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Changing feeding habits and ontogenetic dimorphism in juveniles and adults *Aplysia punctata* (Cuvier, 1803) (Mollusca, Gastropoda, Heterobranchia) in the Mediterranean Sea

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Abstract

Specimens of *Aplysia punctata* inhabiting the Catalonian coast (NE Spain, Western Mediterranean Sea) display two different color patterns, which have been thought to represent two distinct species. However, molecular analyses conducted by other authors and confirmed herein show that there are no genetic differences between individuals of the two color morphs. At the same time, these color morphs display distinct life history traits, including different size, mating behavior, and egg mass and larvae coloration. In this paper, it is hypothesized that distinct diets are responsible of these differences. The results of this study confirm that small red specimens of *A. punctata* feed on red algae and primarily *Sphaerococcus coronopifolius* and *Plocamium cartilagineum*; pigments from these algae give small specimens of *A. punctata* a very similar morphological appearance to adults of the *Aplysia parvula* species complex. In contrast, adult specimens of *A. punctata* feed preferentially on green algae of the species *Ulva lactuca*. This change in feeding behavior is probably related to defensive strategies involving camouflage. In addition, the egg masses take on a pink to reddish color that is retained in the larval stage of *A. punctata* juveniles.

Keywords: *Aplysia punctat;* dietary preference; dimorphism; coloration; Catalonian coast.

Introduction

The adoption of molecular phylogenetic methods has resulted in significant classification and taxonomic changes among diverse groups of living organisms (Field *et al*., 1988), confirming the limitations of morphological data for phylogenetic reconstruction (Doyle, 1992; Knowlton, 2000). Some examples involve species previously considered to have wide geographic distributions (Andrews *et al*., 2014; Gaither *et al*., 2016) or organisms with reduced morphological diversification (Fukami *et al*., 2004). Among the others, Cooke *et al*. (2014), Kienberger *et al*. (2016), Lindsay and Valdés (2016) indicated that molecular analyses have been very useful to clarify the classification of sea slugs in cases where several species with similar morphology could not be discerned by traditional methods, and were formerly considered to be single widespread species. In line with that, molecular analyses have confirmed that most species with large geographical ranges across different ocean basins are in fact pseudo-cryptic or cryptic species complexes of closely related taxa, and several authors provide hypotheses on the mechanisms of evolution resulting in their current distribution (Ornelas-Gatdula *et al*., 2012; Carmona *et al*., 2014; Valdés *et al*., 2017; Uribe *et al*., 2018).

Species of the genus *Aplysia* Linnaeus, 1767, known as sea hares (Heterobranchia, Aplysiida), live in the intertidal or upper subtidal zones where they feed, mate, and lay their egg masses on different species of algae (Ribero *et al*., 1998). According to Bouchet and Gofas (2022), there are around 45 species of *Aplysia* worldwide and many other species considered *nomina dubia* or *taxa inquirenda*. Species of *Aplysia* live in temperate or tropical waters, and no species has been able to colonize the cold waters of the Arctic or the Antarctic oceans. Medina and Walsh (2000) carried out the first phylogenetic analysis of the Aplysiida based on mitochondrial DNA, while Medina *et al*. (2001) carried out a preliminary phylogeny on the genus *Aplysia* in which they include *Aplysia parvula* (Mörch, 1863) and *Aplysia punctata* (Cuvier, 1803) as distinct species.

The identification of species of *Aplysia* based on morphological traits is problematic as some species are highly variable in external coloration (Medina & Walsh, 2000).

An example is *A. punctata*, a very common species in the Mediterranean Sea that displays high levels of chromatic diversity. Ballesteros and Templado (1987) misidentified small specimens of *A. punctata* as *A. parvula*, because of their reduced size and reddish coloration similar to that of *A. parvula*. Ballesteros and Templado (1987) also carried out a morphological study of these small *Aplysia*, analyzing their radular structure and providing data on their biology and reproduction. Since the paper of Ballesteros and Templado (1987), small specimens of *A. punctata* with lengths of 5-20 mm were cited in the western Mediterranean under the name of *A. parvula* (see Martín *et al*., 1990; Sánchez Tocino, 2001; Domènech *et al*., 2002; Cervera *et al*., 2004; Ballesteros, 2007; Coll *et al*., 2010; Ballesteros *et al*., 2016; Gofas *et al*., 2017), although sometimes with reservations (Crocetta *et al.*, 2015; Corsini-Foka *et al.*, 2015; Zenetos *et al.*, 2016). Only recently, Golestani *et al*. (2019) found that there is no evidence of the presence of *A. parvula* in the Mediterranean Sea and that all specimens showing the *A. parvula* phenotype are *A. punctata* or possible hybrids. Finally, Ballesteros *et al*. (2021) provided additional information on these small red specimens of *A. punctata* with numerous photographs illustrating details of their coloration and morphology, as well as how this species changes coloration at different stages of its life cycle.

However, the boundaries in external morphology between the two-color morphs still needs to be clarified, as well as the reasons why the feeding strategy of individuals changes as their size increases. In this paper we attempt to address these questions by examining changes in feeding behavior during different stages of the life cycle of *A. punctata*, as well as the reproductive period, mating behavior, egg mass coloration, and other traits based on natural populations and individuals collected for observation in the laboratory. Moreover, molecular analyses were conducted to verify the identity of the specimens examined and confirm or refute the results by Golestani *et al*. (2019).

Materials and Methods

Morphological analysis

In this paper the abbreviature APJ is used for the small red specimens previously cited as *A. parvula* and the abbreviature APA is used for larger animals typically identified as *A. punctata*. Live specimens of APJ and APA with their egg masses were photographed live in the field or in the laboratory. A binocular Olympus SZ-PT dissecting microscope was used to study their internal anatomy. A dorsal incision was made on the anterior portion of the animal to dissect the distal anterior part of the digestive tract, which includes the buccal bulb, radula, and jaws. The buccal mass was immersed in a potassium hydroxide (KOH) solution (10%) for three hours to dissolve the organic tissues and then rinsed with distilled water. The radula was mounted on a metal stub with bio adhesive tabs, coated with a carbon layer, and examined under a FEI Quanta 200 scanning electron microscope (SEM).

DNA extraction and amplification

For the molecular analyses, 13 specimens of APJ and 13 of APA were collected by SCUBA diving or snorkeling in different locations on the Catalonian coast (NE Spain). Specimens were collected live in the field, immediately transferred to 99% ethanol and stored at -20°C for the subsequent DNA extraction. To complete the dataset, we retrieved all the available sequences of the *A. parvula* and *A. punctata* from Genbank, listed in Table 1. Four specimens of *Bursatella leachi* from Fòrum (Barcelona, Spain) and Ebro Delta (Tarragona, Spain) were used to root the tree.

Total genomic DNA was extracted from the foot of each specimen using the REDExtract-N-Amp™ Tissue-PCR Kit Protocol from Sigma-Aldrich and following the manufacturer's protocol. Partial fragments of two mitochondrial genes and one nuclear gene were sequenced: COI (cytochrome c oxidase subunit I), 16S (ribosomal RNA) and Histone H3. The primers used for amplification are listed in Table 2. The LCOI1490/HCO2198 was the preferred combination, while Nancy was used as a replacement for HCOI2198 (Table 2).

The polymerase chain reaction (PCR) was performed using 5µl 5× MyTaq Reaction Buffer (BIOLINE), 0.4 µl primers forward and reverse (10 µM), 0.2 µl MyTaq Red DNA Polymerase (BIOLINE), 4 µl of diluted DNA and ultrapure distilled water up to a total reaction volume of 20 µl. COI was amplified as a follows: 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature range of 42°C for 35 s, 72°C for 45 s and final extension at 72ºC for 5 min; 16S and Histone H3 were amplified as a follows: 2 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 35 s and extension at 68°C for 1 min and final extension at 68°C for 7 min.

Fragments were visualized by agarose gel electrophoresis at a concentration of 1% (w/v) in 1× TBE buffer (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA) (Panreac AppliChem, Barcelona, Spain) at 110 V for 45 min. PCR products were sequenced in both directions using one of the respective amplification primers at Macrogen services (www.macrogen.com). The chromatograms were assembled and edited in Geneious Prime v2021.1.1. (Drummond *et al*., 2010).

Phylogenetic analysis

Different alignments were built for downstream phylogenetic analyses containing all the available *Aplysia fasciata* Poiret, 1789, *Aplysia nigrocincta* von Martens, 1880, and *A. punctata* sequences and four *Bursatella leachii* Blainville, 1817 sequences, including: the individual COI, 16S, and Histone H3 datasets; the concatenated mitochondrial alignment with COI and 16S sequences; and the concatenated alignment with mitochondrial (COI + 16S) and nuclear (Histone H3) sequences.

Maximum likelihood (ML) and Bayesian inference analysis (BI) phylogenetic analyses were conducted

Table 1. Information on all the specimens used in this study for molecular phylogeny, including the specie, location, voucher (collection code) and the COI GenBank accession number.

Specie	Location	Voucher	COI	16S	H3	Reference
Bursatella leachii	Fôrum, Barcelona, Spain	B1	OK066363	-------------	-------------	present study
B. leachii	Fôrum, Barcelona, Spain	B2	OK066356	-------------	-------------	present study
B. leachii	Ebro Delta, Tarragona, Spain	B6	OK066355	-------------	-------------	present study
B. leachii	Ebro Delta, Tarragona, Spain	B7	OK066359	-------------	-------------	present study
APA	Roses (Costa Brava), Spain	Ap1	OK066334	OK066287	OK073542	present study
APA	Roses (Costa Brava), Spain	Ap2	OK066333	OK066286	OK073541	present study
APA	Fôrum, Barcelona, Spain	Ap3	-------------	OK066285	OK073540	present study
APA	Fôrum, Barcelona, spain	Ap4	-------------	OK066284	-------------	present study
APA	Fôrum, Barcelona, Spain	Ap5	-------------	-------------	OK073539	present study
APA	Cala Fosca (Costa Brava), Spain	Ap6	OK066332	OK066283	OK073538	present study
APA	Cala Fosca (Costa Brava), Spain	Ap7	OK066331	OK066282	OK073537	present study
APA	Es Caials (Costa Brava), Spain	Ap8	-------------	OK066281	OK073536	present study
APA	Cala Sant Antoni (Costa Brava), Spain	Ap9	OK066330	OK066280	OK073535	present study
APA	Fôrum, Barcelona, Spain	Ap10	-------------	OK066279	OK073534	present study
APA	Fôrum, Barcelona, Spain	Ap11	-------------	OK066278	OK073533	present study
APA	Rosas (Costa Brava), Spain	AR	OK066337	OK050558	OK073519	present study
APA	Rosas (Costa Brava), Spain	AR1	-------------	OK050559	OK073520	present study
APJ	Bau Cap Falco (Costa Brava), Spain	A1	OK066349	OK050571	-------------	present study
APJ	Bau Cap Falco (Costa Brava), Spain	A2	OK066348	OK050570	OK073532	present study
APJ	Cala Fosca (Costa Brava), Spain	A3	OK066336	OK050569	OK073531	present study
APJ	Cala Aiguablava-Fornells (Costa Brava), Spain	A4	OK066347	OK050568	OK073530	present study
APJ	Cala Aiguablava-Fornells (Costa Brava), Spain	A5	OK066340	OK050567	OK073529	present study
APJ	Mataró, Spain	A6	OK066339	OK050566	OK073528	present study
APJ	Mataró, Spain	A7	OK066346	-------------	OK073527	present study
APJ	Mataró, Spain	A8	OK066345	OK050565	OK073526	present study
APJ	Mataró, Spain	A9	OK066344	OK050564	OK073525	present study
APJ	Port lligat (Costa Brava), Spain	A10	OK066338	OK050563	OK073524	present study
APJ	Fôrum, Barcelona, Spain	A11	OK066343	OK050562	OK073523	present study
APJ	Fôrum, Barcelona, Spain	A12	OK066335	OK050561	OK073522	present study
APJ	Fôrum, Barcelona, Spain	A13	OK066342	OK050560	OK073521	present study
A. fasciata	Israel	--------------	AF343428	AF192298	-------------	Medina *et al*., 2005
A. fasciata	Sao Sebastiao, Sao Paulo, Brazil (1)	MZSP 103234	KM272290	KM272281	-------------	de Oliveira *et al*., 2014

Continued

Table 1 continued

Specie	Location	Voucher	COI	16S	H3	Reference
A. fasciata	Sao Sebastiao, Sao Paulo, Brazil (2)	MZSP 103233	KM272291	KM272282	-------------	de Oliveira *et al*., 2014
A. fasciata	Sao Sebastiao, Sao Paulo, Brazil (3)	MZSP 103250	KM272292	KM272283	-------------	de Oliveira *et al*., 2014
A. fasciata	Sao Sebastiao, Sao Paulo, Brazil (4)	MZSP 103217	KM272295	-------------	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (1)	MZSP 109903	-------------	KM272284	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (2)	MZSP 113776	KM272293	KM272285	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (3)	MZSP 109975	-------------	KM272286	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (4)	MZSP 109978	-------------	KM272287	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (5)	MZSP 104038	KM272294	KM272288	-------------	de Oliveira *et al*., 2014
A. fasciata	Ponta da Praia, Santos, Sao Paulo, Brazil (6)	MZSP 113777	-------------	KM272289	-------------	de Oliveira *et al*., 2014
A. fasciata	Florida, USA	--------------	AF343426	AF192296	-------------	Medina *et al*., 2005
*A. nigrocinct*a	Madang, Papua New Guinea	CASIZ 191092	-------------	MK422792	-------------	Golestani *et al*., 2019
A. nigrocincta	Espiritu Santo, Vanuatu	CASIZ 176792	MK422893	-------------	MK422693	Golestani *et al*., 2019
A. punctata	North Sea (1)	--------------	KR084671	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (2)	--------------	KR084707	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (3)	--------------	KR084714	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (4)	--------------	KR084740	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (5)	--------------	KR084809	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (6)	--------------	KR084865	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (7)	--------------	KR084890	-------------	-------------	Barco *et al*., 2016
A. punctata	North Sea (8)	--------------	KR084908	-------------	-------------	Barco *et al*., 2016
A. punctata	Norwegian archipelago	--------------	KT952472	-------------	-------------	Ware *et al*., 2015
A. punctata	Azores, Portugal (1)	CPIC 01363	MK422894	MK422795	MK422695	Golestani *et al*., 2019
A. punctata	Azores, Portugal (2)	CPIC 01364	MK422895	MK422796	MK422696	Golestani *et al*., 2019
A. punctata	Azores, Portugal (3)	CPIC 01366	MK422896	MK422798	MK422698	Golestani *et al*., 2019
A. punctata	Azores, Portugal (4)	CPIC 01365	-------------	MK422797	MK422697	Golestani *et al*., 2019
A. punctata	Azores, Portugal (5)	CPIC 01356	-------------	MK422794	MK422694	Golestani *et al*., 2019
A. punctata	Cadiz, Spain	CPIC 01359	MK422897	-------------	MK422699	Golestani *et al*., 2019
A. punctata	Santander, Spain	CPIC 01424	MK422898	MK422799	MK422700	Golestani *et al*., 2019
A. punctata	Pontevedra, NW Spain	--------------	AY345019	-------------	-------------	Grande *et al*., 2004
A. punctata	Brittany, France (1)	ZSM Mol 20180005	MK422899	MK422800	MK422701	Golestani *et al*., 2019
A. punctata	Brittany, France (2)	ZSM Mol 20180005	MK422900	MK422801	MK422702	Golestani *et al*., 2019

Continued

Table 1 continued

Specie	Location	Voucher	COI	16S	H3	Reference
A. punctata	Brittany, France (3)	ZSM Mol 20180006	MK422901	MK422802	MK422703	Golestani *et al*., 2019
A. punctata	Banyuls sur Mer, France (1)	CPIC 01360	MK422902	MK422803	MK422704	Golestani *et al*., 2019
A. punctata	Banyuls sur Mer, France (2)	CPIC 01360	MK422903	MK422804	MK422705	Golestani *et al*., 2019
A. punctata	Banyuls sur Mer, France (3)	CPIC 01362	MK422904	MK422805	MK422706	Golestani *et al*., 2019
A. punctata	Giannutri Island, Italy (1)	--------------	MK422905	MK422806	-------------	Golestani *et al*., 2019
A. punctata	Giannutri Island, Italy (2)	--------------	MK422906	MK422807	MK422707	Golestani *et al*., 2019
A. punctata	Giannutri Island, Italy (3)	--------------	MK422907	MK422808	MK422708	Golestani *et al*., 2019
A. punctata	Giannutri Island, Italy (4)	CPIC 01422	MK422908	MK422809	MK422709	Golestani *et al*., 2019
A. punctata	Livorno, Italy (1)	--------------	MK422909	MK422810	MK422710	Golestani *et al*., 2019
A. punctata	Livorno, Italy (2)	--------------	MK422910	MK422811	-------------	Golestani *et al*., 2019
A. punctata	Capraia Island, Italy (1)	--------------	MK422911	MK422813	MK422712	Golestani *et al*., 2019
A. punctata	Capraia Island, Italy (2)	--------------	-------------	MK422812	MK422711	Golestani *et al*., 2019
A. punctata	Capraia Is., Italy (3)	CPIC 01423	-------------	MK422814	MK422713	Golestani *et al*., 2019
A. punctata	Gallipoli, Italy (1)	CPIC 01353	-------------	MK422815	MK422714	Golestani *et al*., 2019
A. punctata	Gallipoli, Italy (2)	CPIC 01358	-------------	MK422816	MK422716	Golestani *et al*., 2019
A. punctata	Gallipoli, Italy (3)	CPIC 01357	-------------	-------------	MK422715	Golestani *et al*., 2019
A. punctata	Levanto, Italy	CPIC 01421	MK422912	MK422817	MK422717	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (1)	--------------	MK422913	MK422818	MK422718	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (2)	--------------	MK422914	MK422819	MK422719	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (3)	--------------	MK422915	MK422820	MK422720	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (4)	--------------	MK422916	MK422821	MK422721	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (5)	--------------	MK422917	MK422822	MK422722	Golestani *et al*., 2019
A. punctata	Rhodes, Greece (6)	--------------	MK422918	MK422823	MK422723	Golestani *et al*., 2019
A. punctata	Heraklion, Crete, Greece	CPIC 01388	MK422919	MK422824	MK422724	Golestani *et al*., 2019
A. punctata	Cape Kamenjak, Croatia (1)	ZSM Mol 20090003-1	MK422920	MK422825	MK422725	Golestani *et al*., 2019
A. punctata	Cape Kamenjak, Croatia (2)	ZSM Mol 20090003-2	MK422921	MK422826	MK422726	Golestani *et al*., 2019
A. punctata	Cape Kamenjak, Croatia (3)	ZSM Mol 20090003-3	MK422922	MK422827	MK422727	Golestani *et al*., 2019
A. punctata	Cape Kamenjak, Croatia (4)	--------------	MK422923	MK422828	MK422728	Golestani *et al*., 2019
A. punctata	Umago, Croatia	CPIC 01374	MK422913	MK422829	MK422729	Golestani *et al*., 2019
A. punctata	Helgoland, North Sea	--------------	-------------	AF249253	-------------	Wollscheid-Lengeling *et al*., 2001
A. punctata	Southwest Turkey (1)	--------------	MF784857	-------------	MK184485	Yokes, 2018
A. punctata	Southwest Turkey (2)	--------------	MF784858	-------------	MK184486	Yokes, 2018

Continued

Table 1 continued

Specie	Location	Voucher	COI	16S	H3	Reference
A. punctata	Southwest Turkey (3)	--------------	MF784859	-------------	MK184487	Yokes, 2018
A. punctata	Southwest Turkey (4)	--------------	MF784860	-------------	MK184488	Yokes, 2018
A. punctata	Southwest Turkey (5)	--------------	MF784861	-------------	MK184490	Yokes, 2018
A. punctata	Southwest Turkey (6)	--------------	MF784862	-------------	MK184491	Yokes, 2018
A. punctata	Southwest Turkey (7)	--------------	MF784863	-------------	MK184492	Yokes, 2018
A. punctata	Southwest Turkey (8)	--------------	MF784864	-------------	MK184493	Yokes, 2018
A. punctata	Southwest Turkey (9)	--------------	-------------	-------------	MK184489	Yokes, 2018

Table 2. Primers used for each gene.

Gene	Primers Name	Direction	Secuencia	Reference
COI	LCO1490	Forward	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer *et al*. 1994
COI	HCO2198	Reverse	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al*. 1994
COI	Nancy	Reverse	5′-CCCGGTAAAATTAAAATATAAACTTC-3′	Simon *et al* 1994
16S	16Sar	Forward	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi *et al*. 1991
16S	16Sbr	Reverse	5′-CCGGTYTGAACTCAGATCAYGT-3′	Palumbi *et al*. 1991
H3	H3F	Forward	5′-ATGGCTCGTACCAAGCAGACVGC-3′	Bernhard 1999
H3	H3R	Reverse	5′-CTTCTGGCGAGTTGCATATCCT-3′	Bernhard 1999

using the best partition scheme and evolutionary model for each partition selected with Partition Finder v2.1.1. (Lanfear *et al*., 2017). ML analyses were performed with RAxML 8.0 (Stamatakis, 2014) with 10000 replicates, random initial trees, and estimated parameters evolutionary models. Branches with bootstrap values above 70% were considered supported (Hillis & Bull, 1993). The first 10% of the generations were discarded as a burn-in for the analyses. Posterior probability node values (PP) higher than 0.9 were considered supported (Huelsenbeck & Rannala, 2004). The resulting trees were visualized with iToL (https://itol.embl.de/itol.cgi).

Species delimitation under the ASAP algorithm and Haplotype network

The p-genetic distances and intraspecific divergence analyses was calculated for the COI dataset using MEGA X (Kumar *et al*., 2016) using the Kimura 2-parameter model (Kimura, 1980). COI sequences were also used for species delimitation analyses with Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al*., 2021). Jukes Cantor (JC69), Kimura (K80) and Simple Distance substitution models were tested. Finally, a haplotype network for the COI dataset was constructed using the TCS algorithm (Clement *et al*., 2000) implemented in the program PopArt 1.7 program v4.8.4 (Leigh & Bryant, 2015).

Biological data

Most of the observations of APJ and APA have been carried out in different locations on the Costa Brava (NE Spain) (Fig. 1). Data on the biology of APJ and APA in the Catalonian coast is based on long-term field observations by one of the authors (MB), compiled for more than 40 years. Moreover, records of specimens from other sources, mainly from members of the GROC (Grup de Recerca dels Opistobranquis de Catalunya) as well as photographs published on the internet have been examined. All data are provided in Table 3. Data collected include the color pattern of the specimen, date, locality. When possible, the number of specimens, the substrate, and any noteworthy behavior such as copulation and the presence of egg masses, were recorded both in the field observations or photographs. In total, all the observational data correspond to approximately 150 hours of scuba diving by different divers. Due to the small size and camouflage of APJ specimens, masses of frondose red algae such as *Sphaerococcus coronopifolius* Stackhouse and *Plocamium cartilagineum* (Linnaeus) P.S. Dixon were collected in different locations and the specimens were separated from the algae in the laboratory, by placing them in small-capacity aquaria. Some specimens of APJ and APA were induced to ink by touching them, others were collected with their respective algae that serve as food and habitat to observe their behavior in the laboratory. The

Fig. 1: Specimens capture locations of APJ and APA. In red the zone of the Costa Brava that is between 41.84194 °N, 3.10583 °W and 42.43638 °N, 317611 °W.

Table 3. Observations of APJ (as *A. parvula*) and APA on the Catalonian coast. Notes: the observations not reported by M. Ballesteros, M. Pontes and E. Madrenas correspond to members of the GROC (Grup de Recerca dels opistobranquis de Catalunya, https://opistobranquis.org/es/home) and are documented with photographs. CB= localities on the Costa Brava. * means observation not quantified in number of individuals. Blank substrate cells = no data.

Species/Size	Observer	date	locality	other data	substrate
APJ	M. Ballesteros	27/02/1985	Punta de Santa Anna (Blanes, CB)	*	on *Codium* and *Halopteris*
APJ	M. Pontes	01/03/1985	Punta de Santa Anna (Blanes, CB)	*	
APJ	M. Pontes	27/05/1985	Pedra de Deu (Medes Is., CB)	*	
APJ	M. Pontes	27/05/1985	Punta de Santa Anna (Blanes, CB)	*	
APJ	M. Ballesteros	27/05/1985	Meda Gran (Illes Medes, CB)	*	on hidrarians and red algae
APJ	M. Ballesteros M. Pontes	07/06/1985	Mar Menuda (Tossa de Mar, CB)	*	
APJ	M. Pontes	09/03/1986	Mar Menuda (Tossa de Mar, CB)	*	
APJ	M. Ballesteros	12/05/1999	Cadaqués, CB	* copulate	
APJ	M. Ballesteros	22/05/2000	Roses, CB	*	
APJ	M. Pontes	01/02/2002	Illa Mateua (L. Escala, CB)	*	
APJ	M. Ballesteros	21/03/2002	Punta de Santa Anna (Blanes, CB)	*	
APJ.	M. Ballesteros	15/05/2002	Es Caials (Cadaqués, CB)	1	between red algae
APJ	M. Ballesteros	17/05/2002	L'Almadrava (Roses, CB)	2	between algae

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APJ	M. Ballesteros	23/05/2002	Es Caials (Cadaqués, CB)	1	
APJ	M. Ballesteros	21/05/2003	Es Caials (Cadaqués, CB)	1	
APJ	M. Ballesteros	23/05/2003	Punta de la Creu (Roses, CB)	1	
APJ.	M. Ballesteros	14/05/2004	Cala dels Gats (Palamós, CB)	*	
APJ	M. Ballesteros	24/05/2004	Punta de Santa Anna (Blanes, CB)	*	
APJ	M. Ballesteros	16/05/2005	L'Almadrava (Roses, CB)	*	
APJ	M. Pontes	26/01/2008	Es Caials, CB	*	
APJ	M. Pontes	02/02/2008	Es Caials, CB	*	
APJ	J. Regás	02/03/2008	Illa Mateua (L´ Escala, CB)	*	
APJ	M. Pontes	03/05/2008	Es Caials, CB	*	
APJ	M. Ballesteros	19/05/2008	Bau Cap Falcó (Roses, CB)	*	
APJ	M. Ballesteros	18/05/2009	El Bisbe de Norfeu, CB	1	
APJ	M. Ballesteros	19/05/2010	Punta Falcó, CB	7	between algae
APJ	M. Pontes	20/02/2010	Es Caials, CB	*	
APJ.	M. Ballesteros	23/02/2010	Cala Sant Francesc (Blanes, CB)	*	on *Halopteris*
APJ	M. Ballesteros	23/02/2010	Cala Sant Francesc (Blanes, CB)	*	on *Sphaerococcus*
APJ	M. Pontes	06/03/2010	Es Caials, CB	*	
APJ	A. López-Arenas	06/03/2010	Mar Menuda (Tossa de Mar, CB)	*	
APJ	M. Pontes	17/04/2010	Cala Aiguafreda, CB	*	
APJ	GROC 2010	03/07/2010	Ferranelles (Illes Medes, CB)	*	
APJ	M. Pontes	22/01/2011	Es Caials, CB	*	
APJ	M. Pontes	16/04/2011	La Caleta (Palamós, CB)	*	
APJ	M. Ballesteros	23/05/2011	Mar Menuda (Tossa de Mar,)	*	on *Halopteris*
APJ	M. Pontes	28/05/2011	Es Caials (Cadaqués, CB)		
APJ	M. Pacareu	21/04/2012	Pota del Llop (Medes Is., CB)	*	
APJ	M. Ballesteros	21/05/2012	El Bisbe de Norfeu, CB	11	
APJ	M. Ballesteros	04/06/2012	Es Caials (Cadaqués, CB)	*	
APJ	M. Ballesteros	05/06/2012	Cap Norfeu (Roses, CB)	*	
APJ	M. Pontes	30/06/2012	El Port de la Selva, CB	*	
APJ	L. Toll	10/11/2012	Salpatxot (Medes Is., CB)	*	
APJ	E. Madrenas	18/01/2013	Punta del Romaní (L'Escala, CB)	*	
APJ	E. Madrenas	17/04/2013	Mataró	*	
APJ	M. Ballesteros	07/05/2013	Mar Menuda (Tossa de Mar, CB)	1	

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APJ	E. Madrenas	19/05/2013	Punta del Romaní (L'Escala, CB)	*	
APJ	M. Ballesteros	23/05/2013	Els Caials (Cadaqués, CB)	*	on *Sphaerococcus*
APJ	M. Ballesteros	10/06/2013	Punta Falconera (Roses, CB)	1	
APJ	M. Ballesteros	11/06/2013	El Gat de Norfeu (Roses, CB)	1	
APJ	E. Madrenas	25/06/2013	Escollera Port (Tarragona)	3	on *Codium*
APJ	E. Madrenas	05/07/2013	Cala dels Gats (Palamós, CB)	*	
APJ	X. Salvador	10/12/2013	Palamós, CB	*	
APJ	X. Salvador	14/12/2013	Cala Bramant, CB	*	
APJ	X. Salvador	04/01/2014	Llançá, CB	*	
APJ	M. Pontes	22/02/2014	Cala Aiguafreda, CB	*	
APJ	M. Pontes	08/03/2014	Cala Aiguafreda, CB	*	
APJ	M. Pontes	18/04/2014	Cala Margarida, CB	*	
APJ	M. Pontes	26/04/2014	Cala Sa Tuna (Begur, CB)		
APJ	G. Morera	14/05/2014	La Caleta (Palamós, CB)	* copulate	on *Sphaerococcus*
APJ	M. Ballesteros	26/05/2014	Punta de la Creu (Roses, CB)	2	
APJ	M. Ballesteros	10/06/2014	Bau de Punta Falconera (Roses, CB)	15	on *Sphaerococcus*
APJ	M. Ballesteros	11/06/2014	Punta de la Creu (Roses, CB)	1	
APJ	X. Lindo	06/09/2014	Cala Margarida (Palamós, CB)	*	on *Flabellia*
APJ	M. Pontes	24/01/2015	Es Caials (Cadaqusés, CB)	*	
APJ	M. Pontes	28/02/2015	Es Caials (Cadaqués, CB)	*	
APJ	M. Pontes	14/03/2015	Es Caials (Cadaqués, CB)	*	
APJ	M. Pontes	16/05/2015	Es Caials (Cadaqués, CB)	*	
APJ	M. Ballesteros	25/05/2015	Bau de Punta Falconera (Roses, CB)	5	
APJ	M. Ballesteros	26/05/2015	Es Caials (Cadaqués, CB)	3	
APJ	M. Ballesteros	08/06/2015	La Trona (Roses, CB)	3	
APJ	M. Ballesteros	09/06/2015	Punta Falconera (Roses, CB)	7	
APJ	X. Salvador	14/07/2015	Les Sofreres (Sant Feliu de Guíxols, CB)	*	
APJ	G. Mas	12/09/2015	Sa Tuna, CB	*	
APJ	F. Asensio	29/11/2015	Es Caials (Cadaqués, CB)	*	
APJ	E. Madrenas	05/12/2015	Punta del Falaguer (Medes Is., CB)	*	
APJ	E. Madrenas	16/04/2016	Salpatxot (Medes Is., CB)	*	
APJ	M. Ballesteros	23/05/2016	Cap Falcó (Roses, CB)	1	
APJ	M. Pontes	14/01/2017	Es Caials (Cadaqués, CB)	*	

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APJ	X. Salvador	02/03/2017	La Caleta (Palamós, CB)	*	
APJ	M. Casanovas	06/03/2017	Tossa de Mar, CB	*	on green algae
APJ	X. Salvador	15/04/2017	Llafranc, CB	*	on red algae
APJ	X. Salvador	18/04/2017	Cala Aiguaxelida, CB	*	on red algae
APJ	X. Salvador	08/05/2017	Cala Ventosa (Sant Feliu de Guíxols, CB)	*	on red algae
APJ	S. Fuertes	20/05/2017	El Guix (Medes Is., CB)	*	
APJ	X. Salvador	26/12/2017	Cala Montgó (L´Escala, CB)	*	on *Sphaerococcus*
APJ	X. Salvador	31/01/2018	Cala Aiguafreda, CB	*	
APJ	X. Salvador	08/03/2018	Cala Sa Tuna, CB	*	on *Ulva*
APJ	X. Salvador	24/04/2018	Punta del Romaní (L´Escala, CB)	*	on red algae
APJ	X. Salvador	27/04/2018	El Fòrum (Barcelona)	*	on red algae
APJ	G. Biscop	16/05/2018	Tascó Gros (Medes Is., CB)	*	on green algae
APJ	X. Salvador	25/05/2018	Cala Aiguafreda, CB	*	on red algae
APJ	M. Ballesteros	04/06/2018	Cap Falcó (Roses, CB)	2	on *Sphaerococcus*
APJ	M. Ballesteros	05/06/2018	La Trona (Roses, CB)	5	on *Sphaerococcus*
APJ	A. Mares	05/07/2018	Cala El Bofill (Cadaqués, CB)	*	
APJ	J. Fernández	08/08/2018	Platja del Pas (El Port de la Selva, CB)	*	
APJ	X. Lindo	09/02/2019	Cala Sa Tuna, CB	*	
APJ	X. Salvador	13/03/2019	Tamariu, CB	*	
APJ	M. Ballesteros	21/05/2019	El Bisbe (Roses, CB)	5	on *Sphaerococcus*
APJ	M. Ballesteros	27/05/2019	Bau Cap Falcó (Roses, CB)	4	on *Sphaerococcus*
APJ	M. Ballesteros	28/05/2019	Cala Sant Antoni (Cadaqués, CB)	4	on *Sphaerococcus*
APJ	M. Ballesteros	03/06/2019	Bau Cap Falcó (Roses, CB)	3	
APJ	X. Salvador	07/05/2020	Punta d´en Bosch (Sant Feliu de Guíxols, CB)	*	
APJ	X. Salvador	30/01/2021	Coves Cala Maset (Sant Feliu de Guíxols, CB)	*	
APJ	M. Ballesteros	25/05/2021	Bau Cap Falcó (Roses, CB)	13	
APA	M. Ballesteros	26/05/1977	Cubelles	61 + egg masses + copulate	under stones, on *Dictyota*
APA	M. Ballesteros	09/11/1978	Mar Menuda (Tossa de Mar, CB)	*	
APA	M. Ballesteros	14/03/1979	Punta de Santa Anna (Blanes, CB)	*	
APA	M. Ballesteros	25/05/1979	Cubelles	*	under stones
APA	M. Ballesteros	14/06/1979	Cubelles	*	under stones
APA	M. Ballesteros	25/11/1979	Cala Sant Francesc (Blanes, CB)	*	

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APA	M. Ballesteros	10/04/1982	Cubelles	*	under stones
APA	M. Ballesteros	11/04/1982	Cubelles	*	under stones
APA	M. Ballesteros	22/05/1985	Cubelles	*	under stones
APA	M. Ballesteros	07/05/1986	Cubelles	*	under stones
APA	M. Ballesteros	01/05/1993	Roses, CB	*	
APA	M. Pontes	01/08/2000	Torre Valentina (Calonge, CB)	*	
APA	M. Ballesteros	06/05/2002	Cala Fosca (Palamós, CB)	*	
APA	M. Ballesteros	27/08/2003	Punta de Santa Anna (Blanes, CB)	*	
APA	M. Ballesteros	28/08/2003	Punta de Santa Anna (Blanes, CB)	*	
APA	M. Ballesteros	18/05/2005	Es Caials (Cadaqués, CB)	1	
APA	M. Pontes	08/04/2006	Es Caials (Cadaqués, CB)	*	
APA	E. Madrenas	20/02/2010	Sa Tuna (Begur, CB)	*	
APA	E. Madrenas	27/03/2010	La Caleta (Palamós, CB)	*	
APA	A. López-Arenas	29/03/2010	La Caleta (Palamós, CB)	*	
APA	GROC	03/07/2010	Ferranelles (Illes Medes, CB)	*	
APA	A. López-Arenas	25/02/2012	Cala Aiguafreda, CB	*	
APA	M. Ballesteros	22/05/2012	Es Caials (Cadaqués, CB)	1 juv.	under stones
APA	M. Ballesteros	01/06/2012	Cala Fosca (Palamós, CB)	*	
APA	M. Ballesteros	05/06/2012	Cap Norfeu (Roses, CB)	*	
APA	E. Madrenas	25/11/2012	Punta del Romaní (L’Escala, CB)	*	
APA	E. Madrenas	25/11/2012	Punta del Romaní (L’Escala, CB)	*	
APA	E. Madrenas	26/03/2013	La Caleta (Palamós, CB	*	
APA	E. Madrenas	31/03/2013	Aiguafreda (Begur, CB)	*	
APA	M. Ballesteros	28/05/2013	Cala Fosca (Palamós, CB)	3	under stones
APA	M. Ballesteros	29/05/2013	Cala Fosca (Palamós, CB)	3	under stones
APA	E. Madrenas	01/06/2013	Cala Margarida (Palamós, CB)	*	
APA	E. Madrenas	25/06/2013	Barcelona breakwater	*	
APA	M. Pontes	29/03/2014	Cala Sa Tuna (Begur, CB)	*	
APA	X. Salvador	11/04/2014	La Llosa (Palamós, CB)	*	
APA	G. Mas	02/08/014	Cala Margarida, CB	*	
APA	X. Salvador	10/04/2015	Cova d´Ariadna (Sa Riera, CB)	*	on algae
APA	E. Madrenas	18/04/2015	Tascons Petits (Medes Is., CB)	*	
APA	M. Ballesteros	26/05/2015	Es Caials (Cadaqués, CB)	3	under stones

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APA	M. Ballesteros	27/05/2015	Cala Fosca (Palamós, CB)	*	under stones
APA	M. Ballesteros	01/07/2015	Cubelles	12	under stones
APA	M. Badía	28/07/2015	Sant Pol	*	
APA	M. Codina	01/05/2016	Cala Santa Anna, CB	*	
APA	M. Ballesteros	24/05/2016	Es Caials (Cadaqués, CB)	10 + copulate	under stones
APA	M. Ballesteros	25/05/2016	La Fosca (Palamós, CB)	11 + eggs masses	under stones
APA	M. Ballesteros	27/05/2016	La Fosca (Palamós, CB)	10	under stones
APA	M. Ballesteros	8/06/2016	Es Caials (Cadaqués, CB)	4 + copulate	under stones
APA	X. Salvador	18/04/2017	Cala Aiguaxelida, CB	*	on algae
APA	E. Madrenas	18/11/2017	Llosa del Falaguer (Medes Is., CB)	*	
APA	M. Bosch	29/12/2017	La Gavina (Sant Feliu de Guixols, CB)	*	
APA	X. Salvador	27/04/2018	El Fòrum (Barcelona)	*	
APA	A. Mares	16/05/2018	Cala El Bofill (Cadaqués, CB)	*	
APA	G. Alvarez	16/05/2018	El Forum (Barcelona)	*	
APA	M. Ballesteros	17/05/2018	El Forum (Barcelona)	> 150 + copulate	under stones
APA	M. Ballesteros	24/05/2018	La Fosca (Palamós, CB)	7	under stones
APA	X. Salvador	24/05/2018	Punta del Romaní (L´Escala, CB)	*	
APA	X. Salvador	25/05/2018	Cala Aiguafreda, CB	*	on red algae
APA	M. Ballesteros	04/06/2018	Cap Falcó (Roses, CB)	1	
APA	M. Ballesteros	06/06/2018	Es Caials (Cadaqués, CB)	5	
APA	M. Ballesteros	05/07/2018	El Fòrum (Barcelona)	7	under stones
APA	M. Ballesteros	12/07/2018	El Fòrum (Barcelona)	*	
APA	X. Salvador	09/03/2019	Cala Sa Tuna, CB	*	
APA	A. Parera	02/04/2019	El Fòrum (Barcelona)	*	
APA	M. Bosch	16/04/2019	Cala Aiguablava, CB	*	on algae
APA	A. Parera	13/05/2019	El Fòrum (Barcelona)	*	on red algae
APA	M. Ballesteros	22/05/2019	Es Caials (Cadaqués, CB)	1	under stones
APA	M. Ballesteros	24/05/2019	La Fosca (Palamós)	13 + egg masses	under stones
APA	M. Ballesteros	05/06/2019	Es Caials (Cadaqués, CB)	5	under stones
APA	A. Parera	15/06/2019	El Fòrum (Barcelona)	*	
APA	J. Vilanova	06/07/2019	Tamariu, CB	*	
APA	C. Escarré	13/07/2019	El Fòrum (Barcelona)	*	

Continued

Table 3 continued

Species/Size	Observer	date	locality	other data	substrate
APA	M. Ballesteros	18/07/2019	El Fòrum (Barcelona)	4	under stones
APA	X. Salvador	15/06/2020	Coves Cala Maset (Sant Feliu de Guíxols, CB)	*	on algae
APA	A. Mares	30/06/2020	Cala El Bofill (Cadaqués, CB)	*	on algae
APA	X. Salvador	12/07/2020	Cala Aiguafreda, CB	*	on algae
APA	M. Ballesteros	2/06/2021	Es Caials (Cadaqués, CB)	7 + egg masses	under stones
APA	E. Badosa	06/04/2021	Cala Aiguaxelida, CB	*	on algae
APA	J. M. Flamarich	09/08/2021	Es Caials (Cadaqués, CB)	*	on algae

feeding and reproductive behavior, as well as the deposition of egg masses, were observed in the laboratory.

Results

Phylogenetic trees

The mitochondrial alignment and the concatenated COI, 16S and H3 alignment included, respectively, 1113 bases and 1441 bases with 103 sequences. All the trees designed showed a topology with a similar or very similar behavior, giving us Bayesian analysis as maximum likelihood where clades were recovered; well defined as that of *A. fasciata* from the western Atlantic with Israel (Bs = 87), as well as another well-defined clade from *A. nigrocincta* from the Indo-Pacific (Bs = 97), another clade between *A. punctata* from the eastern Atlantic (only those from Azores, Portugal) and a specimen from southwest Turkey and a large clade where all the specimens collected by us on the Catalonian (Mediterranean) coast (APJ and APA) nest with the vast majority of the *A. punctata* of the eastern Atlantic and the Mediterranean (Bs = 72) (Fig. 2).

Species delimitation

The species recovered in the ASAP species delimitation analysis correspond to well-supported clades that include four different hypothetical “groups” of which it stands out; group four: with all the APJ and the APA collected by us together with all the *A. punctata* of the Mediterranean and the great majority of the eastern Atlantic (represented with the red bar); the other groups are constituted as follows: group one with the *A. fasciata* from the western Atlantic and Israel (Green Bar), group two: the *A. nigrocincta* from the Indo-Pacific and finally group three: the *A. punctata* from the eastern Atlantic (only those of Azores, Portugal) together with a specimen from southwest Turkey (blue bar) (Fig. 2).

Haplotype network

The COI dataset included 668 bases in the species *A. punctata*. The haplotype network showed five different haplotypes, differentiated by several substitutions from each other. The *A. punctata* clade has a haplotype that differs by many substitutions from the eastern Atlantic (only those from Azores, Portugal), and one specimen from southwest Turkey; a few substitutions are also observed in specimens of *A. punctata* collected by us, together with specimens of the same species found both in the Mediterranean and Eastern Atlantic; the haplotype network shows a clear geographic structure in specimens of *A. punctata* from the Mediterranean, those from the eastern Atlantic and all the specimens collected by us (APJ and APA) in two well-defined groups: APJ7 which includes the specimens collected by us on the Catalonian coast such as APJ4, APJ5, APJ11, APJ12, APA2 and APA3; together with *A. punctata* from the Mediterranean of France, Italy, Turkey, Greece and Croatia; and other *A. punctata* from the eastern Atlantic, North Sea and the Norwegian archipelago; the other group is *A. punctata* from Livorno, Italy, which groups *A. punctata* from the Mediterranean of Spain, Greece and Turkey, with *A. punctata* from the eastern Atlantic of the North Sea and the APJ6 collected by us on the Catalonian coast (Fig. 3).

External morphology

The smallest specimens (APJ), about 4-20 mm long, have a reddish-brown coloration due to a dense crosslinking of this color that delimits polygonal areas in the center, where there is usually an irregular white spot; also, we found some juveniles of length between 20–25 mm of brown coloration with white spots and morphology very similar to the adult specimens of *A. punctata* (Fig. 4). There are also usually larger white spots scattered along the lateral walls of the body and parapodium, which may be grouped into larger whitish spots. When the animal is moving, the body is elongated and slender and the head is joined to the rest of the visceral mass by a long neck, which can be shrunk and stretched. To the right of the body, the spermatic sulcus is clearly visible, which runs

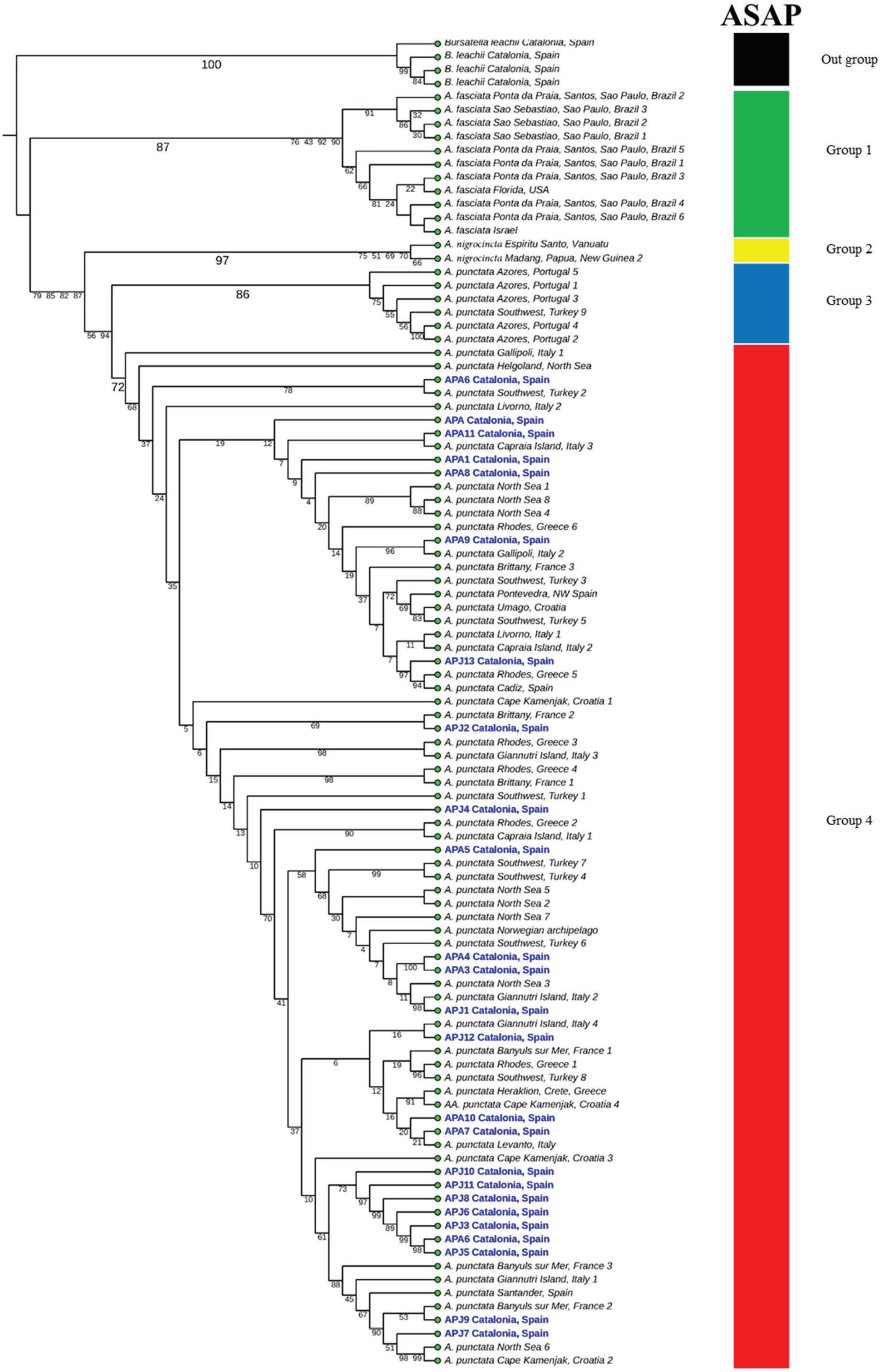

Fig. 2: Phylogenetic tree from the obtained sequences and those of Genbak. Consensus tree topology of mtDNA haplotypes and ASAP species delineation colored clusters; the specimens placed by us (APJ and APA), are in blue.

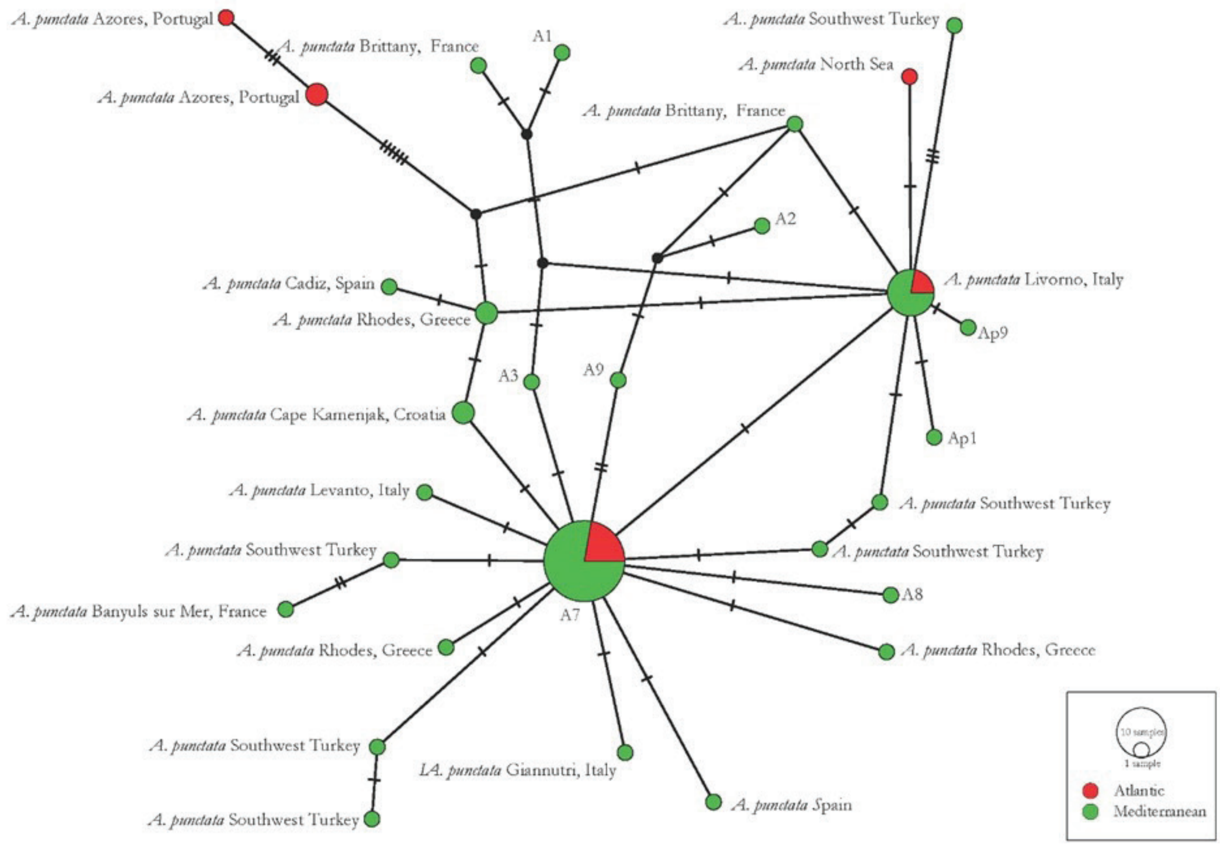

Fig. 3: Haplotype networks based on COI sequence data, the geographic region of origin of the specimens is represented in color. The relative size of the circles is proportional to the number of sequences of the same haplotype.

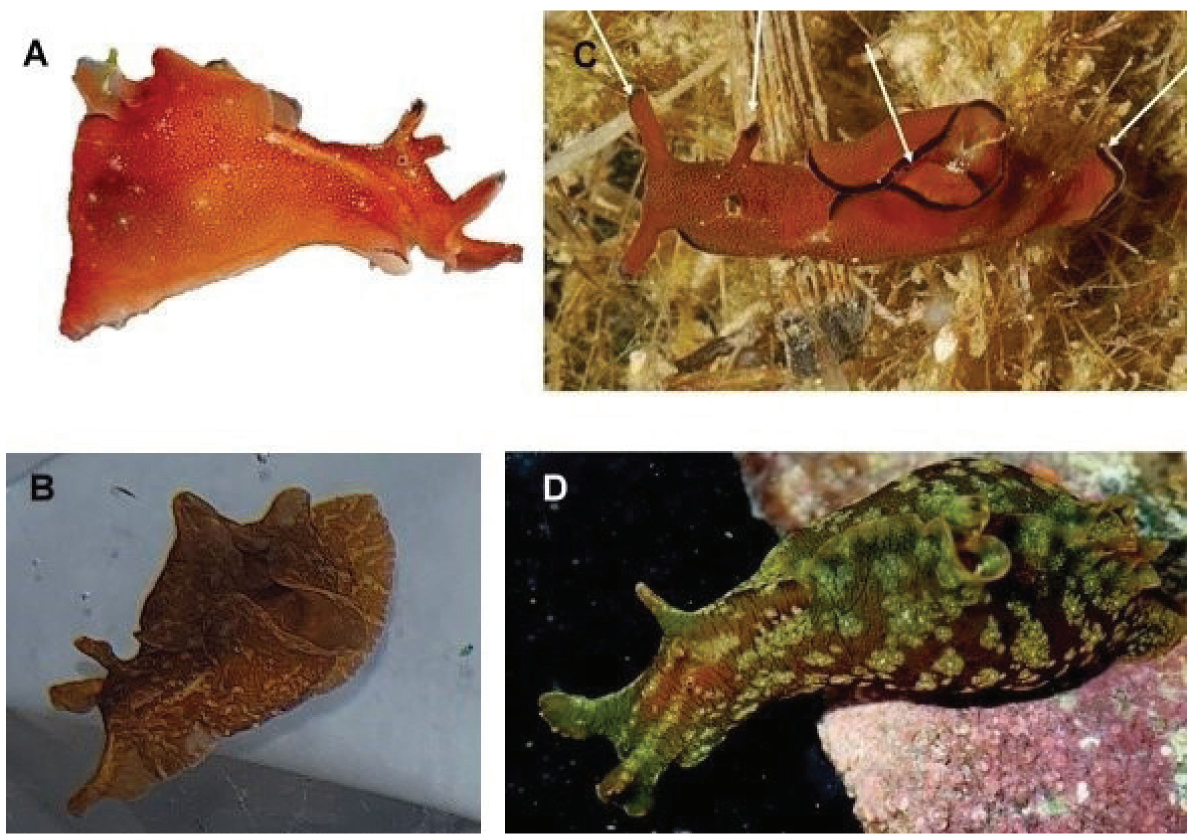

Fig. 4: Comparison between A and C. the "typical" small reddish APJ specimens with a length between 5-20 mm (cited as *A. parvula* in the Mediterranean), B. with juvenile *A. punctata* in the intermediate process to pass to the adult phase with a length between 20-25 mm and D. APA adult specimens with a length between 25-100 mm.

from the anterior end of the parapodium to almost the base of the right cephalic tentacle. The cephalic tentacles are curled throughout and the rhinophores have a slit in their lateral area; both are the same color as the body except for its apical area, which is usually black. The black eyes are located in a slightly prominent whitish areas somewhat to the right and in front of the base of the rhinophores. The mantle has a wide oval foramen through which the shell can be seen. The parapodia are not very wide, they are slightly undulating and they are fused behind. The edge of each parapodium is black, sometimes interrupted by white spots and in some specimens a fine white marginal line is observed. The parapodia are joined in the posterior end, which prevents the animal from making swimming movements. On the dorsal inside of the parapodium there are usually white granulations that form a spot of this very apparent color and that also adorn the anal siphon. The gill is pink and semitransparent. The foot is pink except in the anterior and posterior areas whose margins are black. There may also be white granulations on the rest of the foot margin (Fig. 5). The penis is spoon-shaped and pink and semi-transparent.

Above ~ 25 mm in length up (APA), specimens are more or less dark olive green with numerous white marks throughout the body, including the rhinophores and the dorsal area of the foot, sometimes gathered in specific

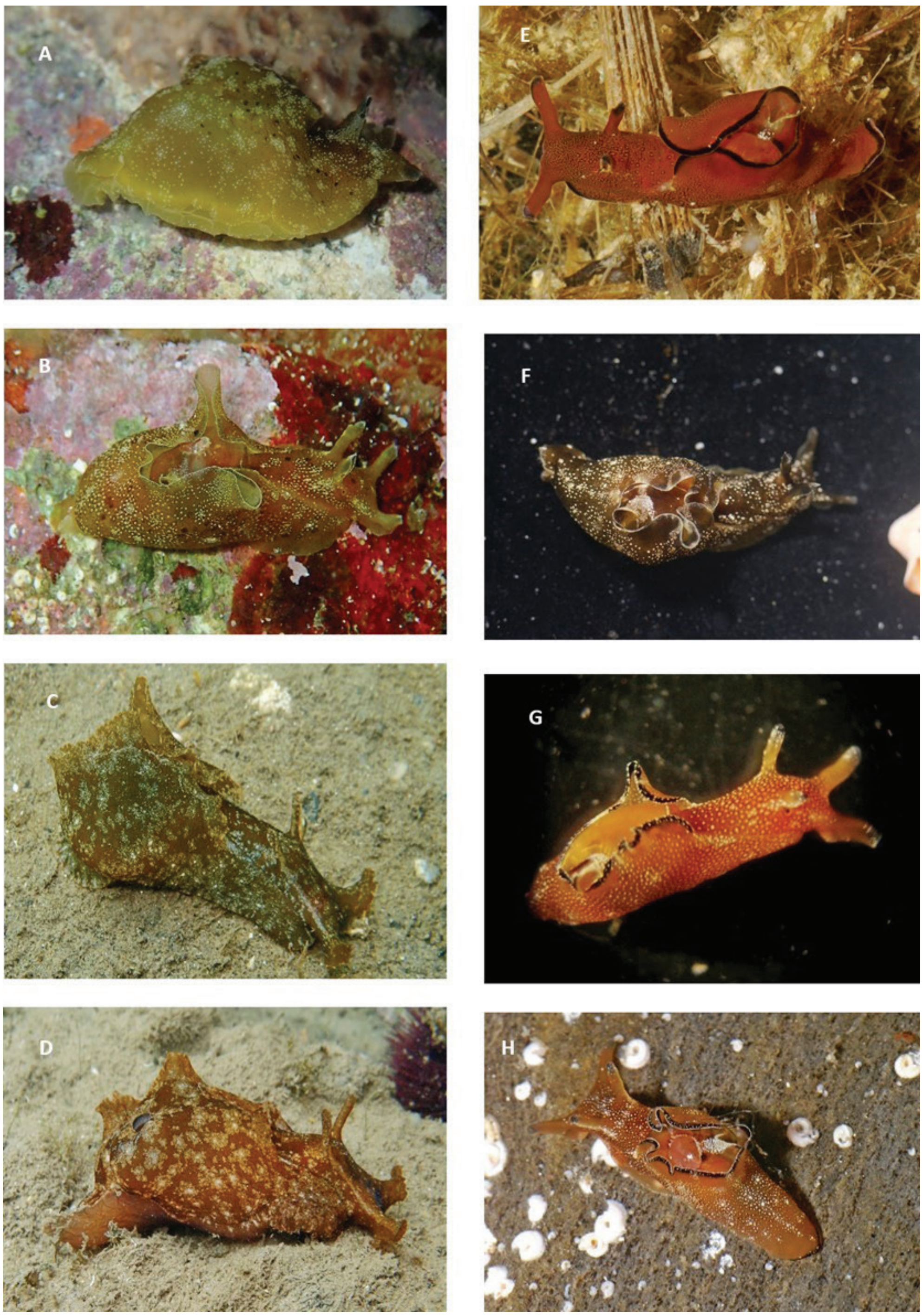

Fig. 5: Specimens of APA 65 mm length (left) and APJ 20 mm length (right). Localities. A: Es Caials; B: La Fosca; C: Fòrum; D: Fòrum; E: Bau del Cap Falcó; F: La Fosca; G: Cap Falcó; H: Bau del Cap Falcó.

areas. In these larger specimens the black pigmentation of cephalic tentacles, rhinophores, margin of the parapodium and anterior and posterior borders of the foot is no longer observed. In some darker specimens a white line is very apparent on the edge of the parapodium. The largest specimens (10-15 cm) are usually brownish in color and the white spots can cover a large part of the body or be very small and widely dispersed. In some of the larger specimens the spots are cream-colored with a whitish central area. The foot is wide behind and acts like a suction cup.

The shells of the specimens of *A. punctata* (Fig. 6) measure 10 mm in a 25 mm living animal and 35 mm in a 65 mm specimen, they are oval, wider about half their length, with a curved posterior end, the right part adjacent to the protoconch is slightly concave.

Radular and jaw structure

The radular formula of a 20 mm APJ specimen collected alive in La Fosca, Palamos, Barcelona, NE Spain was 35×5.10.1.10.5. The central tooth has a geometric shape similar to a trapezium with a very elongated base, a central triangular cusp and several lateral protuberances without clearly defined denticles, the teeth measure on average 54.91 wide × 111.95 µm long; the lateral teeth are narrow and have a main cusp with an inner lateral denticle and 2-3 denticles on the outer side; the outermost teeth are undeveloped, have an outer margin and an inner margin with a well-defined stalk, 79.49 µm long × 12.34 µm wide (Fig. 7 A, B and C right).

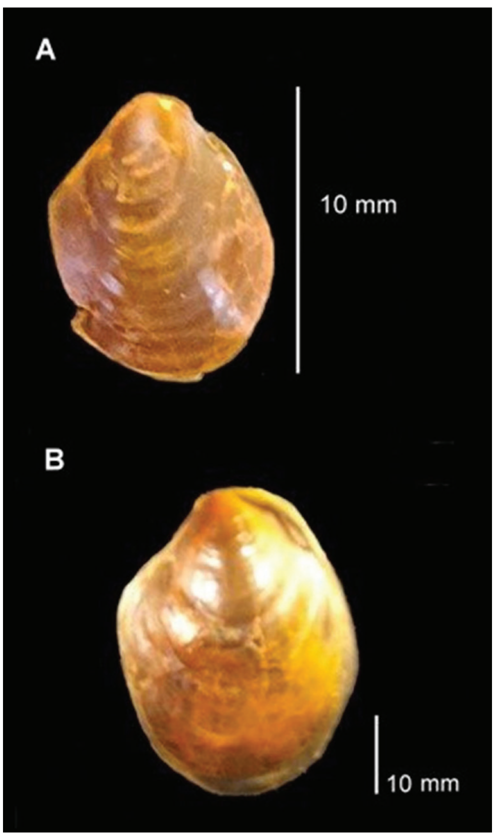

Fig. 6: Shells of specimens. A: APJ (A3). Collected in La Fosca, Barcelona. B: APA (Ap3) from El Fòrum, Barcelona.

In a 85 mm APA specimen collected alive at the Forum, Barcelona, NE Spain the radular formula was 51×8.10.1.10.8. The central tooth has a trapezoidal geometric shape, with a serrated main cusp flanked with 2 denticles on each side; this rachidian tooth measures 94.22 µm long × 54.91 µm wide; lateral teeth are narrow and have a serrate main cusp, inner flanking cusp and reduced outer flanking cusp; they are 94.09 µm long × 48.56 µm wide; outermost they are 126.20 µm long × 16.99 µm wide (Fig. 7A, B and C left). The jaws of the APA and APJ specimens are formed by tiny curved and smooth hook-shaped rods called uncinas that serve to scrape food, having two to four cusps at the apex (Fig, 7D).

Biology

On the Catalonian coast, JPA grow up to a size of about 20 mm (Fig. 8B, C and D), preferentially live among the fronds of red soft algae such as *S. coronopifolius* (Fig. 8A) and *P. cartilagineum*. APJ specimens feed on different red algae and presumably obtain pigments that help them camouflage themselves to hide from predators. Specimens change from APJ (reddish) to APA (olive green) as they get larger.

Due to their larger size, APA they move to a different habitat, living under the rocks; at the same time, they begin to ingest green algae of the genus Ulva Linnaeus (which includes *Enteromorpha* Link), preferably *Ulva lactuca* Linnaeus which is a very common green algae in the Mediterranean; their bodies change from reddish to an olive-green hue with white spots, as they probably acquire pigments from their food and incorporate them into their tissue. Therefore, APA specimens acquire other green or brown tones depending on the algae available to feed on, in addition to the typical white spots that characterize the species. When specimens are disturbed, they can secrete a defensive whitish fluid, sometimes mixed with purple ink (Fig. 8C).

APJ and APA specimens reproduce at the end of spring (April-June), both in the field and in the laboratory, forming aggregations of up to 9-10 individuals (APA) and 4–5 individuals (APJ) (Fig. 9). During this period numerous APA specimens migrate to shallow depths to copulate and lay eggs. The breeding period is simultaneous in various locations such as Cubelles, Cadaqués and Fòrum de Barcelona; in this last place we have counted up to more than 150 APA specimens in one hour of sampling copulating and depositing their egg masses under stones at a depth of about 2 m. After copulation, the animals lay a row of orange-yellow eggs (Fig. 10) sometimes reddish to pink and sometimes mauve or orange, forming a compact mass. Inside the ribbon are capsules containing 3 or 4 eggs, each about 100 µm in diameter. Only APAs mate and lay eggs; APJs only copulate, but do not lay eggs (Fig. 11). In the last 40 years, on the Catalonian coast, 366 observations, 225 copulations and 92 APA clutches, 182 observations and two APJ mating events were recorded (Fig. 11, Table 3).

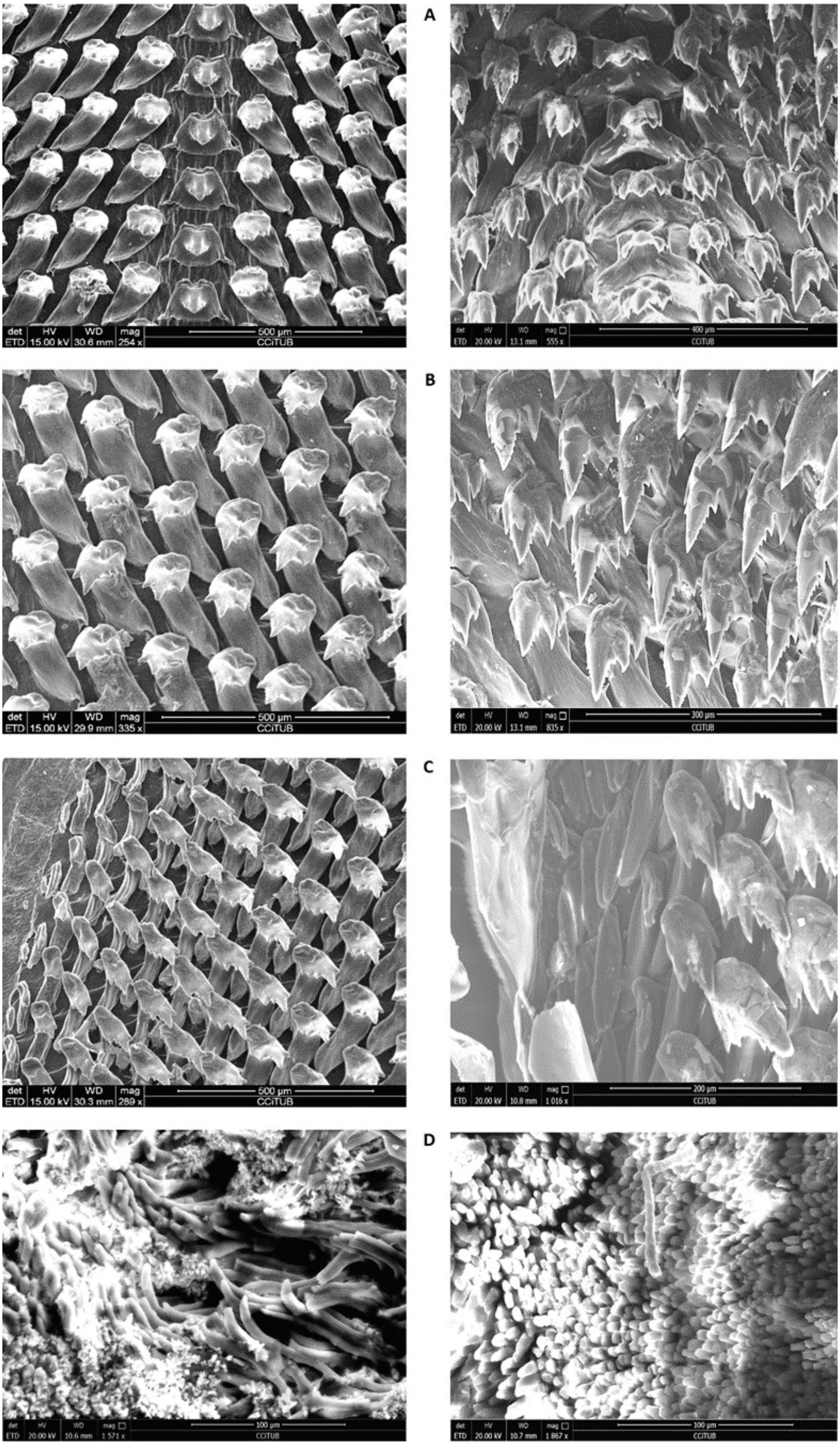

Fig. 7: Scanning electron micrographs of the radular teeth and jaw elements of APA (left) from Fòrum (Barcelona, NE Spain) and APJ (right) from La Fosca (Palamós, NE Spain). A. Rachidian and first lateral teeth, B. lateral teeth, C. marginal and external lateral teeth D. uncinas of the jaw.

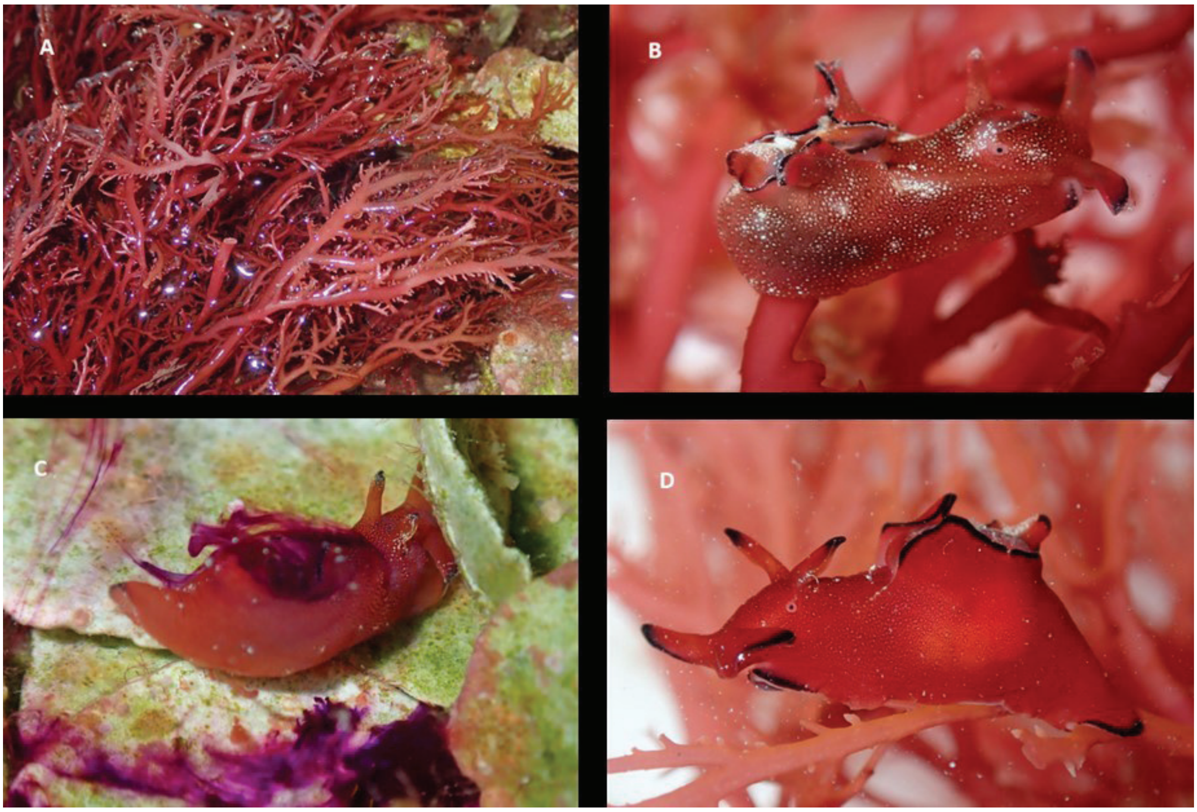

Fig. 8: A: the red algae *Sphaerococcus coronopifolius*; B, C and D: APJ of 20 mm length (known until recently as *A. parvula*) on the same alga, note the black margins of oral tentacles, rhinophores, parapodia, and anterior and posterior end of the foot; in C a specimen emitting the defensive violaceous substance after being disturbed.

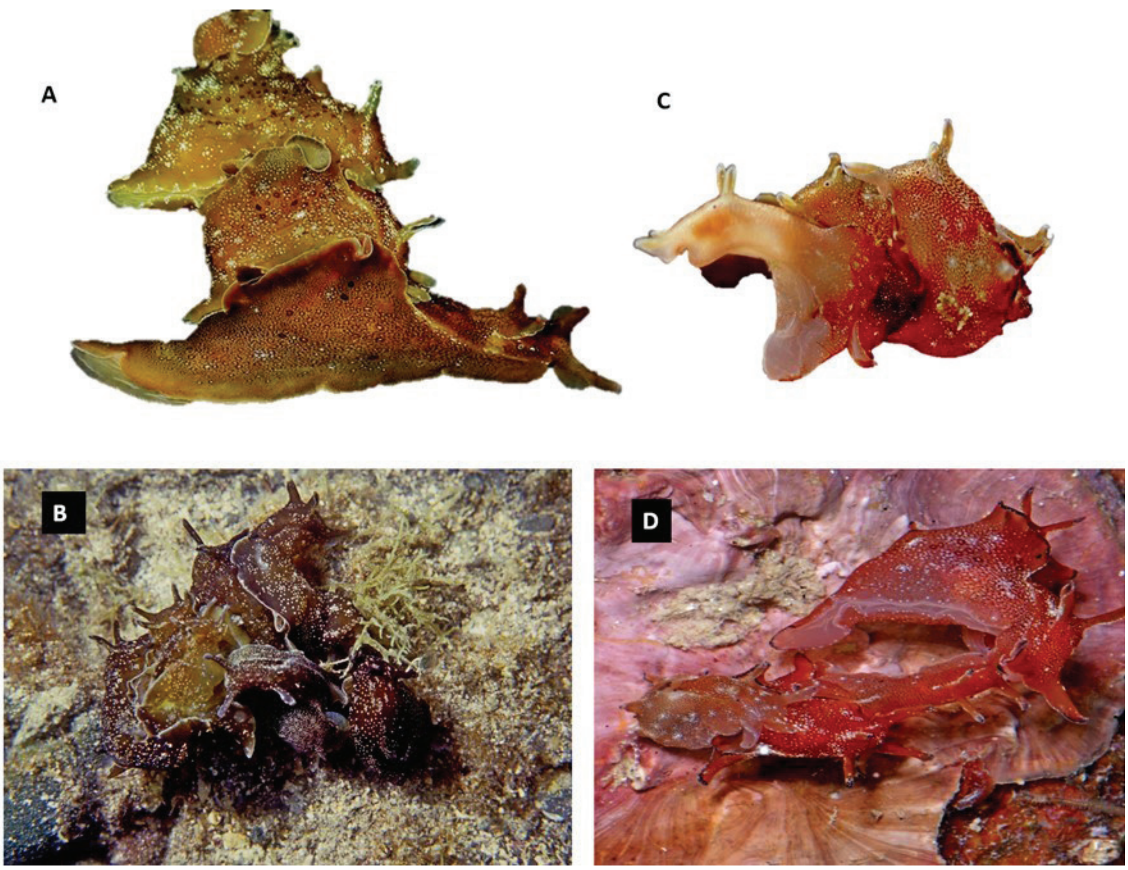

Fig. 9: Reproduction in *Aplysia punctata*. A and B: multiple copulas in APA of 65 mm length; C and D: multiple copulas in APJ of 20 mm length (known until recently as *A. parvula*).

Fig. 10: Typical specimen of *A. punctata* (APA) of 65 mm length with its eggs mass.

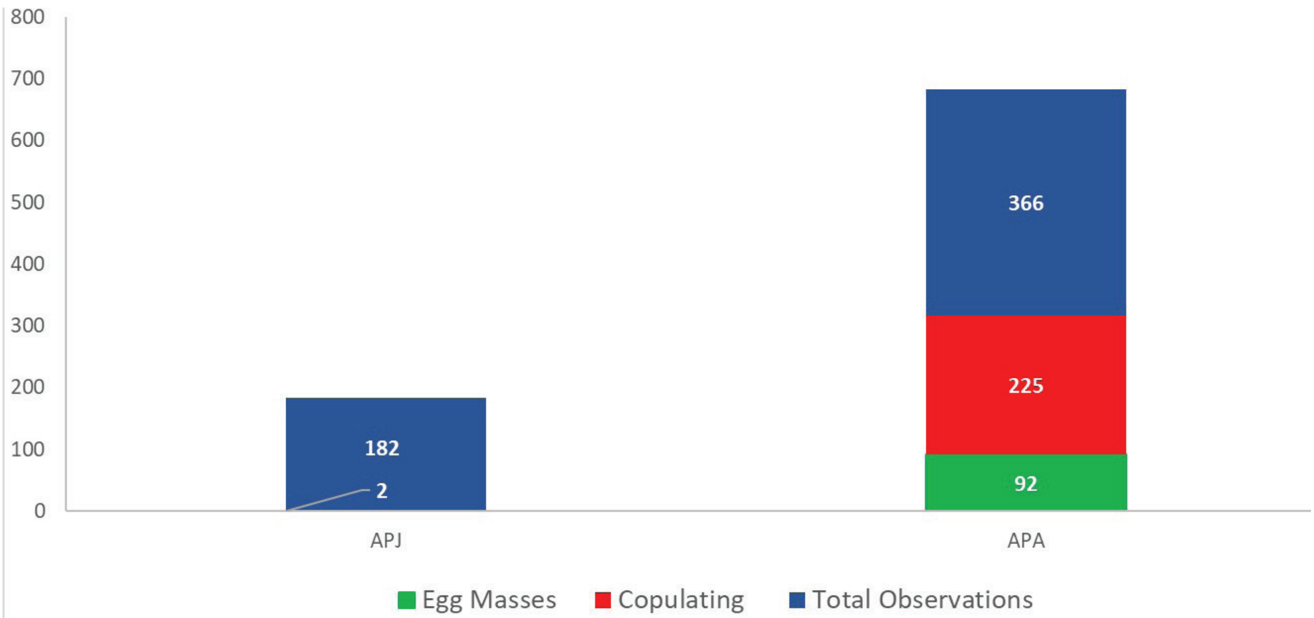

Fig. 11: Comparison of sightings, mating and spawning of APJ and APA, on the left vertical axis is the number of observations and on the right vertical axis the copulas and egg masses.

Discussion

The morphological differences between APJ and APA in body color (Fig. 5), shell (Fig. 6), and radular and jaw morphology (Fig. 7) examined herein suggest that they could be different species, and even that APJ could be a possible new species of *Aplysia*. However, the results of the molecular analyses, including the phylogenetic tree, the species delimitation analysis with the ASAP algorithm (Fig. 3), and the haplotype network (Fig. 4), clearly show that all the specimens from the Catalonian coast of APJ and APA belong to a single species that corresponds to *A. punctata*. Although in Figure 3 there is divergence in the phylogenetic results between the intraspecific genetic biodiversity of some *A. punctata* collected by us with others from the eastern Atlantic (Azores, Portugal), due to the few substitutions observed among these specimens (Fig. 4), genetically they are all still *A. punctata* (both APJ and APA) from the Mediterranean and the eastern Atlantic.

Our data agree with Golestani *et al*. (2019) in that there is no molecular evidence of the presence of *A. parvula* in the Mediterranean and in the eastern Atlantic, and the phenotypical resemblance of the APJ to the adults of *A. parvula* is possibly due to a protective mechanism of APJ from predators, to be able to hide better in red algae.

Regarding interspecific diversity, it is very evident in Figure 3 that the APJ, APA and the other *A. puncta-*

ta from the Mediterranean and the Eastern Atlantic are very different from *A. fasciata* of the Eastern Atlantic, *A. nigrocincta* from the Indo-Pacific, and *A. punctata* recorded in Azores, Portugal; this can also be seen in Figure 4, where numerous substitutions occur between the APJ, APA and the other *A. punctata* from the Mediterranean and the Eastern Atlantic with *A. fasciata* from the Eastern Atlantic, *A. nigrocincta* from the Indo-Pacific, including *A. punctata* recorded in Azores, Portugal; the latter according to this paper and Golestani *et al*. (2019) constitute a different species, despite the fact that in our paper we found an *A. punctata* from southwest Turkey within the clade of *A. punctata* from Azores, Portugal (Fig. 3). Although all specimens from southwest Turkey were mistakenly recorded by Yokes (unpublished work) as *A. parvula*, the paper of Golestani *et al*. (2019), cites all specimens from southwest Turkey as *A. punctata*.

Garstang (1890) and Eales (1921) found that *A. punctata* in the Mediterranean feeds on eight species of algae, red algae in the juvenile stage and green algae as adults. On the other hand, Niell (1977) pointed out that *A. punctata* can feed on up to 32 species of red and green algae in the Atlantic Ocean, but almost always prefers *Ulva gigantea* Bliding and Eales (1921) indicated that this species prefers *Fucus cartilagineus* Linnaeus and species of the genera *Delesseria* J.V. Lamouroux, *Laminaria* J.V. Lamouroux, and *Ulva* Linnaeus. Carefoot (1967a) found that juveniles of *A. punctata* in the British Islands feed preferentially on red algae of different genera, such as *Plocamium* Lamouroux, *Heterosiphonia* Montagne, *Cryptopleura* Kützing, and *Delesseria* Lamouroux, (in that order) when available. Also, Carefoot (1967b) used a combination of these red algae in the laboratory to feed specimens of *A. punctata* and found that they used the energy obtained by ingesting these algae for growth, nitrogen production, carbohydrates, and amino acid synthesis, and that they grew faster feeding on the species *P. cartilagineum* (as *Plocamium coccineum* Lyngbye).

In this paper, we have observed that APJ on the Catalonian coast feed on a great variety of types of red algae, such as *S. coronopifolius* and *P. cartilagineum*, acquiring a reddish hue phenotypically similar to the adults of *A. parvula* due to the fact that they incorporate the pigments of the algae into their body. We hypothesize that as they increase in size, they no longer go unnoticed by predators in the red algae and have to implement alternative defensive strategies. One of such strategies is to feed on the green alga *U. lactuca*, which is available in their environment, and as they intake pigments from the algae, their body changes to an olive-green hue with many white spots, becoming APA. They also move to a different habitat that may provide better protection, such as under rocks. These APA specimens acquire other green or brown tones, in addition to the typical white spots that characterize the species in its adult state, which help it to go unnoticed by predators, contributing to the ecological success of the species, which is very common in the Mediterranean.

Lo Bianco (1909) found that in the Mediterranean *A. punctata* reproduces from February to July, while Eales (1921) indicated that the reproductive period is from spring to summer, Miller (1960) from March to August, Carefoot (1967a) from May to October and Thompson (1976) in spring; all of them for the specimens of the Atlantic. We found Mediterranean APA copulating and laying eggs, and APJ only copulating in April and June, but not copulating with each other, forming reproductive aggregates of 9-10 individuals of APA (Figs 9A, 9B) and 4-5 individuals of red APJ (Figs 9C, 9D), confirming that APJ specimens are sexually mature and therefore smaller adults. Carefoot (1987) observed breeding APA groups of 22 to 30 individuals.

Ballesteros and Templado (1987) indicated that the eggs in APA are laid forming a ribbon of 0.6 mm in diameter with abundant reddish or pinkish eggs, each with a diameter between 80 and 105 µm. We found that the egg masses vary in color depending on the size of the animal, probably a defensive mechanism against predators. Our APA specimens produced pink or reddish egg masses, very similar in color to the algae they feed on (Fig. 10). They also produce egg masses that vary from yellow to pink, and also mauve (Carefoot, 1967b) or orange (Eales, 1921), the latter coinciding with those observed in this study; APJs do not produce egg masses, they were only observed copulating (Fig. 11), because despite maturing early, they may not be able to fertilize their eggs.

In conclusion, based on the results of this study, it appears that changes in the diet of *A. punctata* determine body color, providing this species with a dynamic, adaptable defensive mechanism that would explain the dominance of this species in the Mediterranean Sea.

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