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DNA-barcoding of sympatric species of ectoparasitic gastropods of the genus *Cerithiopsis* (Mollusca: Gastropoda: Cerithiopsidae) from Croatia

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The ectoparasitic gastropod genus Cerithiopsis *Forbes & Hanley, 1850 was nominally based on* Murex tubercularis *Montagu, 1803. We have used the DNA barcode COI sequences to assay sympatric samples of morphotypes recently described as distinct species of the* Cerithiopsis tubercularis*-complex. Our results demonstrated that, in the Croatian waters, the gastropods usually called* C. tubercularis *in fact comprise a complex of cryptic species, which can be reliably diagnosed only by examining the soft parts. In the present study we have demonstrated that the colour pattern of the head-foot is diagnostic at the species level in this complex and, coupled with genetic data, may provide a sounding base for a revision of the cerithiopsids of the European coasts.*

Keywords: Mollusca, Cerithiopsidae, *Cerithiopsis*, DNA-barcoding, Mediterranean Sea, molecular taxonomy

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INTRODUCTION

Cerithiopsidae are a large family of marine gastropods, probably including hundreds of species, living mostly from the intertidal zone down to about 200 m, nearly invariably associated with, and feeding on sponges (see Marshall 1978, for a summary of feeding records).

The alpha-taxonomy of the Cerithiopsidae (here conservatively including also the bathyal and abyssal *Cerithiella*, *Eumetula* and *Laiocochlis* (Gofas & Le Renard, 2012)), still has to be solved all over the world, but it is currently accepted that many different species occur on European coasts, the exact number still being uncertain; 40 species listed by the *European Register of Marine Species* (Costello *et al.*, 2008); 49 species listed by *WoRMS* (Appeltans *et al.*, 2012); 48 species listed by *CLEMAM* (Gofas & Le Renard, 2008). The type genus of the family, *Cerithiopsis* Forbes & Hanley, 1850, comprises a group of small or very small species (3 to 15 mm long) usually less than 10 mm long. The slender, pupoid to sub-cylindrical, highly spired shell has a reticulated sculpture of axial and spiral ribs with tuberculated or beaded intersections, a small aperture and short siphonal canal, and has no varices or umbilicus (Figure 2). The genus *Cerithiopsis* was nominally based on *Murex tubercularis* Montagu, 1803. Recently, Prkić & Mariottini (2010) have shown that in the Croatian waters the gastropods usually called *Cerithiopsis tubercularis* in fact comprise a complex of cryptic species, which can be easily diagnosed only by examining the coloration of the soft parts. However, doubts on the actual status of these morphotypes as distinct species have been raised (Cecalupo & Robba, 2010; Scuderi & Criscione, 2011). Therefore, we have specifically tested this hypothesis, and in the present paper we provide the genetic basis (COI partial sequences) for the existence of three distinct sympatric species of this complex, corresponding to the species morphologically identified by Prkić & Mariottini (2010), and discuss the implications of the resulting taxonomic pattern for the systematics and nomenclature of the genus *Cerithiopsis*.

Although the types of *Murex tubercularis* Montagu, 1803 are not conspecific with any of the species in this complex (and the nomenclatural issue is under examination by the International Commission on Zoological Nomenclature (ICZN): see Cecalupo & Robba, 2011, and Prkić *et al.*, 2012, for a recent review on this subject), we will conservatively use hereafter the binomen '*Cerithiopsis tubercularis*' in the sense adopted by nearly all authors in the past 160 years.

MATERIALS AND METHODS

A total of over 4800 live specimens were collected along the Croatian coast and observed alive before fixing and preserving them in EtOH. Samples used for DNA extraction have been collected at three Croatian localities: Vinišće, Split—loc. Bene and Split—loc. Treća Voda (Figure 1). The two sampling sites at Split (Bene and Treća Voda) are less than one kilometre apart, and samples can be thus considered as sympatric. Specimens were collected by handpicking them from

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Fig. 1. Location of the sampling sites (Bene, Split, 43°30′50″N 016°24′02″E; Treća Voda, Split, 43°30′53″N 016°24′40″E; Vinišće, 43°29′20″N 016°06′44″E).

unidentified sponges under stones in the intertidal, or by washing the sponge/algal coverage of stones at low depth (1–8 m).

Two species were readily identified by shell morphology: *C. minima* (Brusina, 1865) (Figure 2P–T), and *C. ladae* Prkić & Buzzurro, 2007 (Figure 2U–Y). Species of the '*Cerithiopsis tubercularis*'-complex were classified by the colour pattern of the head-foot, according to Prkić & Mariottini (2010). Lots of voucher specimens have been given an ID BAU (Department of Biology and Biotechnologies, 'La Sapienza' University) and two vouchers of each lot have been deposited at MNHN (Muséum National d'Histoire Naturelle, Paris) (Table 1). Specimens for future anatomical studies have been preserved in 75% EtOH, while specimens for DNA extraction have been preserved in pure EtOH.

Due to their very small size, the shell of specimens for DNA extraction was cracked to extract the animal. Total genomic DNA was extracted using a standard proteinase K phenol–chloroform method with ethanol precipitation as reported in Oliverio & Mariottini (2001). The DNA-barcode fragments of the mitochondrial cytochrome oxidase I (COI) was amplified by polymerase chain reaction (PCR) using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). PCR conditions: 30 amplification cycles (30 seconds at 94°C, 30 seconds at 60°C, 1 minute and 30 seconds at 72°C). PCR products were purified using Exosap-IT (USB Corporation) and sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were readily aligned by hand. Base composition of nucleotide sequences was analysed with MEGA 5.0 (Tamura *et al.*, 2011). Nucleotide homogeneity was tested with the χ^2 statistics implemented in PAUP* (v. 4.0b10, Swofford, 2002). Genetic variation (nucleotide diversity, haplotype diversity and nucleotide differences) was calculated using DnaSP 5.10 (Librado & Rozas, 2009).

Phylogenetic relationships among the sequences were inferred by neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) methods, using the Kimura-2-parameters (K2p) nucleotide substitution model, by the softwares MEGA 5.0 (Tamura *et al.*, 2007, 2011), Treefinder (v. October 2008, Jobb *et al.*, 2004; Jobb, 2008) and MrBayes (four chains run twice in parallel for 10^7 generations, and trees sampled every 1000 generations: v. 3.1.2, Ronquist & Huelsenbeck, 2003).

RESULTS

Among the ~3000 specimens of *C. minima* (2270 specimens) and *C. ladae* (709 specimens), a diagnostic feature was observed in the head-foot: the opercular lobe and small dots behind the eyes are yellow in *C. minima*, while in *C. ladae* they are white. Among the over 1800 specimens of the '*Cerithiopsis tubercularis*'-complex collected alive and screened for taxonomic identification, the three distinct

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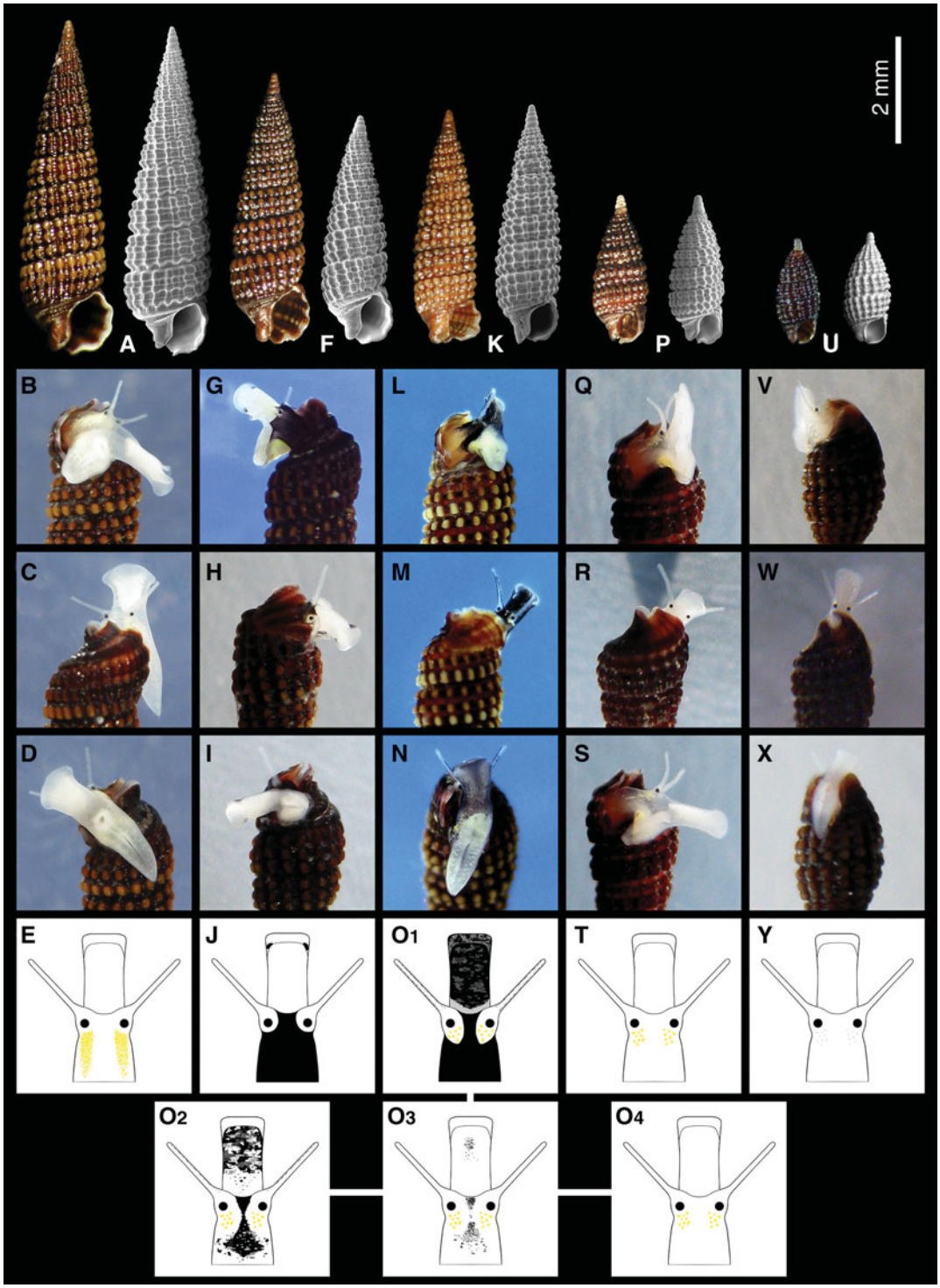

Fig. 2. Morphology of voucher specimens of the assayed species. (A–E) *Cerithiopsis petanii*; (F–J) *Cerithiopsis oculisfictis*; (K–O) *Cerithiopsis* sp.; (P–T) *Cerithiopsis minima*; (U–Y) *Cerithiopsis ladae*. Scale bar = 2 mm.

patterns already described by Prkić & Mariottini (2010) were present, with no intermediates. Specimens have been accordingly classified as either *C. petanii* Prkić & Mariottini, 2010 (723 specimens: Figure 2A–E), *Cerithiopsis oculisfictis* Prkić & Mariottini, 2010 (361: Figure 2F–J), or *C.* sp. (759 specimens: Figure 2K–O), the latter corresponding to '*C. tubercularis* auct. nec Marshall' of Prkić & Mariottini (2010). The number of specimens are not exactly proportional to the respective frequency in nature, very roughly indicating that *C. minima* is the most common species and *C. oculisfictis* the less frequent.

Partial sequences of the mtDNA encoding the COI were obtained from 10 specimens of *C. minima* and 2 of *C. ladae*, and from 21 specimens of the '*Cerithiopsis tubercularis*'-complex (morphologically ascribed to *C. oculisfictis*, *C. petanii* and *C.* sp.). *Cerithiopsis ladae* served as outgroup. The sequences have been deposited at the European Molecular Biology Laboratory (EMBL, accession numbers:

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Table 1. Voucher numbers, collection localities and European Molecular Biology Laboratory sequence accession numbers of the cerithiopsid dataset. Lot vouchers are stored at the Department of Biology and Biotechnology 'Charles Darwin', Rome, Italy (BAU) and two specimens of each lot have been deposited at the Muséum National d'Histoire Naturelle, Paris, France (MNHN).

Species	Voucher ID BAU	Voucher ID MNHN	Locality	Accession numbers
Cerithiopsis oculisfictis	1095	IM2009 22754-5	Bene, Split, 43°30′50″ N 016°24′02″E, 2–3 m depth, amidst seaweeds	HE862991–96
Cerithiopsis petanii	1092	IM2009 22756-7	Treća Voda, Split, 43°30′53″N 016°24′40″E, intertidal, under stones	HE862962–71
Cerithiopsis sp.	1094	IM2009 22758-9	Bene, Split, 43°30′50″N 016°24′02″E, intertidal, under stones	HE862982–90
Cerithiopsis minima	1093	IM2009 22760-1	Treća Voda, Split, 43°30′53″N 016°24′40″E, intertidal, under stones	HE862972–81
Cerithiopsis ladae	1090	IM2009 22762-3	Vinišće, 43°29′20″N 016°06′44″E, intertidal, under stones	HE862960–61

HE862960–HE862996), and at BOLDSYSTEM (www.boldsystem.org; project BCER). A total of 658 base pairs were unambiguously aligned, without gaps, with 200 variable position, resulting in 27 distinct haplotypes (total haplotype diversity, Hd: 0.9; total nucleotide diversity, π: 0.03849; see Table 2 for haplotype and nucleotide diversity in each species).

All three phylogenetic analyses (NJ, ML and BI) yielded the same topology (Figure 3). In Figure 3 the ML tree is reported with bootstrap (ML) and posterior probability (BI) supports at the nodes. The specimens of *C. minima* were the sister to a monophyletic '*Cerithiopsis tubercularis*'-complex. The specimens morphologically ascribed to either species in the complex all fell within their respective monophyletic clade. *Cerithiopsis oculisfictis* and *C.* sp. were always more closely related to each other than they were to *C. petanii*.

The intraspecific distance (K2p) ranged 0.009–0.004 in *C. petanii*, 0.025–0.004 in *C. oculisfictis*, 0.005–0.001 in *C.* sp., 0.015–0.003 in *C. minima*, and 0.000 in *C. ladae*, with an overall range of 0.000–0.025. The interspecific K2p distance ranged 0.186–0.215 (Table 3; Figure 3).

DISCUSSION

A complex of species

Although the marine malacofauna of the Mediterranean Sea is commonly considered as the best known in the world (Oliverio, 2003), new species are still being described at a remarkable pace; over 10 new species per year (see also Crocetta *et al.*, 2012). Nearly all these new taxa have been originally based on diagnostic morphological features, but in many cases, intra- and interspecific morphological variation overlap making genetic data essential for differentiation. Traditionally, shell characters are primarily employed for the taxonomy of gastropods, yet the identification of *Cerithiopsis* species by shell morphology alone can be very difficult or impossible because of their small size, frequent encrustations and damage, loss of the protoconch (larval apical whorls), the extreme variability within some species and, in other cases, the extreme similarity of shell features between different species. By contrast, the flesh colour of the living molluscs often proves to be of clear diagnostic value, similar to what is observed in the closely related Triphoridae (Bouchet, 1985, 1997). However, doubts have been raised on the validity of the head-foot chromatism in the taxonomy of these species by Scuderi & Criscione (2011) who—referring to the species identified by Prkić & Mariottini (2010)—reported the finding in Sicily of 'not only the same three forms described by these authors but also intermediate forms connecting to each other. We consider the presence of these intermediates as the evidence of the expression of an intraspecific variability in the colour pattern of the head-foot of *C. tubercularis*' (Scuderi & Crisione, 2011: 45). Our genetic data, unequivocally confirmed that the three morphotypes, identified by Prkić & Mariottini (2010) as *Cerithiopsis oculisfictis*, *C. petanii* and *C. tubercularis* (here called *Cerithiopsis* sp.), do actually represent distinct species that occur sympatrically in the Croatian waters (Prkić & Mariottini, 2010; J.P., unpublished observations). Since the presumed intermediate specimens have not been figured by Scuderi & Criscione (2011), and based on the evidence of our genetic data, we argue that either they observed specimens of *Cerithiopsis* sp. being misled by the high variability of its animal background colour, ranging from completely white to almost completely black (Figure 2L–O; Prkić & Mariottini, 2010), or a re-examination of their material may reveal the presence of more species within their samples. In fact, distinct head-foot patterns (without intermediates) have been observed throughout most of the range of the '*Cerithiopsis tubercularis* AA complex' in the Mediterranean Sea and in the Atlantic (our data, unpublished; Gofas (2011) and personal communication; Ian Smith at http://conchsoc.org/spaccount/Cerithiopsis-tubercularis). The present data show that the DNA-barcode sequences (COI) can be a very reliable tool for species identification when live collected specimens cannot be scrutinized in the field. The values of the estimated K2p genetic distance fall into two distinct groups, intra- and interspecific estimates, respectively, with

Table 2. Number of haplotypes, haplotype diversity (Hd, Nei, 1987), and nucleotide diversity (Pi, Nei, 1987) calculated within the species with more than 2 sequences.

Species	N	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Cerithiopsis oculisfictis	5	5	1	0.00511
Cerithiopsis petanii	4	4	1	0.03125
Cerithiopsis sp.	8	7	0.964	0.00508
Cerithiopsis minima	10	10	1	0.01548
Cerithiopsis ladae	2	1	0	0

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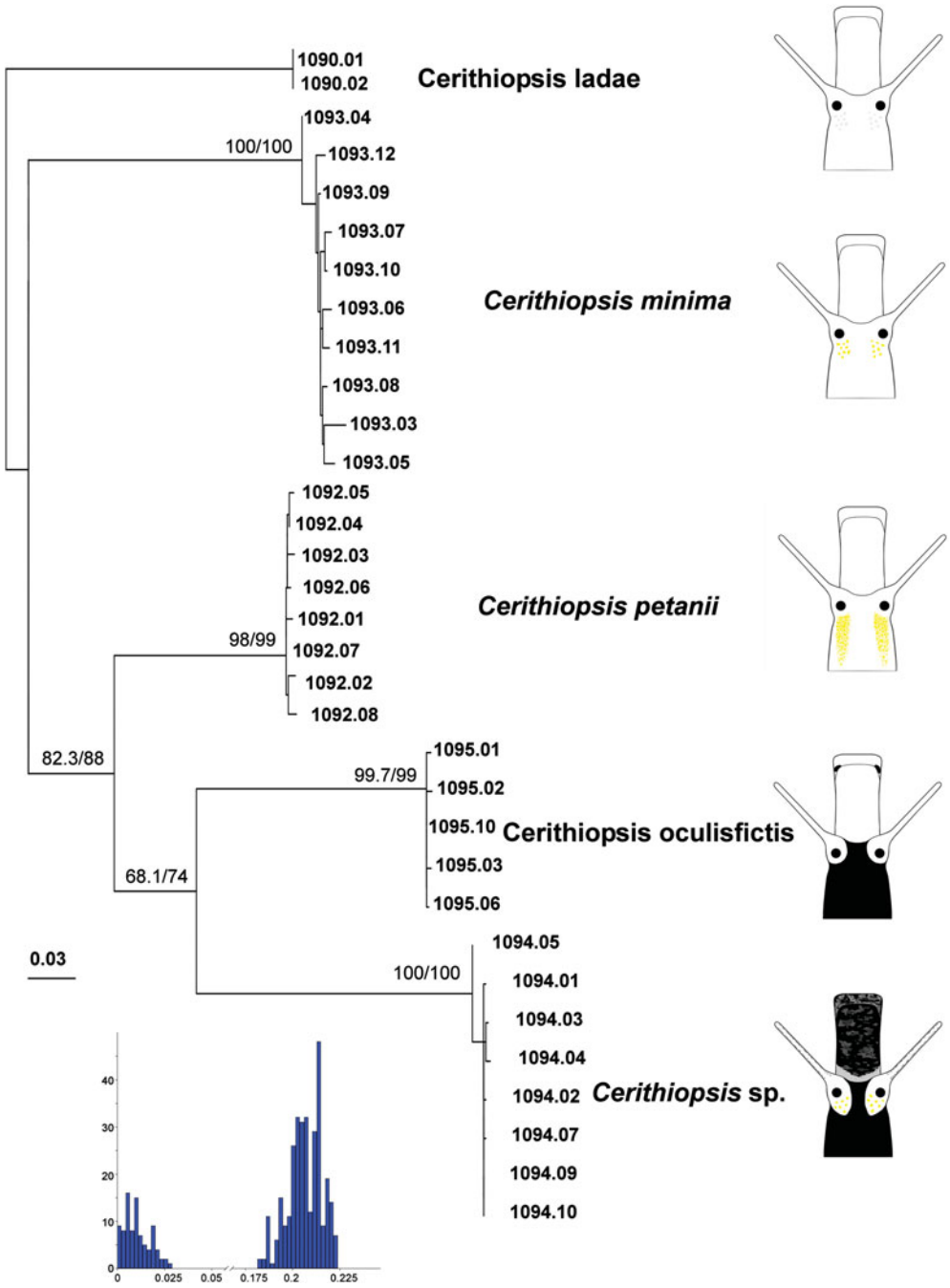

Fig. 3. Maximum likelihood (ML) tree of the cerithiopsid COI sequences. Scale is calibrated ML distance. The histogram shows the distribution of the pairwise estimated genetic distances (K2p) in intraspecific (left) and interspecific (right) comparisons.

Table 3. Genetic distances estimated using the Kimura 2 parameter substitution model. Inter- and intraspecific values are reported as ranges, with mean and standard deviation in parentheses.

Species	*C. oculisfictis*	*C. petanii*	*C.* sp.	*C. minima*	*C. ladae*
Cerithiopsis oculisfictis	0.002–0.008 (0.005–0.002)				
Cerithiopsis petanii	0.184–0.202 (0.191–0.005)	0.003–0.011 (0.008–0.003)			
Cerithiopsis sp.	0.190–0.208 (0.200–0.005)	0.201–0.215 (0.208–0.003)	0.000–0.013 (0.005–0.003)		
Cerithiopsis minima	0.202–0.220 (0.211–0.005)	0.194–0.210 (0.202–0.004)	0.202–0.224 (0.215–0.005)	0.006–0.028 (0.016–0.005)	
Cerithiopsis ladae	0.201–0.207 (0.203–0.002)	0.183–0.192 (0.187–0.003)	0.212–0.216 (0.213–0.001)	0.194–0.207 (0.200–0.004)	0.000

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the intraspecific values always smaller than the usual barcoding gap, i.e. <3% divergence.

The status of *Murex tubercularis* Montagu, 1803

Montagu's (1803) description of *Murex tubercularis* fits at least 10 recent European cerithiopsid species. Marshall (1978: figure 13C) selected a lectotype among two (very likely conspecific) specimens, which have been recently re-figured by Cecalupo & Robba (2010: figure 1C–F). However, these two shells have little to do with what has nearly invariably been called *Cerithiopsis tubercularis* in the past one and half centuries, being two specimens of *Cerithiopsis barleei* Jeffreys, 1867, a fact noticed by Marshall himself, and accepted—at least for the lectotype—by the recent specialists of the European malacofauna (e.g. van Aartsen *et al.*, 1984). The modern (erroneous) concept of *Cerithiopsis tubercularis* arose when Forbes & Hanley (1850: pls 00, 91, 92) introduced the genus *Cerithiopsis*, nominally for *Murex tubercularis* Montagu, but in fact not for the species indicated by the types. This concept of *C. tubercularis* has been followed nearly invariably by all subsequent authors, including Jeffreys (1867) who, describing his *Cerithiopsis barleei*, compared it with *C. tubercularis* (Montagu, 1803) (*sensu* Forbes & Hanley (1850–1851)), and obviously found them to be different.

Cecalupo & Robba (2011) have applied to the ICZN (under Article 75.6 of the Code), with the aim to conserve the current usage of the name *Cerithiopsis tubercularis* (Montagu, 1803), and the Commission has been asked to set aside the previous type fixation (Marshall, 1978) for *Murex tubercularis* Montagu, 1803 and to designate as neotype a 'probable syntype BMNH 20090384' (consistent with the current usage).

A serious problem is raised by the fact that the 'current usage' of *Cerithiopsis tubercularis* (Montagu, 1803), actually comprised a complex of cryptic species, impossible to identify by examining only the shell characters. Forbes & Hanley (1851: 364), while describing the type species of the genus *Cerithiopsis* ('*Cerithiopsis tubercularis*') may have mixed more than one species, as can be deduced from their notes on the coloration of the head-foot. This is the reason why we (Prkić *et al.*, 2012) have not supported Cecalupo & Robba's application, and have recommended that before the Commission takes any decision, the intricate puzzle of the species complex of *C. tubercularis sensu* Auct., must be solved by the study of live collected specimens, with types designed on morphologically and genetically characterized specimens. In the present study we have demonstrated that the colour pattern of the head-foot is diagnostic at the species level in this complex and, coupled with genetic data (the DNA-barcode COI seems to work very well in this regard), may provide a sound basis for a revision of the cerithiopsids of the European coasts.

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