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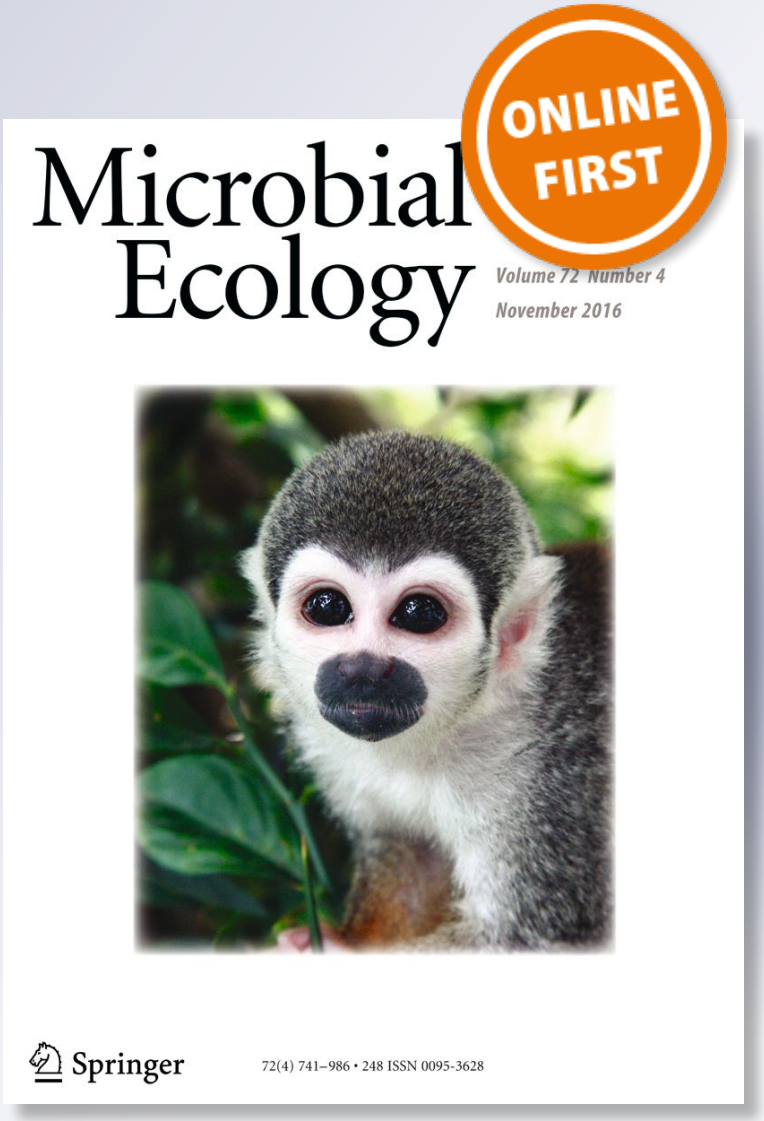

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Core and Dynamic Microbial Communities of Two Invasive Ascidians: Can Host–Symbiont Dynamics Plasticity Affect Invasion Capacity?

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Abstract

Ascidians (Chordata, Ascidiacea) are considered to be prominent marine invaders, able to tolerate highly polluted environments and fluctuations in salinity and temperature. Here, we examined the seasonal and spatial dynamics of the microbial communities in the inner-tunic of two invasive ascidians, *Styela plicata* (Lesueur 1823) and *Herdmania momus* (Savigny 1816), in order to investigate the changes that occur in the microbiome of non-indigenous ascidians in different environments. Microbial communities were characterized using next-generation sequencing of partial (V4) 16S rRNA gene sequences. A clear differentiation between the ascidian-associated microbiome and bacterioplankton was observed, and two distinct sets of operational taxonomic units (OTUs), one core and the other dynamic, were recovered from both species. The relative abundance of the dynamic OTUs in *H. momus* was higher than in *S. plicata*, for which core OTU structure was maintained independently of location. Ten and seventeen core OTUs were identified in *S. plicata* and *H. momus*, respectively, including taxa with reported capabilities of carbon fixing, ammonia oxidization, denitrification, and heavy-metal processing. The ascidian-sourced dynamic OTUs clustered in response to site and season but significantly differed from the bacterioplankton community structure. These findings suggest that the associations between invasive ascidians and their symbionts may enhance host functionality while maintaining host adaptability to changing environmental conditions.

Keywords Introduced species · Microbiome · Tunicate · Lessepsian invasion · *Herdmania momus* · *Styela plicata*

Introduction

Marine invertebrates are considered holobionts, a functional ecological unit combining the host and its various taxa of microbial communities (bacteria, archaea, fungi, microalgae, and viruses) [1, 2]. These microorganisms are associated with a wide range of host-beneficial functions: synthesis of metabolic substances in the carbon [3, 4]; nitrogen [5–7] and sulfur cycles [8], contribution to reproductive processes [9–11], and support of structural rigidity [12]. Symbiotic bacteria may also supply growth factors such as vitamins and amino acids to their host [13], assist in food digestion [14, 15], produce secondary metabolites for chemical defenses [16–20], and protect their host from pathogens [21–23].

Invasive species are considered a major cause of species extinction worldwide [24]. Marine ecosystems are especially vulnerable to colonization since only a few physical barriers exist to inhibit introductions. Previous studies have demonstrated the importance of a suitable microbiome for the successful establishment of an introduced species in a new ecosystem [25, 26]. The fungal parasite *Fibrillanosema crangonycis*, for example, contributed to the invasion of its host, the amphipod *Crangonyx pseudogracilis*, into the UK by inducing an increased female-to-male ratio of the host which may influence reproductive output [27]. The rhizosphere of invasive plants in the introduced range have also been reported to promote plant fitness by increasing the invasive plant biomass and nutrient acquisition, as well as assisting in

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disease suppression [28]. Furthermore, an introduced host that is able to maximize the contribution of the microbiome to enhance its own functionality (enhanced metabolic substances, expanded tolerance thresholds, etc.) may gain a competitive advantage over the local species [29]. It is therefore intriguing to determine whether a host invades the new ecosystem carrying its associated community of microorganisms or whether it acquires beneficial local bacteria following its invasion. Vertical transmission provides symbiont assurance, guaranteeing symbiont functionality for the offspring, yet may reduce adaptability since the symbionts may not be optimally adapted to the new local environment. Horizontal transfer may promote faster acclimation of an introduced host into new habitats by enabling them to establish relationships with beneficial local bacteria [30] but at the risk of limited establishment if the required symbionts are not available in the new ecosystem [31]. Zurel et al. [26] and Roterman et al. [32] concluded that the bivalves *Chama pacifica* and *Spondylus spinosus* had co-invaded the eastern Mediterranean Sea (EMS) along with their symbiont taxa that were tolerant of the temperature fluctuations typical to the Mediterranean, thus assisting their hosts' acclimation to the new environment. In contrast, Erwin et al. [30] found highly variable bacterial lineages in the tunic of *Styela plicata* in the western Mediterranean, most of which were probably acquired with each host generation. Those authors further suggested that a dynamic microbial community structure may promote the acclimation of an introduced host into new habitats by enabling them to establish relationships with beneficial local bacteria. Increased plasticity is one of the characteristics of successful invasive species, allowing high tolerance to environmental heterogeneity [33]. Plasticity of the symbiont microbial community enables the holobiont to adapt to such heterogeneity more rapidly and with greater versatility than a process that is dependent on genetic mutation and selection of the host [11].

Ascidians (Phylum: Chordata, Class: Ascidiacea) are sessile marine filter-feeders inhabiting both natural and artificial substrates [34, 35]. The class Ascidiacea comprises ~3000 species found in all marine habitats [36]. Most ascidians are hermaphroditic [37] and produce short-lived, motile lecithotrophic larvae [38, 39]. The introduction rate of non-indigenous ascidians has been rising in the last few decades [40], mostly due to increasing marine traffic and aquaculture activities [37, 41]. Marinas and harbors play a particularly important role in the establishment of newly introduced ascidians as they act not only as the gateways to invasion but also offer favorable conditions—unoccupied substrate, reduced wave action, and enhanced nutrients resulting from anthropogenic activities [42, 43]. Invasive ascidians have been known to thrive in harsh environments and polluted marinas, exhibiting tolerance to fluctuating temperature and salinity [44–46], as well as to pollution from sewage, land runoff, and heavy metals [43, 47]. Studies have shown that some of these resilient qualities may be partially attributed to symbiotic microorganisms living within the ascidian tunic [48, 49].

Next-generation sequencing has revealed that the ascidian inner-tunic is enriched with diverse microorganisms and considered a hotspot for symbiont-associated processes [30, 50]. These include the production of certain secondary metabolites that serve to reduce predation and assist in competition over substrate [48, 51] and the synthesis of metabolic substances [6, 52]. The inner-tunic microbiome includes taxa previously described in other marine invertebrate hosts as well as ascidian-specific microorganisms and is clearly differentiated from the ambient bacterioplankton community [50]. Both vertical and horizontal transmissions appear to play a role in the construction of the ascidian microbiome [29, 51]. Stable species-specific symbiotic microbial associations may point to the potential role of vertical transfer, especially for colonial ascidians in which the developing embryo is often brooded [53, 54], while selective enrichment of the rare biosphere bacteria points to a horizontal acquisition of, at least, some of these symbionts [30, 50].

Herdmania momus (Savigny 1816) and *Styela plicata* (Lesueur 1823) are two solitary ascidians with documented invasion records. *H. momus* is considered native to the Red Sea (RS) and was introduced into the EMS via the Suez Canal in 1958 [55–57], while the invasion route of *S. plicata* is still unresolved [58]. Until recently, *H. momus* had been restricted to artificial substrates at depths of > 1 m along the Mediterranean coast of Israel [57, 59], but in 2013, a large population was observed on natural rocky substrate [60]. *S. plicata* has been introduced into harbors of temperate and subtropical waters world-wide and exhibits well-established populations in many regions of the world [58, 61]. However, its origin is no longer traceable due to recurrent colonization events and shuffling among populations [58]. Of particular relevance for this study is *S. plicata's* well-established presence along the Atlantic coast of North Carolina (NC) [44, 58, 62]. Along the Mediterranean coast of Israel, in contrast, the presence of a large number of individuals on artificial substrates has only recently been reported for several marinas [63]. The population at the current study site, Jaffa Port, which was first reported 4 to 5 years ago, is currently the highest among the marinas in Israel [63, 64].

Here, we characterize the seasonal and spatial dynamics of the microbial communities in the inner-tunic of two introduced ascidians: *H. momus* collected from both its native (RS) and introduced ranges (EMS) and *S. plicata* from a well-established population in NC and a recently introduced population in the EMS. We hypothesize that some fractions of the microbial symbiont community may undergo change in composition in response to different environmental conditions, while others will remain stable as part of an obligatory functional microbiom.

Methods

Sample Collection

Sampling of *S. plicata* was conducted in Jaffa Port, EMS, Israel (32° 03′ 06.3″ N, 34° 44′ 58.1″ E; recently introduced population) and Wilmington, North Carolina, USA (34° 8′ 27.47″ N, 77° 51′ 59.16″ W; well-established population). Sea-surface temperature (SST) and salinity were recorded during sampling at both locations. Three individuals were collected from each location (total $n = 24$) on the first day of February (winter), May (spring), August (summer), and November (autumn) 2016 from a depth that ranged from zero to 1 m. During each sampling, the individuals were collected by carefully detaching them from submerged ropes or floating docks and placing them in separate Ziploc plastic bags containing seawater. Three seawater samples of 500 ml each were collected at the same time for each location in separate sterile plastic bags or jars, to provide background data on the bacterioplankton. Ascidian identification in the field was based on external morphological characteristics and confirmed later via barcoding of a fragment of the cytochrome *c* oxidase I (COX1) gene (GenBank accession numbers MH383107–MH383130) from the siphon. Samples were placed in a cooler and immediately transported to the lab, where they were separated into body and tunic using a sterile scalpel. A piece of the inner tunic (i.e., tunic tissue connected to the body wall which is not in contact with the ambient seawater) was removed using sterile scalpel and scissors, fixed in 100% ethanol, and stored at 4 °C prior to DNA extraction. Water samples were filtered through 0.2 μM polycarbonate filters (PCTE filters, Maine Manufacturing, ME, USA), which were stored at − 80 °C.

Sampling of *H. momus* was conducted at the Akhziv National Park, EMS, Israel (33° 2′ 55.10″ N, 35° 6′ 0.33″ E) and the Elat Marina, RS, Israel (29° 33′ 10.51″ N, 34° 57′ 37.02″ E) for the introduced and native range, respectively. In order to investigate the effect of temperature on the microbial community stability of *H. momus* in its native and introduced ranges, sampling was conducted at both sites concomitantly when SST at both places were minimum (17 and 20 °C in Akhziv and Elat, respectively), transitional (25 and 27 °C), and maximum (29 and 28 °C). Samples ($n = 18$) were collected during January, June, and July of 2016 (to reflect winter, spring, and summer seasons). At each site three individuals were collected and identified first morphologically and later via COXI barcoding of a piece of the siphon (GenBank accession numbers MH383131–MH383137).

Sampling collection methods were suited to the sites' different characteristics. In Elat seawater and ascidians were collected from the docks at ~ 0.5 m depth and immediately transported to the Interuniversity Institute for Marine Sciences (IUI, Elat) in a cooler for handling (as described for the *S. plicata* samples). In Akhziv National Park, the 2016 January and July samplings were conducted by SCUBA diving at a depth of 14–18 m, while the June sampling was carried out by snorkeling at a depth of 0.5 m due to bad diving conditions. Individuals were collected from rocks and immediately handled in situ in the same manner as described above for *S. plicata*. Three 500 ml seawater samples were collected concomitantly and sent to Tel-Aviv in a cooler for filtering as described above.

DNA Extraction and Next-Generation Illumina MiSeq Sequencing of 16S rRNA

All DNA extractions were performed using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). DNA was amplified using the universal bacterial/archaeal primer set 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) [65]. PCR cycling conditions included an initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, 50 °C for 15 s, and 72 °C for 20 s; and a final extension at 72 °C for 2 min. Both DNA and PCR products were visualized on 1% agarose gels to ensure quality and presence of 16S rRNA amplicons. DNA samples were sent to Molecular Research LP (Shallowater, TX, USA) for Illumina MiSeq amplification and sequencing. A fragment of approximately 300 bp of the V4 region of the 16S rRNA gene was obtained using the same primer set as above.

Next-Generation Sequence Data Processing

Raw sequences were processed using the Mothur software package [66] following a modified version of the Illumina MiSeq SOP pipeline [67] as described in Weigel and Erwin [68]. Briefly, raw sequence reads were filtered for quality reads, aligned with the SILVA reference database (v119), and then trimmed to the V4 region. To reduce sequencing errors, putative chimeras were removed using the precluster and UChime [69] algorithms. Sequences were then classified using a naive Bayesian classifier and bootstrap algorithm [70], non-target reads (chloroplasts, mitochondria, and eukarya) were removed, and 97% sequence similarity OTUs were constructed. To standardize sampling depths (i.e., number of sequence reads) among samples, each dataset was subsampled to the lowest good-quality read count ($n = 29{,}810$ for *S. plicata*, $n = 43{,}181$ for *H. momus*). Two low-quality seawater samples that had been collected with the *H. momus* samples from Elat in July and Akhziv in January were removed, and all subsequent data analyses were based on the resulting subsampled dataset.

Microbial Community Diversity and Structure Analysis

The following microbial community analyses were performed using the R package phyloseq [71] for each of the species separately. The alpha diversity indices for OTU richness (S), expected OTU richness (Chao1), Shannon-Weaver diversity index (H), and inverse Simpson index (D) were calculated for ascidian and seawater samples. Analyses of variance (two-way ANOVA) were used to statistically compare all indices between locations and seasons. Tukey's HSD tests were conducted for multiple pairwise post hoc comparisons of means for significantly different ANOVA results. To compare the microbial community structures among sources, Bray–Curtis similarity (BCS) matrices were constructed and then visualized using principal coordinates analysis (PCoA) plots.

Permutational multivariate ANOVA (PERMANOVA) were calculated using the R package vegan [72] (999 permutations) and used to test for significant differences in microbial community structure among the different sources (ascidian vs. seawater), locations (Jaffa vs. Wilmington and Akhziv vs. Elat), and seasons. Multiple pairwise comparisons were used to test significant PERMANOVA results. Pairwise comparison values were corrected using the Benjamini–Yekutieli (BY) false-discovery rate control, setting an error rate of $p = 0.05$ [73]. Permutational multivariate analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA results to ensure that results were due to structural differences and not to unequal dispersion of variability among groups. Similarity percentage analyses (SIMPER) were calculated using the R package vegan and used to determine the main contributors to the observed dissimilarity in microbial community structure. Venn diagrams were created using the R package VennDiagram to visualize OTU overlap among sampling sources.

All analyses were performed for five different datasets of OTUs: overall, core, dynamic, abundant, and rare. To define the "rare" and "abundant" microbial taxa, a 0.1% threshold [74] was employed, resulting in a cut-off value of 29 reads for the *S. plicata* samples ("abundant" OTUs featured > 29 total reads, "rare" OTUs featured ≤ 29 total reads) and 43 for the *H. momus* samples. "Core" OTUs were defined as taxa shared by all host individuals with more than ten reads in each sample. "Dynamic" OTUs were defined as abundant taxa (> 0.1%) that were absent from the core. The core OTUs were further identified using BLASTn search based on highest percentage identity matches and reported host sources.

Data Availability Identification guides and genetic barcodes of *Styela plicata* and *Herdmania momus* are available for public use through the iMESA Website: http://www.imesalab.com/iMESA/Ascidian_barcode.html. New sequences are deposited at NCBI GenBank under accession numbers MH383107–MH383137

Results

Styela plicata Overall Microbial Community Composition

Table 1 summarizes the diversity and richness metrics for *S. plicata* and seawater samples in Jaffa (Israel) and Wilmington (NC) across seasons. Two-way ANOVA analysis revealed no significant difference between locations for all indices ($p > 0.05$, $F > 2.1$, $df = 3$). Significant differences in the richness indices (S, $p = 0.02$, $F = 4.2$, $df = 3$; Chao, $p < 0.0001$, $F = 52.2$, $df = 3$) were observed between seawater and ascidian samples from both locations (Table 1a). Examining the ascidian samples separately, no significant difference between locations or seasons was detected in any of the alpha diversity indices (Table 1b). A significant interaction between location and season was found only for the richness indices (S and Chao, $p < 0.05$, $F > 2.1$, $df = 3$; Table 1b).

Analyses of 16S rRNA sequences derived from *S. plicata* and ambient seawater in Jaffa and Wilmington revealed a high richness and diversity of microbial communities. In total, 25,332 unique OTUs were recovered from *S. plicata* ($n = 11{,}936$) and seawater samples ($n = 19{,}246$; Fig. 1a). More than two thirds of the OTUs (67.2%, $n = 17{,}029$) were detected exclusively in one source: Jaffa seawater ($n = 5072$), Wilmington seawater ($n = 6959$), Jaffa *S. plicata* ($n = 2724$), and Wilmington *S. plicata* ($n = 2274$), and only 1616 OTUs (6.3%) were present in all four sources.

The seawater communities comprised 55 phyla in Jaffa and 62 phyla in Wilmington and were dominated by Proteobacteria (67.6 and 54.9% for Jaffa and Wilmington, respectively), Bacteroidetes (27.5 and 19.2%), and Actinobacteria (1.4 and 13.5%). The *S. plicata*–associated microbiota was represented by 48 and 51 phyla (Jaffa and Wilmington, respectively), with the most abundant being Proteobacteria (62 and 70.4%), Crenarchaeota (7.9 and 4.5%), Bacteroidetes (7.3 and 7.8%), and Planctomycetes (6.4 and 4.4%); 3727 OTUs (31.2% of ascidian OTUs) were shared by *S. plicata* samples from both locations, of which 29% ($n = 1088$) were exclusive to the ascidians (i.e., not found in the surrounding seawater). Ascidian-exclusive OTUs were composed of 29 bacterial phyla, with the most abundant being Proteobacteria (37.9%), Planctomycetes (21.9%), and Bacteroidetes (12.2%), and one archaeal phylum (Crenarchaeota).

Herdmania momus Overall Microbial Community Composition

Table 2 summarizes all diversity and richness metrics calculated for *H. momus* and seawater samples in Elat (RS) and Akhziv (EMS) across seasons. Two-way ANOVA analyses revealed no significant difference between locations for all

Table 1 Diversity metrics for microbial communities associated with *S. plicata* at overall (a) and seasonal (b) data partitions

Source	S	Chao	D	H′
a. Overall				
S. plicata Jaffa	1810.3 ± 589.7 [a]	2675.0 ± 810.2 [a]	29.8 ± 27.0 [a]	4.8 ± 1.0 [a]
S. plicata Wilmington	1548.1 ± 470.5 [a]	2279.1 ± 540.9 [a]	23.4 ± 24.4 [a]	4.3 ± 1.3 [a]
Seawater Jaffa	1783.1 ± 360.9 [b]	4797.3 ± 807.7 [b]	12.0 ± 4.0 [a]	3.6 ± 0.2 [a]
Seawater Wilmington	2230.1 ± 452.2 [b]	5550.2 ± 858.8 [b]	27.4 ± 8.23 [a]	4.6 ± 0.2 [a]
b. Seasonal				
Jaffa fall	1105.3 ± 219.4 [a]	1624.1 ± 186.1 [a]	8.2 ± 7.8 [a]	3.7 ± 1 [a]
Jaffa spring	1844.3 ± 838.3 [a]	2915.3 ± 1055.7 [a]	34.6 ± 31.6 [a]	5 ± 0.9 [a]
Jaffa summer	2121.6 ± 251.4 [a]	3001.2 ± 396.6 [a]	36 ± 33.9 [a]	5.2 ± 0.7 [a]
Jaffa winter	2170 ± 110 [a]	3159.3 ± 221.7 [a]	40.3 ± 29.3 [a]	5.4 ± 0.5 [a]
Wilmington fall	1944 ± 326.1 [a]	2750 ± 303.7 [a]	13.4 ± 8.9 [a]	4.5 ± 0.9 [a]
Wilmington spring	1293.3 ± 581.2 [a]	2071.8 ± 722.3 [a]	17.9 ± 27.9 [a]	3 ± 2.2 [a]
Wilmington summer	1267.6 ± 523.8 [a]	1833.5 ± 387.9 [a]	45.5 ± 37.3 [a]	4.9 ± 0.9 [a]
Wilmington winter	1687.6 ± 139.4 [a]	2461.3 ± 316.2 [a]	16.8 ± 6.6 [a]	4.8 ± 0.3 [a]

Average values (± 1 SD) are shown, with different letters denoting significantly different means among sources
S, observed; *Chao1*, expected richness; *D*, inverse Simpson; *H′*, Shannon-Weaver

indices ($p > 0.05$, $F > 0.04$, $df = 2$), while a significant difference between ascidian and seawater samples was found for most indices, except expected richness (Chao, $p = 0.8$, $F = 0.03$, $df = 1$) (Table 2a).

Examination of the ascidian samples only, revealed a significant difference in the inverse Simpson index (D) between seasons and locations (two-way ANOVA, $p = 0.02$, $F = 3.81$, $df = 5$). Pairwise comparisons revealed significant differences between Akhziv and Elat in the summer ($p = 0.03$) and between summer and winter in Elat ($p = 0.03$) (Table 2b).

A total of 18,771 OTUs were recovered from *H. momus* ($n = 13{,}687$) and the seawater samples ($n = 10{,}895$; Fig. 1b). Of the OTUs, 58.1% ($n = 10{,}910$) were detected exclusively in one source: Akhziv seawater ($n = 2647$), Elat seawater ($n = 1826$), Akhziv *H. momus* ($n = 3460$), and Elat *H. momus* ($n = 2977$), and only 1597 OTUs (8.5%) were present in all four sources.

The seawater communities comprised 42 phyla in Akhziv and 45 phyla in Elat and, like the Jaffa and Wilmington seawater, contained a high number of Proteobacteria OTUs (66.8 and 57.9% for Akhziv and Elat respectively), Bacteroidetes (8.3 and 13.6%), and Actinobacteria (4.3 and 12%). However, *Cyanobacteria* (8.6 and 6.3%) and Planctomycetes (7.4 and 0.7%) were also prevalent here. The *H. momus* microbiome comprised 46 and 45 phyla (Akhziv and Elat, respectively) and, as in *S. plicata*, it was dominated by Proteobacteria (59.5 and 64.3%), Planctomycetes (7.8 and 7.9%), and Bacteroidetes (4.6 and 12.2%). The archaea Crenarchaeota were prevalent in Akhziv (13.1%) but less so in Elat (0.6%).

H. momus ascidian-exclusive OTUs (i.e., not found in the surrounding seawater) exhibited a similar phyla composition to that of *S. plicata*–exclusive OTUs, with a dominance of the bacterial phyla Proteobacteria (41.2%), Planctomycetes

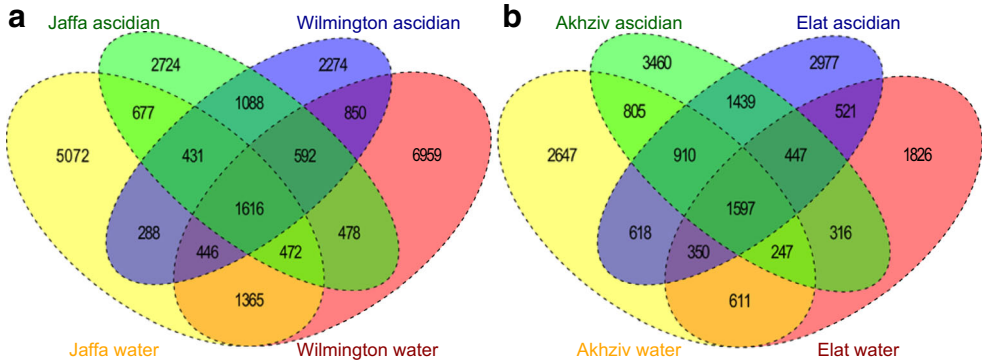

Fig. 1 Venn diagram depicting OTU richness and overlap in the total-set microbial communities of *S. plicata* (**a**) and *H. momus* (**b**). Introduced ascidian (green), native/established ascidian (blue), introduced region seawater (yellow), and native/established region seawater (red). Total OTU richness was 25,332 and 18,771 (*S. plicata* and *H. momus*, respectively) among the four sources

Table 2 Diversity metrics for microbial communities associated with *H. momus* at overall (a) and seasonal (b) data partitions

Source	S	Chao	D	H′
a. Overall				
H. momus Akhziv	2545.3 ± 901.5 [a]	3881.0 ± 1261.9 [a]	31.4 ± 26.5 [a]	5.0 ± 0.9 [a]
H. momus Elat	2305.2 ± 869.1 [a]	3787.5 ± 1152.8 [a]	94.1 ± 109.8 [a]	5.3 ± 0.9 [a]
Seawater Akhziv	1862.5 ± 525.4 [b]	3963.7 ± 1568.5 [a]	20.2 ± 7.4 [b]	4.2 ± 0.3 [b]
Seawater Elat	1353.8 ± 367.9 [b]	3876.9 ± 1250.9 [a]	17.6 ± 7.6 [b]	3.7 ± 0.4 [b]
b. Seasonal				
Akhziv summer	2030 ± 1175.1 [a]	3206.7 ± 1450.1 [a]	26.1 ± 22 [ac]	4.7 ± 0.9 [a]
Akhziv winter	3232.6 ± 106.8 [a]	5153.9 ± 8.5 [a]	33.4 ± 13.3 [a]	5.4 ± 0.4 [a]
Akhziv spring	2373.3 ± 840.8 [a]	3282.3 ± 785.1 [a]	34.7 ± 45.7 [ab]	4.8 ± 1.4 [a]
Elat summer	2147.6 ± 1288.1 [a]	3127.5 ± 1281.8 [a]	204.3 ± 128.7 [b]	6 ± 0.8 [a]
Elat winter	2529 ± 820.4 [a]	4240.7 ± 1398.2 [a]	25.3 ± 20.3 [ac]	4.8 ± 0.8 [a]
Elat spring	2239 ± 820.4 [a]	3994.3 ± 832.1 [a]	52.9 ± 58.5 [abc]	5.1 ± 0.8 [a]

Average values (± 1 SD) are shown, with different letters denoting significantly different means among sources
S, observed; *Chao*, expected richness; *D*, inverse Simpson; *H′*, Shannon-Weaver

(31.6%), and Bacteroidetes (9.7%) and the archaeal phylum Crenarchaeota.

Overall Microbial Community Structure

Statistical analyses of the overall microbial community structure (i.e., the total dataset including ascidian and water samples) (PERMANOVA) revealed significant differences for both ascidian species between sample types (*S. plicata* vs. seawater, $R = 0.34$, $p = 0.001$; *H. momus* vs. seawater, $R = 0.27$, $p = 0.001$), locations (Jaffa vs. Wilmington, $R = 0.07$, $p = 0.001$; Akhziv vs. Elat, $R = 0.11$, $p = 0.001$), and their interaction (*S. plicata*, $R = 0.07$, $p = 0.001$; *H. momus*, $R = 0.08$, $p = 0.001$) (Fig. 2). Statistical analyses of dispersion (PERMDISP) revealed no significant differences between locations ($p = 0.6$). However, a significant difference between *H. momus* and seawater ($p = 0.007$) was found. SIMPER analyses identified OTU 01 (13%, Family Hyphomicrobiaceae) and OTU 02 (5.2%, Family Rhodobacteraceae) as the main contributors to the dissimilarity between *S. plicata* and seawater and OTU 01 (9.5%) as the main contributor to the dissimilarity between locations (Table 3). For the *H. momus* samples, SIMPER analyses could not identify the key driver of dissimilarity between sample types or locations since all OTUs individually contributed less than 5% to the observed dissimilarity (Table 4).

A similar pattern was observed when only the ascidian-associated microbial communities were analyzed. Statistical analyses revealed a significant variation in community structure between locations (PERMANOVA, *S. plicata*, $R = 0.14$, $p = 0.001$; *H. momus*, $R = 0.27$, $p = 0.001$). No significant difference in disparity was detected in either species (PERMDISP, $p > 0.05$). SIMPER analyses identified OTU 01 as the main contributor (12.1%) to the dissimilarity between the locations of *S. plicata* (Table 3b) but could not identify a key contributor to dissimilarity between the locations for *H. momus* (Table 4b).

Seawater bacterial communities in all four locations clustered in response to both location and season (PERMANOVA, *S. plicata*, $R = 0.3$, $p = 0.001$; *H. momus*, $R > 0.27$, $p = 0.001$), whereas the ascidian-associated microbiome displayed seasonal stability for the total dataset (PERMANOVA, *S. plicata*, $R = 0.13$, $p = 0.16$; *H. momus*, $R = 0.11$, $p = 0.12$). When examining the Akhziv-sourced *H. momus* separately, minor seasonal clustering was detected (PERMANOVA, $R = 0.34$, $p = 0.05$) and no significant differences in dispersion were found (PERMDISP, $p = 0.16$).

Core Community Composition and Structure

The core symbiont community (i.e., OTUs shared by all host individuals with > 10 reads) comprised 10 and 17 OTUs for *S. plicata* and *H. momus*, respectively (Online resource Fig. S1). Core OTUs accounted for ~ 0.1% of ascidian-associated OTUs but were disproportionately abundant, representing 36.2 and 26% (*S. plicata* and *H. momus*, respectively) of ascidian bacterial sequence reads. NCBI GenBank BLASTn search of Core OTU sequences of both species revealed a high similarity to known environmental and invertebrate-associated microbes (Online resource Table ST1).

The core community of *S. plicata* was noticeably different in composition to that of *H. momus*. The *S. plicata* core comprised two phyla: Proteobacteria (77.4% of the core) and Crenarchaeota (22.5%). The top-most abundant families were Hyphomicrobiaceae (61.5%), Cenarchaeaceae (22.5%; all from the genus *Nitrosopumilus*), Rhodobacteraceae (8.6%), and Piscirickettsiaceae (4.5%) (Online resource Fig. S2a). The

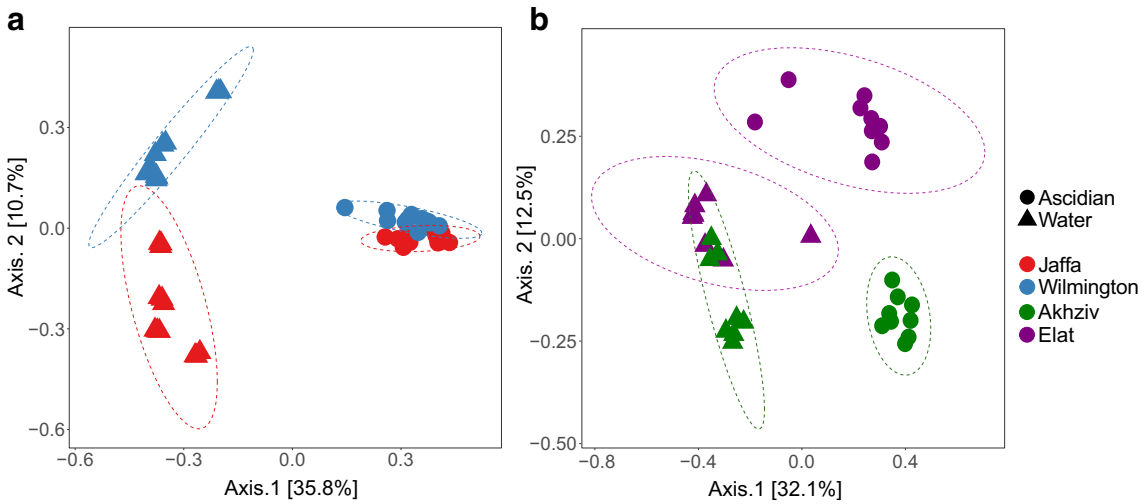

Fig. 2 PCoA plot based on Bray–Curtis similarity of the total microbial fauna associated with *S. plicata* (**a**) from Jaffa (recently introduced population; in red) and Wilmington (well-established population; in blue) and *H. momus* (**b**) in its newly introduced region (Akhziv; in green) and its native region (Elat; in purple). Ascidians (in circles) and seawater (in triangles). Ellipses depict normal distribution around replicate source samples and indicate both host and geographical specificity

H. momus core comprised six bacterial phyla and was dominated by the phylum Proteobacteria (88% of the core). The topmost abundant families were Rhodobacteraceae (36.1%), an unclassified family from the order Kiloniellales (22.1%), Hyphomicrobiaceae (9.5%; all genus *Hyphomicrobium*), Piscirickettsiaceae (8.9%), and Puniceicoccaceae (5.4%; all genus *Coraliomargarita*) (Online resource Fig. S2b).

Although the *S. plicata* individuals at each sites were assigned to different haplotypes [64], core communities maintained a similar community structure in both locations (PERMANOVA, $R = 0.07$, $p = 0.14$; Fig. 3a). SIMPER analysis identified OTU 01 (25%) and OTU 09 (6.6%) as the main contributors to dissimilarity between locations (Table 3c). However, for *H. momus*, core OTUs displayed significant differences between locations (PERMANOVA, $R = 0.3$, $p = 0.001$; Fig. 3b). SIMPER analysis revealed a dissimilarity of 78% between locations in the core, with OTUs from the phyla Proteobacteria and Crenarchaeota as the main drivers of this dissimilarity (Table 4c).

Table 3 Taxonomic classification and relative abundance of symbiont OTUs contributing to > 2% dissimilarity in microbial community structure (SIMPER analyses) between *S. plicata* and seawater (a), *S. plicata* in Jaffa and Wilmington: all (b) and core (c)

Dissimilarity (%)	OTU	Phylum	Lowest taxonomy	Relative abundance (%)		Contribution to dissimilarity (%)
a.				Water	Ascidian	
94	1	Proteobacteria	F. Hyphomicrobiaceae	1.7	26.3	13
	2	Proteobacteria	F. Rhodobacteraceae	10.9	0.5	5.2
	9	Crenarchaeota	G. *Nitrosopumilus*	0.09	5.8	2.8
	3	Proteobacteria	G. *Glaciecola*	5.4	1.8	2.7
	6	Bacteroidetes	F. Flavobacteriaceae	5.2	1.1	2.5
	4	Proteobacteria	F. Pelagibacteraceae	4.8	0.08	2.3
	5	Bacteroidetes	F. Rhodobacteraceae	4.4	1	2.1
b.				Jaffa	Wilmington	
69	1	Proteobacteria	F. Hyphomicrobiaceae	22	30	12.1
	9	Crenarchaeota	G. *Nitrosopumilus*	7.7	4	2.8
c.				Jaffa	Wilmington	
50	1	Proteobacteria	F. Hyphomicrobiaceae	35.2	50.5	25
	9	Crenarchaeota	G. *Nitrosopumilus*	14.7	8.7	6.6

K, kingdom; *P*, phylum; *C*, class; *O*, order; *F*, family; *G*, genus; *S*, species

Table 4 Taxonomic classification and relative abundance of symbiont OTUs contributing to > 2% dissimilarity in microbial community structure (SIMPER analyses) between *H. momus* and seawater (a) and *H. momus* in Akhziv and Elat: all (b) and core (c)

Dissimilarity (%)	OTU	Phylum	Lowest taxonomy	Relative abundance (%)		Contribution to dissimilarity (%)
a.				Water	Ascidian	
91	1	Proteobacteria	F. Pelagibacteraceae	11.1	0.18	5.4
	2	Proteobacteria	F. Rhodobacteraceae	8.59	3.5	4.3
	3	Proteobacteria	P. Proteobacteria	7.24	0.89	3.4
	5	Proteobacteria	O. Kiloniellales	0.09	6.61	3.2
	4	Cyanobacteria	G. *Synechococcus*	6.67	0.22	3.2
	6	Proteobacteria	G. *Amaricoccus*	0.09	5.94	2.9
	7	Crenarchaeota	G. *Nitrosopumilus*	0.09	5.1	2.5
b.				Akhziv	Elat	
84	6	Proteobacteria	G. *Amaricoccus*	11.79	0.1	5.8
	7	Crenarchaeota	G. *Nitrosopumilus*	9.6	0.6	4.5
	5	Proteobacteria	O. Kiloniellales	5.74	7.48	4.4
	2	Proteobacteria	F. Rhodobacteraceae	0.57	6.43	2.9
	13	Proteobacteria	G. *Hyphomicrobium*	5.15	0.05	2.5
c.				Akhziv	Elat	
78	6	Proteobacteria	G. *Amaricoccus*	17.43	0.2	11.6
	7	Crenarchaeota	G. *Nitrosopumilus*	14.89	1.06	9.7
	5	Proteobacteria	O. Kiloniellales	8.19	11.98	8.9
	13	Proteobacteria	G. Hyphomicrobium	8.41	0.11	5.4
	2	Proteobacteria	F. Rhodobacteraceae	0.92	9.49	5.2

K, kingdom; *P*, phylum; *C*, class; *O*, order; *F*, family; *G*, genus; *S*, species

Core OTUs exhibited seasonal stability of ascidian-associated microbial community structure for both ascidian species (PERMANOVA, *S. plicata*, $R = 0.14$, $p = 0.3$; *H. momus*, $R = 0.08$, $p = 0.3$).

Dynamic Microbial Community

The dynamic microbial community (i.e. abundant taxa that are absent from the core) comprised 257 (18.7%) and 399 (30.7%) OTUs of *S. plicata* and *H. momus* sequence reads, respectively. As can be seen in the overall dataset (Fig. 1), the ascidian dynamic dataset is clearly differentiated from that of the bacterioplankton (PERMANOVA, $R > 0.29$, $p = 0.001$ for both species) and clustered in response to location (PERMANOVA, $R > 0.1$, $p = 0.001$ both species) for both ascidian species (Fig. 4). SIMPER analyses identified OTU 07 of the genus *Nitrosopumilus* as the main driver of the difference (13.3%) between locations for *H. momus* but could not pinpoint specific contributors to the observed dissimilarity in the *S. plicata* samples.

Examination of the dynamic community in the ascidian samples revealed significant seasonal and geographical clustering (PERMANOVA, *S. plicata*, $R > 0.2$, $p = 0.001$ location, season, and interaction; *H. momus*, $R > 0.16$, $p < 0.002$ season, location, and interaction; Fig. 5). For *S. plicata* from Jaffa, SIMPER analyses could not identify a specific OTU as the main contributor to the differences between seasons, but in Wilmington OTUs, 70 (family Piscirickettsiaceae), 82 (Rhodobacteraceae), and 132 (Rhodobacteraceae) were found to be the main drivers of change between seasons. No pairwise differences between seasons were found and no significant differences in dispersion were detected (PERMDISP, $p = 0.48$). For *H. momus*, the main drivers of change between seasons in Akhziv were OTU 07 (genus *Nitrosopumilus*; SIMPER, > 11% contribution for all seasons), and OTU 36 (Cenarchaeaceae; SIMPER, > 3% contribution for all seasons), while in Elat no distinct drivers for the detected differences among seasons were identified. No pairwise differences were found and no significant differences in dispersion were detected (PERMDISP, $p = 0.3$ Akhziv, $p = 0.7$ Elat).

Rare Microbial Community

Rare OTUs found in both ascidian species (< 0.1% relative abundance) accounted for the vast majority of microbial OTUs (83.4%, $n = 9964$ and 87.8%, $n = 12{,}024$, *S. plicata*

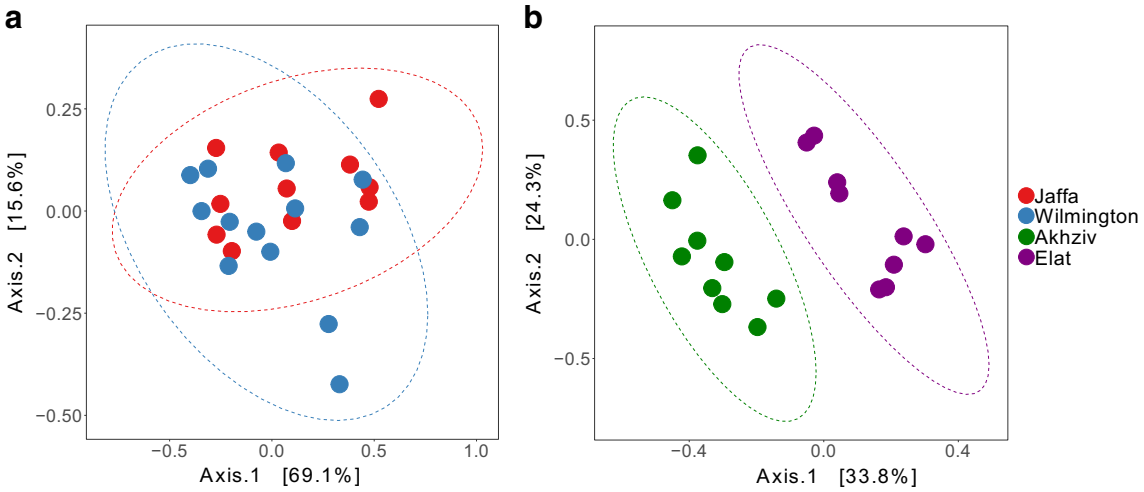

Fig. 3 PCoA plot of the core microbial fauna associated with *S. plicata* (**a**) from Jaffa (recently introduced population; in red) and Wilmington (well-established population; in blue) and *H. momus* (**b**) in its newly introduced region (Akhziv; in green) and its native region (Elat; in purple)

and *H. momus*, respectively), while constituting a minority (7.5 and 8.1%, *S. plicata* and *H. momus*, respectively) of the total sequence reads, post-singleton removal. Significant differences between locations and seasons were found in the rare dataset of both ascidian species (PERMANOVA, $R > 0.07$, $p = 0.001$ location; $R = 0.13$, $p < 0.01$ season; $R = 0.13$, $p < 0.02$ interaction for both species). In contrast with the core and dynamic microbial communities, the differentiation between locations and seasons in the rare dataset was driven by a large number of taxa, each contributing less than 1% to dissimilarity.

Discussion

Although it is now generally agreed upon that symbiotic microorganisms are critical to their hosts' survival, the specific interactions that take place between introduced ascidians and their symbionts are still poorly understood [30, 75]. Here, we characterized the microbial communities associated with two species of introduced ascidians in order to investigate the changes that occur in the microbiome of the same species under different environmental conditions. Employing an original analysis methodology, we have shown that the microbial communities are composed of both core and dynamic components located within the ascidian's inner tunic. The relative proportion of these two components is not constant and appears to shift with changing environmental conditions.

As previously reported for *S. plicata* and other ascidian species [29, 30, 50, 51, 54, 63, 75, 76], the microbiome of both *S. plicata* and *H. momus* was clearly differentiated from that of the surrounding bacterioplankton. Furthermore, the overall microbial community structure of both species

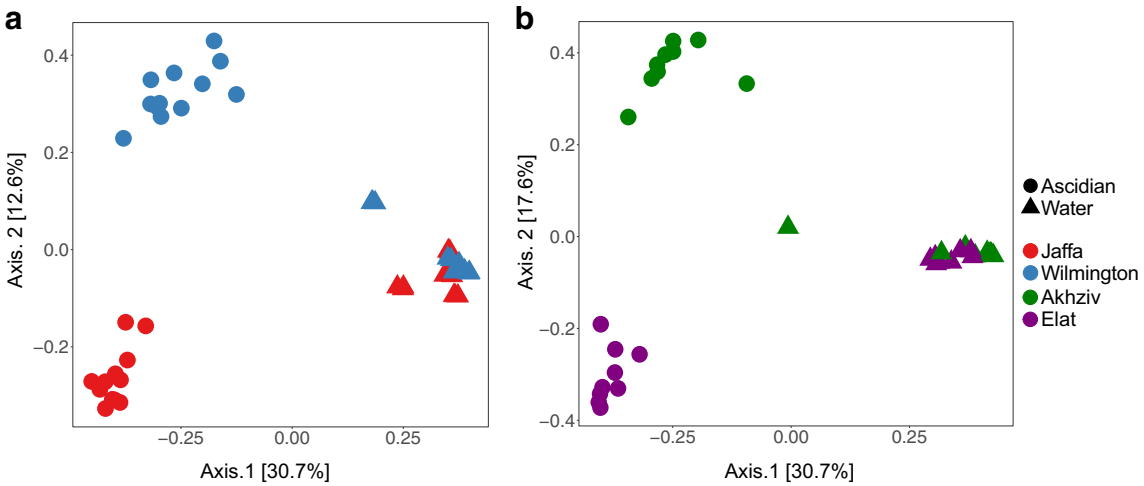

Fig. 4 PCoA plot of the dynamic microbial community associated with *S. plicata* (**a**) from Jaffa (recently introduced population; in red) and Wilmington (well-established population; in blue) and *H. momus* (**b**) in its newly introduced region (Akhziv; in green) and its native region (Elat; in purple). Ascidians (in circles) and seawater (in triangles). The dynamic community is differentiated from seawater and exhibits geographical clustering

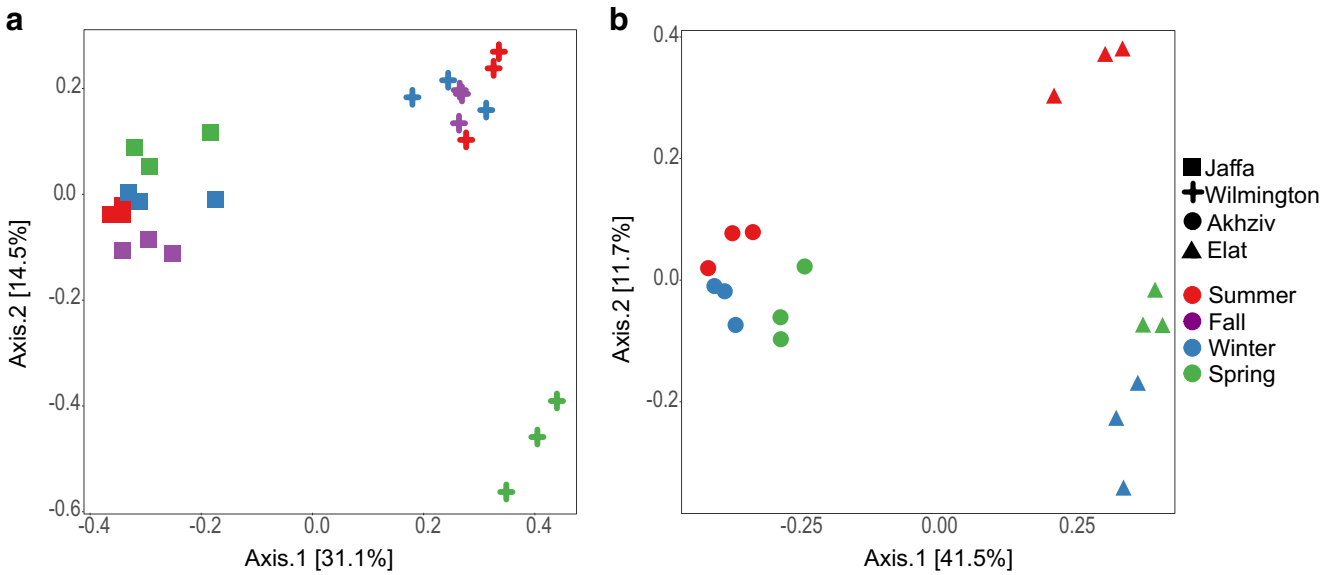

Fig. 5 PCoA plot of the dynamic microbial community associated with *S. plicata* (a) and *H. momus* (b) across locations (in shapes) and seasons (in color). The dynamic microbial community clusters in response to location and season

clustered in response to site. The ascidian microbiome was conserved at the phylum level and was dominated by Proteobacteria and Bacteroidetes, as also reported for various ascidian species worldwide [30, 50, 51, 54, 63, 77–79]. Additional abundant phyla found in this study that have been previously detected in other ascidians include the archaea Crenarchaeota [6, 30, 50] and the bacteria Planctomycetes [30, 63, 78, 79]. However, in agreement with previous studies, OTU-level comparisons among replicates of *S. plicata* revealed a high variability in community structure [30, 63], reaching up to 60% dissimilarity between replicates within seasons. For *H. momus*, intra-specific variability among replicates was also high, reaching values of 70% dissimilarity. These findings suggest that both species are able to establish new associations with a wide range of environmental microorganisms, which may prove beneficial to the species' establishment in a new environment.

A detailed examination of the ascidian microbiome revealed both a core (stable) community and a dynamic community in the two ascidian species, as has also been reported in corals and marine algae [80]. While spatial and seasonal stability were observed for the core microbial symbionts in *S. plicata*, for *H. momus* the structure of the core microbial symbionts differed greatly between sites. Although differences in the sampling depth of *H. momus* between sites may lead to differences in their microbial community, many ascidian species nonetheless displayed a constant core community structure across distances and depths [50, 75]. Despite the high microbial diversity found within the ascidian tunic, only a small fraction (< 0.2%) of the OTUs were present in all hosts of each species and comprised the core (17 and 10 for *H. momus* and *S. plicata*, respectively). The relative abundance of these few core OTUs was very high, representing between 26 and 36% of the microbial reads (*H. momus* and *S. plicata*, respectively). A high degree of species-specificity had been previously reported in *S. plicata* and other ascidian species [29, 30, 50, 51], supporting the hypothesis that the inner tunic provides unique microhabitat conditions that foster specific microbial communities [81, 82]. Microbial host-specificity across wide geographic distances has been described in corals, sponges, and mussels, as well as in other invertebrate species [22, 26, 83–86], suggesting that vertical transmission plays an important role in microbiome structuring. Additionally, species-specific selective chemical constraints (e.g., oxygen levels and light exposure) may also play a significant role in microbial community composition and structuring [32, 51, 81, 87–89]. In the two ascidian species studied here, horizontal transfer and selective enrichment constitute a probable source of symbiotic microbes as we observed a high degree of intra-species variation in OTU composition within and between locations and since in a previous study of gonad sections in *S. plicata* no bacterial cells were revealed that could be transmitted to the progeny [30].

The core community of *H. momus* not only differed in structure among sites but was also relatively smaller than the core community of *S. plicata* (36.2 and 26% of total reads in *S. plicata* and *H. momus*, respectively). In addition, in *H. momus*, each core OTU contained a relatively low number of sequence reads (< 6.5% of total reads). These findings may indicate a more facultative affiliation between host and symbiont in *H. momus* than that in *S. plicata* [2] or suggest a time-adaptation situation. Following its introduction into the Mediterranean several decades ago, *H. momus* appears to have been restricted to

artificial structures and has only recently undergone niche expansion from an artificial to a natural and more pristine substrate [59, 60]. We hypothesize that the relatively small core community and the difference in core structure are the result of profound differences in environmental factors resulting from this recent "two-step introduction": first from the Red Sea into the Mediterranean on artificial structures, and then onto the natural rocky reef, to which *H. momus* has adapted over a relatively short time-scale in comparison with *S. plicata*. Aires et al. [90] have shown that the community composition of algal-associated microorganisms significantly differed between disturbed and undisturbed habitats. A future experimental set-up based on samples from both natural substrate and artificial substrate in both native and invaded habitats may contribute to a better understanding of time-scale shifts in recently introduced microbial communities. It may well be that a larger set of dynamic microbial communities is typical to *H. momus*. Indeed, an examination of changes in the relative composition of core and dynamic communities of *H. momus* on a broader time scale, may contribute to our hypothesis that these two components not only shift in a way that supports host adaptability to the changing environmental conditions but also according to the host's introduction stage.

Core OTUs for both species were associated with a range of potentially host-beneficial functional capabilities, including metabolic cycling of carbon, nitrogen, and sulfur, and resistance to heavy metals. A highly abundant core OTU found in both *S. plicata* and *H. momus*, and which was also the main contributor to the dissimilarity between groups in *S. plicata*, was affiliated with the family Hyphomicrobiaceae. Strains belonging to this family have been identified as important denitrifiers in sponges [91, 92] and demonstrate remarkable resistance to various heavy metals [93]. Another bacterial family known to be associated with heavy metal resistance is the Alteromonadaceae [94], which was part of the core microbiome of *S. plicata* (OTU 03). Marinas and harbors are frequently polluted with toxic heavy metals, hydrocarbons, and high levels of nitrogenous compounds [95]. Such a close interaction between host and symbiont may contribute to the physiological ability of these two ascidian species to cope with the polluted conditions present in harbor ecosystems. However, it is hard to distinguish between cause and effect: does the possession of such core functional microbial groups facilitate host invasion into polluted environment; or does facilitating the acquisition of such groups enhance invasion ability? Other key core OTUs found in both species belonged to the phototrophic family Rhodobacteraceae. Strains from this family have been previously found in association with many marine organisms, including *S. plicata* [30] and are involved in sulfur and carbon cycling and the synthesis of other bioactive substances [96, 97]. The most common archaeal core symbiont in *S. plicata* was assigned to the genus *Nitrosopumilus*, an ammonia-oxidizing lineage [98] that has been previously reported in other ascidian species [6, 29, 30, 50]. In contrast, *Nitrosopumilus* was only found in high abundance in the Akhziv samples of *H. momus* but was rare in the Elat ones. Whenever it occurs, this association is probably beneficial for both host and symbiont, since *Nitrosopumilus* utilizes ammonia-rich concentrations for its metabolic needs, which it may obtain from the nitrogenous waste of the host. Additional interesting core OTUs found only in *H. momus* were from the orders Kiloniellales and Chromatiales. The order Kiloniellales has been reported as a dominant component of the sponge microbiome [68, 91], with a potential role in denitrification and the production of anti-microbial compounds. OTUs belonging to this order have also been previously reported in ascidians [99] and mussels [92]. Chromatiales are purple sulfur bacteria, a photosynthetic, anaerobic microorganism that is usually found in anoxic environments such as marine sediment, but that has also been reported in ascidians [51]. Active carbon and nitrogen-fixing purple sulfur bacteria have been reported in association with pelagic copepods [100].

Dynamic OTUs have been defined here as abundant OTUs (> 20% of ascidian reads) that are not present in all replicate samples and thus are not part of the "core." The ascidian dynamic dataset varied independently of the seawater seasonal community structure dynamics and demonstrated significant clustering in response to location as well as to seasons within each of the locations for both *S. plicata* and *H. momus*. Similarly, Hester et al. [80] described two bacterial community types associated with corals and algae: the stable symbionts, which are associated with a particular host species; and the sporadic symbionts, whose abundance varies between individual hosts. The latter study determined that sporadic symbionts can be either the product of stochastic events or a response to particular environmental pressures that may contribute to host adaptability. Here, we have shown that the dynamic microbial community of both ascidian species is not stochastic. Reshef et al. [22] posits that coral microbial symbionts undergo changes that aid the holobiont fitness when environmental conditions undergo change. Other studies have shown that changes in the microbial community structure of corals may increase host adaptability to specific biogeochemical conditions such as depth or nutrient levels [101, 102]. Seasonal changes reported in the symbiont microbial composition of marine invertebrates [32, 103] were associated with maintaining holobiont homeostasis and even increasing invasive capabilities [26, 32, 104]. However, it is also possible that the dynamic community is not random and yet not entirely beneficial for the host. It may reflect seasonal shifts in composition of the source assemblages of bacterioplankton or serve as a reservoir for commensal and opportunistic pathogens at times, when their abundance in the seawater decreases

[104].Whether these dynamic OTUs act as an opportunistic community, an adaptive one, or both, requires further investigation.

In summary, we have demonstrated here a new analysis methodology by which to examine microbial community datasets, enabling a more detailed examination of the microbiome. We found both stable and dynamic (location- and season-specific) associations within the ascidian tunic. The dynamic OTUs were not as abundant as the core OTUs but nonetheless significant in terms of abundance in addition to enabling plasticity for the host. A revised examination of the microbial communities of additional ascidian species applying this methodology may reveal that the dynamic community plays a more prominent role than previously assumed. Further work should seek to determine whether core and dynamic OTUs are beneficial to the ascidian host and contribute to its successful establishment. Alternatively, most of these OTUs may instead constitute merely opportunistic microorganisms, reacting to changes in the biochemical conditions of the inner tunic. Additional studies targeting this unique physiological niche may advance our understanding of ascidians as holobionts.

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Author Contributions H.D., N.S. and S.L.L. designed this study. H.D., L.N., and J.S.E. collected samples. J.S.E. assisted in sequence processing. H.D. conducted the analyses and led the writing of the manuscript. All authors contributed critically to all drafts and gave final approval for publication.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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