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Seeing double: pseudocryptic diversity in the *Doriopsilla albopunctata-Doriopsilla gemela* species complex of the north-eastern Pacific

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Molecular analyses of samples of *Doriopsilla albopunctata* and its 'twin' species *Doriopsilla gemela* collected from their entire geographic ranges in the north-eastern Pacific revealed an unexpected level of species diversity. Species delimitation analyses recovered five distinct species in the complex. When the specimens were re-examined morphologically in the light of the molecular results, some external and internal differences between them became evident; thus, these species are considered pseudocryptic. The ranges of the three species previously named *D. albopunctata* overlap partially or entirely, and this along with anecdotal evidence of prezygotic isolation suggests sympatric speciation may be at play. On the contrary, the ranges of the two species previously named *D. gemela* do not overlap and their developmental modes differ. A review of the literature was conducted to clarify the taxonomy of the species complex, and we concluded that two available names (*D. fulva* and *D. gemela*) can be confidently assigned to two of the species. The original description of *D. albopunctata* is ambiguous, but the common usage of the name is fixed herein with the designation of a neotype for the most common intertidal southern California species. Finally, two new names are introduced for the remaining two species.

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Introduction

Four species of nudibranchs from the Pacific coast of North America have a similar colour pattern consisting of a yellowish orange to brown background colour and numerous white spots. These include the closely related species *Doriopsilla albopunctata* (Cooper, 1863) and *Doriopsilla gemela* Gosliner, Schaefer & Millen, 1999 (both members of the Dendrodorididae) as well as the more distantly related *Baptodoris mimetica* Gosliner, 1991 and *Peltodoris lancei* Millen & Bertsch, 2000 (both members of the Discodorididae). The specific names *D. gemela* (meaning twin in Spanish) and *B. mimetica* (meaning imitation or dramatization in Latin) refer to their striking similarity with *D. albopunctata* (Gosliner 1991; Gosliner *et al.* 1999). The shared colour pattern between these four species, similar habitat and overlapping ranges suggest they may constitute a mimicry complex.

Another intriguing aspect of the biology of this group of species is the fact that two of them, *D. albopunctata* and *D. gemela*, have been historically reported to have disjunct ranges with populations in California and the Pacific coast of Baja California as well as in the Sea of Cortez (Gulf of California), but are absent from the southern Baja California Peninsula (Angulo-Campillo 2005). Furthermore, dif-

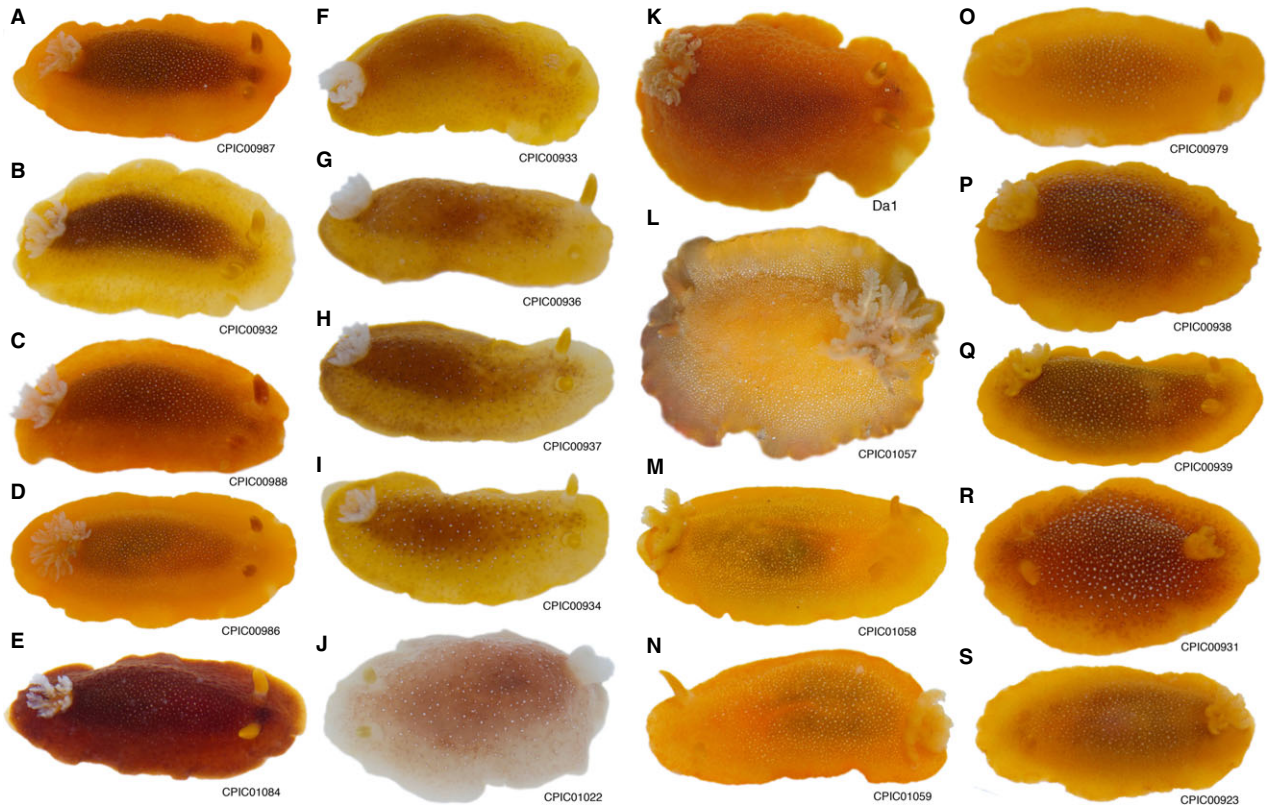

Fig. 1 Photos of living animals studied in this study showing the range of colour variation in all the specimens examined and including voucher numbers. (A–E) *Doriopsilla albopunctata*; (F–J) *Doriopsilla fulva*; (K–L) *Doriopsilla davebehrensi*; (M–N) *Doriopsilla bertschi*; (O–S) *Doriopsilla gemela* (photos by CH).

ferences in egg size and mode of development between Gulf of California and California specimens of *D. gemela* (Goddard 2005) as well as colour pattern variation across the range of *D. albopunctata* (Gosliner *et al.* 1999) (Fig. 1) suggest the taxonomy of these species could be more complex than previously thought.

In this study, we use genetic data to study the population structure of *D. albopunctata* and *D. gemela* along their ranges and compare Gulf of California individuals with their counterparts on the Pacific coast. We also substantiate the molecular differences using morphological data of the live animals and the anatomy of the digestive and reproductive systems.

Material and methods

Source of specimens

Most specimens examined were collected by the authors or donated by colleagues over the entire range of the species complex (Fig. 2). Specimens were collected by SCUBA diving, on floating docks or during low tide. Specimens were photographed and preserved in 95% ethanol. Non-type specimens were deposited at the California State Polytechnic University Invertebrate Collection (CPIC), whereas type material was deposited at the Natural History Museum of Los Angeles County (LACM). Additional specimens were obtained from the LACM collections (Table 1).

Morphological examination

All specimens collected were examined morphologically based on the photographs of live animals taken in the field. The colour pattern and external morphology were observed and recorded. The number of rhinophoral lamellae and branchial leaves were counted on the preserved specimens. At least two specimens of each putative species were dissected. Based on Valdés & Gosliner's (1999) description of the internal anatomical variability among species of *Doriopsilla* and diagnostic traits for species in this group, the digestive anatomy and reproductive anatomy were used as the main internal traits for species identification and characterization. The digestive organs were examined by performing a dorsal incision along the entire length of the animal and drawn under a Nikon SMZ-100 dissecting microscope with a *camera lucida* attachment. The reproductive organs were dissected, examined and drawn with the same dissecting microscope. The penises were

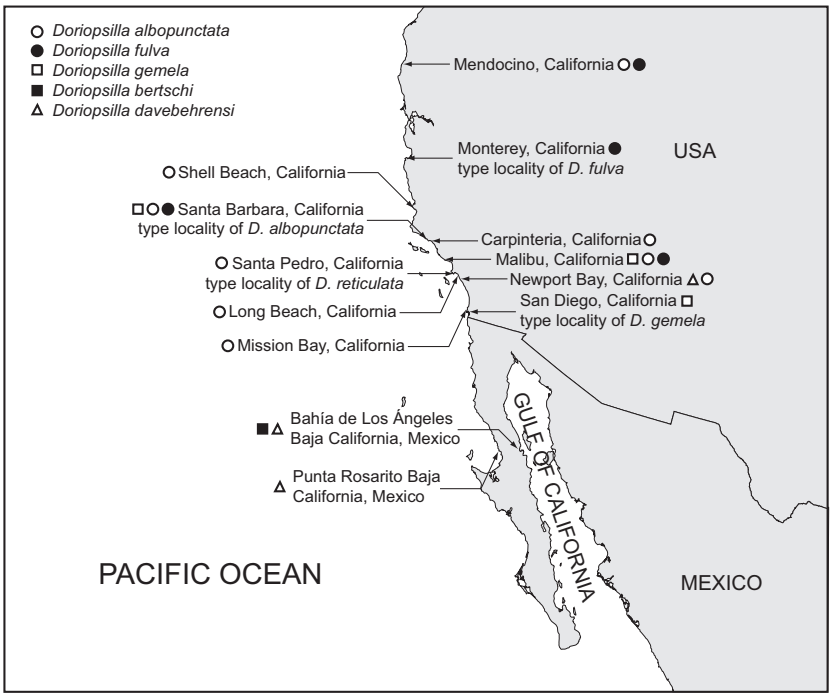

Fig. 2 Map of localities where specimens studied were collected and type localities of all nominal species in the *Doriopsilla albopunctata* species complex.

removed, mounted on a microscope slide and drawn while viewed with an Olympus CX31 compound microscope utilizing a *camera lucida* attachment.

Development

Several egg masses were collected in the field or spawned in a laboratory aquarium from specimens collected in southern California and the Sea of Cortez. The egg masses were photographed with a Leica EZ4D microscope. One of us (JG) observed and measured embryonic development and hatching stages according to the methods described by Goddard (2004) and Goddard & Green (2013). We also examined unpublished notes on the development and egg mass morphology, including 35-mm photographic slides and illustrations, in the James R. Lance collection deposited at the Invertebrate Zoology sand Geology Collection of the California Academy of Sciences (CASIZ).

DNA extraction, amplification and sequencing

A total of 55 specimens were sequenced for this study (Table 1), collected from several localities spanning the entire range of *D. albopunctata* and *D. gemela*. DNA extractions were performed using approximately 1–3 mg of tissue taken from the foot of the animals followed by a hot Chelex® extraction protocol with minor modifications. Tissue samples were placed into 1.7-mL tubes containing 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) and allowed to incubate at room temperature on a rotator overnight to rehydrate the tissue and allow cells to begin dissociating. Samples were then vortexed followed by centrifugation for 3 min at 23 897.25 ***g***. After samples were centrifuged, 975 μL of the original 1 mL of TE buffer in each tube was carefully removed without disturbing the pellet of tissue. The Chelex® solution was then added (175 μL) and samples were heated in a 56°C water bath for 20 min, then immediately placed in a 100°C heating block for 8 min. The supernatant was the final product used for PCR.

The polymerase chain reaction (PCR) was used to amplify portions of the mitochondrial cytochrome c oxidase 1 (COI) and 16S ribosomal RNA (16S) genes, as well as the nuclear histone 3 (H3) gene. The following universal primers were used to amplify the regions of interest for all specimens: COI (LCOI490 5′-GGTCAACAAATCATAAA GATATTGG-3′, HCO2198 5′TAAACTTCAGGGTGAC CAAAAAATCA-3′ developed by Folmer *et al.* 1994), 16S rRNA (16S ar-L 5′-CGCCTGTTTATCAAAAACAT-3′, 16S br-H 5′-CCGGTCTGAACTCAGATCACGT-3′ developed by Palumbi 1996) and H3 (H3 AF 5′-ATGG CTCGTACCAAGCAGACGGC-3′, H3 AR 5′-ATATCC TTGGGCATGATGGTGAC-3′ developed by Colgan *et al.* 1998). Confirmation of amplification was done using agarose gel electrophoresis with ethidium bromide to detect the presence of DNA. PCR products were sent to Source Bioscience Inc. (Santa Fe Springs, CA, USA) for sequencing. Sequences were assembled and edited using GENEIOUS PRO 4.8.3 (Drummond *et al.* 2010).

Table 1 List of specimens examined in this study for which sequences were obtained, including locality, isolate numbers, voucher collection numbers and GenBank accession numbers for the three genes sequenced; (*) tissue sample only, no voucher specimen; (**) sequence shorter than 200 bp, not accepted by GenBank

				GenBank accession numbers		
Species	Locality	Isolate	Voucher number	COI	16S	H3
Doriopsilla spaldingi	San Pedro, California, USA	EB006	CPIC 00908	KR002479	KR002427	KR002523
Doriopsilla albopunctata	Long Beach, California, USA	EB002	CPIC 00909	KR002480	KR002428	–
Doriopsilla albopunctata	Long Beach, California, USA	EB003	CPIC 00915	KR002481	KR002429	KR002524
Doriopsilla albopunctata	Long Beach, California, USA	EB004	CPIC 00916	KR002482	KR002430	–
Doriopsilla albopunctata	Long Beach, California, USA	EB005	CPIC 00917	KR002483	–	KR002525
Doriopsilla albopunctata	Long Beach, California, USA	TL029	CPIC 00918	KR002484	–	KR002526
Doriopsilla albopunctata	Malibu, California, USA	TL036	CPIC 00930	KR002485	KR002431	KR002527
Doriopsilla albopunctata	Malibu, California, USA	TL037	CPIC 00932	KR002486	KR002432	KR002528
Doriopsilla albopunctata	Carpinteria, California, USA	TL082	CPIC 00986	KR002487	KR002433	–
Doriopsilla albopunctata	Carpinteria, California, USA	TL083	CPIC 00987	KR002488	KR002434	KR002529
Doriopsilla albopunctata	Carpinteria, California, USA	TL084	LACM 3420	KR002489	KR002435	KR002530
Doriopsilla albopunctata	Newport Beach, California, USA	Da2	*	KR002490	KR002436	KR002531
Doriopsilla albopunctata	Newport Beach, California, USA	Da3	*	KR002491	KR002437	KR002532
Doriopsilla albopunctata	Newport Beach, California, USA	Da4	*	KR002492	KR002438	KR002533
Doriopsilla albopunctata	Mendocino, California, USA	CH001	CPIC 01239	KR002493	KR002439	KR002534
Doriopsilla albopunctata	Shell Beach, California, USA	CH002	CPIC 01254	KR002494	KR002440	KR002535
Doriopsilla albopunctata	Shell Beach, California, USA	CH003	CPIC 01255	KR002495	KR002441	KR002536
Doriopsilla albopunctata	Mission Bay, California, USA	CH004	CPIC 01084	KR002496	KR002442	–
Doriopsilla albopunctata	Redondo Beach, California, USA	CH005	CPIC 01083	KR002497	KR002443	–
Doriopsilla fulva	Malibu, California, USA	TL038	CPIC 00933	KR002498	KR002444	KR002537
Doriopsilla fulva	Malibu, California, USA	TL039	CPIC 00934	KR002499	KR002445	KR002538
Doriopsilla fulva	Malibu, California, USA	TL041	CPIC 00936	KR002500	KR002446	KR002539
Doriopsilla fulva	Malibu, California, USA	TL042	CPIC 00937	KR002501	KR002447	–
Doriopsilla fulva	Palos Verdes, California, USA	TL089	CPIC 01022	KR002502	KR002448	KR002540
Doriopsilla fulva	Mendocino, California, USA	CH006	CPIC 01240	KR002503	KR002449	KR002541
Doriopsilla gemela	Malibu, California, USA	CH007	CPIC 00923	–	KR002450	KR002542
Doriopsilla gemela	Malibu, California, USA	CH008	CPIC 00924	KR002504	KR002451	KR002543
Doriopsilla gemela	Malibu, California, USA	CH009	CPIC 00931	KR002505	KR002452	–
Doriopsilla gemela	Malibu, California, USA	CH010	CPIC 00938	KR002506	KR002453	KR002544
Doriopsilla gemela	Malibu, California, USA	CH011	CPIC 00939	KR002507	KR002454	KR002545
Doriopsilla gemela	Carpinteria, California, USA	CH012	CPIC 00978	KR002508	KR002455	KR002546
Doriopsilla gemela	Carpinteria, California, USA	CH013	CPIC 00979	KR002509	KR002456	KR002547
Doriopsilla gemela	Carpinteria, California, USA	CH014	CPIC 00980	KR002510	KR002457	**
Doriopsilla gemela	Carpinteria, California, USA	CH015	CPIC 00981	KR002511	KR002458	–
Doriopsilla gemela	Carpinteria, California, USA	CH016	CPIC 00982	KR002512	KR002459	KR002548
Doriopsilla gemela	Carpinteria, California, USA	CH017	CPIC 00983	KR002513	KR002460	KR002549
Doriopsilla gemela	Carpinteria, California, USA	CH018	CPIC 00984	KR002514	KR002461	KR002550
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL079	CPIC 00976	KR002515	**	KR002551
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL080	CPIC 00977	KR002516	**	**
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	CH019	CPIC 01058	KR002517	KR002462	KR002552
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	CH020	CPIC 01059	KR002518	KR002463	–
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL103	LACM 3421	–	KR002464	KR002553
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL104	LACM 140782	–	KR002465	KR002554
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL105	LACM 140782	–	KR002466	KR002555
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL106	LACM 140782	–	KR002467	KR002556
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL109	LACM 140782	–	KR002468	KR002557
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL110	LACM 140782	–	KR002469	KR002558
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL111	LACM 140782	–	KR002470	KR002559
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL114	LACM 140785	–	KR002471	KR002560
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL115	LACM 140785	KR002519	KR002472	KR002561
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL116	LACM 140785	–	KR002473	KR002562
Doriopsilla bertschi	Bahía de los Ángeles, Baja California, Mexico	TL117	LACM 140785	–	KR002474	KR002563
Doriopsilla davebehrensi	Bahía de los Ángeles, Baja California, Mexico	TL141	CPIC 01038	KR002520	KR002475	KR002564

Table 1 Continued

Species	Locality	Isolate	Voucher number	GenBank accession numbers		
				COI	16S	H3
Doriopsilla davebehrensi	Bahía de los Ángeles, Baja California, Mexico	CH021	LACM 3419	KR002521	KR002476	KR002565
Doriopsilla davebehrensi	Newport Beach, California, USA	Da1	*	KR002522	KR002477	–
Doriopsilla davebehrensi	Bahía de los Ángeles, Baja California, Mexico	TL118	LACM 76-5.6	–	KR002478	KR002566

Phylogenetic analyses

To assess whether the three genes have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich & Farris 1981; Farris *et al.* 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford 2002), was calculated for all genes combined with 2000 replicates. The levels of saturation for each gene and for the first and second vs. third codon positions of COI were investigated using the substitution saturation test developed by Xia *et al.* (2003) and Xia & Lemey (2009) implemented in the program DAMBE 5.3 (Xia 2013).

Phylogenetic analyses were conducted for all genes concatenated and each gene individually. For 16S, gaps were included in the analyses. The best-fit models of evolution (HKY+I for COI first and second codon positions, GTR+G for COI third codon positions, HKY+G for 16S, F81 for H3 first and second codon positions, HKY+I for H3 third codon positions, and GTR+I+G for the entire concatenated data set) were determined using the Akaike information criterion (Akaike 1974) implemented in JMODELTEST 2 (Darriba *et al.* 2012). Bayesian analyses were conducted using MRBAYES 3.2 (Ronquist *et al.* 2012), partitioned by gene and codon position (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for 20 million generations, with sampling every 100 generations. The default 25% burn-in was applied before constructing the majority-rule consensus tree. Convergence was confirmed by eye using the 'Trace' function in TRACER 1.5 (Rambaut & Drummond 2007). Maximum-likelihood analyses were conducted for the entire concatenated alignment with GARLI 0.951 GUI (Zwickl 2006), with 2000 bootstrap repetitions, and the GTR+I+G model. Maximum parsimony analyses were conducted in PAUP (Swofford 2002) with 2000 bootstrap replicates, using the heuristic algorithm (100 searches per replicate, TBR branch swapping option); 50% majority-rule trees were generated.

Species delimitation analysis

The Species Delimitation plugin (Masters *et al.* 2011) for Geneious was used to provide a statistical framework to help determine whether in-group clades obtained in the Bayesian phylogenetic analysis have identity as distinct species. The statistics implemented were the ratio between the mean distance within the members of the clade and the mean distance of those individuals to the nearest clade and the *P* ID, which represents the mean probability and 95% confidence interval for a member of the putative species to fit inside (strict *P* ID), or at least to be the sister group (liberal *P* ID) of the clade made up by other individuals belonging to the species.

Automatic barcode gap discovery (ABGD) analysis

ABGD analysis was run on the in-group sequences to provide further corroboration for the delimitation of species identified through the phylogenetic and species delimitation analyses. ABGD infers the number of species present in a set of sequence data (and assigns individuals to the putative species) based on gaps in the distribution of pairwise distances between each sequence in a data set (Puillandre *et al.* 2012). The analysis was run twice for each gene individually, once using Kimura 2-parameter (K2) and once using Tamura–Nei (TN) distance matrices. The matrices (available in Data S1) were loaded into the online ABGD webtool (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). The default relative gap width (x) of 1.5 and a range of prior values for maximum divergence of intraspecific diversity (*P*) from 0.001 to 0.1 were used.

Results

Morphological examination

Morphological examination of specimens revealed that *D. albopunctata* and *D. gemela* constitute a species complex totalling five different species, some with overlapping geographic ranges. These species are named below in the Systematics section, and their morphological differences are discussed.

Development

Specimens originally identified as *Doriopsilla albopunctata* with different colour patterns and internal anatomies and collected in different geographic areas differed little in their egg mass and development (Table 2). Specimens from the Gulf of California (Fig. 3B) and morphologically similar species from California (Fig. 3A) laid a crenulate egg ribbon compared to slightly crenulate or simple ribbon in the other two forms from California (Fig. 3C). Gulf of

Table 2 Comparison of egg ribbon morphology and developmental mode of different forms of *Doriopsilla albopunctata* and *D. gemela*. Means are based on 3–15 measurements from separate egg masses; a range in means is therefore based on measurements from at least two egg masses

Species	Locality	Egg mass morphology	Mean egg diameter (μm)	No. eggs per capsule	Mean shell or juvenile length at hatching (μm)	Mode of development	References
D. fulva	Carmel Point, Monterey Co. and Cayucos, San Luis Obispo Co.	Upright coil	<110	1–3	193–198	Planktotrophic	Goddard & Green (2013); this study
D. albopunctata	Mission Bay, San Diego	Upright coil	108	1–3	198	Planktotrophic	Goddard (2004); present study
D. davebehrensi	Bahía de los Ángeles	Upright coil with wavy free edge	No data	2–4	184–190	Planktotrophic	Present study
D. gemela	Point Loma, San Diego	Flat coil	111	1	173–184	Planktotrophic	Goddard (2005)
D. bertschi	Bahía de los Ángeles	Flat coil	≈260	1	800	Ametamorphic direct	Mulliner (1972); Goddard (2005), Table 1 (cited as yellow porostome)

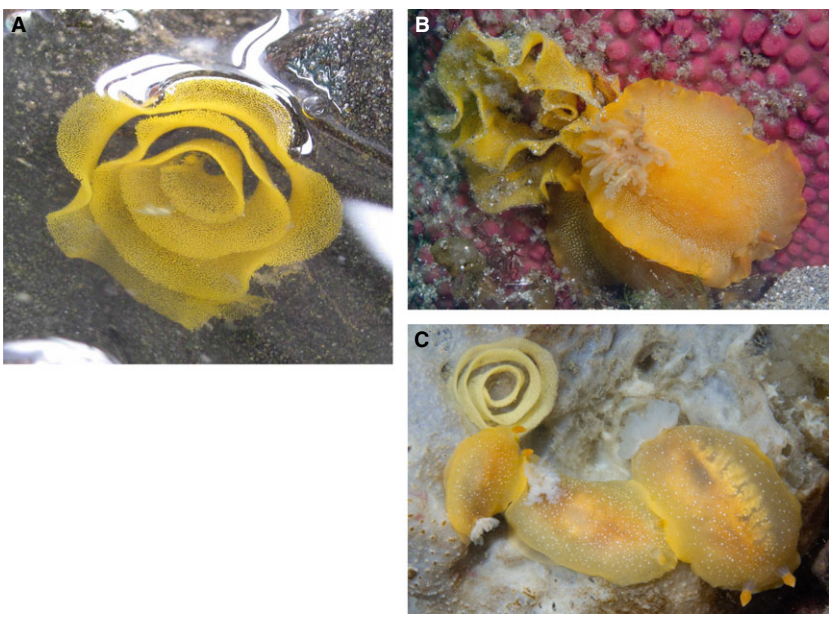

Fig. 3 Egg mass morphology of *Doriopsilla albopunctata*, *D. fulva* and *D. davebehrensi*. (A) *D. albopunctata* egg mass from Newport Bay, California. (B) *D. davebehrensi* specimen and egg mass from Bahía de Los Ángeles, Mexico. (C) *D. fulva* specimens and egg mass (photos by CH).

California specimens also had slightly more embryos per egg capsule (2–4, compared to 1–3) and had a slightly shorter veliger shell at hatching (184–190 μm, compared to 193–198 μm). *Doriopsilla albopunctata* from Mission Bay, San Diego, and those from Bahía de los Ángeles, Mexico, both developed to hatching in 18–19 days at 14–15°C. Hatching larval *D. albopunctata* from California and the Gulf of California had similar anatomy characterized by a yellow to golden brown left digestive gland, two large round anal cells and a large, translucent larval kidney comprised of three flask-shaped cells filled with glistening vacuoles.

In contrast, there were substantial differences in the egg size and development in specimens of *Doriopsilla gemela* (Table 2). Both populations (California and Gulf of California) deposited flat coiled egg masses (Fig. 4A–B, E), but the eggs of the Gulf of California animals were much larger (about 260 μm in diameter in average, vs. 111 μm in California) (Fig. 4E). Gulf of California specimens hatched as 800 μm long juveniles (Fig. 4C), whereas California specimens hatched as planktonic larvae (Fig. 4D).

Phylogenetic analyses

Analyses of the sequence data indicate adequacy for phylogenetic inference. The results of the ILD test show non-significant conflicting signals between the genes combined (COI-16S $P = 0.57$, 16S-H3 $P = 0.64$, H3-COI $P = 0.16$).

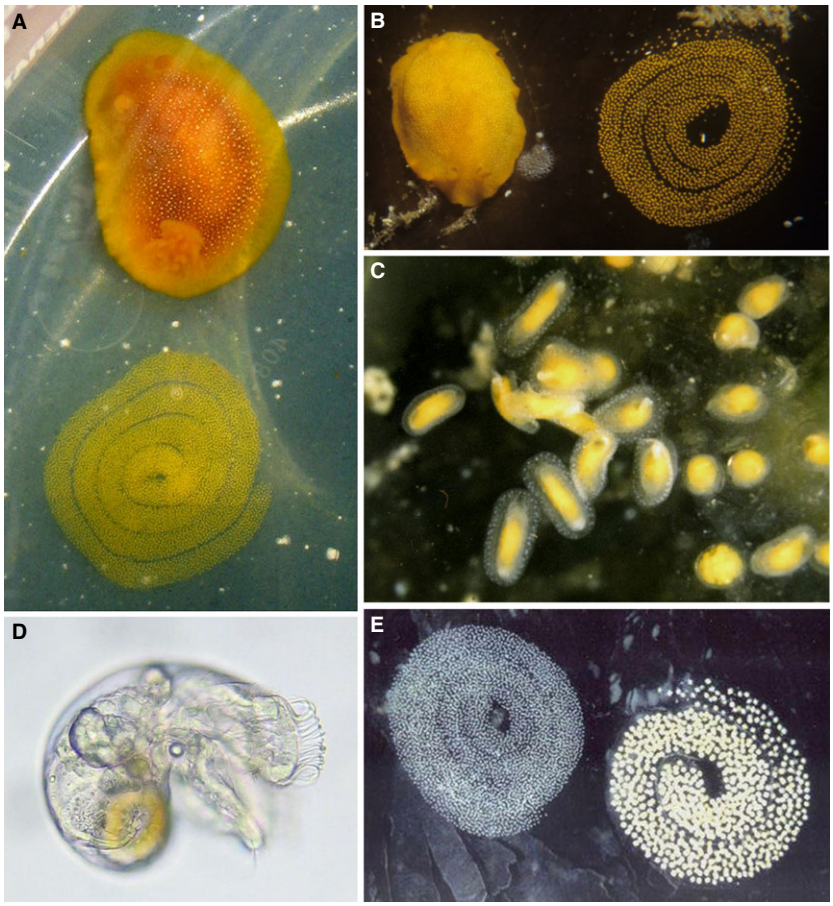

Fig. 4 Development and eggs of *Doriopsilla bertschi* and *D. gemela*. (A) *Doriopsilla gemela*, egg mass and live animal from Pt. Loma, San Diego, California (photo by JG). (B) *Doriopsilla bertschi*, egg mass and live animal from Cuevitas, Bahía de los Ángeles, Mexico (photo by Hans Bertsch). (C) *Doriopsilla bertschi*, hatching juveniles from Bahía de Los Ángeles, Mexico (photo by Jim Lance). (D) *Doriopsilla gemela*, hatching veliger from Pt. Loma, San Diego, California (photo by JG). (E) Comparison of the egg masses of the two yellow-gilled species, *D. gemela* (left) and *D. bertschi* (right) (photo by Jim Lance).

The results of the saturation tests show that all three genes display little saturation (16S: Iss = 0.25 < Iss.c = 0.695, $P = 0.0000$; COI: Iss = 0.219 < Iss.c = 0.718, $P = 0.0000$; H3: Iss = 0.272 < Iss.c = 0.6755, $P = 0.0000$).

Bayesian, maximum-likelihood and maximum parsimony consensus trees (Fig. 5) have similar topologies and recovered the same clades. Bayesian posterior probabilities (pp), maximum-likelihood bootstrap (mlb) and maximum parsimony bootstrap (mpb) values are shown above the corresponding branches. Bayesian pp values ≥0.95 were considered significant (Alfaro *et al.* 2003), and mlb and mpb values ≥70 were considered significant (Hillis & Bull 1993). There are two main clades in both trees: one includes specimens previously identified as *D. gemela* in recent publications (see Gosliner *et al.* 1999; Behrens & Hermosillo 2005) [pp = 1; mlb = 100; mpb = 100] and the other includes specimens identified as *D. albopunctata* in recent publications (see Gosliner *et al.* 1999; Behrens & Hermosillo 2005) [pp = 1; mlb = 100; mpb = 100]. The first main clade is subdivided into two well-supported clades: one includes specimens of *D. gemela* from California [pp = 1; mlb = 81; mpb = 100] and the other specimens from the Gulf of California [pp = 1; mlb = 100; mpb = 100]. The second main clade contains three clades. One of them, which includes specimens identified as *D. albopunctata* with wider bodies and found in both in California and the Gulf of California [pp = 0.99; mlb = 99; mpb = 95], is sister to another clade [pp = 0.94; mlb = 81; mpb = 69] containing two sister clades: one including specimens identified as *D. albopunctata* from California with the dorsum bearing numerous white spots [pp = 1; mlb = 99; mpb = 100] and the other including specimens from California with few white spots [pp = 1; mlb = 100; mpb = 100]. The individual gene trees recovered the same topologies with similar support values and are not shown.

Species delimitation analysis

The results of the species delimitation and barcoding gap analyses (Table 3) provided additional support for the delineation of the clades recovered in the phylogenetic analysis into separate species. Values of the ratio of the average distance among samples of one group (intra dist) to the average distance between those samples and the closest clade (inter dist) are small, below 0.16 (see

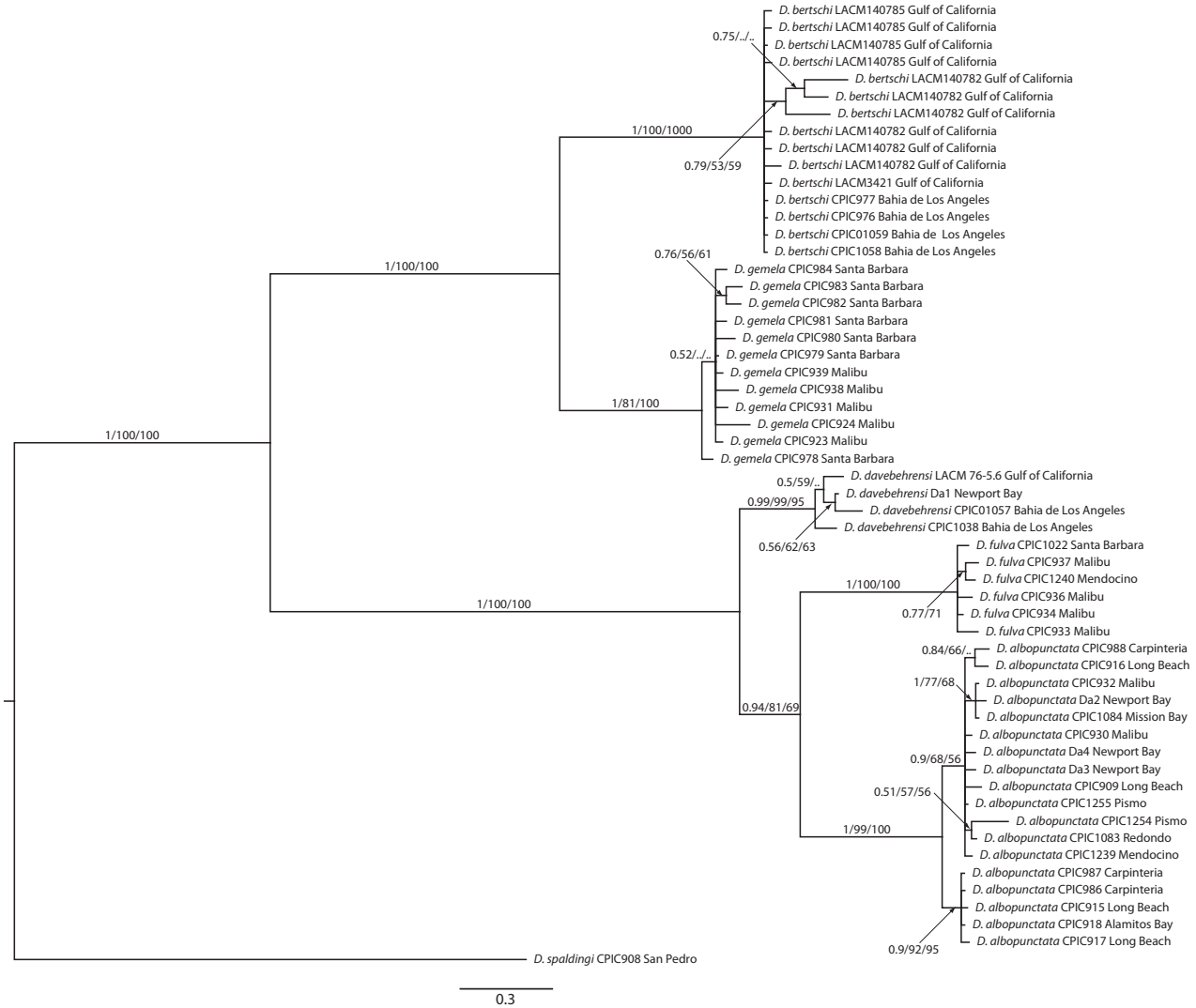

Fig. 5 Bayesian consensus tree of the concatenated analysis including posterior probabilities (first), bootstrap values from the maximum-likelihood analysis (second) and bootstrap values from the maximum parsimony analysis (third). Only values >0.5 (pp) or 50 (mlb, mpb) are provided; dots indicate values <0.5 (pp) or 50 (mlb, mpb).

Table 3 Species delimitation results from the Bayesian tree, including the species names proposed in this study and the codes for the morphology of the male reproductive anatomy described in the results section

Species	Closest species	Monophyly	intra dist	inter dist closest	Intra/Inter	*P* ID (strict)	*P* ID (liberal)
D. bertschi	*D. gemela*	Yes	0.12	1.286	0.09	0.95	0.99
D. gemela	*D. bertschi*	Yes	0.109	1.286	0.08	0.95	0.99
D. davebehrensi	*D. fulva*	Yes	0.154	1.104	0.14	0.77	0.95
D. albopunctata	*D. davebehrensi*	Yes	0.139	1.117	0.12	0.95	0.98
D. fulva	*D. davebehrensi*	Yes	0.095	1.104	0.09	0.88	0.97

López-López *et al.* 2012 for interpretation). This suggests that genetic differences within all clades are small relative to the differences between clades, so that there is a well-defined separation of clades. The probability (*P* ID) of a new sequence fitting inside (strict) or at least as sister group (liberal) of its clade is >0.77 in all cases. Therefore, these clades are here considered distinct species and are formally described in the Systematics section.

Automatic barcode gap discovery (ABGD) analysis

The number of species identified in the analysis of each gene is shown in Table 4. Using both K2 and TN distance matrices (see Data S1), the COI and H3 sequences showed a major barcode gap between a priori genetic distance thresholds of 0.01 and 0.02. Using a value of *P* between this range (0.0129), five species were identified in the COI and H3 sequence sets, and assignment of individuals to the species matched the Bayesian and maximum-likelihood phylogenies. For 16S, the first major barcode gap occurred at an a priori genetic distance threshold of 0.01, which corresponds with only two unique species. Importantly, however, the two species identified are not polyphyletic in relation to those identified using COI or H3.

Table 4 ABGD results, with the prior interspecific divergence (*P*), the number of species identified (*N*) and identity of the species based on the Bayesian phylogeny. Results were the same using both Kimura 2-parameter (K2) and Tamura–Nei (TN) matrices

Gene	*P*	*N*	Species
16S	0.0129	2	*D. albopunctata, D. gemela*
COI	0.0129	5	*D. albopunctata, D. gemela, D. fulva, D. bertschi, D. davebehrensi*
H3	0.0129	5	*D. albopunctata, D. gemela, D. fulva, D. bertschi, D. davebehrensi*

Systematics

Doriopsilla albopunctata (Cooper, 1863): Figs 1A–E, 3A, 6A, 7A, 8A–B, 9A

Doris albopunctata Cooper 1863: 58.

Doriopsis reticulata Cockerell *in* Cockerell & Eliot 1905: 41–42; pl. 7, Fig. 5.

Type material

Holotype lost (MacFarland 1905). Neotype, here designated, one specimen 14 mm preserved length (LACM 3420; ex. CPIC 00988).

Material examined

Mission Bay, San Diego, California, USA, 7 July 2014, one specimen 11 mm preserved length, 3 m depth (CPIC 01084). Alamitos Bay, Long Beach, California, USA, 14 February 2014, one specimen 11 mm preserved length, 1 m depth (CPIC 00909), one specimen 30 mm preserved length, 1 m depth (CPIC 00915), one specimen 26 mm

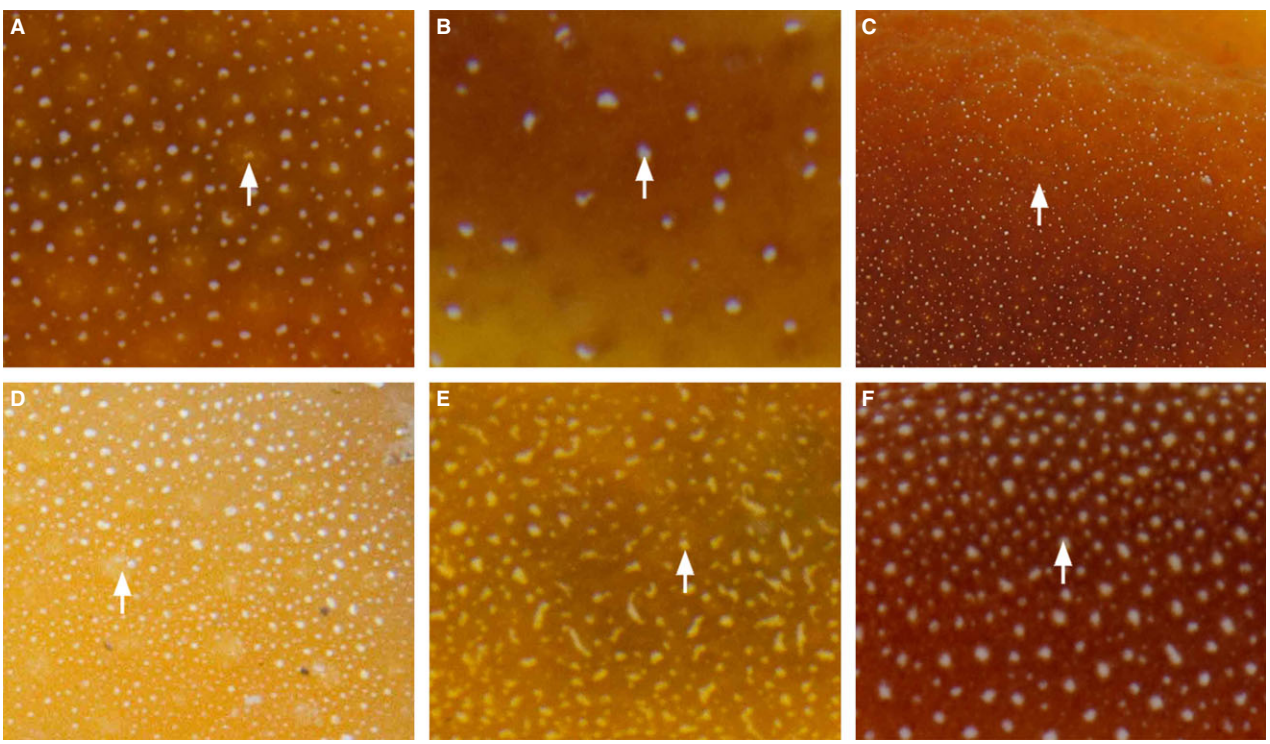

Fig. 6 Close-up photographs of the dorsal tubercles and arrangement of dorsal white pigment in all species studied. (A) *Doriopsilla albopunctata* (CPIC00986). (B) *Doriopsilla fulva* (CPIC00934). (C) *Doriopsilla davebehrensi* from California (Da1). (D) *Doriopsilla davebehrensi* from the Gulf of California (CPIC01057). (E) *Doriopsilla bertschi* (CPIC01058). (F) *Doriopsilla gemela* (CPIC00939). Arrows indicate the centre of tubercles.

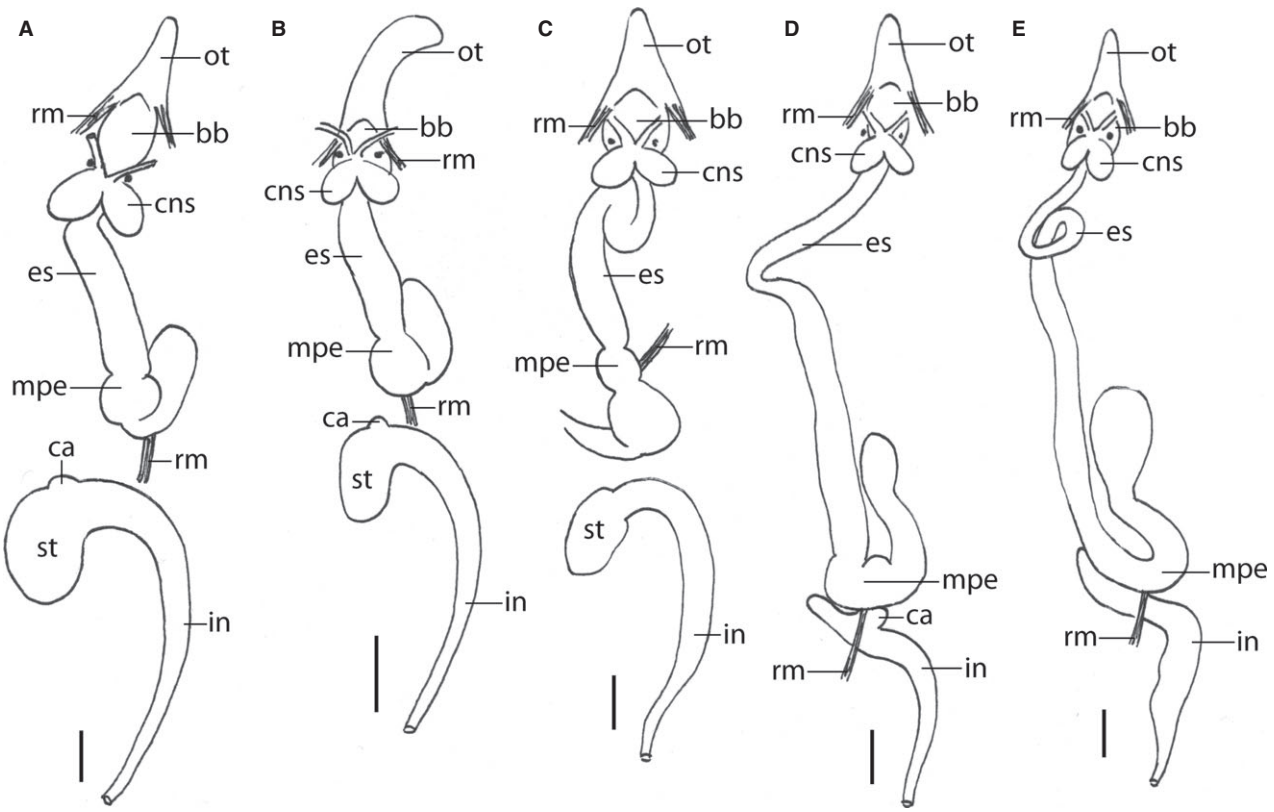

Fig. 7 Digestive systems of the species recognized in this study. (A) *Doriopsilla albopunctata* (CPIC00918). (B) *Doriopsilla fulva* (CPIC00937). (C) *Doriopsilla davebehrensi* (CPIC01038). (D) *Doriopsilla bertschi* (CPIC00976). (E) *Doriopsilla gemela* (CPIC00939). Scale bars = 1 mm. bb, buccal bulb; cns, central nervous system (stylized); ca, caecum; es, oesophagus; in, intestine; mpe, muscular portion of the oesophagus; ot, oral tube; rm, retractor muscle; st, stomach.

preserved length, 1 m depth, dissected (CPIC 00916), one specimen 21 mm preserved length, 1 m depth (CPIC 00917), one specimen 32 mm preserved length, 1 m depth, dissected (CPIC 00918). Redondo Canyon, Los Angeles, California, USA, 10 July 2014, one specimen 15 mm preserved length, 10 m depth (CPIC 01083). Malibu, California, USA, 14 March 2014, one specimen 16 mm preserved length, 14 m depth (CPIC 00930), one specimen 15 mm preserved length, 18 m depth (CPIC 00932). Carpinteria, California, USA, 6 April 2014, one specimen 29 mm preserved length, 9 m depth, dissected (CPIC 00986), one specimen 22 mm preserved length, 9 m depth, dissected (CPIC 00987). Shell Beach, California, USA, 8 October 2014, one specimen 36 mm preserved length, 0 m depth (CPIC 01254), one specimen 32 mm preserved length, 0 m depth (CPIC 01255). Mendocino, California, USA, 7 August 2014, one specimen 24 mm preserved length, 7 m depth (CPIC 01239).

Diagnosis

Body oval with a relatively wide mantle margin. Colour variable, from light yellow to dark orange or brown with numerous white dots. Larger white dots are located on the apex of the tubercles, and smaller white dots form circles around the tubercles. Gill usually white, sometimes yellow.

External morphology

Body oval with a relatively wide mantle margin in relation to the body width, approximately one-fourth of the total animal width (Fig. 1A–E). Dorsum covered with numerous small conical tubercles, becoming lower and more scattered towards the mantle margin. Background colour variable, from light yellow to dark orange or dark brown, with numerous white dots. Larger white dots are located on the apex of the tubercles, and smaller white dots form circles around the tubercles (Fig. 6A). Rhinophores club shaped, with the same colour as the rest of the body, and having 16–18 lamellae. Gill usually white, sometimes yellow, with five branchial leaves.

Internal anatomy

Digestive system (Fig. 7A) with a relatively short and triangular oral tube in comparison with other species. Buccal bulb short, oval in shape. Oesophagus relatively short in

comparison with other species, expanding proximally into a small, round muscular portion. Muscular portion of oesophagus connecting into the digestive gland through a wider tube, curving towards the right-hand side of the body. A retractor muscle connects to the muscular portion of the oesophagus. Stomach oval, wide, with a single caecum, narrowing into a relatively long and narrow intestine in comparison with other species that curves to the right and opens proximally into the anal papilla.

Reproductive system (Fig. 8A–B) with an elongate ampulla, joining with a short, narrow tube coming from the prostate and connecting into the female gland complex. Vagina as wide as the deferent duct, short, slightly curved, connecting into the large, oval bursa copulatrix. At the point where the vagina inserts into the bursa copulatrix, two other ducts emerge: one is a long duct expanding into the elongate and folded seminal receptacle and the other is the uterine duct. Deferent duct wide and relatively short in comparison with other species, expanding into a large, flat, glandular prostate that covers about one-third of the surface of the reproductive system. Penis armed with numerous penial hooks, with a short base and a very elongate, curved cusp (Fig. 9A).

Range

California, from Mendocino to San Diego (present study); possibly on the outer coast (Pacific) of the Baja California Peninsula (Gosliner *et al.* 1999).

Remarks

Doriopsilla albopunctata is the most variable species in external coloration, from pale yellow to dark brown. It is recognizable because of the presence of a white gill (usually) and numerous white dots on the dorsum, some on the tip of the tubercles and some forming circles around them.

Doriopsilla fulva (MacFarland, 1905): Figs 1F–J, 3C, 6B, 7B, 8C–D, 9B

Doriopsis fulva MacFarland 1905: 45.

Type material

Holotype (USNM 181286), tide pools near Pacific Grove, Monterey Bay, California, June 1893.

Material examined

Palos Verdes, California, USA, 19 April 2014, one specimen 21 mm preserved length, 20 m depth, dissected (CPIC 01022). Malibu, California, USA, 30 March 2014, one specimen 16 mm preserved length, 18 m depth, dissected (CPIC 00933), one specimen 12 mm preserved length, 18 m depth (CPIC 00934), one specimen 12 mm preserved length, 18 m depth (CPIC 00936), one specimen 10 mm preserved length, 18 m depth (CPIC 00937). Mendocino, California, USA, 7 August 2014, one specimen 33 mm preserved length, 7 m depth (CPIC 01240).

Diagnosis

Body oval with a relatively narrow mantle margin. Colour typically pale yellow, occasionally white. Large white dots located on the apex of most tubercles, no white dots present on space between tubercles. Gill white.

External morphology

Body oval with a relatively narrow mantle margin in relation to the body width, approximately one-fifth of the total animal width (Fig. 1F–J). Dorsum covered with small conical tubercles, becoming lower and more scattered towards the mantle margin. Background colour pale yellow, rarely brownish, some specimens are white. The centre of the dorsum can be darker than the margin, giving the animal a dual colour pattern. Large white dots located on the apex of most tubercles, and no white dots present on space between tubercles (Fig. 6B). Rhinophores club shaped, with a pale yellow club and a white base, and having 10–12 lamellae. Gill white with five branchial leaves.

Internal anatomy

Digestive system (Fig. 7B) with an elongate oral tube. Buccal bulb short, oval in shape. Oesophagus relatively short in comparison with other species, expanding proximally into a small, round muscular portion. Muscular portion of oesophagus connecting into the digestive gland through a wider tube, curving towards the right-hand side of the body. A retractor muscle connects to the muscular portion of the oesophagus. Stomach oval, wide, with a single caecum, narrowing into a relatively long and narrow intestine in comparison with other species, which curves to the right and opens proximally into the anal papilla.

Reproductive system (Fig. 8C–D) with an oval ampulla, joining with a long, narrow tube coming from the prostate and connecting into the female gland complex. Vagina wider than the deferent duct, relatively long and convoluted in comparison with other species, connecting into the large, oval bursa copulatrix. At the point where the vagina inserts into the bursa copulatrix, two other ducts emerge: one is a long duct expanding into the oval seminal receptacle and the other is the uterine duct. Deferent duct wide and relatively long in comparison with other species, expanding into a large, flat, glandular prostate that covers about half the surface of the reproductive system. Penis armed with numerous penial hooks, with a short base and an elongate, straight cusp (Fig. 9B).

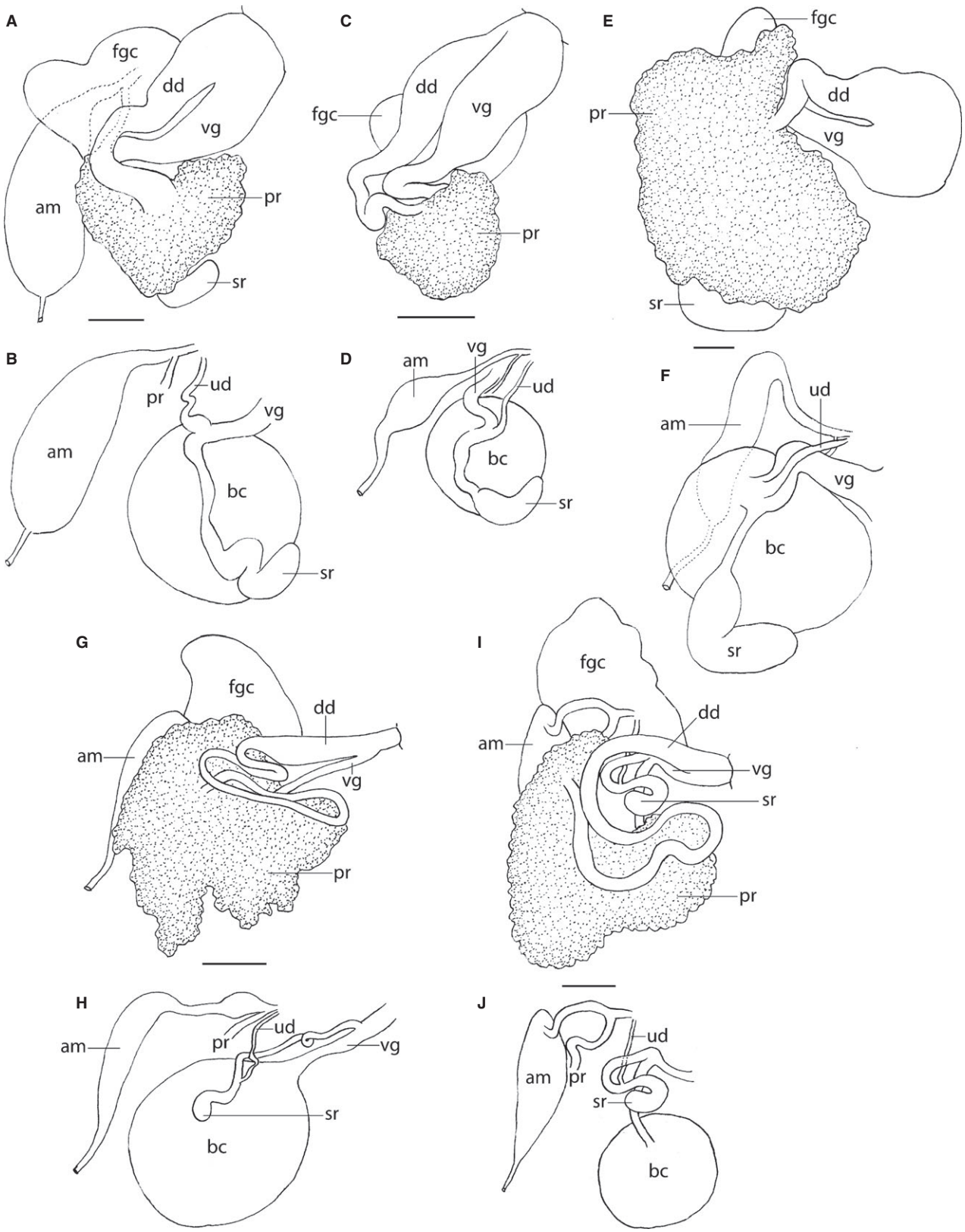

Fig. 8 Reproductive systems of the species recognized in this study. (A–B) *Doriopsilla albopunctata* (CPIC00987), dorsal view of the dissected reproductive system (A) and dorsal view of the reproductive organs after removal of the prostate and deferent duct (B). (C–D) *Doriopsilla fulva* (CPIC00935), dorsal view of the dissected reproductive system (C) and dorsal view of the reproductive organs after removal of the prostate and deferent duct (D). (E–F) *Doriopsilla davebehrensi* (CPIC01037), dorsal view of the dissected reproductive system (E) and dorsal view of the reproductive organs after removal of the prostate and deferent duct (F). (G–H) *Doriopsilla bertschi* (CPIC01058), dorsal view of the dissected reproductive system (G) and dorsal view of the reproductive organs after removal of the prostate and deferent duct (H). (I–J) *Doriopsilla gemela* (CPIC00939), dorsal view of the dissected reproductive system (I) and dorsal view of the reproductive organs after removal of the prostate and deferent duct (J). Scale bars = 1 mm. am, ampulla; bc, bursa copulatrix; dd, deferent duct; fgc, female gland complex; pr, prostate; sr, seminal receptacle; ud, uterine duct; vg, vagina.

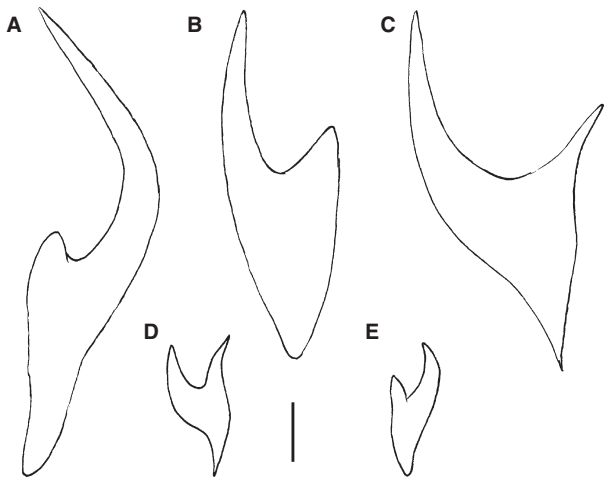

Fig. 9 Penial spines of the species recognized in this study. (A) *Doriopsilla albopunctata* (CPIC00987). (B) *Doriopsilla fulva* (CPIC00935). (C) *Doriopsilla davebehrensi* (CPIC01037). (D) *Doriopsilla bertschi* (CPIC01058). (E) *Doriopsilla gemela* (CPIC00939). Scale bars = 10 μm.

Range

California, probably from Point Loma, San Diego (see remarks) to Humboldt County (Jaeckle 1984).

Remarks

Doriopsilla fulva is distinguishable from *Doriopsilla albopunctata* by several external and internal traits enumerated in the next few sentences. Whereas the external background colour of *D. albopunctata* typically varies from light yellow to dark orange or brown, *D. fulva* is almost always pale yellow, although some specimens can be white and some may have a dual colour pattern described above. In *D. albopunctata*, there are many tubercles as well as numerous white dots on the dorsum; larger white dots are located on the apex of the tubercles, and smaller white dots form circles around the tubercles. On the contrary, in *D. fulva*, the dorsal tubercles are fewer, and the white dots are located exclusively on the apex of most tubercles, with no white dots present on the space between tubercles. Internally, the two species can be distinguished by the presence of a more elongate oral tube in *D. fulva*.

In this study, we have confirmed the presence of *D. fulva* in southern California as far south as Malibu, based on the examination of specimens (Fig. 2). However, online photographs of specimens found in Point Loma, San Diego (Lee 2011), taken in August 2011 indicate the limit of the range of this species is further south.

Doriopsilla davebehrensi sp. n. (Figs 1K–L, 3B, 6C–D, 7C, 8E–F, 9C)

Doriopsilla albopunctata Gosliner, Schaefer & Millen 1999: 202–205 (part), Fig. 2A; Kerstitch & Bertsch 2007: 72, fig. 164.

Holotype

Bahía de los Ángeles, Baja California, Mexico, 12 June 2014, one specimen 34 mm preserved length, 6 m depth (LACM 3419; ex. CPIC 01057).

Material examined

Bahía de los Ángeles, Baja California, Mexico, 15–17 May 1976, one specimen 17 mm preserved length (LACM 76-5.6); 1 April 2000, one specimen 22 mm preserved length, dissected + egg mass, 0 m depth (CPIC 01038); 1 April 2000, one specimen 21 mm preserved length, dissected + egg mass, 0 m depth (CPIC 01039). Punta Rosarito, Baja California, México, 13 March 1994, one specimen 32 mm preserved length + egg mass, 0 m depth (CPIC 01036); 13 March 1994, one specimen 20 mm preserved length, 0 m depth, dissected (CPIC 01037);

Etymology

Named to honour our friend and colleague David Behrens, who was the last person to give up on the name *D. fulva*. The species name is composed with his short first name (Dave) and last name (Behrens) to avoid potential secondary homonymy with the closely related species *Dendrodoris behrensi*.

Diagnosis

Body wide, mantle margin narrow. Dorsum with numerous minute tubercles, almost invisible and few large scattered tubercles. Numerous small white dots mainly found on the

space between the tubercles, but occasionally some dots are on the tubercles. Rhinophores with a lighter tip. Gill off-white or yellow. Muscular portion of oesophagus connecting into the digestive gland through a tube curving towards the left.

External morphology

Body wide, giving the animal a more rounded appearance than in the other species of the complex (Fig. 1K–L). Mantle margin proportionally narrow, approximately one-fifth of the total animal width of the body. Dorsum covered with minute tubercles, almost invisible, and a few large scattered tubercles. Background colour dark yellow to orange. Dorsum with numerous small white dots mainly found on the space between the tubercles (Fig. 6C), but occasionally some dots are on the tubercles (Fig. 6D). Rhinophores club shaped, the same colour as the rest of the body with a lighter tip and having 14 lamellae. Gill variable in colour, typically yellow, sometimes off-white, with five branchial leaves.

Internal anatomy

Digestive system (Fig. 7C) with a relatively short and triangular oral tube in comparison with other species. Buccal bulb short, oval in shape. Oesophagus relatively short in comparison with other species, expanding proximally into a small, round muscular portion. Muscular portion of oesophagus connecting into the digestive gland through a wider tube, curving towards the left-hand side of the body. A retractor muscle connects to the muscular portion of the oesophagus. Stomach oval, wide, lacking a caecum, narrowing into a relatively long and narrow intestine in comparison with other species, which curves to the right and opens proximally into the anal papilla.

Reproductive system (Fig. 8E–F) having an elongate ampulla, with a conspicuous proximal bend, connecting to a long, narrow tube coming from the prostate and into the female gland complex. Vagina wider than the deferent duct, relatively long and convoluted in comparison with other species, connecting into the large, oval bursa copulatrix. At the point where the vagina inserts into the bursa copulatrix, two other ducts emerge: one is a long duct expanding into the oval seminal receptacle and the other is the uterine duct. Deferent duct wide and relatively long in comparison with other species, expanding into a large, flat, glandular prostate that covers most of the surface of the reproductive system. Penis armed with numerous penial hooks, with a short base with lateral extensions and an elongate, curved cusp (Fig. 9C).

Range

Northern Sea of Cortez (Gulf of California; present study) to Newport Beach, California (present study).

Remarks

Doriopsilla davebehrensi is the most recognizable species of the species complex, as its body is typically wider than in the other species. It is also distinguishable from both *D. albopunctata* and *D. fulva* (the other two species usually with a white gill) by having very small dorsal tubercles, almost invisible, few large scattered tubercles and numerous white dots situated mainly on the space between tubercles. Other external characteristics include a lighter tip in the rhinophores and a yellow to off-white gill. Anatomically, *D. davebehrensi* is the only species of the complex in which the muscular portion of oesophagus connects into the digestive gland through a tube curving towards the left-hand side of the body, instead of the right-hand side. Additionally, this species appears to lack a caecum, which is present in both *D. albopunctata* and *D. fulva*.

Because *D. davebehrensi* often has a yellow gill, it can be confused with both *D. gemela* and *D. bertschi* in the field. Even more problematic is the fact that *D. albopunctata* can occasionally have a yellow gill, making the two species particularly challenging to distinguish, at least where their ranges overlap along the California coast. For that reason, the northernmost limit of the range of *D. davebehrensi* remains unclear. We have been able to determine the presence of *D. davebehrensi* in Newport Bay, California, but this species may be present on the California coast. For example, McDonald (2014) identified specimens collected in 1971 from Monterey Bay, California, as *D. gemela* because of the possession of a yellow gill, but in the light of the results of this study, those specimens could be *D. davebehrensi*.

Doriopsilla bertschi sp. n. (Figs 1M–N, 4B–C, 6E, 7D, 8G–H, 9D)

Doriopsilla gemela Gosliner, Schaefer & Millen 1999: 205–207 (in part); Kerstitch & Bertsch 2007: 72, fig. 163.

Holotype

Bahía de los Ángeles, Baja California, Mexico, May 1960, 15 mm preserved length (LACM 3421; ex. LACM 140781).

Material examined

Bahía de los Ángeles, Baja California, Mexico, 15 May 1976, eight specimens 14–22 mm preserved length (LACM 140782); May 1976, four specimens 17–21 mm preserved length (LACM 140785); 11 June 2014, one specimen 22 mm preserved length, 6 m depth, dissected (CPIC 01058), one specimen 17 mm preserved length, 6 m depth (CPIC 01059); date unknown, one specimen 12 mm preserved length, dissected (CPIC 00976), one specimen 12 mm preserved length, dissected (CPIC 00977).

Etymology
Named after our friend and colleague Dr. Hans Bertsch, to acknowledge his seminal contributions to the knowledge of the opisthobranch fauna of the Sea of Cortez. His passion for the natural history and beauty of the Baja California Peninsula and the Mexican people has been an inspiration for many of us.

Diagnosis
Body oval with a relatively narrow mantle margin. Numerous small white dots or flecks, irregularly scattered all over, with no clear distinction in spotting pattern between apices of tubercles and spaces between tubercles. Gill dark yellow to orange, very large, with the leaves oriented towards the posterior end of the live animal. Oesophagus very long, stomach undifferentiated.

External morphology
Body oval with a relatively narrow mantle margin in relation to the body width, approximately one-fifth of the total animal width (Fig. 1M–N). Dorsum covered with minute tubercles, almost invisible. Colour dark yellow to orange. Dorsum with numerous small white dots or flecks, irregularly scattered all over with no clear distinction between those located on the tips of the tubercles or between the tubercles (Fig. 6E). Rhinophores and gill dark yellow to orange. Rhinophores elongate, wider at the base, with 8–9 oblique lamellae. Gill very large, almost as wide as the body, with five branchial leaves oriented towards the posterior end of the animal.

Internal anatomy
Digestive system (Fig. 7D) with a relatively short and triangular oral tube in comparison with other species. Buccal bulb short, oval in shape. Oesophagus long, with a loop posterior to the cerebral ganglia, expanding proximally into a large, elongate muscular portion. Muscular portion of oesophagus connecting into the digestive gland through a wider tube curving towards the right-hand side of the body. A retractor muscle connects to the muscular portion of the oesophagus. Stomach undifferentiated. Intestine with or without a caecum, curving to the right and opening proximally into the anal papilla.

Reproductive system (Fig. 8G–H) with an elongate ampulla that narrows and expands again into a small vesicle. At the end of this vesicle, the ampulla joins with a short, narrow tube coming from the prostate and into the female gland complex. Vagina narrower than the deferent duct, relatively long and straight in comparison with other species, connecting into the large, oval bursa copulatrix. A duct emerges from the vagina at about mid-length, which expands into the elongate seminal receptacle. The uterine duct connects to the seminal receptacle at its base. Deferent duct very long, wider distally and narrowing into a convoluted very long section that expands into a large, flat, glandular prostate that covers about two-thirds of the surface of the reproductive system. Penis armed with numerous small penial hooks, with a short base and a short, curved cusp (Fig. 9D).

Range
Only known from the northern Sea of Cortez (Gulf of California; present study).

Remarks
Doriopsilla bertschi is distinguishable from the other species previously described in this study by having both a yellow gill and narrow body. The only other species with these two characteristics combined is *D. gemela*, described below. *Doriopsilla davebehrensi* can also have a yellow gill, but the body of the animal is much wider. *Doriopsilla bertschi* and *D. gemela* are very similar externally, but in *D. gemela*, larger dorsal white dots are located on the apex of the tubercles, and numerous small white dots are irregularly scattered on the spaces between tubercles, whereas in *D. bertschi*, there is no clear distinction between the white spotting on the apices of the tubercles or on the spaces between tubercles. Also, the gill of *D. bertschi* is very large, with the branchial leaves oriented towards the posterior end of the animal, whereas in *D. gemela*, the gill is much smaller and the leaves are oriented upward. Internally, there are no consistent differences between the two species, except that *D. gemela* appears to lack a caecum, which was not detected in the two specimens dissected. Both *D. gemela* and *D. bertschi* can be distinguished anatomically from other species in the complex by having much more elongate deferent ducts, a different arrangement of the seminal receptacle, which inserts in the vagina instead of in the bursa copulatrix, and much smaller penial spines. Developmentally, *D. bertschi* differs from *D. gemela* in its large eggs and ametamorphic mode of development.

Doriopsilla gemela Gosliner, Schaefer & Millen, 1999: Figs 1O–S, 4A, D, 6F, 7E, 8I–J, 9E

Dendrodoris sp. a McDonald 1983: 171.

Doriopsilla gemela Gosliner, Schaefer & Millen 1999: 205–207 (in part), Figs 1C, 2B–C, 2E, 3C–E, 4B.

Type material
Holotype (CASIZ 111392), intertidal zone, Hill Street, San Diego, California, 1 August 1996.

Material examined
Malibu, California, USA, 14 March 2014, one specimen 15 mm preserved length, 14 m depth (CPIC 00931); 30

March 2014, one specimen 18 mm preserved length, 18 m depth (CPIC 00938), one specimen 25 mm preserved length, 18 m depth, dissected (CPIC 00939). Carpinteria, California, USA, 6 April 2014, one specimen 9 mm preserved length, 9 m depth (CPIC 00978), one specimen 10 mm preserved length, 9 m depth (CPIC 00979), one specimen 8 mm preserved length, 9 m depth (CPIC 00980), one specimen 9 mm preserved length, 9 m depth, dissected (CPIC 00981), one specimen 13 mm preserved length, 9 m depth (CPIC 00982), one specimen 10 mm preserved length, 9 m depth (CPIC 00983), one specimen 9 mm preserved length, 9 m depth (CPIC 00984).

Diagnosis

Body oval with a relatively narrow mantle margin. Colour dark yellow to orange. Larger white dots on the tips of tubercles and numerous small white dots between tubercles. Gill dark yellow to orange and branchial leaves relatively small, oriented upward. Oesophagus very long and stomach undifferentiated.

External morphology

Body oval with a relatively narrow mantle margin in relation to the body width, approximately one-fifth of the total animal width (Fig. 1O–S). Dorsum covered with small conical tubercles, becoming lower and more scattered towards the mantle margin. Colour dark yellow to orange. Larger white dots are located on the apex of the tubercles, and numerous small white dots are irregularly scattered on space between tubercles (Fig. 6F). Rhinophores club shaped, dark yellow to orange, having nine lamellae. Gill dark yellow to orange with five small branchial leaves, covering about one-third of the width of the dorsum; the leaves are oriented upward in the live animal.

Internal anatomy

Digestive system (Fig. 7E) with a relatively short and triangular oral tube in comparison with other species. Buccal bulb short and oval in shape. Oesophagus long, with a loop posterior to the cerebral ganglia, expanding proximally into a large, elongate muscular portion. Muscular portion of oesophagus connecting into the digestive gland through a wider tube curving towards the right-hand side of the body. A retractor muscle connects to the muscular portion of the oesophagus. Stomach undifferentiated. Intestine smooth, lacking caecum, curving to the right and opening proximally into the anal papilla.

Reproductive system (Fig. 8I–J) with an oval ampulla, joining a short tube coming from the prostate and connecting into the female gland complex. Vagina much narrower than the deferent duct, relatively long and curved in comparison with other species, connecting into the large, oval bursa copulatrix. A duct emerges from the vagina at about one-third of the length of the vagina from the distal end, which expands into the oval seminal receptacle. The uterine duct connects to the seminal receptacle at its base. Deferent duct narrow, very long and convoluted, expanding into a large, flat, glandular prostate that covers about half the surface of the reproductive system. Penis armed with numerous small penial hooks, with a short base and a short, curved cusp (Fig. 9E).

Range

Bahía Tortugas, Baja California Sur (Gosliner *et al.* 1999) to probably Monterey, California (see remarks).

Remarks

Gosliner *et al.* (1999) provided morphological and allozyme data to differentiate *D. gemela* from *D. albopunctata*. In the present study, we have confirmed external differences with *D. albopunctata*, such as the presence of a yellow gill and fewer rhinophore lamellae in *D. gemela*, and have substantiated the allozyme differences with sequence data.

Because of the difficulties of identifying specimens of *D. gemela* (and other species studied herein) based on photographs, and the confusion regarding the taxonomy of the group, it is problematic to establish the actual geographic range of this species. A specimen photographed by Bauder (2014) from Monterey in 2014 is the northernmost record of *D. gemela* that we can verify based on photographs. The northernmost record we can establish based on specimens is Santa Barbara, California.

Discussion

History of the problem

Doris albopunctata Cooper, 1863 was described based on a specimen dredged in 20 fathoms (36.5 m) from a rocky bottom a mile from the shore at Santa Barbara, California, plus additional specimens from the NW end of Santa Catalina Island (Cooper 1863). The holotype is lost (MacFarland 1905), and the original description contains no illustrations of the animal. The specimen was described as yellow or orange brown, with the dorsal surface thickly speckled with small white dots, each forming a slightly raised papilla. Years later, Cockerell & Eliot (1905) described *Doridopsis reticulata* based on eight specimens of different sizes and colours collected in San Pedro, California, and Dead Man's Island, near San Pedro (the latter no longer exists, after being demolished in 1928 during construction of the Los Angeles Harbor). The specimens were of a deep chestnut colour with very numerous white spots. The gill was entirely white and the rhinophores pale orange. No illustrations of the living animals were provided, only drawings of the spicules. The same year,

MacFarland (1905) described *Doriopsis fulva* from Monterey, California (USNM 181286), as a rich yellow species with yellowish white gill, covered by low tubercles nearly all of which have a small central white fleck. Although the species was not illustrated in the original description, drawings of the live animal were published 1 year later by MacFarland (1906). The animal illustrated was uniformly pale yellow with several white spots and a white gill.

Steinberg (1961) reviewed the taxonomic status of these three species. In discussions with Jim Lance, Joan Steinberg initially considered the possibility that perhaps there were two separate species, *D. fulva* in central California and *D. albopunctata* (= *D. reticulata*) in southern California. Steinberg (1961) commented that the animals she observed in Monterey Bay were nearly always bright yellow, whereas animals in the San Diego region varied from yellow to warm brown. Additionally, Steinberg (1961) observed that in the San Diego animals, the white 'glands' (spots?) are conspicuous, whereas they are difficult to see in the animals from Monterey. On July 1961, Steinberg collected several specimens from Mission Point and Point Pinos on the Monterey Peninsula, which matched MacFarland's (1906) description and illustration of *D. fulva*. She also collected darker orange specimens but having the white spots typical of *D. fulva*. Specimens representing the colour range observed in Monterey were transported alive by air to La Jolla, southern California. The next morning she collected a number of specimens at Point Loma, also representing the entire colour range observed in southern California. After careful examination of the specimens side by side, Steinberg (1961) concluded that she was dealing with a single species with colour variation along latitude. Further anatomical examination of the reproductive system and comparisons with published data corroborated that all three species *D. albopunctata*, *D. fulva* and *D. reticulata* were synonyms with *D. albopunctata* having priority. Steinberg (1961) also considered that *D. albopunctata* was a member of *Dendrodoris* and that *Doriopsilla* was a synonym. Most authors dealing with California nudibranchs followed this opinion, but some included *D. albopunctata* in *Dendrodoris* (e.g. McDonald 1983) and others in *Doriopsilla* (e.g. Roller 1970; Valdés & Ortea 1997). On the contrary, Behrens (1980, 1991) maintained *D. fulva* as distinct from *D. albopunctata* and members of different genera, *Dendrodoris* and *Doriopsilla*, respectively. Gosliner *et al.* (1999) produced additional evidence to support the synonymy of *D. albopunctata* and *D. fulva* and transferred both species to *Doriopsilla*. Gosliner *et al.* (1999) examined specimens from Baja California to northern California comprising the colour range of *D. albopunctata* and *D. fulva*. They confirmed that specimens from southern California display greater colour variation than those from central and northern California, which are typically lighter. However, they did not observe significant anatomical or developmental differences between them, concluding that *D. albopunctata* and *D. fulva* are synonyms. Later, Behrens & Hermosillo (2005) accepted the synonymy of *D. fulva* and *D. albopunctata*.

Behrens (1980, 1991) as well as McDonald & Nybakken (1980) and McDonald (1983) recognized the existence of another species, which had a yellow gill instead of the white gill typical of the common use of the names *D. albopunctata* and *D. fulva*. Gosliner *et al.* (1999) described this species with a yellow gill as *D. gemela* and differentiated it from *D. albopunctata* based on anatomical, developmental and allozyme comparisons. The type locality of *D. gemela* is Hill Street, San Diego, California, but Gosliner *et al.* (1999) also reported it from the Gulf of California. In his unpublished notes, Jim Lance maintained separate species folders for the 'yellow-gilled porostome' from California (= *Doriopsilla gemela*) and those from the Gulf of California, and in contradiction to Gosliner *et al.* (1999), considered them separate species (JR Lance, personal communication to JG, 2005). This was based in part on his observations of large eggs and hatching juveniles in the Gulf of California egg masses, and small eggs and hatching veliger larvae in the California egg masses. Mulliner's (1972) description of direct development and hatching juveniles in the Gulf yellow-gilled porostome was based on information from Jim Lance, including 35-mm slides of the egg mass and hatching juveniles (these slides are in the James Lance collection at the California Academy of Sciences). Goddard (2005), citing Mulliner (1972) and a personal communication from Jim Lance, also noted that the egg size and development of the Gulf of California *D. gemela* is different from those in southern California. Goddard (2005) stated that because of the rarity of variable developmental mode (poecilogony), the yellow-gilled *Doriopsilla* from Gulf of California could be an undescribed, cryptic species. Goddard (2005) observed planktotrophic larvae in *D. gemela* from San Diego and argued that the evidence presented by Gosliner *et al.* (1999) for this species also supports planktotrophic development, rather than their own determination of lecithotrophic development (and no independent evidence supports the latter determination).

Previous molecular analyses

Two 16S sequences of specimens assigned to *Doriopsilla albopunctata* and *D. gemela*, respectively, have been deposited in GenBank (AF430354, AF430356). These sequences were not included in the present study but were compared with newly obtained sequences. Whereas the *D. albopunctata* belongs to this species, the *D. gemela* sequence is probably a mislabelled *Doriopsilla areolata* sequence.

Taxonomy

As described above, the molecular and morphological data here obtained clearly indicate *D. albopunctata* and *D. gemela* constitute two different species complexes. Both species delimitation analyses confirmed there are five species involved, two in what used to be called *D. gemela* and three on what used to be called *D. albopunctata*. All five species can be distinguished morphologically (see diagnosis of each species above), but these differences were detected only after molecular results became available. Therefore, these are defined as pseudocryptic species (Sáez *et al.* 2003).

One of the main taxonomic tasks of this study is to assign the correct names to all the species involved. In the case of *D. gemela*, we recognize two distinct species: one found in southern California and the northern Pacific coast of the Baja California Peninsula, and another found exclusively in the Sea of Cortez. Although Gosliner *et al.* (1999) examined specimens from southern California and the Sea of Cortez, the type locality of *D. gemela* is San Diego; thus, the name *D. gemela* is retained for the southern California species (Fig. 1O–S) and the new name *D. bertschi* is introduced for the Sea of Cortez species (Fig. 1M–N).

The situation with *D. albopunctata* is much more complex. One problem is that the range of all three species here recognized partially overlaps; the other is that the original description of *D. albopunctata* is ambiguous and the holotype is lost. Moreover, when describing *D. albopunctata*, Cooper (1863) did not indicate the gill colour and the dorsal coloration description is too vague to determine the species placement of the single specimen. In fact, that description could correspond to any of the four species found in southern California including *D. gemela*. To prevent further confusion and fix the common use of the name *D. albopunctata*, we designate one specimen collected from Carpinteria near Santa Barbara (the original type locality) as the neotype (LACM 3420). This specimen represents the species found most commonly intertidally in southern California (Fig. 1A–E) and the most common use of the name *D. albopunctata* in field guides (Behrens 1980, 1991). Another species here recognized with molecular data is more common in central and northern California (north of Santa Barbara) intertidally. This species is characterized by having a lighter body colour and a reduced number of large white dots only on the tips of tubercles (Fig. 1F–J). These characteristics clearly match the original description and illustration of *D. fulva* (MacFarland 1905, 1906), and therefore, this name is assigned to this species. Finally, the last species is found in the Sea of Cortez but is also present in southern California, and it is characterized by having a much wider body than the other species (Fig. 1K–L) as well as other characteristics (see species diagnoses). We could not find an available name in the literature for this species, which is here named *D. davebehrensi*.

Another available name *D. reticulata* was based on specimens collected in southern California (San Pedro) and described as having a deep chestnut colour with very numerous white spots, an entirely white gill and pale orange rhinophores (Cockerell & Eliot 1905). This description most likely corresponds to the species here named *D. albopunctata*, and therefore, we consider *D. reticulata* a synonym. We sequenced specimens with the deep chestnut colour phenotype (Fig. 1E), and they were confirmed to be *D. albopunctata*.

Biogeography and mode of speciation

One of the most intriguing aspects of the results of this study is that three of the species here identified (*D. albopunctata*, *D. fulva* and *D. davebehrensi*) overlap in range in central and southern California and two of them (*D. albopunctata*, *D. fulva*) share most of their range in California. In the Palos Verdes Peninsula and Santa Monica Bay, both *D. fulva* and *D. albopunctata* are found together in large numbers. However, the two species appear to avoid mating, suggesting prezygotic isolation. During the course of this study, one of us (CH) found numerous copulating pairs in this region and all of them were monospecific. Also, *D. albopunctata* and *D. davebehrensi* coexist in Newport Bay, although in this case, there are no observations of mating pairs. All three species also have a similar developmental mode (planktotrophic), which is conducive to long distance larval dispersal. On the contrary, the two other sister taxa (*D. gemela* and *D. bertschi*) have different developmental modes and allopatric ranges.

Further research, including sequences of additional species of *Doriopsilla* from other biogeographic regions, is necessary to confirm whether the pseudocryptic diversity here examined is the result of a single radiation. Although some sequences of *Doriopsilla* are available in GenBank, the coverage is spotty, and a much larger effort, beyond the scope of this study, is necessary to fully understand the phylogeny of this group. Such a phylogeny would be critical to address some intriguing questions such as the mode of speciation of the sympatric species here described. Because prezygotic isolation and range overlap are signatures of sympatric speciation (Coyne & Orr 2004), we speculate that sympatric speciation could explain the observed pattern in *D. albopunctata*, *D. fulva* and *D. davebehrensi* if this group is confirmed to be monophyletic. All three sympatric species have different reproductive anatomies, suggesting that sexual selection may have played a role in isolation (Churchill *et al.* 2013). An alternative hypothesis is that past vicariant events resulted in geographic isolation and speciation; once these hypothetical barriers to dispersal

were removed, the ranges of the three species could spread resulting in the current overlap. This hypothesis is plausible in the case of *D. davebehrensi*, which maintains populations in the Gulf of California; however, in the absence of obvious physical barriers in the north–south-oriented California coastline and considering these organisms are primarily found in the open coast, it seems an unlikely explanation for the divergence of *D. albopunctata* and *D. fulva*. Other possible mechanisms of isolation such as feeding specialization, depth preference in southern California (*D. fulva* is more common subtidally and *D. albopunctata* intertidally) and mimicry with similarly coloured species (see introduction) need to be investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Distance matrices produced for the ABGD analyses generated with MEGA using the Kimura 2-parameter (K2) and the Tamura-Nei (TN) models. Matrices for all 3 genes (COI, 16S, and H3) provided as tabs in a Microsoft Excel document.