miramarae* Ortea & Espinosa, 2000 from the original description and *Okenia picoensis* spec. nov., present study.

Species	*Okenia miramarae* Ortea & Espinosa, 2000	*Okenia picoensis* spec. nov
Coloration	Snow-white background colour with the tips of the lamellae, rhinophores and gill coloured in light orange. Presents a purplish pink stain behind the gill.	Bright yellow background with the ends of the rhinophores, the gill branches and the tail coloured in orange or snow-white specimens with the same ends coloured in yellow
Lateral papillae	5–6	5
Dorsal papillae	1	1
Latero-gill appendages	Extraordinarily large (exceed the length of the tail when the animal moves)	Elongate but not exceeding the length of the tail when the animal moves
Number of lamellae	3 lamellae cup-shaped in the lower half and 5 tubercles in the upper half	7–9 lamellae
Gill branches	4 unipinnate	4 unipinnate
Radular formula	25 × 1.1.0.1.1 (1 specimen)	19 × 1.1.0.1.1 (6 specimens)
Seminal receptacle	Not described	Kidney-shape
Ampulla	Not described	Rounded
Distribution	La Habana, Cuba	Pico Island (Azores, Portugal)

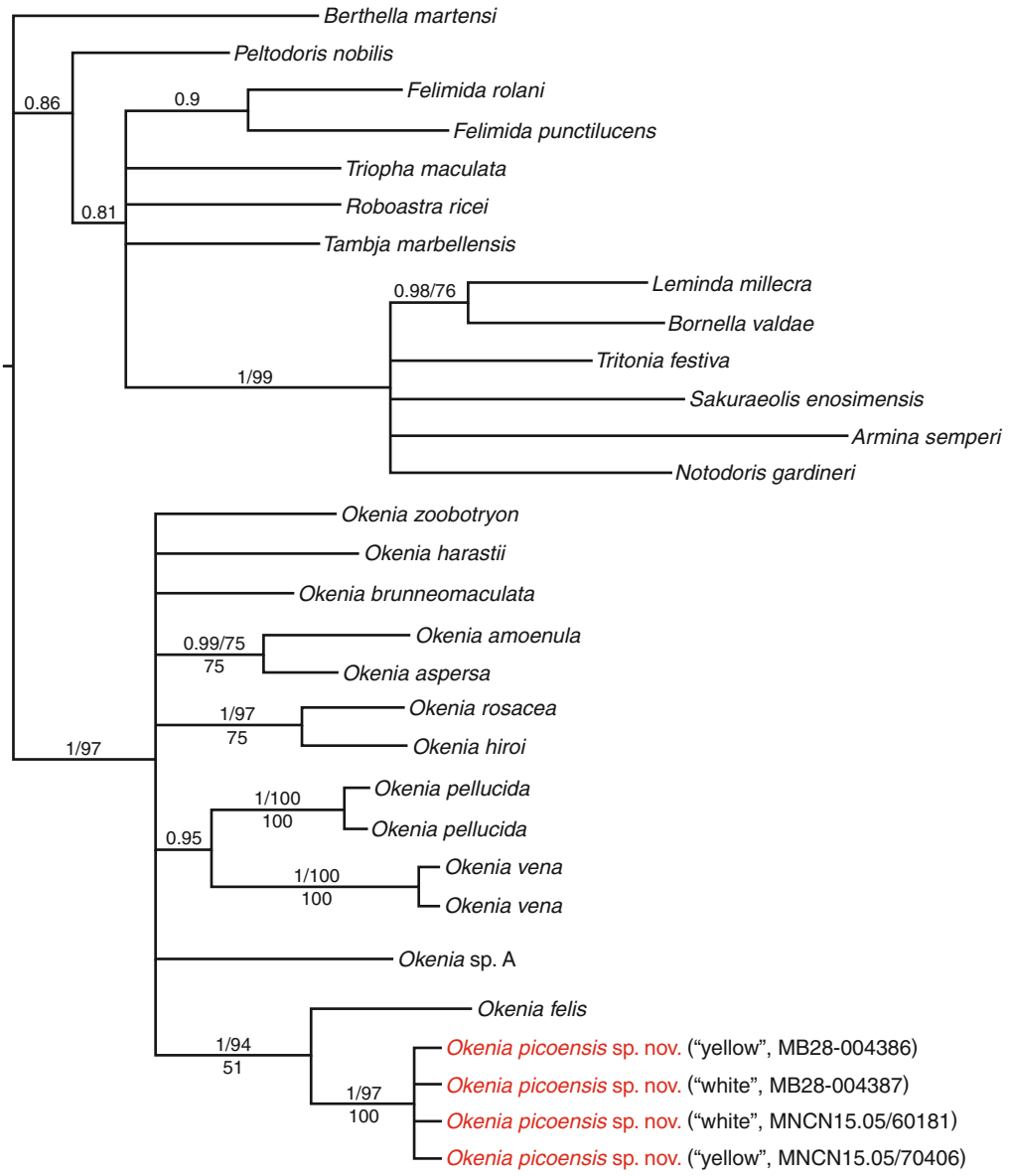

Fig. 4. Phylogenetic hypothesis based on COI. Numbers above branches represent posterior probabilities from BI and bootstrap values for ML. Numbers below branches show values for MP.

O. felis present six (Gosliner 2010) and *O. japonica* six or seven (Gosliner 2004). The most remarkable difference in their external anatomy is the number of lamellae in their rhinophores, being 21–23 in *O. felis*, 7–9 in *O. picoensis* spec. nov., 7 in *O. japonica* and 5–6 in *O. cochimi*. *O. japonica* and *O. felis* show bright white colours (Gosliner 2004, 2010), while *O. cochimi* has yellow body colouration (Gosliner & Bertsch 2004). However, they show external differences: the white morphotype presents yellow colouration in the top of its appendices whereas *O. japonica* and *O. felis* lack this character. Nevertheless, the more remarkable differences are found in the reproductive system and their distribution. There are differences in the shape of the seminal receptacle, being kidney-shape in *O. picoensis* spec. nov., while in *O. japonica*

and *O. cochimi* it is pyriform (Gosliner 2004, Gosliner & Bertsch 2004), and club-shaped in *O. felis* (Gosliner 2010). Also, the shape of the ampulla varies. It is rounded in *O. picoensis* spec. nov., globose in *O. felis* (Gosliner 2010) and ovoid in *O. japonica* and *O. cochimi* (Gosliner 2004, Gosliner & Bertsch 2004). *Okenia hispanica* Valdés & Ortea, 1995 is a species geographically closer that also presents some white and yellow colouration. The background of this species is hyaline white and the papillae are yellow with the apex being white. The notum and the anal area possess several pink patches (Valdés & Ortea 1995). Apart from this different colour pattern, *O. hispanica* does not present any dorsal papillae and the shape of the ampulla and the prostate differ from those of *O. picoensis* spec. nov.

Regarding the molecular results of this study, we are well aware that the relationships showed in Figure 4 might not be real since many *Okenia* species are still absent and may be a product of missing taxa. The sister relationship found between *Okenia brunneomaculata* Gosliner, 2004 and *Okenia pellucida* Burn, 1967 by Pola et al. (2014) is not recovered. The major goal of this phylogeny is to show that all colour patterns found in *Okenia picoensis* spec. nov. are intraspecific variation.

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