Supplementary Material

Table S1. List of the 128 reference samples, with the 20 COI haplotypes obtained

The number of specimens per haplotype is indicated in parentheses. Accession numbers are provided in Table S3 and S4

Species	Country	Population	Provider	N	Haplotypes
***Botrylloides diegensis* (N=84) 4 unique haplotypes**	France	Aber Wrac'h	Laurent Lévêque, FV	9	Bd-H1 (9)
		Brest-MB	Laurent Lévêque, FV	10	Bd-H1 (9), Bd-H6 (1)
		Bloscon	Laurent Lévêque, FV	1	Bd-H6 (1)
		Camaret	Laurent Lévêque, FV	8	Bd-H1 (8)
		Saint Quay	Laurent Lévêque, FV	17	Bd- H1 (16), Bd-H3 (1)
	Italy	Venice	Davide Tagliapietra, Gretchen Lambert	14	Bd-H1 (7), Bd-H2 (7)
	New Zealand	New Zealand	Kirsty Smith	10	Bd-H1 (1), Bd-H3 (9)
	Western USA	Bodega Bay	Marc Rius, Gretchen Lambert	7	Bd-H1 (6), Bd-H3 (1)
		Santa Barbara	Adam Langenbacher, Gretchen Lambert	8	Bd-H1 (8)
***B. violaceus* (N=9) 3 unique haplotypes**	France	Aber Wrac'h	Laurent Lévêque, FV	6	Bv-H1 (3), Bv-H4 (3)
		Saint Quay	Laurent Lévêque, FV	3	Bv- H3 (1), Bv-H4 (2)
***B. leachii*, 'radiata' morph (N=17) 5 unique haplotypes**	France	Ile verte, Roscoff	Laurent Lévêque	1	Bl-Rf-H1 (1)
		Quiberon	Laurent Lévêque, Franck Gentil	1	Bl-Rf-H2 (1)
	UK	Falmouth	JB	1	Bl-Rf-H1 (1)
		Milford Haven	JB	7	Bl-Rf-H2 (1), Bl-Rf-H4 (1), Bl-Rf-H5 (5)
		Queen Anne's Battery, Plymouth	JB	1	Bl-Rf-H1 (1)
		Sutton Harbour, Plymouth	JB	3	Bl-Rf-H1 (3)
	Ireland	Finish Island	Julia Nunn	1	Bl-Rf-H2 (1)
		Omey Island	Julia Nunn	2	Bl-Rf-H2 (1), Bl-Rf-H3 (1)
***Botryllus schlosseri* (N=18) 8 unique haplotypes**	FR	Aber Wrac'h	Laurent Lévêque, FV	1	Bs-H2 (1)
		Bloscon	Laurent Lévêque, FV	4	Bs-H7 (3), Bs-H8 (1)
		Brest-MB	Laurent Lévêque, FV	5	Bs-H1 (5)
	UK	Falmouth	JB	1	Bs-H1 (1)
		Queen Anne's Battery	JB	2	Bs-H4 (2)
		Ocean Village, Southampton	JB	1	Bs-H3 (1)
		Tinside, Plymouth	JB	3	Bs-H5 (1), Bs-H6 (2)
		Weymouth	JB	1	Bs-H1 (1)

Table S2. Dataset used in the analysis of Mediterranean *Botrylloides* sequences, obtained from GenBank (with their source) or new samplings (this study).

Accession number	Initial Identification / code for specimens analysed in this study[1]	Location (and sampling date for specimens analysed in this study)	color pattern	reference
FJ528645	*B. leachii*	NW Spain (natural)	unknown	Pérez-Portela et al (2009)
HF548553[2]	*B. leachii*	Venice Lagoon (Italy)	unknown	Griggio et al (2014)
NC024103[2]	*B. leachii*	Venice Lagoon (Italy)	unknown	Griggio et al (2014)
HG931921[2]	*B. leachii*	Taranto Gulf	unknown	Griggio et al (2014)
HF548552[2]	*B. violaceus*	Venice Lagoon	unknown	Griggio et al (2014)
HF548554[2]	*B. pizoni*	Taranto Gulf	unknown	Griggio et al (2014)
HF548559[2]	*B. niger*[3]	Med coast of Israel	unknown	Rubinstein et al (2013)
KF309549	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309551	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309608	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309609	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309610	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309611	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309641	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309642	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KF309644	*B. leachii*	NW Spain (harbour)	unknown [4]	López-Legentil et al (2015)
KU711782	*B. niger*	Israel coast	unknown	Sheets et al (2016)
KU711783	*B. niger*	Israel coast	unknown	Sheets et al (2016)
MG009581	*B. anceps*	Israel coast	single-color (yellow)	Reem et al (2018)
MG009580	*B. israeliense*	Israel coast	single-color (red)	Reem et al (2018)
MG009579	*B.* aff. *leachii*	Israel coast	unknown	Reem et al (2018)
MG009578	*B. leachii*	Venice Lagoon (Italy)	unknown	Reem et al (2018)
MN076465	DEL1	NW Spain - Ebro Delta (20/01/2016)	single-color (red)	This study
MN076466	VEN1	Venice Lagoon - Italy (06/05/2015)	two-color	This study
MN076467	VEN2	Venice Lagoon - Italy (06/05/2015)	two-color	This study

MN076468	NAP1	Miseno Lagoon - Naples, Italy (22/07/2013)	unknown	This study
MN076469	VEN3	Venice Lagoon - Italy (06/05/2015)	single-color (orange)	This study
MN076470	VEN4	Venice Lagoon - Italy (06/05/2015)	single-color (red)	This study
MN076471	VEN5	Venice Lagoon - Italy (06/05/2015)	single-color (orange)	This study
MN076472	SET1	Sète Marina (Mole Saint-Louis) – France (12/06/2013)	two-color	This study
MN076473	SET2	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076474	SET3	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076475	SET4	Sète Marina (Mole Saint-Louis)– France ((12/06/2013)	two-color	This study
MN076476	SET5	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076477	SET6	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076478	SET7	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076479	SET8	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076480	SET9	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076481	SET10	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076482	SET11	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study
MN076483	SET12	Sète Marina (Mole Saint-Louis)– France (12/06/2013)	two-color	This study

(1) spelling corrected to match WORMS database, (2) complete mitochondrial genome, (3) originally identified as *B.* affinis *leachi*, reassigned by Griggio et al. (2014), (4) one specimen illustrated in the article had a two-color pattern

References:

Griggio, F., Voskoboynik, A., Iannelli, F., Justy, F., Tilak, M.-K., Xavier, T., et al. (2014). Ascidian mitogenomics: comparison of evolutionary rates in closely related taxa provides evidence of ongoing speciation events. Genome Biology and Evolution 6(3), 591-605.

López-Legentil, S., Legentil, M., Erwin, P., and Turon, X. (2015). Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. Biological Invasions 17(6), 1623-1638.

Pérez-Portela, R., Bishop, J.D.D., Davis, A.R., and Turon, X. (2009). Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. Molecular Phylogenetics and Evolution 50(3), 560-570.

Reem, E., Douek, J., and Rinkevich, B. (2018) Ambiguities in the taxonomic assignment and species delineation of botryllid ascidians from the Israeli Mediterranean and other coastlines. Mitochondrial DNA Part A. 29(7), 1073-1080.

Rubinstein, N.D., Feldstein, T., Shenkar, N., Botero-Castro, F., Griggio, F., Mastrototaro, F., et al. (2013). Deep sequencing of mixed total DNA without barcodes allows efficient assembly of highly plastic ascidian mitochondrial genomes. Genome Biology and Evolution 5(6), 1185-1199.

Sheets, E.A., Cohen, S., Ruiz, G.M., and Rocha, R.M. (2016) Investigating the widespread introduction of a tropical marine fouling species. Ecology and Evolution 6(8), 2453-2471

Table S3. Haplotype details and results of the search in BOLD System for *Botrylloides violaceus* and *B. diegensis* colonies sampled in 20 marinas from the English Channel (627 colonies)

COI haplotype frequency (frequency computed over the total number of colonies analysed per taxon) and their count (number) over the 627 colonies are provided together with their GenBank accession number. The outcome of the taxonomic assignment (taxa assigned and percentage of similarity) made in BOLD (May 23rd, 2019) is indicated with the BOLD reference number. The sequences with the best match were all mined from GenBank, and the corresponding Genbank accession numbers are provided with their published source when available. For haplotypes with no match in BOLD (N/A), a search was made on the same date in the NCBI portal; the accession number of the best match is indicated in italics.

	Haplotype code	Haplotype frequency and number	Genbank no.	BOLD species assignment	% similarity	BOLD.no.	Genbank no.	Reference
B. violaceus	Bv-H1	0.524 (130)	MK978800	*B. violaceum*	97.59	GBMIN41973-14	GU946479	Bock et al. (2011)
(N=248)	Bv-H2	0.008 (2)	MK978801	*B. violaceum*	97.59	GBMIN41854-14	GQ365692	Lejeusne et al. (2011)
	Bv-H3	0.004 (1)	MK978802	*B. violaceum*	97.62	GBMIN41854-14	GQ365692	Lejeusne et al. (2011)
	Bv-H4	0.403 (100)	MK978803	*B. violaceum*	97.59	GBMIN41971-14	GU946477	Bock et al. (2011)
	Bv-H5	0.060 (15)	MK978804	*B. violaceum*	97.59	GBMIN41970-14	GU946476	Bock et al. (2011)
***Botrylloides* sp. (N=6)**	BvX-H6	1.00 (6)	MK978805	No match	N/A	N/A	*MG009580* (1)	Reem et al. (2018)
Botrylloides diegensis	Bd-H1	0.879 (328)	MK978806	*B. leachi*	100	GBMIN42957-14	KF309549	Lopez-Legentil et al. (2015)
(N=373)	Bd-H2	0.005 (2)	MK978807	*B. leachi*	100	GBMIN41991-14	HG931921	Griggio et al. (2014)
	Bd-H3	0.091 (34)	MK978808	*B.leachii*	100	GBGC14130-19	KY235402	Unpublished
	Bd-H4	0.003 (1)	MK978809	*B.leachii*	99.76	GBGC14130-19	KY235402	Unpublished
	Bd-H5	0.005 (2)	MK978810	*B.leachii*	99.76	GBGC14130-19	KY235402	Unpublished
	Bd-H6	0.016 (6)	MK978811	*B.leachi*	100	GBMIN43015-14	KF309608	Lopez-Legentil et al. (2015)

(1) This accession number is registered under the name *B. israeliense*. It has only 93.27% similarity with our sequence (91% of query cover 461 base pairs)

References:

Bock, D.G., Zhan, A., Lejeusne, C., MacIsaac, H.J., and Cristescu, M.E. (2011). Looking at both sides of the invasion: patterns of colonization in the violet tunicate *Botrylloides violaceus*. Molecular Ecology 20(3), 503-516.

Lejeusne, C., Bock, D.G., Therriault, T.W., MacIsaac, H.J., and Cristescu, M.E. (2011). Comparative phylogeography of two colonial ascidians reveals contrasting invasion histories in North America. Biological Invasions 13(3), 635-650.

Griggio, F., Voskoboynik, A., Iannelli, F., Justy, F., Tilak, M.-K., Xavier, T., et al. (2014). Ascidian Mitogenomics: Comparison of evolutionary rates in closely related taxa provides evidence of ongoing speciation events. Genome Biology and Evolution 6(3), 591-605.
López-Legentil, S., Legentil, M., Erwin, P., and Turon, X. (2015). Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. Biological Invasions 17(6), 1623-1638.
Reem, E., Douek, J., and Rinkevich, B. (2018). Ambiguities in the taxonomic assignment and species delineation of botryllid ascidians from the Israeli Mediterranean and other coastlines. Mitochondrial DNA Part A 29(7), 1073-1080.

Table S4. GenBank accession numbers for the haplotypes found in this study for the 'radiata' morph of *Botrylloides leachii* and *Botryllus schlosseri*, and used as reference for the phylogenetic analyses

The outcome of the taxonomic assignment (taxa assigned and percentage of similarity) made in BOLD (May 23rd, 2019) is indicated with the BOLD accession number. The sequences with the best match were all mined from GenBank, and the corresponding GenBank accession numbers are provided with their published source when available. For haplotypes with no match in BOLD, a search was made on the same date in the NCBI portal; the accession number of the best match is indicated in italics.

	Haplotype code	Accession Number	BOLD species assignment	% similarity	BOLD.no.	GenBank no.	Reference
***Botrylloides leachii*, 'radiata' morph**							
	Bl-rf-H1	MK978812	No match	N/A	N/A	*KY235402 (1)*	Unpublished
	Bl-rf-H2	MK978813	No match	N/A	N/A	*KY235402 (1)*	Unpublished
	Bl-rf-H3	MK978814	No match	N/A	N/A	*KY235402 (1)*	Unpublished
	Bl-rf-H4	MK978815	No match	N/A	N/A	*KY235402 (1)*	Unpublished
	Bl-rf-H5	MK978816	No match	N/A	N/A	*KY235402 (1)*	Unpublished
Botryllus schlosseri							
	Bs-H1	MK978817	*Botryllus schlosseri*	100	GBGC14058-19	KX500815	Nydam et al. (2017)
	Bs-H2	MK978818	*Botryllus schlosseri*	100	GBGC14050-19	KX500775	Nydam et al. (2017)
	Bs-H3	MK978819	*Botryllus schlosseri*	100	GBGC14060-19	KX500828	Nydam et al. (2017)
	Bs-H4	MK978820	*Botryllus schlosseri*	100	GBGC14048-19	KX500765	Nydam et al. (2017)
	Bs-H5	MK978821	*Botryllus schlosseri*	100	GBGC14075-19	KX500939	Nydam et al. (2017)
	Bs-H6	MK978822	*Botryllus schlosseri*	100	GBGC14064-19	KX500869	Nydam et al. (2017)
	Bs-H7	MK978823	*Botryllus schlosseri*	100	GBGC14075-19	KX500939	Nydam et al. (2017)
	Bs-H8	MK978824	*Botryllus schlosseri*	99.74	GBGC14064-19	KX500869	Nydam et al. (2017)

(1) This accession number is registered under the name *B. leachii*; however the similarity to our *B. leachii* ('radiata' morph) haplotypes is extremely low (85.15% to 85.68%), thus well below the usual threshold used in taxonomic assignment. The source associated with this reference is: "Phan, A. and Mackie, J. 2016. COI barcodes for macroinvertebrates colonizing settlement panels in California."

Reference:
Nydam, M.L., Giesbrecht, K.B., and Stephenson, E.E. (2017). Origin and dispersal history of two colonial ascidian clades in the *Botryllus schlosseri* species complex. PLOS ONE 12(1), e0169944. doi: 10.1371/journal.pone.0169944.

Table S5. Species delimitation inferred by GYMC and mPTP analyses

Entities (presumably 'species') distinguished by General Mixed Yule Coalescent (GMYC) model and Multi-rate Poisson tree processes (mPTP) method are shown, with one number per entity. In bold (and dark color) are the two sequences that were recognized as entities by the GYMC but not by the mPTP analyses. Note that for GYMC, support (the maximum likelihood value) was 1 for each of the entities 2, 3, 5, 7 and 9.

Samples (code as in Fig. 3)	GMYC entities	mPTP entities
MG009581 B anceps	1	1
KU711782 B niger	2	2
HF548559 B niger	2	2
KU711783 B niger	2	2
MG009579 B aff leachii	2	2
SET1-SET9 SET11 SET12	3	3
Bd-H1	3	3
VEN1 VEN2	3	3
DEL1	3	3
KF309549 B leachii	3	3
KF309551 B leachii	3	3
KF309610 B leachii	3	3
KF309611 B leachii	3	3
KF309642 B leachii	3	3
KF309644 B leachii	3	3
FJ528645 B leachii	3	3
HG931921 B leachii	3	3
SET10	3	3
Bd-H2	3	3
MG009578 B leachii	3	3
Bd-H3	3	3
Bd-H4	3	3
Bd-H5	3	3
HF548553 B leachii	3	3
NC024103 B leachii	3	3
KF309641 B leachii	3	3
KF309609 B leachii	3	3
KF309608 B leachii	3	3
Bd-H6	3	3
BvX-H6	**4**	4
MG009580 B israeliense	5	4
NAP1	5	4
HF548554 B pizoni	6	5
Bl-rf-H4	7	6
Bl-rf-H1	7	6
Bl-rf-H5	7	6
Bl-rf-H2	7	6
Bl-rf-H3	7	6
Bv-H5	**8**	7
VEN3 VEN4	9	7
Bv-H1	9	7
Bv-H2	9	7
Bv-H3	9	7
VEN5	9	7
HF548552 B violaceus	9	7
Bv-H4	9	7

Figure S1. Pairwise distance (% of divergence) distribution between the 12 *Botrylloides* spp. COI haplotypes obtained with the English Channel sampling.

Pairs of haplotypes compared within a given species (excluding BvX-H6 for *B. violaceus*), between species and between BvX-H6 and either *B. diegensis* or other haplotypes of *B. violaceus* are pictured with black, white and grey bars, respectively.

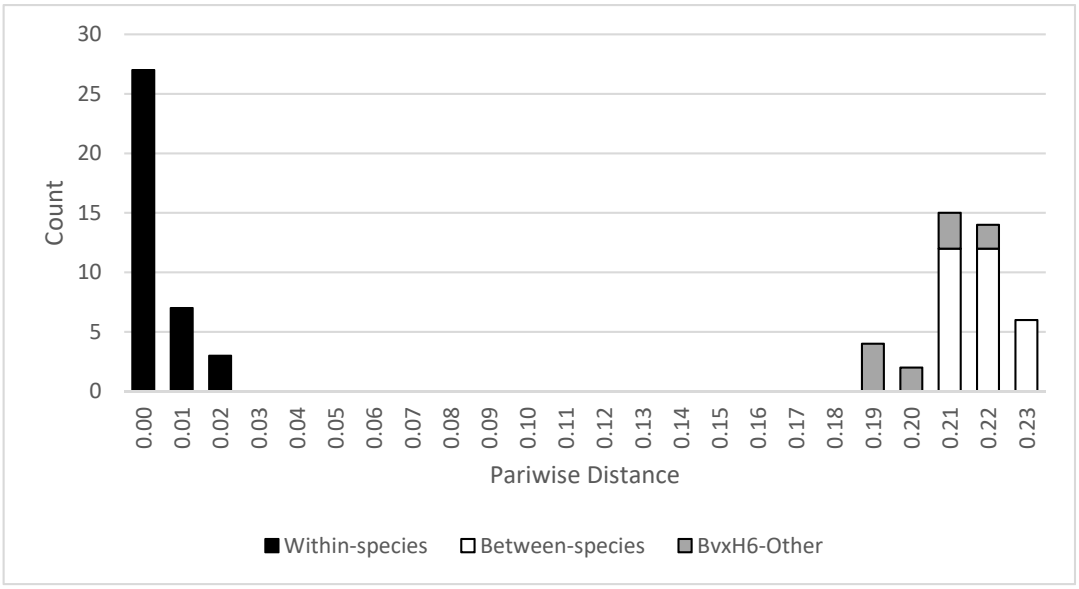


Figure S2. Percentage (and number indicated on bars) of the COI haplotypes found in the second sampling of Southsea and Gosport populations

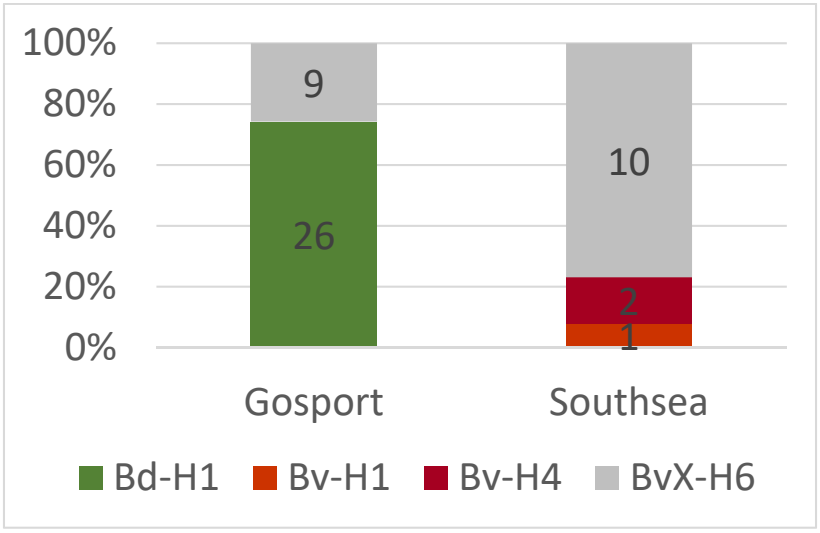


Figure S3. Color morphs of the four *Botrylloides* taxa from NW Europe examined in this study

Color morphs of the four *Botrylloides* species from NW Europe discussed are shown here (pictures on the next page). The different images are not to scale. All specimens were photographed alive, and all except Panel 2 (main picture) are shown in water, but all except the inset to Panel 2 were photographed after collection, i.e. disturbed to some degree and not fully inflated.

Panel 1, *B. diegensis*, single-color (orange) morph. Panel 2, *B. diegensis*, two-color morph (out of water), with inset showing detail of a different two-color combination. Panel 3, details of orange (single-color) *B. diegensis* showing narrow red rim around exhalant (cloacal) colony opening (upper image) and inhalant zooidal openings, forming red spots when fully contracted (lower image) and red rings when partially opened (image on right). These thin red markings do not seem to be shared by orange colonies of *B. violaceus* (subject to confirmation), but are not generally seen over the whole colony of *B. diegensis* and are probably difficult or impossible to see in fully expanded, undisturbed colonies.

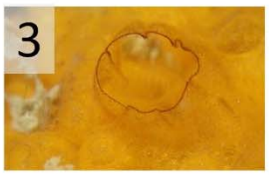
Panel 4, *B. violaceus*, single-color (yellow) colony. This particular colony is brooding numerous large, red larvae (examples indicated by yellow arrowheads); the anatomy and size of the larvae is characteristic of the species.

Panel 5, an orange and yellow example of the 'radiata' color pattern of *B. leachii*. Panel 6, a yellow and dark brown colony of the same form. In the latter, there is some resemblance to the two-color pattern of *B. diegensis*, but the color around each zooidal orifice is not solid, but resembles a spoked wheel (see inset).

Panels 7 and 8, specimens with the BvX-H6 COI haplotype. This apparently cryptic species has wide-looking water channels with relatively transparent roofs, allowing the underlying zooids to be seen with unusual clarity unless the roofs are heavily speckled with white spots. The partially contracted zooidal orifices often appear rectangular. Colonies lack a strongly contrasting color pattern, and are generally pale pink or pinkish-fawn ('flesh pink') or pale orange-brown, but can be yellow. The branchial baskets and/or endostyles of the zooids are often outlined by pale pigment. The roofs of the water channels often have small pale spots, which can be very dense. In some colonies, a larger whitish mark is present on the dorsal edge of the orifice of each zooid, adjacent to the water channel.


1

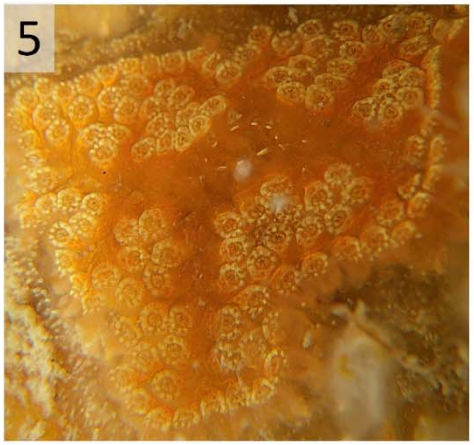

2

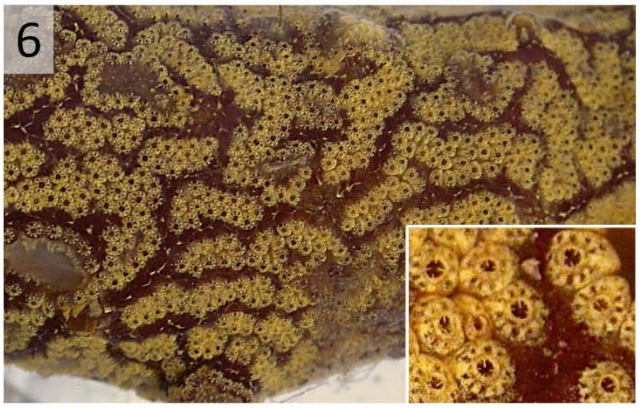

3






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