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Two new highly polymorphic microsatellite loci and inadvertent minisatellite loci for *Lymnaea auricularia*

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Microsatellite loci are useful tools for resolving population genetic histories.¹ Because of their high mutation rates, they often reveal fine-scale variation between recently diverged populations that other markers (e.g. allozymes) may fail to detect.² We report here on the development of species-specific microsatellite primers for *Lymnaea (Radix) auricularia* (Linnaeus, 1758) (Pulmonata, Lymnaeidae) which we undertook in order to recover the history of its arrival in Lake Baikal. *Lymnaea auricularia* is a palaeartic generalist not recorded from Baikal until around 1960, and only recently found in large numbers. Populations in Baikal are morphologically divergent from potential shallow water source populations (M. Stift, E. Michel, T.Ya. Sitnikova, K.Yu. Mamonova & D.Yu. Sherbakov, unpublished observations). This raises questions of population isolation, selection, and invasion dynamics that can be addressed with microsatellites. We also tested cross-species amplification of existing primers that were developed for the closely related *Lymnaea truncatula*³. *L. auricularia*, *Lymnaea truncatula* and *L. peregra* form a well-supported clade in an 18S rDNA-based phylogeny⁴.

Genomic DNA was isolated from parasite-free foot tissue of *L. auricularia* using a modified CTAB method.⁵ For the isolation of the microsatellite loci we used a modified FIASCO method (Fast Isolation by AFLP of Sequences Containing repeats).⁶ DNA was digested with restriction enzymes *MseI* and *TaqI*, followed by adaptor ligation. Fragments were amplified by a polymerase chain reaction (AFLP-PCR). Biotin-labelled di-, tri- and tetra-nucleotide repeat sequences were hybridized to the PCR product and the hybridization complex was lifted out with streptavidin-coated magnetic spheres (Promega)⁷. After washing, the bound DNA was eluted from the magnetic spheres and re-amplified.

The DNA fragments were cloned using the plasmid pGEM-TEasy vector (Promega) and transformed into *Escherichia coli*

JM109 High Efficiency Competent Cells (Promega). We screened the colonies for the presence of a repeat-insert using PCR with universal M13 primers and the biotinylated microsatellite sequence. Using alkaline lysis,⁸ we recovered plasmids from 72 insert-containing colonies and sequenced them using the Amersham sequencing kit. Sequencing reaction products were run on a Li-Cor 4200 automatic sequencer and analysed with E-Seq software (Li-Cor V.1.1) yielding 16 unique repeat-containing sequences of both microsatellites (2-, 3-, 4-nucleotide repeats) and minisatellites (> 20-nucleotide repeats). We used the Primer3 program^{9,10} to select primer pairs for these sequences. A total of nine sequences had suitable flanking regions for forward and reverse primer development. We tested these and the *L. truncatula* primer sets in polymerase chain reactions (PCR) with a total volume of 10 µl containing approximately 10 ng DNA, 0.1 µg BSA, 1 µl PCR buffer (HT Biotechnology), 100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, 500 mM KCl, 1% Triton X-100, 0.1% (w/v) stabilizer, 10 µM primer, 0.25 mM of each dNTP and 1U Taq (HT Biotechnology Ltd).

Each reaction was exposed to the following temperature regime: an initial denaturation of 2 min at 94°C, followed by 35 cycles of amplification at 94°C for 30 s, 30 s at the optimal annealing temperature (Table 1), 45 s at 72°C, and an additional 10 min at 72°C. The PCR-products of all loci were visualized on agarose gels (1%) stained with ethidiumbromide.

The minisatellite loci were immediately scored by hand. All three were monomorphic. For the microsatellite loci that amplified (two presented for the first time here, one of *L. truncatula*³), PCR was repeated with IR-700 labelled primers. The PCR products were visualized on 6.5% polyacrylamide gels and run on a LiCor 4200 automatic sequencer. Three primer pairs produced interperable and repeatable amplification products. We tested variability on samples from 11 populations (sample sizes ranging from five to ten) of *L. auricularia* from Lake Baikal and its

Table 1. Microsatellites for *Lymnaea auricularia* including repeat motif, observed heterozygosity (H_o), expected heterozygosity (H_t), annealing temperature (T_{ann}) and forward (F) and reverse (R) primer sequence.

Locus	Repeat motif	H_o	H_t	T_{ann}	Primer sequence (F and R)
Laurmin1	a: TGTGAGTGAGAGTCGGTGTGTTGG b: TATGGAAG aaaaabaaababababa	0	0	59°C	CTTTGAGCGATTCTCGGTGT TGTCGCACTACTTCAACACACA
Laurmin2	(GGGTGCAAGATTAGAAGAGATGAAG) ₈	0	0	59°C	CATACCAGGCCAGAAAAAC TATTTTAGCGCCCTCACTC
Laurmin3	(CACCCACACCACGCCACACTTTATGA ACTGAGTA) ₉	0	0	60°C	ATCGAGTTCTATGTGGTAGTTGG AATACACGCACACCGTCTCT
Laurmic1	(AG) ₂ GAGAAGG(AG) ₁₅ AC(AG) ₁₅	0.274	0.924	58°C	ATGCTTTGGWACACCTTCGT CGTTCACCTGCTTCGGGATT
Laurmic2	(AC) ₈ A(AC) ₂₉	0.328	0.914	58°C	TCATAACCCTGGCTTCCTTG GCACATTTTACGATTCTAGTGG
52 ³	(AG) ₈	0	0	51°C	GAGGGGGATGCAAAAACAAG TGGGTGGCAATGACGTAG

in- and outflowing rivers, in southern Siberia, Russia. Genotypes were scored by hand. Two microsatellite primers were highly polymorphic, whereas the *L. truncatula* primer was monomorphic. Locus LAURMIC1 had 14 different alleles and locus LAURMIC2 had 18 alleles. A total of 79 individuals were analysed for each locus and the overall observed (H_o) and expected heterozygosity (H_e)¹⁰ was calculated (Table 1) using Fstat, version 2.9.3.¹¹

Although the applied enrichment procedure was specifically designed to enrich for microsatellites, the sequenced fragments often contained minisatellites, highly complex longer stretches of repetitive DNA.¹² Similar patterns of inadvertent minisatellite isolation have been observed in the snails *Bulinus obtusispira*¹³, *Physa acuta* (P. Jarne, personal communication), *Buccinum undatum* and *Potamopyrgus antipodarum* (D. Weetman, personal communication) and the freshwater bivalve *Uttarbackia imbecilis* (J. P. Curole, personal communication). Application of minisatellites is attractive as genotyping of individuals can be achieved on basic agarose gels, without the need of expensive and time-consuming labelling. However, minisatellite evolution is poorly understood (but see^{12,14}) and may thus pose analytical difficulties. Moreover, the minisatellites we analysed were monomorphic, confirming theoretical predictions that minisatellites are less variable than microsatellites.¹⁵

In conclusion, we isolated two useful and highly polymorphic microsatellite loci for *L. auricularia*. The three minisatellite primer sets we developed did amplify, but the loci were monomorphic in our samples. Minisatellites may prove more useful in detecting variation between species.

Our attempt to use primers from the closely related species *L. truncatula* did not yield usable results as only one of the six primer pairs amplified and, unfortunately, this locus was monomorphic in our samples. This underscores the importance of species-specific development of genetic tools such as microsatellite primers in population genetics.

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Extinction risk and harbours as marine reserves?

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The mollusc *Patella ferruginea* Gmelin, 1791 is the most endangered marine invertebrate species on the list of the European Council Directive 92/43/EEC on the Conservation of Natural Habitat of Wild Fauna and Flora,¹ and is considered to be in danger of extinction.² Although its relative abundance in Palaeolithic and Neolithic deposits indicates an extensive former distribution in the Western Mediterranean, its range has progressively contracted.³ These population regressions have been generally attributed to increasing pollution levels along the marine coastline and, especially, to human predation through the collection of specimens for food, fishing bait and decorative purposes, as this mollusc is one of the most attractive

limpets of the Mediterranean. Today, the species has practically disappeared in the Iberian Peninsula and the North African coast of the Strait of Gibraltar. Strikingly, at the coast of Ceuta, and especially inside the harbour, we have found dense and stable populations of *Patella ferruginea* (Fig. 1).

The harbour of Ceuta is unusual from an environmental point of view, differing substantially from other conventional harbours. It is located between two bays connected by a channel, which increases the water movement and exchange, contributing to the maintenance of rich and diverse communities of marine invertebrates (Fig. 2).

After assessing the presence of *P. ferruginea* in Ceuta, a total of 70 stations were selected along the coast, inside the harbour and outside (North Bay and South Bay). The density of *P. ferruginea*

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in each station was measured at low tide by counting the number of specimens found in a transect placed parallel to the coast with a length of 10 m and a width corresponding with the whole intertidal belt⁴ (1.25 m on Ceuta's coast). A mean density of 11.33 individuals/10 m was measured inside the harbour, while outside the harbour the mean was 5.54 (North Bay) and 6.96 (South Bay), respectively. The values registered inside the harbour were significantly higher than those registered outside (one-way ANOVA, $F_{2,69} = 3.50$, $P < 0.05$) (Fig. 1). These values inside the harbour are even higher than the densities recorded in the Mediterranean relict populations of *P. ferruginea*, located

in protected areas (7.9 ind/10m in Corsica⁴ and 7 ind/10 m in Zembra Island, Tunisia⁵).

The unusual environmental structure of the harbour of Ceuta, together with the fact that people consider harbours as 'non-attractive' places to collect specimens for food and fishing, have contributed to the maintenance of high densities of *P. ferruginea*. Taking into account that *P. ferruginea* is considered a *K*-strategist species, with a low rate of growth and reproduction, and that it has been traditionally associated with high hydrodynamism and low levels of pollution,^{3,5} the high densities of this mollusc reported inside a harbour is even more striking.

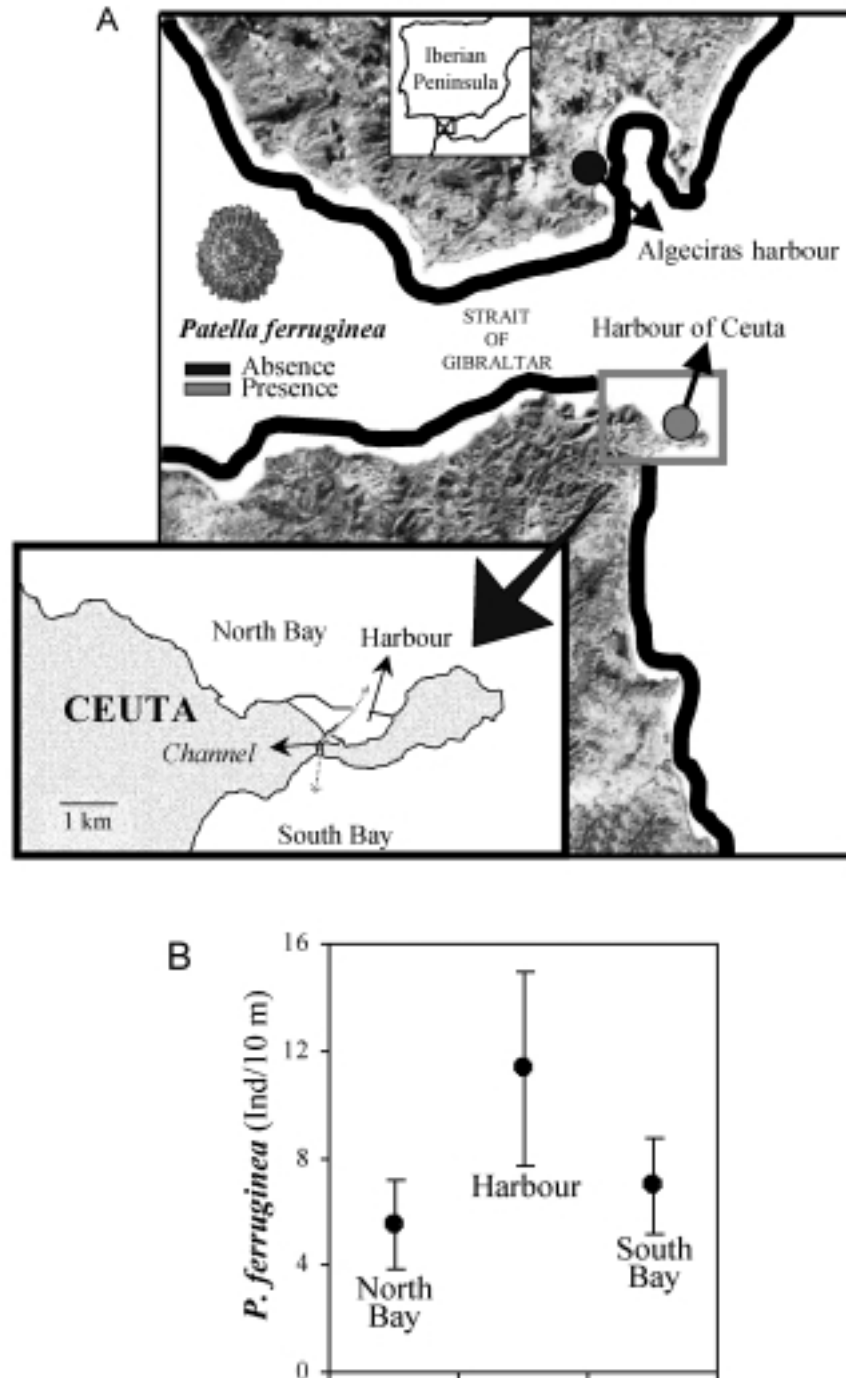


Figure 1. A. Location of Ceuta in North Africa. B. Density of *Patella ferruginea* (mean values \pm standard deviation) in North Bay ($n = 33$), South Bay ($n = 29$) and the harbour ($n = 8$) of Ceuta.

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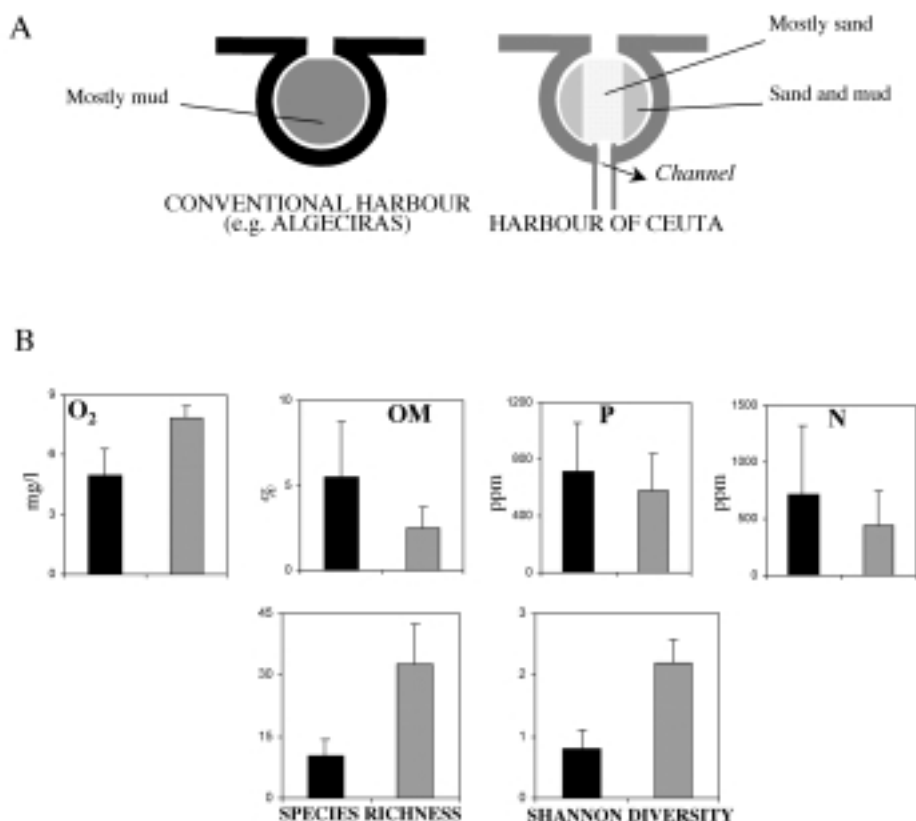


Figure 2. A. Schematic comparison between a conventional harbour and the harbour of Ceuta. B. Comparison of physicochemical and biological parameters between Algeciras harbour⁷ (black) and the harbour of Ceuta⁸ (dotted). Species richness and Shannon diversity were measured for sediment macrofaunal assemblages. Abbreviations: O₂, oxygen in water column; OM, organic matter in sediment; P, total phosphorus in sediment; N, total nitrogen in sediment.

Consequently, the design of the harbour of Ceuta, provided with a channel which increases the water renovation, should be taken into consideration for future civil engineering projects in order to reduce the negative impact of harbour building on marine environments. Furthermore, a harbour displaying adequate environmental features can offer useful monitoring and protection facilities (e.g. vigilance and installation of anti-pollution artificial barriers). This is particularly interesting in areas, like the Strait of Gibraltar, which have a high risk of environmental disaster because of intense maritime traffic.

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Epitonium dendrophylliae (Gastropoda: Epitoniidae) feeding on *Astroides calycularis* (Anthozoa, Scleractinia)

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Epitonium dendrophylliae Bouchet & Warén, 1986 is a rare, thin-shelled, eastern Atlantic and western Mediterranean epitoniid usually found on the deep shelf or in the bathyal zone, associated with the scleractinian corals *Dendrophyllia* and *Balanophyllia* (Dendrophylliidae).^{1,2} It was originally recorded from Madeira, the Mediterranean, the Atlantic coast of Morocco, and as far as Luanda (Angola). All known records of *E. dendrophylliae* are from depths exceeding 40 m, and most of them consist of empty shells. Although the species may reach 15.2 mm, the holotype measures only 5.6 mm. The species is here reported for the first time from shallow water, living on colonies of the scleractinian dendrophylliid *Astroides calycularis* (Pallas, 1766).

A single living specimen was hand collected (by SCUBA diving) on *Astroides calycularis* in Punta de la Mona (Granada province, SE Spain) at 19 m depth, and kept alive on the coral in an aquarium at 20°C for 12 days. Subsequently, it was relaxed in MgCl₂ isotonic with sea water, fixed in 70% ethanol, dissected and studied under the stereomicroscope. The radula was extracted and mounted for SEM.

The shell, of 4 mm length, with about 5 teleoconch whorls and an eroded protoconch of about 3 whorls, matched the original description, and was covered by a thin, light brown periostracum.

The head-foot, mantle and visceral mass of the living animal was golden yellow, with scattered, minute, white spots that were dense on the margin of the foot and the base of the cephalic tentacles (Fig. 1A, B). The hypobranchial gland, yellow with narrow transverse black bands on its anterior part, lay on the right side of the mantle, encompassing the last whorl of the teleoconch. The head possessed a pair of relatively long cephalic tentacles with well-developed black eyes at the base; the proboscis was rather short. A relatively deep transverse groove ran along the anterior margin of the foot, and the posterior end of the foot was tapered and slightly bilobed with a short mid-longitudinal slit. A short cylindrical tentacle with an annular thickening at the base was located on the midline of the dorsal surface of the anterior propodium. This tentacle may be sensory, since the animal, while creeping, retracted and stretched it and changed its orientation. The round operculum was paucispiral, thin and transparent and covered the whole metapodial sole.

Within the buccal apparatus were a pair of fragile lateral jaws, more or less semicircular in outline, minutely serrated on the convex cutting edge and with a reticulate pattern on the surface. Chitinous stylets were absent. The radula (Fig. 1C, D) had a large number (not determined) of slender teeth with long, thin basal shafts and from three to six pointed cusps. The distal cusp was the largest and bent upwards, appearing sinusoid in lateral view; the next two cusps were more or less equally developed and slightly curved upwards (especially the subdistal one), and the basal cusps shorter and less curved.

The animal moved freely on the polyps of *Astroides calycularis*, secreting a thin but resistant mucous filament, whereby it

remained attached to the polyp when the foot lost its attachment. The gastropod fed by attaching the tip of the proboscis to the cenosarc and oral disc of the polyps and biting off minute pieces of tissue; it was never observed introducing the proboscis through the oral aperture or perforating the oral disc. Contraction or relaxation of the polyp column and tentacles were not observed during feeding, suggesting that no anaesthetic was injected by the snail. During 10 days the gastropod restored the broken peristome and secreted eight additional, regularly spaced, axial lamellae, which encompassed slightly less than a quarter whorl, reaching 4.08 mm in shell length. This resulted in a growth rate of 0.008 mm/day.

The shells of the Mediterranean and amphi-Atlantic *Epitonium striatissimum* (Monterosato, 1878) and the Indo-Pacific *E. billeeanum* (DuShane & Bratcher, 1965) closely resemble that of *E. dendrophylliae*^{1,2} in shape and sculpture. The protoconchs of these three species are also very similar. They are multispiral (3–4 whorls), with numerous fine, incised axial lines.^{1,2} *Epitonium billeeanum* feeds and even spawns on the dendrophylliid coral genera *Dendrophyllia* and *Tubastraea*^{2–6} throughout its wide biogeographic Indo-Pacific range, which extends from the Red Sea² to the Galapagos Islands, Ecuador and Gulf of California.^{6–8} This species shows preference for shaded, shallow-water areas (2–14 m),^{2,8} but can be found at depths to 45 m.^{2,5} As in the described specimen of *E. dendrophylliae*, the shell of *E. billeeanum* is covered by a thin periostracum, light brown or of a yellowish buff, and the animal is brightly golden yellow or orange coloured (it is commonly named ‘golden wentletrap’)^{2,4,5–7}; with minute lighter yellow (‘lime’) specks on the whole body², and with a round and thin operculum.^{7,8} Nevertheless, no propodial tentacle has been described for *E. billeeanum*. The polyps of both *Tubastraea* and *Astroides calycularis* are of a bright pink-orange or orange colour. As has been suggested,² the striking colour differences between the light yellow epitoniids and the pink or orange corals do not support the previous hypothesis that the pigments of the snails directly originate from corals⁴ or that snails are cryptic.

A radula very similar to that of *Epitonium dendrophylliae* has been described for small specimens (up to 8.6 mm in length) of *E. billeeanum* from the Great Barrier Reef.⁵ The middle lateral teeth of the studied specimen of *E. dendrophylliae* have from three to six cusps (denticles), whereas small *E. billeeanum* has three to seven cusps. The distal cusp has a more sinusoid shape in *E. dendrophylliae* than in *E. billeeanum*. Large specimens of *E. billeeanum* (>12.3 mm) have smooth middle lateral teeth, whereas intermediate specimens (8.6–12.3 mm) show transitional radulae with either denticulate (1–7) or smooth middle lateral teeth. This suggests an ontogenetic change in the radular morphology that may be related to sex change.⁵ However, the radular teeth of the holotype (7 mm in length) are figured either smooth or with a single denticle,⁷ at a size in which denticulate teeth should be expected; thus, further ontogenetic studies on eastern Pacific material are needed to resolve this matter.⁵ A similar ontogenetic change in the radular teeth of *E. dendrophylliae* is expected to be found when radulae of enough spec-

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imens of different sizes are studied. The radulae of *E. dendrophylliae* and *E. billeeanum* are rather different from those known of other epitoniids.^{2,9,11–19} However, it must be emphasized that no complete descriptions of the teeth for different shell sizes were made.

The similar shell (protoconch and teleoconch), periostracum, operculum, pigmentation pattern of the body, and radula support a possible close relationship between *E. dendrophylliae* and *E. billeeanum*. In addition, these features differ sufficiently from those of the type species of *Epitonium* (*Turbo scalaris* Linnaeus, 1758) to warrant a generic separation. *Epitonium scalare* has a higher shell, with different shape, sculpture and soft part colour (yellowish brown to dark brown), a thick and black operculum, and unicuspid radular teeth.^{13,14} In the currently chaotic state of epitoniid taxonomy (both at the generic and specific levels), it is difficult to propose a suitable genus name for these species. *Epitonium dendrophylliae* has been recently included in the subgenus *Sodaliscala*, whereas the related *E. striatissimum* is included in *Parviscala*, and *E. billeeanum* in *Limiscala*², in all cases without any evidence.²⁰ The latter species has also been included in the genus *Asperiscala*,⁶ and even in *Alora*,²¹ again without discussion. Due to the unclear diagnoses

of all the genera or subgenera included under *Epitonium*, we here use the name in a very broad sense. A worldwide generic review based on anatomical and biological features will probably group *E. dendrophylliae*, *E. billeeanum* and *E. striatissimum* in a genus different from *Epitonium scalare*.

The growth rate measured in *Epitonium dendrophylliae* is much lower than that of recently metamorphosed post-larvae of *Epitonium ulu* (0.2 mm/day at 24–28°C) fed with *Aiptasia* sp.,²² which it is not its natural prey (this species feeds on a variety of *Fungia* species^{23,24}). The growth rate is also lower than that of young individuals of *E. albidum* ranging between 2.2 and 3.5 mm, which over a period of 13.8–15.8 days feeding on its natural prey, the actinarian *Stichodactyla helianthus* (Ellis, 1768), increased on average 0.17 mm/day and secreted 1.5 ribs/day.²⁵ Considering that *E. dendrophylliae* may reach 15.2 mm,¹ the specimen here studied is a young one. Differences in growth rate might be due to the usual rapid growth of post-larvae of carnivorous gastropods,²² the larger size of our individual compared with the young *E. albidum*, or the lower water temperature during our observations. Also, it may reflect the slower growth characteristic of a usually deep-water species or the low energetic value of the scleractinian versus actinarian prey.

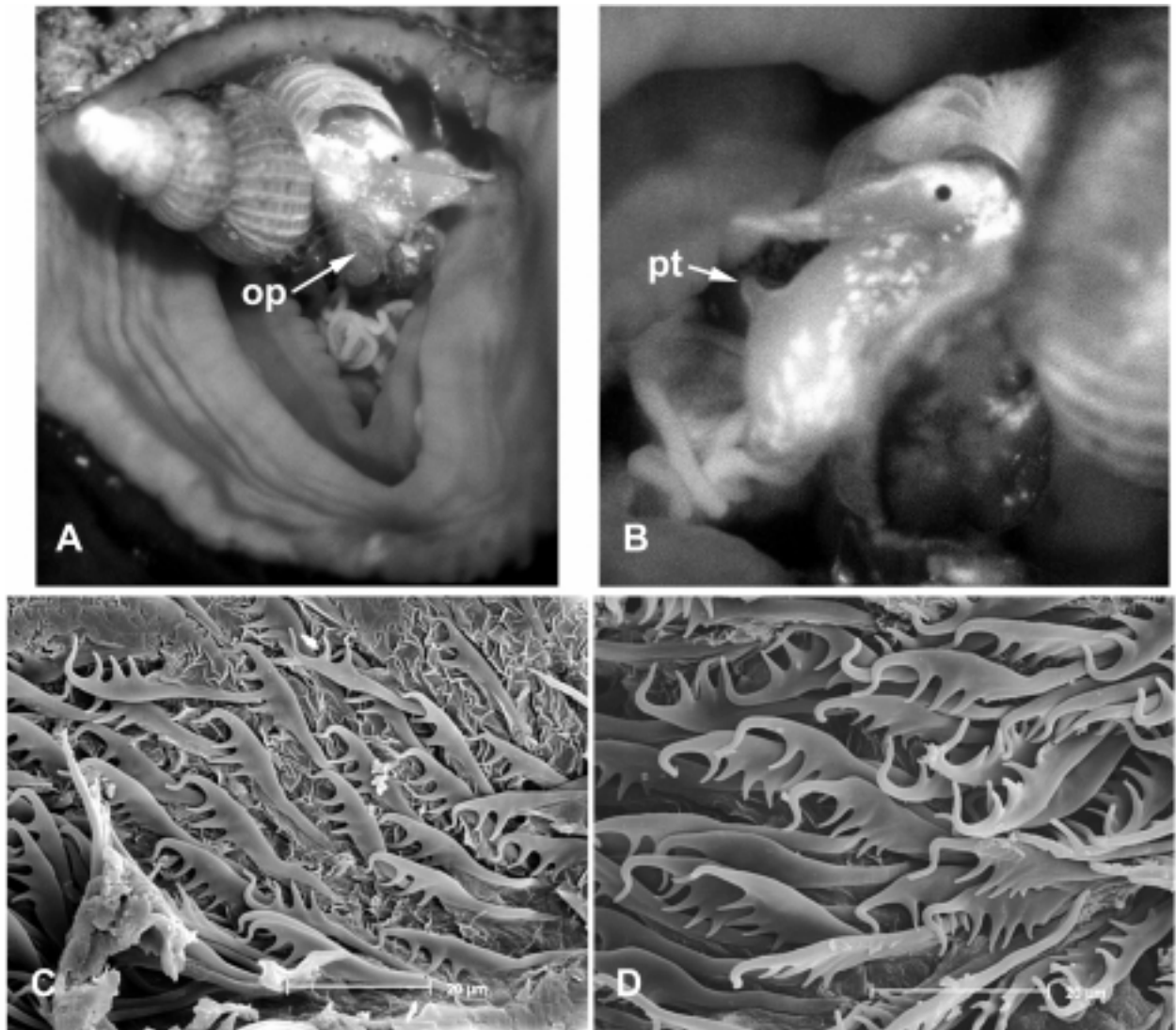


Figure 1. *Epitonium dendrophylliae*. **A.** Living animal on a polyp of *Astroides calycularis*. **B.** Detail of the head-foot, showing the propodial tentacle. **C, D.** Radula. Abbreviations: op, operculum; pt, propodial tentacle. Scale bar = 20 μm.

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Astroides calycularis is included in Annexe II (list of endangered or threatened species) of the Barcelona Convention. It is considered a relict species restricted to the western Mediterranean.²⁶ At least two uncommon gastropods are now known to live and feed on this coral (*Epitonium dendrophylliae* and the coralliophilid *Babelomurex cariniferus* (Sowerby, 1834), personal observation), and this fact reinforces the arguments for its protection.

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