

The genus *Acanthochitona* (Mollusca: Polyplacophora) in the Mediterranean Sea: morphological and molecular data

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SUMMARY: This work represents an attempt to resolve the confused and contradictory taxonomy of Mediterranean chitons of the genus *Acanthochitona* by analysing morphological (SEM observations of aesthetes, radula and girdle) and molecular data (COI, 12S, ITS1). Both analyses support the validity of the three species, *Acanthochitona fascicularis*, *A. crinita* and *A. oblonga*, the latter two of which were previously considered as synonymous.

Keywords: SEM morphology, molecular systematics, Mediterranean Sea, *Acanthochitona*, Polyplacophora.

RESUMEN: EL GÉNERO *ACANTHOCHITONA* (MOLLUSCA: POLYPLACOPHORA) EN EL MAR MEDITERRÁNEO: DATOS MORFOLÓGICOS Y MOLECULARES. – En el presente trabajo se pretende resolver la confusa taxonomía de las especies mediterráneas de los quitones del género *Acanthochitona* a través de su estudio morfológico (observaciones al SEM de aestetes, rádula y cintura) y molecular (COI, 12S, ITS1). En ambos casos se confirma la validez de las tres especies *Acanthochitona fascicularis*, *A. crinita* y *A. oblonga*, las dos últimas consideradas previamente como sinónimas.

Palabras clave: SEM morfología, sistemática molecular, mar Mediterráneo, *Acanthochitona*, Polyplacophora.

INTRODUCTION

Acanthochitona species are characterized by extreme variability of morphological features commonly used in their systematics. Kaas (1985), in his review of the Polyplacophora of Mediterranean and Atlantic coasts, recognizes three living species: *Acanthochitona fascicularis* (Linné, 1767) and *A. crinita* (Pennant, 1777) from the Mediterranean Sea, and *A. discrepans* (Brown, 1827) from the northeastern Irish coast and southern British coast. As regards the Mediterranean Sea, Dell'Angelo and Smriglio (1999) more recently agreed with the view of Kaas. An additional taxon, *Acanthochitona oblonga* (Leloup, 1981), has a strong morphological relation to *A. crinita* with such different marginal issues that Kaas (1985) and

Dell'Angelo and Smriglio (1999) considered them as conspecific; however, this issue remains to be demonstrated convincingly. The aim of this work is to try to resolve the confused systematics position of *A. oblonga*, compared to the other two Mediterranean species of *Acanthochitona*, by analyzing morphological and molecular features.

MATERIALS AND METHODS

The morphological analysis was performed with a scanning electron microscope Jeol jsm 5200 on some traditional systematic characters such as the morphology of granules of tegmentum, radulae and elements of the girdle. Hundreds of specimens from several European and African localities were studied.

Most molecular work was carried out at the molecular laboratory at IRSNB, Brussels, Belgium. Total genomic DNA was isolated from a small piece of tissue taken from the foot of ethanol-preserved specimens. The extractions were carried out using the “Genomic DNA from tissue” kit (Macherey-Nagel) and the Chelex 100 procedure according to Nelson and Savannah River Ecology Lab protocol. When these two methods failed, or in all cases of a low yield of DNA extract, the CTAB method was used instead (Winnepenninckx *et al.*, 1993). All the DNA extractions were kept at 4°C for short-time use. Undiluted or different dilutions (from 1:10 to 1:50, based on the DNA concentration) of each DNA extraction were used as templates for PCR amplification of a portion of each of the three loci: the mitochondrial small subunit ribosomal DNA (mt-12S) and the cytochrome oxidase subunit 1 (mt-COI) genes, and the nuclear Internal Transcribed Spacer 1 (nu-ITS1) region. For the COI gene the primers used were LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer, 1994), which amplified a region of 658 bp; the PCR conditions involved an initial denaturation step at 95°C for 5 minutes; then 35 cycles of denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds and extension at 72°C for 1 minute and 30 seconds; followed by a final extension step at 72°C for 5 minutes. For the 12S gene the primers used were 12SaL (5'-AAACTGGGATTAGATACCCCATAT-3') and 12SaH (5'-GAGGGTGACGGGCGGTGTGT-3') (Kocher, 1989), which amplified a region of 362 bp; the PCR conditions involved an initial denaturation step at 95°C for 5 minutes; then 40 cycles of denaturation at 95°C for 45 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 5 minutes. For the ITS1 region the primers used were ITS1L (5'-TCCGTAGGTGAACCTGCGGAAGGAT-3') and 58C (5'-TGCGTTCAAGATATCGATGTTCAA-3') (Hillis and Dixon, 1991), which amplified a fragment of 641 bp; the PCR conditions involved an initial denaturation step at 95°C for 5 minutes; then 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes; followed by a final extension step at 72°C for 5 minutes. PCR reactions were performed in a total volume of 25 µl including 2.5 µl of 2 mM of each dNTP (GE Healthcare), 2.5 µl of 10x load buffer-MgCl₂ (Qiagen), 2.5 µl of 2 µM of each primer, 0.25 µl of 5 U/µl Taq DNA polymerase (Qiagen), 19 µl of demineralized water and 1 µl of the DNA template. Amplified products were purified using the GFX PCR DNA and GEL Band Purification Kit (GE Healthcare), or the NucleoFast PCR plates (Macherey-Nagel), following the protocol provided for the purification of PCR products under vacuum. Cycle sequencing was carried out in both directions of the amplified regions using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and Hitachi 3130x Genetic Analyser (Applied Biosystems).

TABLE 1. – GenBank accession numbers and sequence length values for each outgroup species used in the phylogenetic analysis.

Species	COI	12S	ITS1
<i>Crassostrea gigas</i>	AY397686 551 bp	EF484878 671 bp	AB041761 602 bp
<i>Lottia digitalis</i>	AB238464 661 bp	AB238241 346 bp	DQ248942 558 bp
<i>Portunus pelagicus</i>	FJ812293 598 bp	FJ812311 374 bp	AM410550 688 bp

For each gene, the forward and reverse sequences obtained were imported and read in the program Chromas Lite version 2.01 (http://www.technelysium.com.au/chromas_lite.html), for visual inspection of chromatograms, and subsequently combined in the program Bioedit version 7.0.9.0 (Hall, T.A. 1999) in order to obtain consensus sequences to be used in subsequent analyses. All the sequences were identified by BLAST matching and assembled separately for each gene using Bioedit. The external primer regions and blank ends were subsequently trimmed from each of the three groups of sequences. Each group was converted in MEGA format using the program DnaSP version 5 (Librado *et al.*, 2009) and imported in the program MEGA version 4 (Tamura *et al.*, 2007).

Three species were selected as outgroup taxa for the molecular analysis: two molluscs, the bivalve *Crassostrea gigas* (Thunberg, 1793) and the gastropod *Lottia digitalis* (Rathke, 1833), and a more distant species, the crustacean *Portunus pelagicus* (Linnaeus, 1758). It was not possible to include other chitons or species belonging to the genus *Acanthochitona* due to the lack of data in GenBank; of the three genes considered, only COI sequences are published for chitons (Okusu *et al.*, 2003; Wilson *et al.*, 2010), but not for 12S and ITS1. Published sequences from GenBank for the three selected outgroups were downloaded and then added to each alignment (see Table 1 for their accession number and lengths). All the sequences for each gene were aligned with the multisequence alignment Clustal W software (Thompson *et al.*, 1994), as implemented in the program MEGA 4. The alignments were then refined by eye. Therefore, all the regions larger than 4 nucleotides which were ambiguously aligned with respect to the bordering regions were deleted while the gaps were included in the analysis and scored as missing characters. Pairwise evolutionary divergence between sequences calculated between *A. oblonga* and *A. crinita*, between *A. oblonga* and *A. fascicularis*, and between *A. crinita* and *A. fascicularis*, were calculated using the Kimura-2-parameters method (Kimura, 1980) in MEGA 4, with pairwise deletion of gaps and missing data, and uniform rates among sites. The final three alignments were concatenated in MEGA 4 in all the possible combinations in order to create different datasets to be used in the phylogenetic analysis. These combined data sets contain a smaller number of sequences with respect to the sequences of each single gene because specimens with no data for all three genes were excluded from the combined analyses.

For each data set the phylogenetic analysis was performed in the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using the Bayesian Inference method (Holder and Lewis, 2007 and Huelsenbeck and Ronquist, 2001). Each data set was examined assuming the most complex evolution model (GTR+I+ Γ) and using the Markov Chain Monte Carlo (Yang and Rannala, 1997; Larget and Simon, 1999) as the tree sampling procedure. The analyses were run for three million generations with every hundredth tree and parameter value stored. The plot of likelihood scores versus generations was generated to establish how many generations were necessary to reach stationarity. The likelihood values clearly plateaued after 20000 generations, that is, after 20000 generations the changes in the tree topology and parameter values did not continue to improve the tree likelihood scores. Therefore, the first 200 trees (from the first 20000 generations) were excluded from the analysis as burn-in and the remaining trees were used to make a 50% majority rule consensus and to estimate the Bayesian Posterior Probability (BPP) to give support to tree nodes. All the trees were drawn with the program Dendroscope version 1.4 (Huson *et al.*, 2007).

Abbreviations

MNHN Museum National d'Histoire Naturelle, Paris,
CT Catania province, Sicily
LE Lecce province, Apulia
ME Messina province, Sicily
MZB Museum of Zoology, University of Bologna
RSMNH Royal Scottish Museum of Natural History, Edinburgh
IRSNB Royal Belgian Institute of Natural Sciences
TA Taranto province, Apulia

RESULTS

Taxonomic remarks

Several papers deal with the systematics and morphology of the investigated species; here we summarize some data and the main references.

Acanthochitona fascicularis (Linné, 1767)

Chiton fascicularis Linné, 1767: 1106.

Acanthochitona fascicularis Kaas, 1985: 585, Figs. 1-6 (bibliography and synonymy). Dell'Angelo and Smriglio, 1999: 192, Figs. 113-123, pls 64-65 (bibliography and synonymy). Öztürk *et al.*, 2000: 72. Anistratenko V.V. and O.Y., 2001: 51, Fig. 17. Öztürk *et al.*, 2004: 52. Sigwart, 2005: 19. Peñas *et al.*, 2006: 34, Fig. 419. Trono, 2006: 75. Cecalupo *et al.*, 2008: 54, pl. 1, Figs. 10-12.

Type. The species is not preserved at the Linnean Society of London (fide Dodge, 1952). Neotype designated and figured by Kaas (1985: Fig. 1), MNHN.

Type locality. Algeria, Oran.

Material examined. Campomarino (TA): 5 specimens (used for the present study). Capo Molini (CT): 1 specimen (used for the present

study). Torre Ovo (TA): 6 specimens (used for the present study). Hundreds of specimens and valves from diverse European (Spain, France, Italy, Croatia, Greece, Turkey, Malta, Canary Islands), African (Morocco, Algeria, Tunisia) and eastern Mediterranean (Israel, Lebanon) localities (BDA, MZB).

Remarks. *Acanthochitona fascicularis* is a highly variable species with a puzzling synonymy; see Kaas (1985) and Dell'Angelo and Smriglio (1999) for a good description, synonymy and literature. Owing to the variability and convergence of the morphological characters of the *Acanthochitona* species it would be useful to refer to all specimens of *A. fascicularis* as being mainly characterized by:

tegumentum with dense, round, rather small granules arranged in arched lines; quadrangular apophyses anteriorly well developed; girdle densely covered with small and thin spicules and some others scattered which are longer and thicker, with 18 tufts of white bristles; and rather small, triangular apical area.

The outline and profile of intermediate and tail valves are very variable, and a representative range of this variability was nicely figured in Leloup (1941: Fig. 2).

Distribution. The Mediterranean Sea, the Black Sea, and the eastern Atlantic Ocean from the Channel and Britain to Azores and Canary Islands.

Acanthochitona crinita (Pennant, 1777)

Chiton crinitus Pennant, 1777: 71, pl. 36, Figs. 1, A1.

Acanthochitona crinita: Kaas, 1985: 588, Figs. 7-50 (bibliography and synonymy). Dell'Angelo and Smriglio, 1999: 198, Figs. 124-130, pls 66-68 (bibliography and synonymy). Öztürk *et al.*, 2000: 72. Öztürk *et al.*, 2004: 52. Sigwart, 2005: 19. Peñas *et al.*, 2006: 34. Trono, 2006: 75. Cecalupo *et al.*, 2008: 24.

Type. The three syntypes of *A. crinita* are no longer with the Pennant collection. Neotype designated and figured by Kaas (1985: Fig. 27), RSMNH no. reg. 1978.052.02601.

Type locality. Hebrides Islands.

Material examined. Villafranca Tirrena (ME): 10 specimens (used for the present study). Hundreds of specimens and valves from diverse European (Portugal, Spain, France, Italy, Croatia, Greece, Turkey, Malta, Canary Islands), African (Morocco, Algeria, Tunisia) and eastern Mediterranean (Israel, Lebanon) localities (BDA, MZB).

Remarks. *A. crinita* is also a highly variable species with a puzzling synonymy. See Kaas (1985) and Dell'Angelo and Smriglio (1999) for a good description, synonymy and literature. It differs from *A. fascicularis* mainly in the jugal area, which is generally wider, slightly elevated and not well separated from the lateropleural area, and the granules are less thickly arranged, more spaced, and shaped from oval to a more or less elongated drop.

Distribution. The Mediterranean Sea, a few records from the north African coast: e.g. Jerba Tunisia (Dell'Angelo and Cuppini, 1983; Cecalupo *et al.*, 2008), The North American Atlantic coast

and the eastern Atlantic along the European coast from Norway to Azores, Madeira, Canary and Cape Verde Islands (Dell'Angelo and Smriglio, 1999). As reported by Dell'Angelo and Smriglio (1999), *A. crinita* has been collected in the intertidal zone and as deep as 50 m, and there are records from a depth of 175 m deep. It lives under stones which are often partly buried in sand or pebbles. It may be associated with the barnacle zone or with several kinds of algae.

Acanthochitona oblonga (Leloup, 1981)

Acanthochiton oblongus Leloup, 1981: 1, Figs. 1a-d, pl. 1. Kaas, 1985: 596, Figs. 39-43.

Acanthochitona oblonga Dell'Angelo and Cuppini, 1983: 77. Gaglioli, 1985: xviii, pl. 1, Fig. 6; 1989: 9. Savona and Carnieri, 1985: 36, Figs. 1-3. Dell'Angelo and Smriglio, 1999: 200, Figs. 124-130, pl. 67, Figs. K-L; pl. 68, Figs. Q-V.

Acanthochitona crinita f. *oblonga* Baschieri, 1994: 40, Fig. 2. Baschieri *et al.*, 1992: 68, Fig. 4.

Type. Holotype at the Royal University of Malta.

Type locality. Malta, Salina Bay, "under rocks at 3 meters depth, July 1974".

Material examined: San Foca (LE), 11 specimens (used for the present study); San Foca (LE), 20 specimens, leg. B. Dell'Angelo 08/2004 (BDA); Roca Li Posti (LE), 8 specimens, leg. L. Baschieri 08/1989 (BDA); Campomarino (TA): 6 specimens, leg. B. Dell'Angelo 08/2007 (BDA); Marina di Lizzano (TA), 20 specimens, leg. B. Dell'Angelo 08/2007 (BDA); Malta, Gnejna Bay, 3 specimens, ex coll. C. Mifsud (BDA); Tunisia, Djerba, Menzel, 2 specimens, ex coll. P. Piani (BDA); and Tunisia, Djerba, Sidi Garus: 1 specimen, leg. A. Germanà 04/2007 (BDA).

Remarks. This species, described by Leloup based on 4 specimens collected at Salina Bay, Malta, is characterized by the very extended sharp granules of the tegmentum. After the original description, several specimens were reported from the Mediterranean Sea (Dell'Angelo and Cuppini, 1983; Baschieri, 1994), showing that this species is very common in some localities of the South Apulian Adriatic coast where specimens exhibit granules that vary from typical drop shaped to a shape that is even more extended than what was originally described by Leloup.

Distribution. Southern Mediterranean Sea in scattered Italian localities (Apulia, Sicily, Lampedusa Is.), Malta, Cyprus, Tunisia (Jerba) (Dell'Angelo and Cuppini, 1983; Dell'Angelo and Smriglio, 1999). It is worth noting that in Bruno Dell'Angelo's collection, there are specimens of both *A. crinita* and *A. oblonga* from Jerba; however, he did not collect them directly and the information on the label does not specify either the locality along the coast of the isle, or the habitat. The two species are surely sympatric and are clearly discriminated by the shape of granules in S. Foca (LE) where they were collected on a sandy bottom 2 - 3 m deep with sparse rocky fissured tables. Green algae grow in these fissures and the two *Acanthochitona* species live under them.

A third species of *Acanthochitona*, *A. discrepans* (Brown, 1827), lives along the northeastern Irish and southern English coasts but not in the Mediterranean Sea. This species has been studied by Kaas (1985) and differs from *A. crinita* mainly due to:

valves carinated (not carinated in *A. fascicularis* and *A. crinita*); "velvety" perinotum covered by short spicules 40 µm long; and short tufts of spicules sometimes with 1 to 3 extra tufts around the posterior valve.

Kaas (1985) writes that these extra tufts, which were considered typical of *Chiton gracilis* Jeffreys, 1859, are present only in a few specimens (mostly only one tuft) and "... on the other hand I possess a fine and in all respects normal specimen of *crinita* from Normandy, with one extra tuft".

Morphological analysis

In all *Acanthochitona* species the perinotum is characterized by rounded or drop shaped elevated granules; those of *A. fascicularis* (Fig. 1) are rounded, rather elevated, in some cases heart shaped, concave, with a characteristic incision on the anterior margin. The single central macroaesthete is generally surrounded by 1 to 5 microaesthetes, (sometimes 0) irregularly arranged.

In *A. crinita* the granules (Figs. 2A, B) are lower with respect to *A. fascicularis*, ovoid and drop-shaped, more or less elongated, flat or slightly concave. The single posteriorly located macroaesthete is surrounded by a different number of microaesthetes, ranging from 12 to over 16, irregularly grouped mainly in the antero-central part of the granule.

In *A. oblonga*, the granules (Figs. 3A, B) appear lower if compared to *A. crinita*, more elongated, narrow, lanceolate and slightly concave anteriorly. The single posteriorly located macroaesthete is surrounded by a lower number of microaesthetes, from 6 to 9, irregularly grouped mainly in the middle part of the granule.

Radulae

The symmetrical radulae (Figs. 4A, B, C) are quite similar with minor quantitative differences. The central tooth is squarish in outline, medially constricted with a strong median crista in each species and only in *A. oblonga* (Fig. 4C) appears very slightly bilobated at the apex. The centro-lateral tooth is smaller as usual, being very arched in *A. fascicularis* (Fig. 4A) and more or less straight in the other two species (Figs. 4B, C). Its tip is distinctly concave in *A. fascicularis* (Fig. 4A) and slightly concave in *A. crinita* (Fig. 4B) giving the impression of a truncated end, while it is convex and spatuliform in *A. oblonga* (Fig. 4C). The major lateral tooth has a large head with mesocone larger than the endo- and ectocone in *A. fascicularis* (Fig. 4A), while in both *A. crinita* (Fig. 4B) and *A. oblonga* (Fig. 4C) the endo- and mesocone are of similar size and the ectocone is smaller.

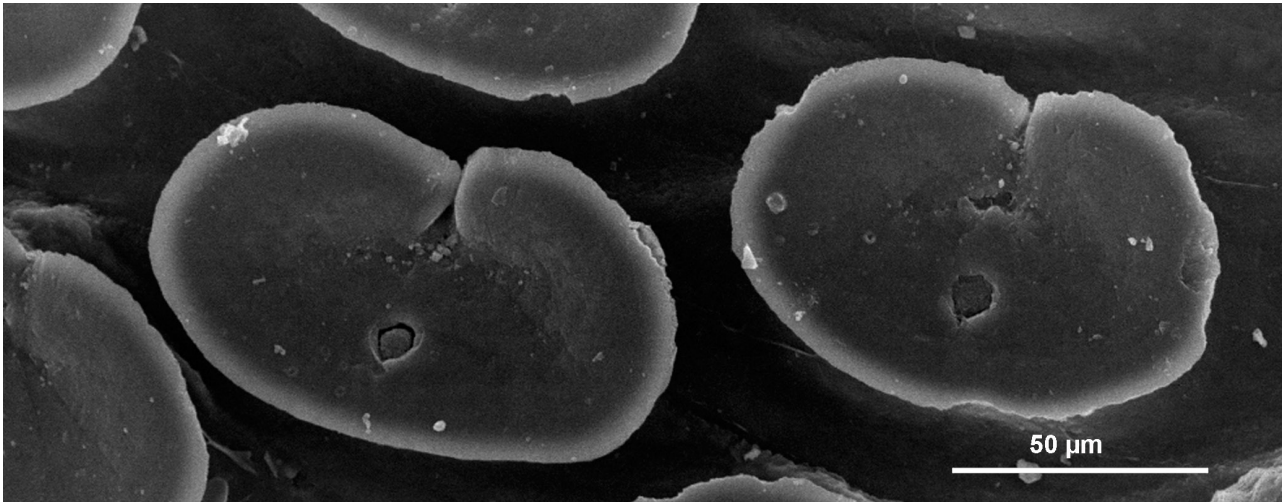


FIG. 1. – Morphology of the granules of tegmentum in *Acanthochitona fascicularis*; on the right side their proximal end. Specimen from Torre Ovo (TA).

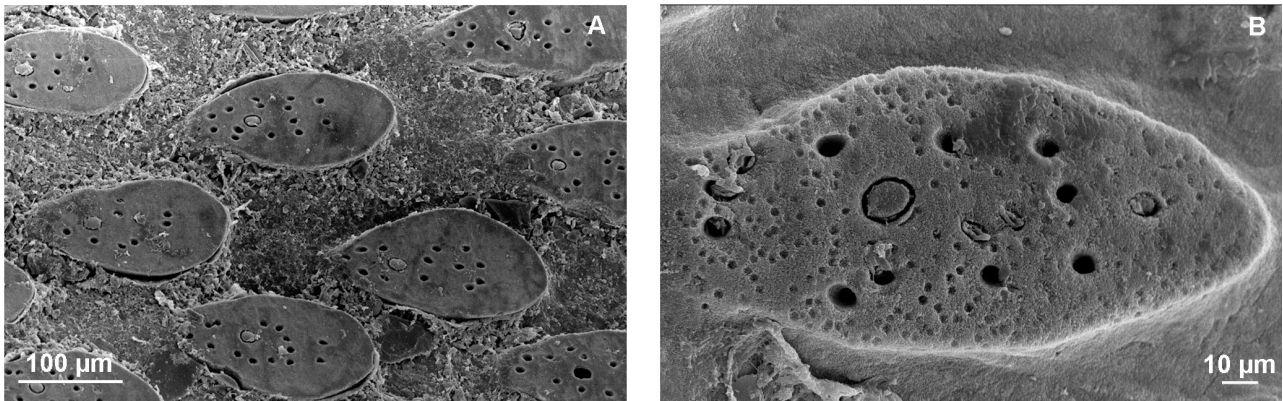


FIG. 2. – A, morphology of the granules of tegmentum in *Acanthochitona crinita*. B, close-up of a single granule of the same species. On the right side its proximal end. Specimens from Villafranca Tirrenia (ME).

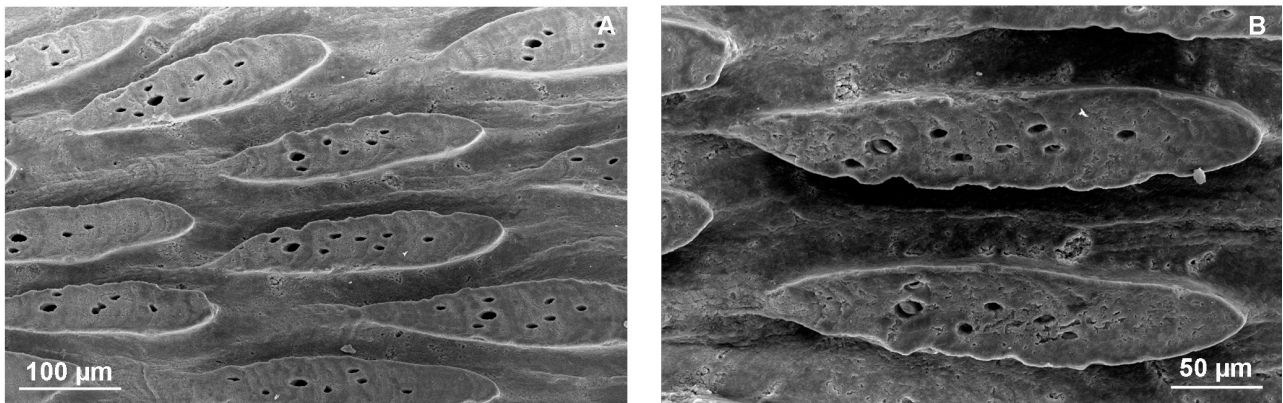


FIG. 3. – A, morphology of the granules of tegmentum in *Acanthochitona oblonga*. B, close-up of two granules in the same species; on the right side their proximal end. Specimens from San Foca (LE).

Perinotum

All species share a broad perinotum with 18 large tufts of usually colourless spines that vary in length from 500 to 1000 µm and more (Fig. 5). The dorsal

surface is covered by short more or less curved spicules among which other thicker and longer spicules are scattered. According to Dell'Angelo and Smriglio (1999) in *A. fascicularis* the short curved spicules are 100-150 µm in length, the others are up to 500 µm;

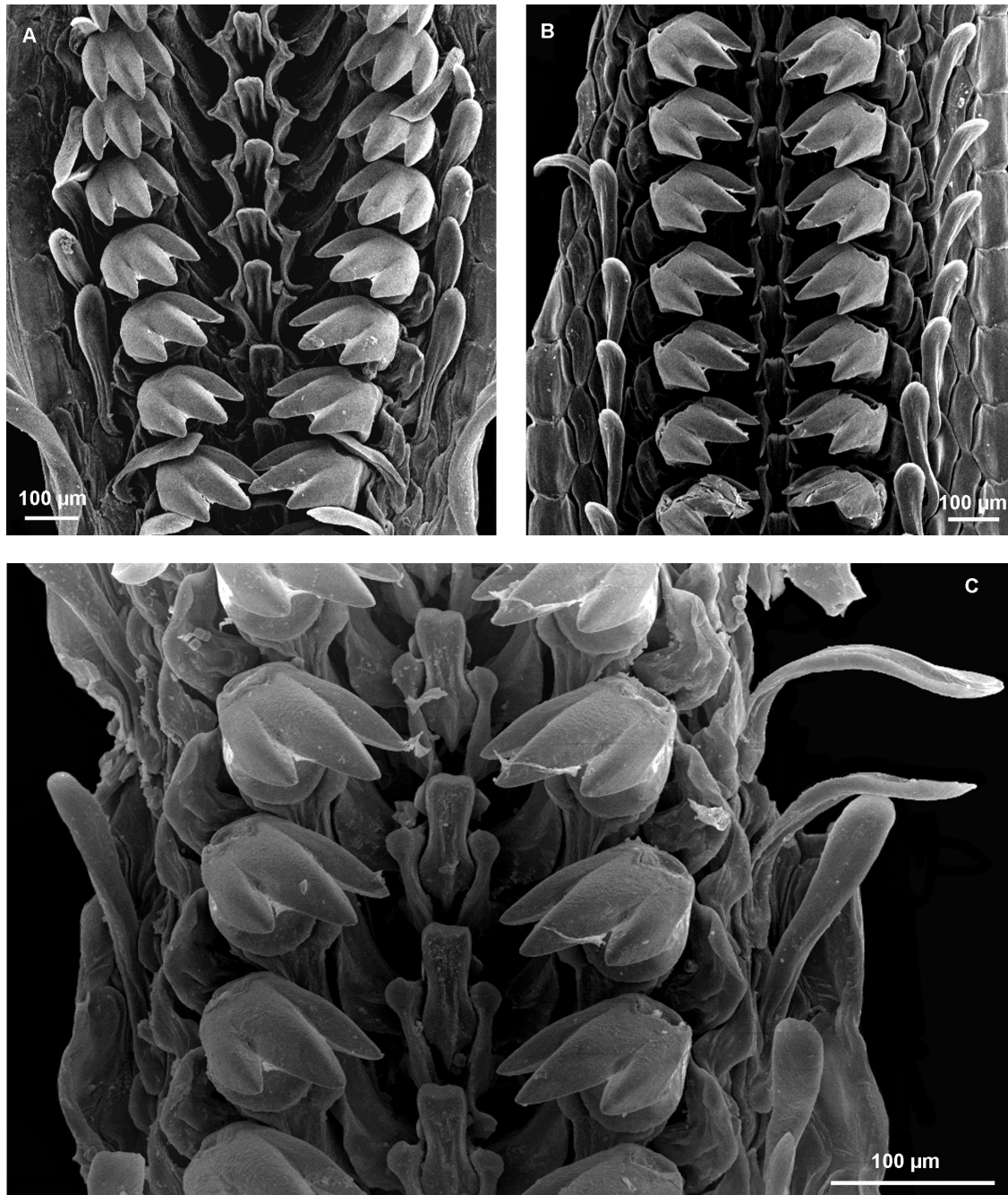


FIG. 4. – Portion of the radular ribbon of *Acanthochitona fascicularis* (A), *A. crinita* (B) and *A. oblonga* (C). Specimens from Castiglioncello (LI), Arenzano (GE) and San Foca (LE) respectively.

in *A. crinita* they are respectively 50 µm and 320 µm and in *A. oblonga* 60-70 µm and 400 µm. A marginal fringe of longitudinally finely striated spicules of 300-400 µm is always present and on the ventral surface the spicules are shorter and also finely striate. We can add that in *A. fascicularis* the perinotum doesn't exhibit the marked difference between the two kinds of spicules as in the other two species. The specimens we examined (Figs. 6A, B, C) agree in general with this description

Molecular analysis, sequence data

A total of 33 DNA sequence fragments, 12 belonging to *A. fascicularis*, 10 to *A. crinita* and 11 to *A. oblonga*, were obtained for COI, 12S and ITS1 loci (Table 2). In addition, for each gene, 3 sequences for the three selected outgroups (*Crassostrea gigas*, *Lotia digitalis* and *Portunus pelagicus*) were downloaded from GenBank. After removing identical haplotypes from each alignment, the number of sequences was

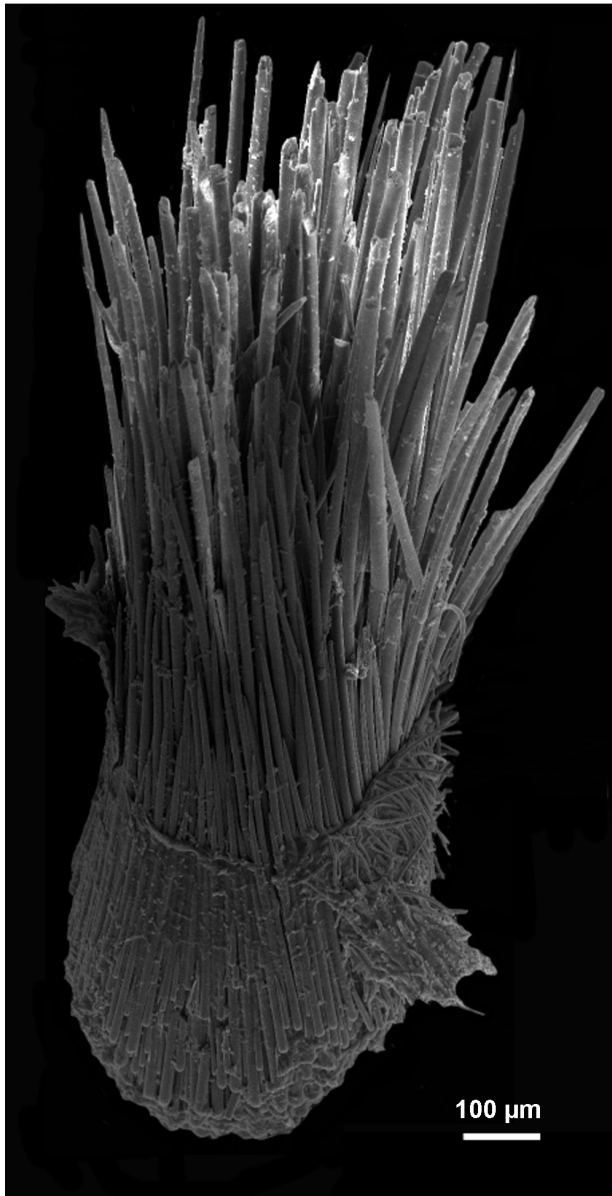


FIG. 5. – *Acanthochitona fascicularis*; one of the 18 tufts of spicules of the girdle. Torre Ovo (TA).

reduced to 20 for COI, 16 for 12S and 18 for ITS1. As the four combined analyses performed required the same haplotypes to be included for each species, the total number of sequences examined was further reduced to 3 for *A. fascicularis*, 4 for *A. crinita* and 4 for *A. oblonga*. The COI data included 658 aligned positions, leaving a final data set of 648 characters. 131 variable sites were identified and 128 of them were parsimony informative. There was no length variation among our COI sequences and gaps were not found. The amino acid translation with invertebrate code did not show stop codons, making us confident that our sequences are mitochondrial in origin and not contaminated by nuclear copies. Using all 648 nucleotides, the pairwise distances were calculated and were as fol-

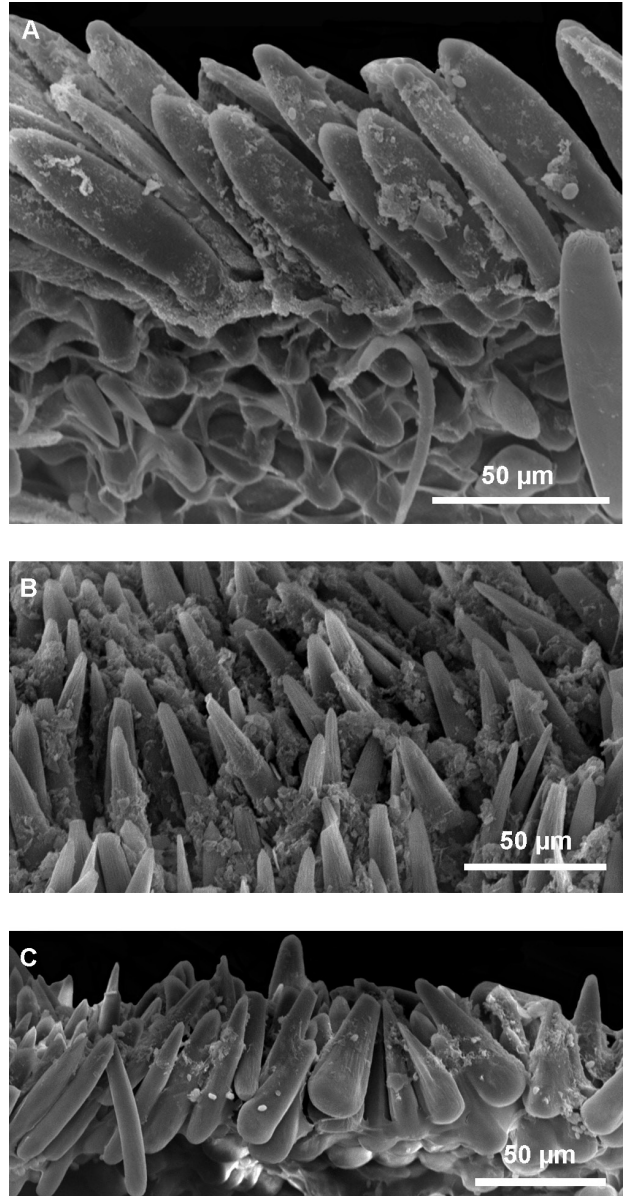


FIG. 6. – Spicules of the upper side of the girdle in *Acanthochitona fascicularis* (A), in *A. crinita* (B) and of the margin (on the left) and the upper side of the girdle in *A. oblonga* (C). In the lower part of the figure the undersurface of the perinotum. Specimens from Torre Ovo (TA), Villafranca Tirrenia (ME) and San Foca (LE) respectively.

TABLE 2. – Source of specimens used.

Species	specimens	locality
<i>A. fascicularis</i>	5	Campomarino (TA, Apulia)
<i>A. fascicularis</i>	1	Capo Molini (CT, Sicily)
<i>A. fascicularis</i>	6	Torre Ovo (TA, Apulia)
<i>A. crinita</i>	10	Villafranca Tirrenia (ME, Sicily)
<i>A. oblonga</i>	11	San Foca (LE, Apulia)

lows: 9% between *A. oblonga* and *A. crinita*, from 16% to 17% between *A. oblonga* and *A. fascicularis*, and 15% between *A. crinita* and *A. fascicularis*. The 12S alignment contained 364 aligned positions, only

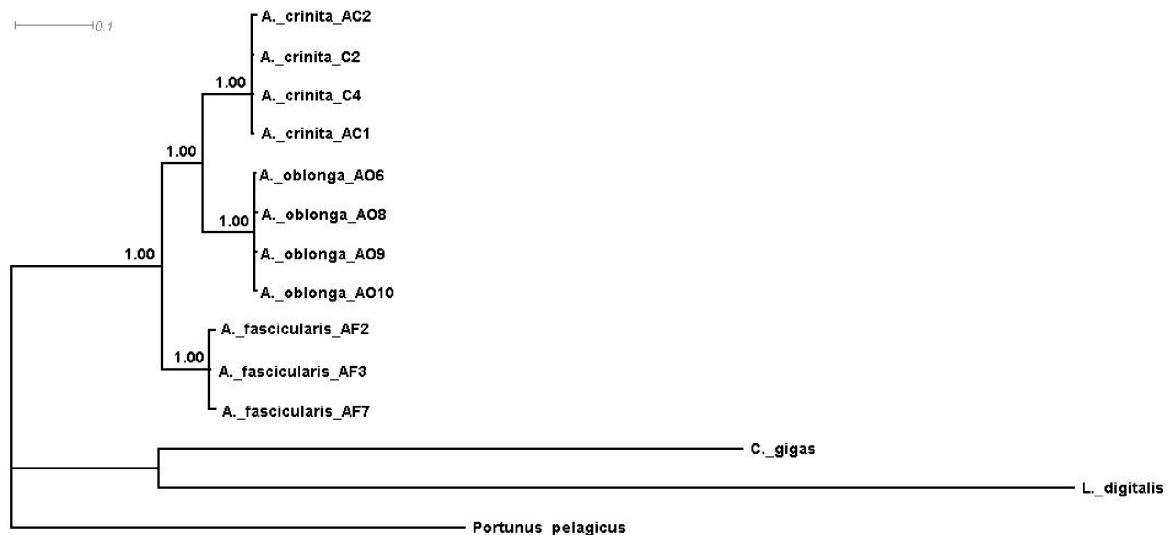


FIG. 7. – Bayesian Inference phylogram of the relationship among *A. oblonga*, *A. crinita* and *A. fascicularis* based on COI, 12S and ITS1 concatenated sequences. Fifty percent majority rule consensus tree, rooted using *Portunus pelagicus* as designate outgroup. Posterior probability values (only values above 95% are statistically significant) provided at the branches. Numbers at the end of the haplotypes represent the sample names used for this study.

a seven sites region was excluded due to its ambiguity; therefore, the final data set was 357 bp. The alignments were relatively simple as the sequence lengths were again conserved and only four gap regions (corresponding to single nucleotide substitutions) were found. The number of variable sites was 53 and almost all of them (52) were parsimony informative. The values of uncorrected divergences were 7% for *A. oblonga* and *A. crinita*, 14% for *A. oblonga* and *A. fascicularis*, and 13% for *A. crinita* and *A. fascicularis*. The ITS1 alignment shows considerable length variations (the maximum observed was equal to 624 positions (all four haplotypes of *A. crinita*) while the minimum was equal to 499 positions, belonging to *A. oblonga* haplotype AO8. 124 sites were variable, 97 of which were informative. The sequence length was too variable to be globally aligned, particularly from position 255 to 280. Therefore, this and all the other highly variable regions were excluded and many gaps were added to align sequences locally. The final data set was 624 bp. The uncorrected divergences ranged from 5% to 10% between *A. oblonga* and *A. crinita*, from 12% to 15% between *A. oblonga* and *A. fascicularis*, and from 14% to 17% between *A. crinita* and *A. fascicularis*. The total number of sites considered in the combined analyses were as follows: 1005 (COI+12S), 1272 (COI+ITS1), 981 (12S+ITS1) and 1629 (COI+12S+ITS1).

Bayesian inference analysis

Phylogenetic Bayesian estimates made for each gene separately could not resolve well the phylogenetic relationship among *A. oblonga*, *A. crinita* and *A. fascicularis*. In all three trees obtained (data not shown), the three species were always separated as distinct monophyletic groups but with low levels of support

(BPP values less than 95%), especially in the 12S tree. The deepest internal nodes of the trees were found to be less robust.

However, the four combined Bayesian analyses provide good resolution among the three species and recover identical consensus tree topologies with nearly identical posterior probabilities for each clade. The consensus tree obtained through the Bayesian Inference based on the combined data set of the three genes (COI+12S+ITS1) is shown in Figure 7. The tree clearly reveals two major clades, one consisting of *A. fascicularis* and one including *A. crinita* and *A. oblonga*, each supported by strong BPP values (100%). The latter clade clearly resolved into two branches: one with *A. crinita* and *A. oblonga* so that *A. crinita* and *A. oblonga* are clearly identified as monophyletic groups, again highly supported by 100% BPP scores.

DISCUSSION

After the SEM analysis, the morphology of the tegmentum appears to be the most useful morphological character for distinguishing the three taxa. The shape of the granules and the distribution of the aesthetes of *A. fascicularis* were studied in detail by Fischer and Renner (1979), and Dell'Angelo and Smriglio (1999) also made some observations of both *A. fascicularis* and *A. crinita*; however, contrary to what they stated, the aesthetes are absent in the intertubercular areas of the tegmentum in all examined specimens of each taxon.

Apart from the shape of the granules, based on which Leloup (1981) separated *A. oblonga* from *A. crinita*, which may exhibit some degree of variation, the distribution of anterocentral microaesthetes in the second species vs the central microaesthetes in the first

species and the completely different numerical macro/microaesthete ratio (1-5/1 in *A. fascicularis*, 12-16/1 in *A. crinita* and 6-9/1 in *A. oblonga*) probably represent the best characters for identifying Mediterranean *Acanthochitona* well.

Even if the differences in the radulas are not particularly remarkable, the three species can be distinguished mainly by the shape of the centrolateral teeth, which are only very arched in *A. fascicularis* and their tip is only convex and spatuliform in *A. oblonga*. Moreover, a slight difference can be observed in the three cusps of the major lateral teeth: the central cusp is larger in *A. fascicularis* while in *A. crinita* and *A. oblonga* it is the same size as the internal one and the external cusp is always the smallest.

The perinotum is the most variable character; Leloup (1941, 1968), Kaas (1985) and Dell'Angelo and Smriglio (1999) described and illustrated in detail its spicules showing some differences, but we observed high variability in shape and also minor variability in the size of the dorsal spicules. This led us to consider this character to be of minor importance, at least for discriminating between the two disputed species *A. crinita* and *A. oblonga*.

The molecular analysis reported here represents the first estimate of the relationship within the genus *Acanthochitona* (Gray, 1821). The uncorrected pairwise distance values calculated among the three species show an evident close genetic relationship between *A. oblonga* and *A. crinita*, while *A. fascicularis* appears more distant. The topologies of the trees based on the four combined data sets show that *A. oblonga* and *A. crinita* always clustered separately from *A. fascicularis* and resolve each one as a monophyletic group. Therefore, all the genetic data definitely highlights that *A. oblonga* is closely related to *A. crinita* but it is an independent lineage. The pattern shown by the trees obtained from the analysis of the single genes can be explained by a rapid speciation (very low BPP values at deep nodes) followed by a slower process of differentiation of the three species (higher values of BPP). These data might be in agreement with the reports of many suspected neo-endemic species of the southern Mediterranean Sea and in particular the gulf of Gabes (Cecalupo *et al.*, 2008). The lower BPP values observed in the 12S tree were expected due to the more conservative nature of this gene with respect to COI and ITS1. The fact that good resolution is obtained only by the combined data sets and not by the single genes was not surprising as it has been recognized that a single marker is often insufficient for reconstructing the phylogeny of a group of living animals (Nichols, 2001). Combining data sets can in fact recover hidden signals because the true signal is not randomly distributed across data sets. In general, the results of our investigation clearly demonstrate the usefulness of molecular data to delineate the relationship among the species of the genus *Acanthochitona*, and in particular the status of *A. oblonga* as a valid species.

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