Species specificity of symbiosis and secondary metabolism in ascidians

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Supplementary information index

Methods S1

DNA extraction. Ascidian tissue (~0.5 cm^3) was ground in liquid nitrogen in sterile mortar and pestle. The powder obtained was resuspended in TE (5 mL) with lysozyme (2 mg ml^{-1}) and incubated at 30 ºC for 1 hour. EDTA (0.5 M, 1.2 mL) was added to the solution and gently mixed, after which proteinase K (0.2 mg mL^{-1}, Qiagen) was added and the solution was incubated at 30 ºC for 5 minutes. For cell lysis, SDS (10%, 0.65 mL) was added and the solution was incubated at 37 ºC for 3-4 hours. After visibly complete lysis (clear solution), NaCl (5M, 1.2 mL) was added, mixed, and CTAB/NaCl (1 mL) solution was added before incubation at 65 ºC for 1 hour. Standard phenol/chloroform extraction was then performed (Sambrook and Russell 2006).

Methods S2

Processing and analysis of ascidian chemical extracts. Each ascidian was extracted for 1 hour with methanol (5 mL), and the methanol was removed and dried under vacuum. Each extract was dissolved in methanol:water (1:9; 1.1 mL) and loaded into a Gilson GX-271 liquid handling system. Samples (0.9 mL) were subjected to automated SPE (ThermoFisher Scientific HyperSep C18, 50 mg absorbent mass, 1 mL reservoir volume), washed with H_2O (1 mL), and eluted with 95% methanol (1 mL) directly into an LC/MS-certified vial. Following SPE, each extract was quantified using an evaporative light-scattering detector (ELSD) according to previously published methods.(Adnani et al 2012) Each extract was diluted with 95% methanol to a concentration of 30 ng/mL.

Methods S3

Representative samples from different locations were analyzed for putative lipid components on an Agilent MS 6520 Q-TOF mass spectrometer fitted to an Agilent 1290 UPLC. The extract components were separated on Acquity UPLC CSH C18 column (1.7 μM, 2.1 X100 mm) using the following gradient at 0.3 mL min^{-1} (mobile phase A:B): t = 0, 85:15; t = 0-4 min, gradient to 70:30; t = 4-5, gradient to 48:52; t = 5-22, gradient to 18:82; t = 22-23, gradient to 1:99; t = 23-30, 1:99. Mobile phase A consisted of acetonitrile:water (60:40 v/v) in 10 mM ammonium formate and 0.1% formic acid, and mobile phase B consisted of isopropyl alcohol:water (90:10 v/v) in 10 mM ammonium formate and 0.1% formic acid. The column temperature was set to 65 ^{o}C. Acquisition was performed in positive ESI mode acquiring from *m/z* 100-1700. The mass spectrometer was operated using the following parameters: dry gas temperature, 350 ^{o}C; dry gas flow, 11.1 L/min; nebulizer pressure, 24 psig; Vcap, 5000V; fragmentor, 250 V. Additional parameters (precursors per cycle, 2; absolute threshold, 200) were used for data-dependent MS/MS.

Methods S4

Antimicrobial broth dilution assays. The assay was performed as previously described(Koch et al 2010) with the exception that four wells per compound were used for each dilution for MIC determination. DMSO served as a negative control. Bacterial strains used, along with the positive control antibiotic for each strain, were *M. tuberculosis* H37Ra ATCC 25177 (rifampicin); *E. coli* ATCC 25922 (gentamicin); *B. subtilis* ATCC 6633 (gentamicin); and *S. aureus* ATCC 25923 (gentamicin) and *C. albicans* ATTC 90028 (itraconazole).

Cytotoxicity assay. Cytotoxicity testing was performed using CEM-TART lymphoblastoid cells(Chen et al 1992) cultured in RPMI-1640 with 20% fetal bovine serum and antibiotic/antimycotic supplement at 37 °C with 5% CO_2 in moisture saturated atmosphere in 96-well culture clusters as previously described (Lin et al 2013).

Table S1 : Sample collection details and 18S rRNA gene BLAST hits. * Indicates codes used in preliminary taxonomic identification of samples.

Code	ollection Date	Collection Site, coordinates	BLAST hits, GenBank accession number	% identity	18S primers
CA_Ascidia_sp_4-19	20-Feb-11	Catalina Island, 33^o 26.95 ' N, 118^o 29.94' W	*Ascidia ceratodes*, L12378.2	99	F1/R1
CA_Botryllus_sp_5-19	12-Oct-11	Catalina Harbor	*Bottrylus planus*, DQ346653.1	99	F3/R3
CA_Didemnum_sp_4-17	20-Feb-11	Catalina Island, 33^o 26.95' N, 118^o 29.94' W	*Didemnum molle*, AB211071.1	94	AscF2/AscR5
CA_Didemnum_sp_4-2	19-Feb-11	Catalina Island, 33^o 26.92' N, 118^o 28.64' W	*Didemnum molle*, AB211071.1	92	F3/R3
CA_Didemnum_sp_4-7	19-Feb-11	Catalina Island, 33^o 26.92' N, 118^o 28.64' W	*Didemnum* sp. DidSA/57, AB211072.1	92	AscF2/AscR5
CA_Didemnum_sp_5-1	10-Oct-11	Catalina Island, 33^o 26.69' N, 118^o 28.92' W	*Didemnum* sp. DidSA/57, AB211072.1	92	AscF2/AscR5
CA_Didemnum_sp_5-11	11-Oct-11	Catalina Island, 33^o 26.87' N, 118^o 28.61' W	*Didemnum molle*, AB211071.1	94	AscF2/AscR5
CA_Didemnum_sp_5-18	12-Oct-11	Catalina Island, 33^o 26.21' N, 118^o 27.48' W	*Didemnum molle*, AB211071.1	90	AscF2/AscR5
CA_Pyura_sp_4-21	19-Feb-11	Catalina Island, 33^o 26.67' N, 118^o 29.417' W	*Pyura haustor*, AY903926.1	99	F3/R3
CA_Styela_sp_5-16	11-Oct-11	Catalina Harbor	*Styela plicata*, L12444.2	99	F3/R3
FJ_Lissoclinum_patella_06037		Fiji, 17^{o}55' S, 177^{o}16' E	*Lissoclinum patella*, AB211085.1	99	F1/R1
FL_Didemnum_sp_TB3 *(*D.psammathode*)	15-Aug-11	Florida Keys, 24 ^{o} 41.672' N, 81^o 26,797' W	*Didemnum* sp. Whangamata-EM-2003, AJ579861.1	89	AscF2/AscR5
FL_Ecteinascidia_turbinata_5	10-Aug-11	Florida Keys, 24 ^{o} 39.591' N, 81 ^{o} 25.217' W	*Ecteinascidia turbinata*, FM244848.1	99	F3/R3
FL_Ecteinascidia_turbinata_TB2	15-Aug-11	Florida Keys, 24 ^{o} 39.453' N, 81 ^{o} 25.247' W	*Ecteinascidia turbinata,* FM244848.1	99	F3/R3
FL_Eudistoma_sp. _004 *(*E. hepaticum*)	28-Oct-10	Florida Keys, 24 ^{o} 41.078 N, 81 ^{o} 26.957 W	*Eudistoma gilboviride,* AB211069.1	89	AscF2/AscR5
FL_Eudistoma_sp_TB1 *(*E. olivaceum*)	1-Sep-11	Florida Keys, 24 ^{o} 41.685' N, 81 ^{o} 26.840' W	*Eudistoma gilboviride,* AB211069.1	95	AscF2/AscR5
FL_Tridi demnum_sp_001BB *(*T. orbicatum)*	11-Oct-10	Florida Keys, 24 ^{o} 37.487' N, 81 ^{o} 27.443' W	*Trididemnum paracyclops,* AB211077.1	92	F1/R1
FL_Trididemnum_sp_TB4 *(*T. palmae*)	15-Aug-11	Florida Keys, 24 ^{o} 37.487' N, 81 ^{o} 27.443' W	*Trididemnum paracyclops,* AB211077.1	92	AscF2/AscR5
PNG_Didemnum_sp_11038	9-Nov-11	Papua New Guinea, 10° 16' S, 145° 38' E	*Didemnum molle*, AB211071.1	94	AscF2/AscR5
PNG_Didemnum_sp_11055	10-Nov-11	Papua New Guinea, 10° 2' S, 145° 33' E	*Didemnum* sp. DidSA/57, AB211072.1	95	AscF2/AscR5
PNG_Didemnum_sp_11089	12-Nov-11	Papua New Guinea, 9° 36' S, 147° 18' E	*Didemnum molle,* AB211071.1	94	AscF2/AscR5
PNG_Lissoclinum_patella_11033	9-Nov-11	Papua New Guinea, 10° 16' S, 145° 38' E	*Lissoclinum patella*, AB211085.1	99	F1/R1
PNG_Lissoclinum_patella_11040	9-Nov-11	Papua New Guinea, 10° 16' S, 145° 38' E	*Lissoclinum patella*, AB211085.1	99	F1/R1
PNG_Lissoclinum_vareau_11047	10-Nov-11	Papua New Guinea, 10° 8' S, 145° 35' E	*Lissoclinum badium*, AB211078.1	86	F3/R3
PNG_Lissoclinum_vareau_11062	11-Nov-11	Papua New Guinea, 10° 2' S, 145° 32' E	*Lissoclinum badium*, AB211078.1	86	F3/R3
PNG_Lissoclinum_vareau_11068	11-Nov-11	Papua New Guinea, 10° 2' S, 145° 32' E	*Lissoclinum badium*, AB211078.1	86	F3/R3

PNG_Lissoclinum_vareau_11083	12-Nov-11	Papua New Guinea, 9° 35' S, 147° 17' E	*Lissoclinum badium*, AB211078.1	87	F3/R3
PNG_Lissoclinum_bistratum *(*L. voeltzkowi*)	05-Oct-07	Papua New Guinea, 4° 8' S, 151° 34' E	*Lissoclinum bistratum,* AB211084.1	99	F1/R1
VN_Cystodytes_sp_08002	28-Oct-08	Vanuatu, 15° 32.496' S, 167°12.895' E	*Cystodytes* sp., FM244842.1	94	AscF2/ AscR5
VN_Cystodytes_sp_08019	1-Nov-08	Vanuatu, 15° 36.675' S, 167° 01.258' E	*Cystodytes* sp., FM244842.1	91	AscF2/ AscR5
VN_Cystodytes_sp_08041	4-Nov-08	Vanuatu, 15° 25.416' S, 167° 12.476' E	*Cystodytes* sp., FM244842.1	94	AscF2/ AscR5

Table S2. Microbiome statistics

Number of tunicate samples	32
Number of filtered reads	217073
Number of 97 % OTUs	3982
Counts statistics (min/max/average)	572/31660/6784
OTUs statistics (min/max/average)	10/804/207

Table S3. List of primers used in this study

Primer	Sequence (5’-3’)	Source
AscF1	CTGGTTGATCCTGCCAG	Yokobori, 2006 (Yokobori et al 2006)
AscR1	CACCTACGGRWACCTTG	Yokobori, 2006 (Yokobori et al 2006)
AscF3	GATCCTGCCAGTAGTBATAT	Yokobori, 2006 (Yokobori et al 2006)
AscR3	TGATCCTTCTGCAGGTTCA	Yokobori, 2006 (Yokobori et al 2006)
AscF2new	CAAGGAAGGCAGCAGGCGCGCAAAT	This study
AscR5new	GCGGTGTGTACAAAGGGCAGGGA	This study
AscR3new	AAGGAATTGACGGAAGGGCACCACCAGGA	This study
18S1	CCTGGTTGATCCTGCCAG	Tsagkogeorga, 2009 (Tsagkogeorga 2009)
18S2	TAATGATCCATCTGCAGG	Tsagkogeorga, 2009 (Tsagkogeorga 2009)
27F	AGAGTTTGATCMTGGCTCAG	Lane, 1991 (Lane 1991)
1492R	CGGTTACCTTGTTACGACTT	Turner, 1999 (Turner et al 1999)
939F	TTGACGGGGGCCCGCACAAG	RTL in house primer

Table S4. Sequence counts and alpha diversity values of ascidian microbiomes measured using different diversity indices.

Sample	Counts	OTU	Shannon	Simpson	Chao1
CA_Ascidia_sp_4-19	2213	248	6.19	0.96	255.40
CA_Botryllus_sp_5-19	2406	220	5.68	0.94	229.67
CA_Didemnum_sp_4-17	3992	136	3.27	0.77	156.53
CA_Didemnum_sp_4-2	2959	106	2.87	0.68	117.12
CA_Didemnum_sp_4-7	2681	160	4.09	0.82	168.22
CA_Didemnum_sp_5-1	2478	170	4.86	0.92	182.00
CA_Didemnum_sp_5-11	3312	140	3.59	0.83	167.04
CA_Didemnum_sp_5-18	5068	149	3.15	0.67	152.89
CA_Pyura_sp_4-21	2841	58	2.54	0.73	60.50
CA_Styela_sp_5-16	2037	163	4.97	0.92	169.88
FJ_Lissoclinum_patella_06037	1594	209	4.35	0.84	393.53
FL_Didemnum_sp._tb3	7454	429	5.62	0.92	446.73
FL_Ecteinascidia_turbinata_5	10015	234	3.60	0.80	268.55
FL_Ecteinascidia_turbinata_tb2	31660	804	4.56	0.85	836.25
FL_Eudistoma_sp_004	13886	41	0.78	0.17	50.00
FL_Eudistoma_sp_tb1	12822	667	5.27	0.86	699.72
FL_Trididemnum_sp_001BB	1007	74	3.72	0.84	78.57
FL_Trididemnum_sp_tb4	572	10	0.61	0.15	10.25
PNG_Didemnum_sp_11038	14155	166	5.09	0.93	178.55
PNG_Didemnum_sp_11055	3372	290	6.79	0.98	300.44
PNG_Didemnum_sp_11089	5585	236	5.38	0.95	241.70
PNG_Lissoclinum_badium_11062	3916	92	4.20	0.90	98.18
PNG_Lissoclinum_badium_11068	8993	104	3.24	0.80	108.62
PNG_Lissoclinum_badium_11083	20324	278	2.40	0.56	284.36
PNG_Lissoclinum_badium_11047	1899	44	3.25	0.81	48.50
PNG_Lissoclinum_patella_11033	12851	347	3.08	0.65	368.72
PNG_Lissoclinum_patella_11040	13778	186	2.55	0.64	195.60
PNG_Lissoclinum_sp_7062	5528	114	4.39	0.88	121.00
PNG_Lissoclinum_bistratum	11015	475	3.68	0.64	779.59
VN_Cystodytes_sp_08002	2578	106	4.51	0.90	109.00
VN_Cystodytes_sp_08019	1610	97	4.49	0.92	100.62
VN_Cystodytes_sp_08041	2472	110	4.19	0.89	114.50

Table S5. Host species maps for networks analysis of ascidian microbiomes

SampleName	map1	map2	map3	map4
CA_Botrylloides_sp_5-19	Botryllus	Botryllus	Botryllus	Botryllus
CA_Pyura_sp_4-21	Pyura	Pyura	Pyura	Pyura
CA_Styela_sp_5-16	Styela	Styela	Styela	Styela
FL_Ecteinascidia_turbinata_5	Ecteinascidia_turbinata	Ecteinascidia_turbinata	Ecteinascidia_turbinata	Ecteinascidia_turbinata
FL_Ecteinascidia_turbinata_tb2	Ecteinascidia_turbinata	Ecteinascidia_turbinata	Ecteinascidia_turbinata	Ecteinascidia_turbinata
CA_Ascidia_sp_4-19	Ascidia	Ascidia	Ascidia	Ascidia
FL_Eudistoma_sp_004	Eudistoma_sp_1	Eudistoma_sp_1	Eudistoma_sp_1	Eudistoma_sp_1
FL_Eudistoma_sp_tb1	Eudistoma_sp_2	Eudistoma_sp_2	Eudistoma_sp_2	Eudistoma_sp_2
VN_Cystodytes_sp_08019	Cystodytes_sp	Cystodytes_sp_g2		
VN_Cystodytes_sp_08002	Cystodytes_sp	Cystodytes_sp_g1		
VN_Cystodytes_sp_08041	Cystodytes_sp	Cystodytes_sp_g1		
PNG_Lissoclinum_badium_11083	Lissoclinum_badium	Lissoclinum_badium_g1		
PNG_Lissoclinum_badium_11047	Lissoclinum_badium	Lissoclinum_badium_g2		
PNG_Lissoclinum_badium_11062	Lissoclinum_badium	Lissoclinum_badium_g2		
PNG_Lissoclinum_badium_11068	Lissoclinum_badium	Lissoclinum_badium_g2		
FJ_Lissoclinum_patella_06037	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella
PNG_Lissoclinum_patella_11033	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella
PNG_Lissoclinum_patella_11040	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella	Lissoclinum_patella
PNG_Lissoclinum_vareau_7062	Lissoclinum_sp	Lissoclinum_sp	Lissoclinum_sp	Lissoclinum_sp
PNG_Lissoclinum_bistratum	Lissoclinum_bistratum	Lissoclinum_bistratum	Lissoclinum_bistratum	Lissoclinum_bistratum
FL_Trididemnum_sp_001BB	Trididemnum_sp_1	Trididemnum_sp_1	Trididemnum_sp_1	Trididemnum_sp_1
FL_Trididemnum_sp_tb4	Trididemnum_sp_2	Trididemnum_sp_2	Trididemnum_sp_2	Trididemnum_sp_2
FL_Didemnum_sp_tb3	Didemnum_sp	Didemnum_sp_g1	Didemnum_sp	Didemnum_sp_g1
PNG_Didemnum_sp_11089	Didemnum_sp	Didemnum_sp_g1	Didemnum_sp	Didemnum_sp_g1
CA_Didemnum_sp_4-17	Didemnum_sp	Didemnum_sp_g1	Didemnum_sp	Didemnum_sp_g1
CA_Didemnum_sp_4-2	Didemnum_sp	Didemnum_sp_g1	Didemnum_sp	Didemnum_sp_g1
CA_Didemnum_sp_5-11	Didemnum_sp	Didemnum_sp_g1	Didemnum_sp	Didemnum_sp_g1
CA_Didemnum_sp_5-18	Didemnum_sp	Didemnum_sp_g2	Didemnum_sp	Didemnum_sp_g2
PNG_Didemnum_sp_11038	Didemnum_sp	Didemnum_sp_g2	Didemnum_sp	Didemnum_sp_g2
PNG_Didemnum_sp_11055	Didemnum_sp	Didemnum_sp_g2	Didemnum_sp	Didemnum_sp_g2
CA_Didemnum_sp_5-1	Didemnum_sp	Didemnum_sp_g2	Didemnum_sp	Didemnum_sp_g2
CA_Didemnum_sp_4-7	Didemnum_sp	Didemnum_sp_g2	Didemnum_sp	Didemnum_sp_g2
G(Host)	4.99	2.18	5.51	2.42
Pvalue	0.012	0.099	0.009	0.05
G(Location)	6.22	6.22	5.28	5.28
Pvalue	0.006	0.006	0.01	0.01

Table S6. Table of shared microbes (top 10) in locations (A-C) and hosts species (D-F) and their percent abundance.

A. California ascidians

#OTU ID	Consensus lineage	Average % abundance in samples	Present in % samples
denovo3071	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Chromatiales ; ; ;	0.16%	70
denovo116	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Alteromonadales ;Alteromonadaceae ;Glaciecola ;	0.10%	60
denovo3294	Bacteria ;Bacteroidetes ;Flavobacteriia ;Flavobacteriales ;Flavobacteriaceae	0.15%	60
denovo3389	Bacteria ;Cyanobacteria ;Chloroplast ;Stramenopiles ; ; ;	0.09%	60
denovo27	Bacteria ;Bacteroidetes ;Flavobacteriia ;Flavobacteriales ;Flavobacteriaceae ; ;	0.06%	50
denovo1982	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Rhizobiales	0.05%	50
denovo2251	Bacteria ;Acidobacteria ;Sva0725 ;Sva0725 ; ; ;	0.21%	50
denovo2984	Bacteria ;Proteobacteria	0.06%	50
denovo3340	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Alteromonadales ;OM60 ; ;	0.04%	50

B. Florida ascidians

#OTU ID	Consensus Lineage	Average % abundance in samples	Present in % samples
denovo701	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.17%	67
denovo766	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Vibrionales ;Vibrionaceae ;Vibrio	0.11%	67
denovo1101	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.08%	67
denovo1332	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Desulfobacterales ;Desulfobulbaceae ;	0.08%	67
denovo1589	Bacteria ;Proteobacteria ;Gammaproteobacteria	7.31%	67
denovo1834	Bacteria	0.38%	67
denovo1931	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Oceanospirillales ;Litoricolaceae ;Litoricola	0.38%	67
denovo2290	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Xanthomonadales ; ;	0.02%	67
denovo2327	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.43%	67

C. Papua New Guinea ascidians

#OTU ID	Consensus Lineage	Average % abundance in samples	Present in % samples
denovo866	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Alteromonadales ;Alteromonadaceae ;Alteromonas ;	0.30%	42
denovo1357	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	2.85%	42
denovo2569	Bacteria ;Proteobacteria ;Alphaproteobacteria	1.72%	33
denovo2672	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Desulfobacterales ;Nitrospinaceae ;Nitrospina ;	3.33%	33
denovo2769	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Oceanospirillales ;Oceanospirillaceae ;Marinomonas ;	0.04%	33
denovo3942	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Sva0853 ; ; ;	9.92%	33
denovo217	Bacteria ;Bacteroidetes ;Sphingobacteriia ;Sphingobacteriales ;Ekhidnaceae ; ;	0.10%	25
denovo283	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Kordiimonadales ;Kordiimonadaceae ; ;	0.02%	25
denovo352	Bacteria	0.09%	25

D. *Lissoclinum patella* (group 1, Figure 3B)

#OTU ID	Consensus Lineage	Average % abundance in samples	Present in % samples
denovo1567	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	46.08%	100
denovo2569	Bacteria ;Proteobacteria ;Alphaproteobacteria	6.86%	100
denovo2404	Bacteria ;Proteobacteria ;Gammaproteobacteria	6.17%	100
denovo1357	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	10.76%	100
denovo883	Bacteria ;Proteobacteria ;Alphaproteobacteria	0.14%	100
denovo832	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Rhodospirillales	2.77%	100
denovo1977	Bacteria ;Cyanobacteria ;Chloroplast ;Stramenopiles ; ; ;	0.12%	100
denovo231	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	0.10%	100
denovo3369	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	0.34%	100

E. *Lissoclinum badium* (group 2, Figure 3B)

#OTU ID	Consensus lineage	Average % abundance in samples	Present in % samples
denovo3942	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Sva0853 ; ; ;	39.67%	75
denovo3967	Bacteria ;Proteobacteria ;Alphaproteobacteria	4.08%	75
denovo1567	Bacteria ;Cyanobacteria ;Oscillatoriophycideae ;Chroococcales ;Prochloraceae ;Prochloron ;	2.52%	75
denovo2672	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Desulfobacterales ;Nitrospinaceae ;Nitrospina ;	9.99%	75
denovo279	Bacteria ;Proteobacteria	1.81%	75
denovo1477	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Rhizobiales ;Phyllobacteriaceae ;Mesorhizobium ;	2.98%	75
denovo1436	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.78%	75
denovo3289	Bacteria	0.72%	75
denovo2829	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Kordiimonadales ;Kordiimonadaceae ; ;	0.50%	75

F. *Didemnum* sp. (group 3, Figure 3B)

#OTU ID	Consensus lineage	Average % abundance in samples	Present in % samples
denovo2476	Bacteria ;Proteobacteria ;Alphaproteobacteria	17.42%	100
denovo2071	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Pseudomonadales ;Pseudomonadaceae ;Pseudomonas	4.20%	100
denovo1698	Bacteria ;Proteobacteria ;Deltaproteobacteria ;Bdellovibrionales ;Bdellovibrionaceae ;Bdellovibrio ;	1.11%	100
denovo42	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Pseudomonadales ;Pseudomonadaceae	0.18%	100
denovo2984	Bacteria ;Proteobacteria	0.13%	100
denovo1982	Bacteria ;Proteobacteria ;Alphaproteobacteria ;Rhizobiales	0.09%	100
denovo3206	Bacteria ;Proteobacteria ;Gammaproteobacteria ;Oceanospirillales ;Endozoicimonaceae ; ;	0.15%	75
denovo980	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.04%	75
denovo3508	Bacteria ;Proteobacteria ;Gammaproteobacteria	0.12%	75

Table S7. BLAST hits of 18S rRNA sequences of eukaryotes associated with ascidians

Ascidian sample	**Symbiotic eukaryote**	**Genbank accession number**
Eudistoma sp. 004	*Aiptasia mutabilis*	FJ489438.1
	Prostheceraeus vittatus	AJ312272.1
	Aiptasia pulchella	AY297437.1
Didemnum sp. 4-7	*Grateloupia luxurians*	U33132.1
Didemnum sp. 4-17	*Clytia gracilis*	DQ068053.1
	Clytia sp. AGC-200	AF358074.1
Didemnum sp. 5-1	*Baeria nivea*	AF182191.1
Didemnum sp. 5-11	*Selaginopsis cornigera*	Z92899.1
	Uncultured eukaryote clone SS1_E_02_11	EU050966.1
Didemnum sp. 5-18	*Igernella notabilis*	EU702420.1
	Rhacostoma atlantica	EU305501.1
	Melicertum octocostatum	AY920757.1
Cystodytes sp. 08002	*Lankesteria ascidiae*	JX187607.1
	Uncultured eukaryote clone SS1_E_02_1	EU050966.1
Cystodytes sp. 08019	*Corallochytrium limacisporum*	L42528.1
	Notodelphys prasina	JF781536.1
	Cyclopina gracilis	JF781537.1
Cystodytes sp. 08041	*Symbiodinium* sp.	AY165766.1
	Notodelphys prasina	JF781536.1
	Lankesteria ascidiae	JX187607.1
Didemnum sp. 11038	Uncultured eukaryote clone CC02A105.033	KF031747.1
	Cyclopina gracilis	JF781537.1
	Doridicola agilis	JF781541.1
Didemnum sp. 11055	*Cyclopina gracilis*	F781537.1
	Anticomidae sp. JCC29	HM564572.1
Didemnum sp.11089	*Melicertum octocostatum*	AY920757.1
	Dictyota sp. YMGTB002	AB087109.1
	Uncultured alveolate	FN263032.1
Eudistoma sp. TB1	*Calomicrolaimus parahonestus*	AY854218.1
	Sycettusa aff. hastifera OV-2012	JQ272322.1
	Spongia tubulifera	KC902150.1
	Grantessa sp. OV-2012 voucher GW979	JQ272312.1
Didemnum sp. TB3	*Posidonia australis*	GQ497582.1
	Spyridia filamentosa	EU718707.1
	Attheyella crassa	EU380307.1
Trididemnum sp. TB4	*Biemna fistulosa*	FR819688.1
	Thelepus crispus	JN936473.1
	Euplotes charon voucher JJM08102201	JF694043.1

Table S8. Summary of secondary metabolites and associated microbes in ascidians from this data set and related species.

Sample Code	Host Taxonomy	Location	Major associated secondary metabolites	Major symbiont characterized	Previous microbiome analysis of related species
CA_Ascidia_sp_4-19	*Ascidia ceratodes*	California			
CA_Botryllus_sp_5-19	*Bottrylus planus*	California			
CA_Didemnum_sp_4-17	*Didemnum* sp.	California			
CA_Didemnum_sp_4-2	*Didemnum* sp.	California			
CA_Didemnum_sp_4-7	*Didemnum* sp.	California			
CA_Didemnum_sp_5-1	*Didemnum* sp.	California			
CA_Didemnum_sp_5-11	*Didemnum* sp.	California			
CA_Didemnum_sp_5-18	*Didemnum molle*	California			
CA_Pyura_sp_4-21	*Pyura haustor*	California			
CA_Styela_sp_5-16	*Styela plicata*	California	plicatamide		Erwin et al, 2013
FL_Didemnum_sp_tb3	*Didemnum* sp.	Florida			
FL_Ecteinascidia_turbinata_5	*Ecteinascidia turbinata*	Florida	ecteinascidins	*Ca.* Endolisoclinum frumentensis	Rath 2011, Perez 2007, Moss 2003
FL_Ecteinascidia_turbinata_tb2	*Ecteinascidia turbinata*	Florida	ecteinascidins	*Ca.* Endolisoclinum frumentensis	Rath 2011, Perez 2007, Moss 2003
FL_Eudistoma_sp_004	*Eudistoma* sp.	Florida	eudistomins		
FL_Eudistoma_sp_tb1	*Eudistoma* sp.	Florida	eudistomins		
FL_Trididemnum_sp_001BB	*Trididemnum* sp.	Florida			
FL_Trididemnum_sp_tb4	*Trididemnum* sp.	Florida			
PNG_Didemnum_sp_11038	*Didemnum* sp.	Papua New Guinea			
PNG_Didemnum_sp_11055	*Didemnum* sp.	Papua New Guinea			
PNG_Didemnum_sp_11089	*Didemnum* sp.	Papua New Guinea			
PNG_Lissoclinum_patella_11033	*Lissoclinum patella*	Papua New Guinea	cyanobactins, patellazoles	*Prochloron* spp., *Ca.* Endolissoclinum faulkneri	Schmidt 2005, Donia 2008, Kwan 2012, Behrendt 2012
PNG_Lissoclinum_patella_11040	*Lissoclinum patella*	Papua New Guinea	cyanobactins, patellazoles	*Prochloron* spp.,	Schmidt 2005, Donia 2008, Kwan 2012, Behrendt 2012
FJ_Lissoclinum_patella_06037	*Lissoclinum patella*	Fiji	cyanobactins, patellazoles	*Prochloron* spp., *Ca.* Endolissoclinum faulkneri	Schmidt 2005, Donia 2008, Kwan 2012, Behrendt 2012
PNG_Lissoclinum_sp_7062	*Lissoclinum* sp.	Papua New Guinea			
PNG_Lissoclinum_badium_11047	*Lissoclinum badium*	Papua New Guinea	pyridoacridine alkaloids	*Nitrospina* sp.	Erwin 2013
PNG_Lissoclinum_badium_1	*Lissoclinum*	Papua New	pyridoacridine	*Nitrospina* sp.	Erwin 2013

1062	*badium*	Guinea	alkaloids		
PNG_Lissoclinum_badium_11068	*Lissoclinum badium*	Papua New Guinea	pyridoacridine alkaloids	*Nitrospina* sp.	Erwin 2013
PNG_Lissoclinum_badium_11083	*Lissoclinum badium*	Papua New Guinea	pyridoacridine alkaloids	*Nitrospina* sp.	Erwin 2013
PNG_Lissoclinum_bistratum	*Lissoclinum bistratum*	Papua New Guinea	cyanobactins, bistramides	*Prochloron* spp.,	
VN_Cystodytes_sp_08002	*Cystodytes* sp.	Vanuatu	pyridoacridine alkaloids		
VN_Cystodytes_sp_08019	*Cystodytes* sp.	Vanuatu	pyridoacridine alkaloids		
VN_Cystodytes_sp_08041	*Cystodytes* sp.	Vanuatu	pyridoacridine alkaloids		

Table S9. Statistical comparisons of ascidian microbiomes across species, and within the same species across locations and over time (at the same location). There was a statistically significant difference in ascidian microbiomes across species, and within the same species across locations, but no difference in microbiomes within a species collected at two different time points. Analyses were performed only on groups with a minimum of two samples using the ANOSIM method in the vegan R package(ANOSIM R, P-value1) with 100,000 permutations. A sensitivity analysis was performed using a second permutation ANOVA method "adonis" also available in vegan (R^2, P-value2).

Samples/Groups	# Samples	ANOSIM R	P-value1	R^2	P-value2
All Papua New Guinea	**11**	**0.762**	**0.002**	**0.426**	**0.002**
Didemnum	3				
Lissoclinum_badium	4				
Lissoclinum_patella	2				
All *Didemnum*	**9**	**0.846**	**0.011**	**0.252**	**0.012**
Papual New Guinea	3				
California	6				
California *Didemnum*	**6**	**-0.037**	**0.499**	**0.171**	**0.701**
Fall collection	3				
Spring collection	3				

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