

Host Phylogeny Determines the Gut Microbial Landscape of Cephalopods

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Research

Keywords: cephalopod, gut microbiota, Mollusca, phyllosymbiosis, Photobacterium, Mycoplasma

Posted Date: May 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-556214/v1>

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Abstract

Background: Compared to vertebrate gut microbiomes, little is known about the factors shaping the gut microbiomes in invertebrates, especially in non-insect invertebrates. Class *Cephalopoda* is the only group in the phylum *Mollusca* characterized by a closed circulatory system and a well-differentiated digestive system to process their carnivorous diet. Despite their key phylogenetic position for comparative studies as well as their ecological and commercial importances, analyses of the cephalopod gut microbiome are limited. In this study, we characterized the gut microbiota of six species of wild cephalopods by Illumina MiSeq sequencing of 16S rRNA gene amplicons.

Results: Each cephalopod gut consisted of a distinct consortium of microbes. *Photobacterium* and *Mycoplasma* were prevalent in all cephalopod hosts and were identified as core taxa. The gut microbial composition reflected host phylogeny. The importance of host phylogeny was supported by a detailed oligotype-level analysis of operational taxonomic units assigned to *Photobacterium* and *Mycoplasma*, although *Photobacterium* typically inhabited multiple hosts, whereas *Mycoplasma* tended to show host-specific colonization. Further, we showed that class *Cephalopoda* has a distinct gut microbial community from those of other molluscan groups. The gut microbiota of the phylum *Mollusca* was determined by host phylogeny, diet, and environment (aquatic vs. terrestrial).

Conclusion: We provide the first comparative analysis of cephalopod and mollusk gut microbial communities. The gut microbial community of cephalopods is composed of the distinctive microbes and strongly associated with their phylogeny. The genera *Photobacterium* and *Mycoplasma* are core taxa in the cephalopod gut microbiota. Collectively, our findings of this study provide evidence that cephalopod and mollusk gut microbiomes reflect phylogeny, environment, and the diet of the host and these data can be suggested to establish future directions for invertebrate gut microbiome research.

Background

Interactions between animal hosts and the gut microbiota are essential for host immune responses [1] and metabolic regulation [2]. The structure of the gut microbiota is associated with the host diet [3], lifestyle [4], habitat [5], and genetic factors [6]. In vertebrates, the composition of gut microbiota is influenced by host phylogeny and host dietary shifts during evolution [7]. In contrast to vertebrates, little is known about the factors shaping the gut microbiome in invertebrates, especially in non-insect invertebrates [8, 9], even though invertebrates account for roughly 90% of all animal species [10]. Owing to this selection bias, our understanding of the animal gut microbiome and its evolution is inadequate.

The phylum *Mollusca* is the second largest invertebrate group after *Arthropoda* in terms of the number of extant species [11]. Mollusks are estimated to include 120,000 species [12], which is more than the total number of vertebrate species, and are highly diverse with respect to anatomical structures, feeding, and habitat [13]. The phylum *Mollusca* includes eight living classes, *Caudofoveata*, *Solenogastres*, *Polyplacophora*, *Monoplacophora*, *Gastropoda*, *Cephalopoda*, *Bivalvia*, and *Scaphopoda*, among which

Cephalopoda is one of the oldest and most successful groups in the phylum *Mollusca* [14]. Over 11,000 species of cephalopods have been documented, including about 800 extant species. They are found in all of the oceans and at most depths, from the surface to the deep sea [15]. Cephalopods are the only group of mollusks with a closed circulatory system, analogous to that of vertebrates [16], and they have the most advanced nervous system among invertebrates [17]. Furthermore, cephalopods have a well-differentiated digestive system to process their carnivorous diet [18]. Thus, cephalopods are expected to have a unique gut microbiome that differs from those of other mollusks. Furthermore, they are major component in the marine food chain as predators of zooplankton, crustaceans, and small fishes and as prey of vertebrate predators [19]. Cephalopods are an important fishery resource for many countries [20]. Despite these unique biological properties and the ecological and commercial importance of cephalopods, few studies have evaluated the cephalopod gut microbiome. S. Lehata, F. Valenzuela and C. Riquelme [21] described the *Octopus mimus* gut microbiome using a 16S rDNA clone library and Á. Roura, S.R. Doyle, M. Nande and J.M. Strugnell [22] characterized the gut microbiomes of wild and captive *Octopus minor* paralarvae. Notably, studies focused on the adult cephalopod gut microbiota using a high-throughput sequencing approach are lacking, thereby limiting our understanding of the co-evolution of animals and the gut microbiome.

Here, we characterized the gut microbiota of six species of wild cephalopods, cuttlefish (*Sepia esculenta*), beka squid (*Loliolus beka*), inshore squid (*Uroteuthis edulis*), Japanese flying squid (*Todarodes pacificus*), common octopus (*Octopus vulgaris*), and whiparm octopus (*Octopus variabilis*), by 16S rRNA gene sequencing using the Illumina MiSeq platform. We investigated associations between host phylogeny and the gut microbiome and compared our data with previously reported data for mollusks and other aquatic animals to investigate the factors that shape the gut microbial composition in the phylum *Mollusca*.

Methods

Sampling

Cuttlefish, beka squid, inshore squid, Japanese flying squid, common octopus, and whiparm octopus were captured from offshore waters in the Republic of Korea. All samples were transferred to the laboratory directly in the living state and sacrificed in the laboratory by an adequate anesthetic method. The dorsal mantle length and weight of each individual were determined. Samples were then dissected to obtain the stomach, cecum, and other digestive organs. In Supplementary Table S1, detailed metadata for the cephalopod samples are presented.

Identification of cephalopod hosts by Cytochrome oxidase I sequencing

Cephalopod subjects were initially subjected to basic taxonomic identification based on morphological characteristics. For the detailed identification of cephalopods, genomic DNAs were extracted from the

flesh of specimens aseptically. A fragment of each tissue sample was suspended in 750 ml of lysis buffer and homogenized by FastPrep-24 (MP Biomedicals, Santa Ana, CA, USA) with glass beads (0.5 mm diameter) for 45 s at 5.0 m/s. Standard phenol–chloroform DNA extraction was performed after lysis. The extracted DNAs were PCR-amplified using cytochrome c oxidase subunit I (CO1) primers designed for diverse metazoan invertebrates [67]. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the standard protocol and bidirectionally sequenced using an automated DNA analyzer system (PRISM 3730XL DNA Analyzer; Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequence fragments were assembled using SeqMan (DNASTAR). The assembled CO1 gene sequences were compared with other CO1 gene sequences in the nucleotide collection (nr/nt) in GenBank by a BLAST search (Additional file 2: Supplementary Table S1) [68]. The CO1 gene sequences were aligned using the multiple alignment program CLUSTAL W (v. 1.4), and a phylogenetic tree was constructed with using MEGA 7 [69, 70] using the maximum-likelihood algorithm with 1000 bootstrap replicates [71].

DNA extraction and sequencing of bacterial 16S rRNA genes

From dissected intestinal tracts of collected cephalopods, the cecum was primarily used to investigate gut microbial communities of cephalopods. The luminal contents of dissected cecal samples were also collected and used to extract microbial DNAs. To maximize microbial cell lysis for DNA extraction, the cecum and luminal contents were homogenized by shaking in a sterile screw tube containing zirconia beads (2.3 mm, 0.1 mm diameter) and glass beads (0.5 mm diameter) using FastPrep-24 (MP Biomedical) for 50 s. After lysis, genomic DNAs from the homogenized gut samples were extracted using the Qiagen DNA Stool Mini Kit (Qiagen). The V3-4 hypervariable region of the 16S rRNA gene was amplified with the primers 341F and 805R, and four independently amplified products for each sample were pooled and purified using the QIAquick PCR Purification Kit (Qiagen) to minimize bias. Libraries were prepared using the Nextera XT DNA Library Preparation Kit for Illumina MiSeq platform (Illumina, San Diego, CA, USA). The prepared DNA libraries were sequenced by certified service provider (Macrogen, Seoul, Korea) using the Illumina MiSeq platform with 2 × 300 bp reads, according to the manufacturer's instructions.

Sequence analysis

Raw 16S rRNA sequence data were processed using QIIME 1.9.1 [72]. Paired-end sequence reads were assembled with default parameters and minimally quality filtered with a Phred quality score threshold of 20. Data were then error-filtered using a de novo chimera removal algorithm, USEARCH (v. 7.0.1090) [73]. High-quality sequence reads were assigned to OTUs by an open-reference OTU picking protocol [74] using the QIIME toolkit, where the UCLUST [73], OTU picking algorithm was applied to search sequences against the Greengenes reference database at a 97% sequence similarity threshold [75]. A representative sequence for each OTU was aligned with the Greengenes reference using PyNAST [76]. For bacterial taxonomic assignment, Ribosomal Database Project (RDP) classifier (version 2.3;

<https://rdp.cme.msu.edu/classifier/classifier.jsp>) was used with 80% as a confidence value threshold [77]. An even-depth rarefied OTU table matrix (6000 sequences) was constructed to calculate various α - and β -diversity indices and for further microbial analyses using QIIME pipelines. Sequences belonged to *Mycoplasma* and *Photobacterium*, were re-clustered with minimum entropy decomposition (MED) for sensitive discrimination of closely related organisms [26].

Network-based analysis of *Mycoplasma* and *Photobacterium*

Network maps of *Mycoplasma* and *Photobacterium* were generated using QIIME and visualized using Cytoscape (version 3.4.0) [78, 79]. Briefly, the even-depth rarefied MED tables constructed with *Mycoplasma* and *Photobacterium* and converted to Cytoscape format using a QIIME script (*make_otu_network.py*). In the converted MED network maps, samples and MEDs represented nodes of the network and these nodes were connected by edges, indicating the abundance of the MED in the samples. Edge-weighted spring embedded models were derived for network arrangement.

Comparison of gut microbiomes of cephalopods and various animal

Sequence data for the sea slug (*Elysia chlorotica*) and eastern oyster (*Crassostrea virginica*) gut microbiome were obtained from the MG-RAST server (mgp561 and mgp1994, respectively; <http://metagenomics.anl.gov>) [35, 36]. Sequence data for Hawaiian land snail (*Auriculella ambusta*) gut microbiome was downloaded from NCBI Sequence Read Archive (SRP047488, <https://www.ncbi.nlm.nih.gov/sra>) [34]. Sequence data for the blood clam and marine fish gut microbiome were used from our unpublished data. Since the targeted region and applied sequencing technologies differed among experiments, we assigned taxonomic characteristics against the identical reference database using RDP classifier. After unaligned sequences were discarded, the even-depth rarefied OTU table was generated and used for further analyses. Non-phylogenetic distance metrics (binary Jaccard and Bray–Curtis dissimilarities) were calculated and visualized by a principal coordinates analysis (2D PCoA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (v. 8.43; GraphPad, San Diego, CA, USA). Two-tailed Mann–Whitney *U*-tests were used to compare the β -diversity indices between multiple groups. Analysis of similarities (ANOSIM) and multivariate ANOVA based on similarities (adonis) tests with the β -diversity matrix were performed using the QIIME pipeline (*compare_categories.py*) [80, 81]. Statistical significance for both tests was determined based on 10,000 permutations.

Results

Characteristics of the cephalopod gut microbiota

After sequence quality filtering (and excluding sequences found fewer than 15 times in the entire sample), 3,661,327 high-quality reads from 30 samples were generated with a mean sample depth of 122,044 and a standard deviation of 20,693 reads.

After rarefaction, 76,381 high-quality sequences were clustered into 1,835 OTUs at a 97% sequence identity threshold (357 ± 103 OTUs per sample). The phylogenetic diversity index, an alpha diversity measure, was used to estimate bacterial species richness (Additional file 1: Supplementary Fig. S1). The Chao1 metric reached a plateau after 75000 reads, suggesting that the depth of coverage was sufficient to capture nearly all of the biological diversity within samples (Additional file 1: Supplementary Fig. S2). Cuttlefish and beka squid showed higher gut bacterial diversity than those of other cephalopod species, and Japanese flying squid showed the lowest bacterial diversity. Whiparm octopus and common octopus, members of the order *Octopoda*, had similar diversity levels. Each species of cephalopod had a unique gut microbiota. However, some major taxa, especially *Mycoplasma* and *Photobacterium*, were widely distributed in cephalopod hosts (Fig. 1). Predominant bacteria in the gut microbial community varied among cephalopod host species. The cuttlefish microbiota was dominated by *Mycoplasma* ($57.4 \pm 13.5\%$). Beka squid possessed *Photobacterium* ($58 \pm 16.5\%$), *Aliivibrio* ($14.7 \pm 11.2\%$), and *Psychrilyobacter* ($13.2 \pm 6.9\%$). Inshore squid had *Photobacterium* ($75.9 \pm 7.1\%$) and *Mycoplasma* ($16.6 \pm 3.5\%$). The genera *Mycoplasma* ($84.2 \pm 8.8\%$) and *Arcobacter* ($14.8 \pm 8.7\%$) were enriched in the Japanese flying squid. In whiparm and common octopuses, *Mycoplasma* ($43.7 \pm 7.2\%$ and $97.5 \pm 0.8\%$, respectively) was most abundant. Overall, *Tenericutes* ($50.0 \pm 7.0\%$) and *Proteobacteria* ($43.2 \pm 6.5\%$) were the most frequent phyla in most samples, with high frequencies of *Mycoplasma* ($50.0 \pm 7.0\%$) and *Photobacterium* ($23.8 \pm 6.4\%$).

Cephalopod gut microbial community reflects host phylogeny

Taxonomic profiles clearly show that cephalopod gut microbiotas share core taxa but also include unique components (Fig. 1). A beta diversity analysis supported this observation. The cephalopod gut microbial communities were clustered according to host species in a PCoA of unweighted UniFrac distances (Fig. 1b). Furthermore, average interspecific variation was significantly higher than intraspecific variation in the beta diversity matrix (Fig. 1c). Similarity of the gut microbial composition was higher within the order *Octopoda* compared to within the orders *Sepiida* and *Teuthoidea*. A PCoA based on UniFrac unweighted distances indicated that species belonging to the order *Octopoda* were similar, with significantly lower intra-order beta diversity than those for other orders (Fig. 1b and d).

In vertebrates, phylogeny and host diet influence the structure of gut microbial communities [23, 24]. We evaluated whether there is a similar correlation between host phylogeny and gut microbial composition in cephalopods. We constructed a host phylogenetic tree based on mitochondrial housekeeping genes reported by JE Uribe and R Zardoya [25]. We also generated an unweighted-pair-group method with arithmetic-mean (UPGMA) tree based on 16S rDNA gene sequences from the gut microbial communities (Fig. 2a). The host phylogenetic tree and the UPGMA tree of the gut microbiomes showed identical

topologies, indicating that the gut microbial communities in cephalopods are closely related to the host phylogeny. As shown in Fig. 2b, a heat map indicated that host CO1 similarity and microbial dissimilarity are strongly correlated with cephalopod host species. Furthermore, the order *Octopoda* showed high intra-order CO1 and microbial composition similarity.

A majority of OTUs were matched to the genera *Mycoplasma* and *Photobacterium*, which were regarded as the core taxa of the cephalopod gut microbiota (48.3% and 23.8%, respectively; Additional file 1: Supplementary Fig. S3). Although OTUs belonging to these genera were differentially distributed according to host phylogeny, the limited taxonomic resolution rendered an OTU-level analysis ineffective. Furthermore, sequences included in major OTUs are overestimated during taxonomic stratification, distorting the sequence distribution. To overcome these obstacles, we decomposed the OTUs assigned to identical genera (*Mycoplasma* and *Photobacterium*) and re-clustered the sequences into fine-scale units using nucleotide entropy by the minimum entropy decomposition (MED) method, an unsupervised oligotyping approach [26]. The OTUs belonging to *Mycoplasma* and *Photobacterium* were resolved into 228 oligotypes. Distortion in the sequence distribution was reduced for oligotypes (Additional file 1: Supplementary Fig. S4). We performed a network analysis using oligotypes to evaluate the distribution of the core taxa with better taxonomic resolution (Fig. 3). The distributions of oligotypes among hosts were consistent with the aforementioned results for the core OTUs and showed host-specific connections. In the case of *Mycoplasma*, oligotypes were divided into three sub-clusters according to host: cuttlefish and Japanese flying squid; beka squid and inshore squid; whiparm octopus and common octopus. The majority of *Photobacterium* oligotype nodes were connected to multiple hosts. There was also a striking difference in co-speciation patterns between the genera *Mycoplasma* and *Photobacterium* in the oligotype-level phylogenetic analysis (Additional file 1: Supplementary Fig. S5). In *Mycoplasma*, we found that most oligotypes colonized a single host species. Oligotypes assigned to *Photobacterium* with earlier diverged, were found in multiple host species, whereas those that diverged more recently were host-specific.

Sexual dimorphism is not associated with the gut microbial community in cephalopods

Male and female cephalopods have different growth rates, body sizes [27], and organ structures [28, 29]. Moreover, there is evidence that sexual differences affect the diet and ecological niche [30, 31] of cephalopods. Because the host diet and habitat are critical determinants of the microbiota [32, 33], we investigated whether the cephalopod gut microbiota is shaped by sex (Additional file 1: Supplementary Fig. S6a). Sex did not affect the gut microbial community at the levels of class (Additional file 1: Supplementary Fig. S6b) and order, except in the case of the order *Teuthida* (Additional file 1: Supplementary Fig. S6c).

Host phylogeny, diet, and habitat shape the gut microbiota of mollusks

We further compared the gut microbiota of cephalopods and other mollusks and identified the relative contributions of various environmental and genetic factors to the microbial community composition. We obtained data for the gut microbiomes of four mollusk species from public databases, including the Hawaiian land snail (*Achatinella mustelina*) [34] and emerald sea slug (*Elysia chlorotica*) [35] belonging to the class *Gastropoda* and oyster (*Crassostrea virginica*) [36] and blood clam (*Anadara broughtonii*) (our unpublished data) belonging to the class *Bivalvia*. A marine fish gut microbiome (62 species, our unpublished data) was also included in the analysis for comparison between mollusks and vertebrates.

Each mollusk class showed a largely distinct gut microbial composition (Fig. 4a). The core taxa in cephalopods, *Mycoplasma* and *Photobacterium*, were not predominant in other mollusks or fishes. Moreover, a PCoA based on binary Jaccard distances (Fig. 4b) showed that cephalopods form a single cluster distinct from vertebrates and other molluscan groups, although each species in *Cephalopoda* had a unique microbiome. The classes *Gastropoda* and *Bivalvia* each formed a single cluster as well. Emerald sea slug was most similar to cephalopods (Fig. 4b). The class *Gastropoda*, including the emerald sea slug, was the most recently diverged branch within the class *Cephalopoda* (Fig. 4c). These results suggested that the molluscan gut microbiota reflects host phylogeny. Interestingly, the Hawaiian land snail belonging to class *Gastropoda* had a completely different gut microbiota from those of other marine mollusks, despite their phylogenetic similarity (Fig. 4d and Additional file 1: Supplementary Fig. S7a). We then compared the gut microbiotas of marine fish to those of mollusks to determine the role of the marine environment. The gut microbiotas of marine mollusks were more similar to those of marine fishes than to those of the Hawaiian land snail, despite their evolutionary divergence (Fig. 4d and Additional file 1: Supplementary Fig. S7a). These results indicate that gut microbiota of marine animal is potentially affected by the aquatic environment. Among marine mollusks, diet was also a strong determinant of the gut microbiota. The cephalopod gut microbiota could be categorized according to the host diet in a binary Jaccard analysis (Additional file 1: Supplementary Fig. S7b).

Discussion

Studies of invertebrate microbiomes have largely focused on insects, although *Mollusca* is the second largest invertebrate phylum [11]. In particular, few studies have focused on cephalopod gut microbiomes [21, 22], with a lack of studies of multiple species and analyses of the factors determining the community structure. Thus, we characterized the gut microbiota of six wild cephalopod species (cuttlefish, beka squid, inshore squid, Japanese flying squid, common octopus, and whiparm octopus).

Based on our comparative analysis of 16S rRNA gene sequences obtained by Illumina MiSeq sequencing, we identified the genera *Mycoplasma* and *Photobacterium* as the core taxa in the cephalopod gut microbiota. These genera are the predominant taxa in the digestive tracts of wild Chilean octopus [21] and aquacultured common octopus [22]. *Mycoplasma* is an obligate parasitic bacterial group and is a component of the gut microbiome of many marine animals, such as Norway lobster [37], jellyfish [38], and Atlantic salmon [39]. Their roles in the intestinal ecosystem are typically recognized as pathogenic or opportunistic bacteria in vertebrates [40–42]; however, little is known about their roles in invertebrates,

other than a report of a potential symbiotic *Mycoplasma* in scorpion [43]. The genus *Photobacterium* is well known for its bioluminescence [44] and pathogenicity [45, 46]; however, their phylogeny and taxonomy are not clearly elucidated [47]. Members of the genus *Photobacterium* show ecological diversity, including taxa that are symbiotic [48–50] or parasitic [51, 52] with sea animals, free-living in seawater [53] and in saline lake water [54], and even piezophilic [55]. Light production is a common feature of many genera in *Vibrionaceae*, and *Photobacterium* is one of the most extensively studied groups [56, 57]. In this study, the genus *Photobacterium* was particularly abundant in beka squid (58.0%) and inshore squid (75.9%), members of the suborder *Myopsida*, containing Hawaiian bobtail squid (*Euprymna scolopes*). The Hawaiian bobtail squid is famous for its light-associated symbiosis and symbiont-specific immune tolerance with the bioluminescent bacterium *Aliivibrio fischeri* [58, 59], once assigned to the genus *Photobacterium* [47]. Although beka squid and inshore squid do not have bioluminescence, the dominance of *Photobacterium* in *Myopsida* hosts suggests that there is a general symbiotic relationship between *Myopsida* hosts and *Vibrionaceae* bacteria.

Our results revealed that host phylogeny is reflected in the gut microbiota of cephalopods, indicating that the host phylogeny can be predicted by the gut microbial community. Phylogenetic analyses based on many housekeeping genes in *Cephalopoda* have yielded contradictory results, making evolutionary relationships within the class difficult to define [60, 61]. The cephalopods included in our study have clearly resolved phylogenetic positions based on well-supported consensus trees [25, 60, 62]. Our cephalopod gut microbial composition-based UPGMA tree matched the consensus tree for the host species. Accordingly, the gut microbial composition is a potential target for studies of cephalopod phylogeny.

In microbial community analyses by 16S amplicon sequencing, sequences are typically clustered into OTUs based on similarity, with a typical threshold of 97%. This clustering process is beneficial for downstream analyses; however, with respect to the operational definition of a species, 3% dissimilarity is only a rough approximation. There is a risk that closely related species could be identified as a taxonomic unit in the clustering process. Furthermore, OTU-based analyses show a limited resolution for analyses below the genus level. The MED method overcomes a number of the limitations of the OTU-based approach. MED provides a computationally efficient means to partition marker gene datasets into MED nodes, which represent homogeneous OTUs. We used the MED approach to perform a network analysis at the within-genus level. The oligotyping analysis revealed different co-evolutionary histories between two major cephalopod species. The distribution of oligotypes of *Mycoplasma* was concentrated with host-specific colonization; however, a large number of *Photobacterium* oligotypes were located in multiple cephalopod species. Based on these results, *Mycoplasma* colonization in cephalopods was frequently related to host-specific evolution or biological activities, while *Photobacterium* colonized cephalopods more broadly, and interactions with *Photobacterium* might be essential for survival or adaptation of cephalopod species. This finding agrees with the Atlantic cod gut microbiome study [49].

We expected the cephalopod gut microbiota to differ between sexes based on differences in the growth rate, body size, diet, and space niche between male and female octopuses. However, we did not detect a

significant difference in gut microbial composition according to sex. This lack of a difference has several potential explanations. First, we used cephalopod samples that are similar in size and were collected simultaneously at the same location, thereby minimizing the effects of body size, space niche, and similar variables. Second, there are conflicting results regarding the difference in octopus diet between sexes [63, 64]. Therefore, a meta-analysis is needed to clarify the differences between male and female cephalopods.

This is the first comparative analysis of the cephalopod and mollusk gut microbiota. We identified three factors that influence the gut microbiota of cephalopods and mollusks: host phylogeny, habitat type, and diet. The host phylogeny was the most prominent determinant of the gut microbiota. Although all cephalopod hosts in our study had similar diets and living environments, gut microbial compositions were distinguished by host phylogeny. This was supported by our beta-diversity, phylogenetic, and network analyses. With respect to the living environment, the gut microbiota of aquatic mollusks was more similar to that of fish than to that of terrestrial mollusks, suggesting that environmental conditions overwhelm other factors. However, further research is still needed, including analyses of mollusk samples in a wider range of environments. Lastly, the mollusk gut microbiota was distinguished by diet in our beta-diversity analysis. However, diet was a host phylogeny-dependent factor in our study. Therefore, to assess the independent effect of diet on the gut microbiota of mollusks, follow-up studies are needed.

We found that the features of cephalopod and mollusk gut microbial communities were quite similar to the common features of the vertebrate gut microbiota, which is also affected by host phylogeny [7], evolutionary divergence time [65], living environment [5], and diet [66]. The shared characteristics of the microbiomes suggest that insights from studies of the vertebrate gut microbiota can be applied to invertebrate studies; this can help establish directions for invertebrate gut microbiome research. In addition, new findings based on invertebrate gut microbiome studies have the potential to be applied to vertebrate and human research.

Conclusions

Taken together, we performed the first comparative analysis of the cephalopod gut microbiota by a high-throughput sequencing approach. We revealed that each species in class Cephalopoda has a unique gut microbiota. We identified the genera *Mycoplasma* and *Photobacterium* as core taxa in the gut microbiota of cephalopods. Furthermore, we found that the cephalopod gut microbial community composition was determined by host phylogeny, and host phylogeny is also an important determinant of the gut microbiota of marine mollusks. Diet and habitat also contributed to the mollusk gut microbiota.

Abbreviations

ANOSIM, analysis of similarities;

BLAST, Basic Local Alignment Search Tool;

CO1, cytochrome c oxidase subunit I;

MED, minimum entropy decomposition;

OTU, operational taxonomic unit;

PCoA, principal coordinate analysis;

RDP, Ribosomal Database Project

UPGMA, unweighted-pair-group method with arithmetic-mean.

Declarations

Ethics approval

All experiments were approved by the Institutional Animal Care and Use Committee of Kyung Hee University and performed in accordance with the protocol KHUASP(SE)-18-048.

Consent for publication

Not applicable.

Availability of data and material

The newly generated 16S rRNA sequence datasets are available in the European Nucleotide Archive (ENA) of EMBL-EBI under the accession number PRJEB27490. The cytochrome oxidase subunit 1 (CO1) gene sequences used for identifying host species have been submitted to NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) under accession numbers MH542436-MH542464 (under the title "Factors shaping invertebrates gut microbiota: host phylogeny, habitat, and diet are involved in shaping of gut microbiota of *Cephalopoda*, *Mollusca*").

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202002108 to J-WB); a grant from the Mid-Career Researcher Program (NRF-2020R1A2C3012797 to J-WB) through the National Research Foundation of Korea (NRF).

Authors' contributions

WK, PSK, and J-WB planned and designed the research and experiments. WK, PSK, EJT, HS, J-YL, J-HY and M-JJ undertook the field work and processing of samples. WK, PSK, N-RS, D-WH, TWW, HSK, J-YL, J-HY, and M-JJ performed the experiments and analyzed the data. WK, PSK, and J-WB wrote the paper. All authors read and approved the final manuscript.

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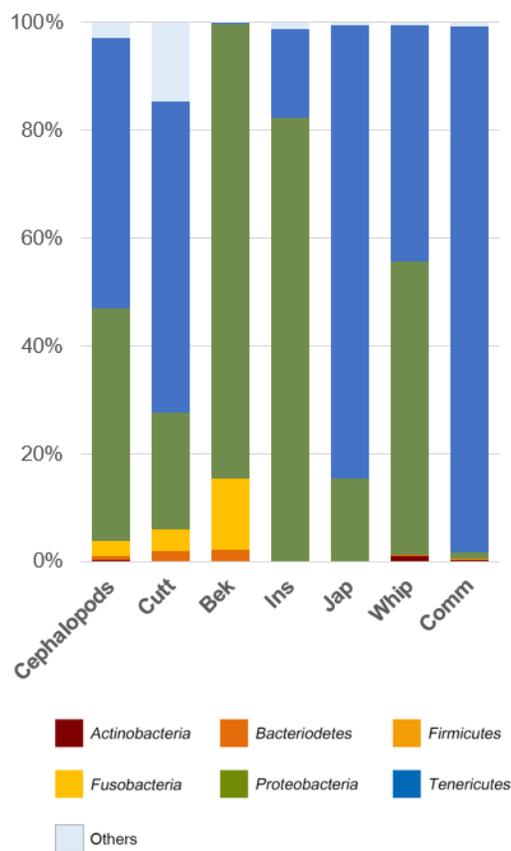
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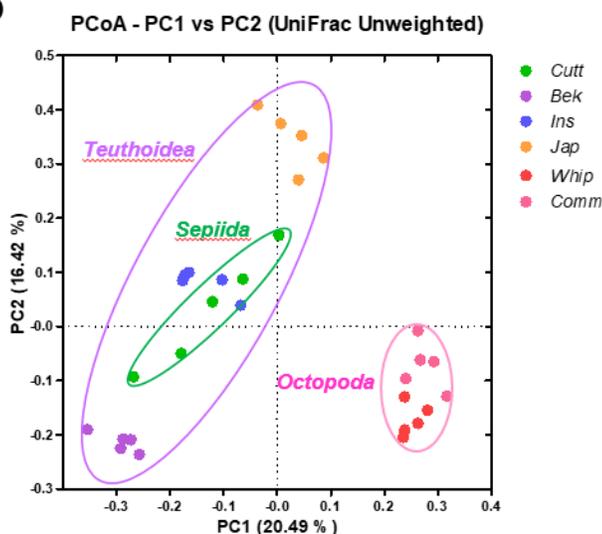
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Figures

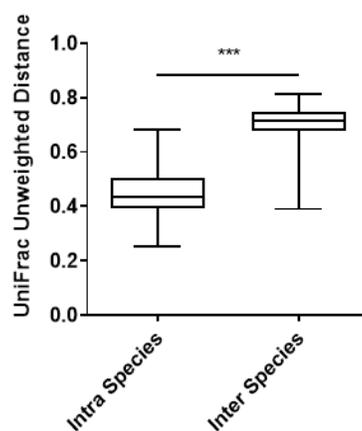
Fig. 1
a



b



c



d

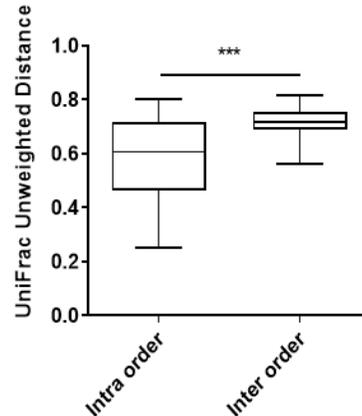


Figure 1

Gut microbial community structure of cephalopods. (a) Bar charts of the relative abundance of bacterial phyla and predominant families, particularly *Vibrionaceae* and *Mycoplasmataceae*, in six cephalopod species as well as the overall gut microbial composition of cephalopods. (b) Principal coordinates analysis (PCoA) of unweighted UniFrac distances between cephalopod samples. Colors of dots and ellipses in PCoA represent the host cephalopod species and their orders (ANOSIM, $R = 0.28$, $p < 0.001$; adonis, $R^2 = 0.28$, $p < 0.001$). (c-d) Comparisons of intra- and inter-specific (c) and intra- and inter-order (d) microbial variation based on unweighted UniFrac distances. Asterisks indicate significant differences between the unweighted UniFrac distances according to two-tailed Mann-Whitney U-tests. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

Fig. 2

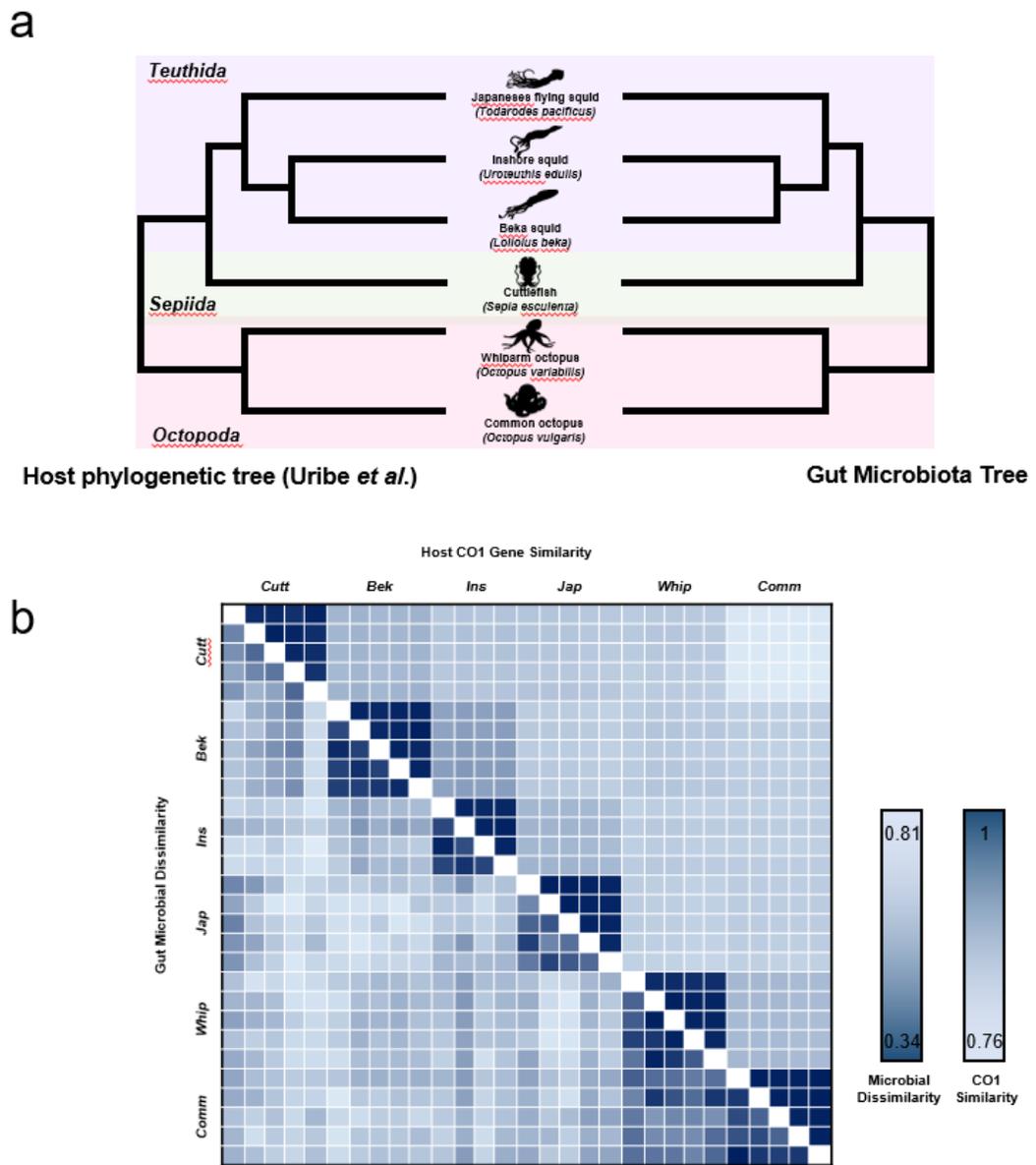
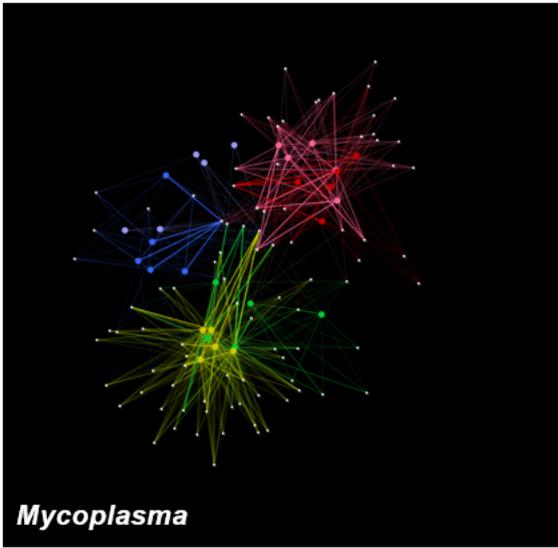


Figure 2

Phylosymbiotic host–gut microbiota assembly in cephalopods. (a) UPGMA-clustering dendrograms for cephalopod gut microbiomes based on unweighted UniFrac distances, compared with the host phylogenetic tree based on complete host mitochondrial genomes (reported by Uribe et al., 2017). Species of respective orders of cephalopods are depicted by a specific background color. (b) Heatmap of gut microbial dissimilarity based on unweighted UniFrac distance metric (left lower half) and host genetic relatedness based on CO1 gene similarity (right upper half). The range of colors indicates the microbial dissimilarity or host CO1 gene dissimilarity: from bright blue (highest gut microbial dissimilarity or lowest host CO1 gene dissimilarity) to dark blue (lowest gut microbial dissimilarity or highest host CO1 gene dissimilarity).

Fig. 3

a



b

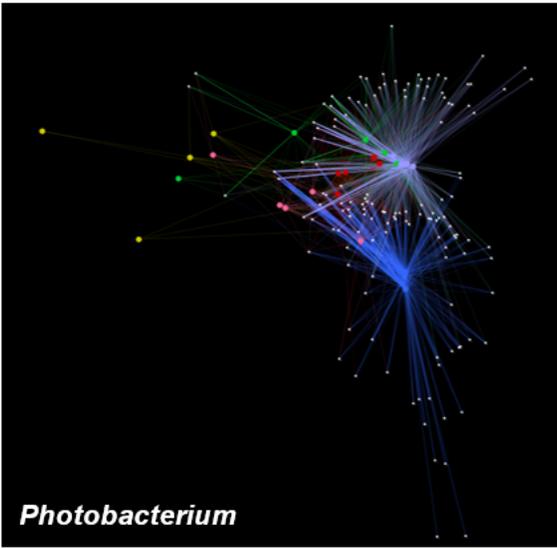


Figure 3

Network analyses of two core genera in cephalopod species constructed by an unsupervised oligotyping approach. The networks of oligotypes belonging to (a) *Mycoplasma* and (b) *Photobacterium* are plotted. The edges connecting nodes representing cephalopod samples (large circles) to identified oligotypes in a particular sample are colored according to the host species (edge-weighted spring embedded model in Cytoscape v. 3.4.0).

Fig. 4

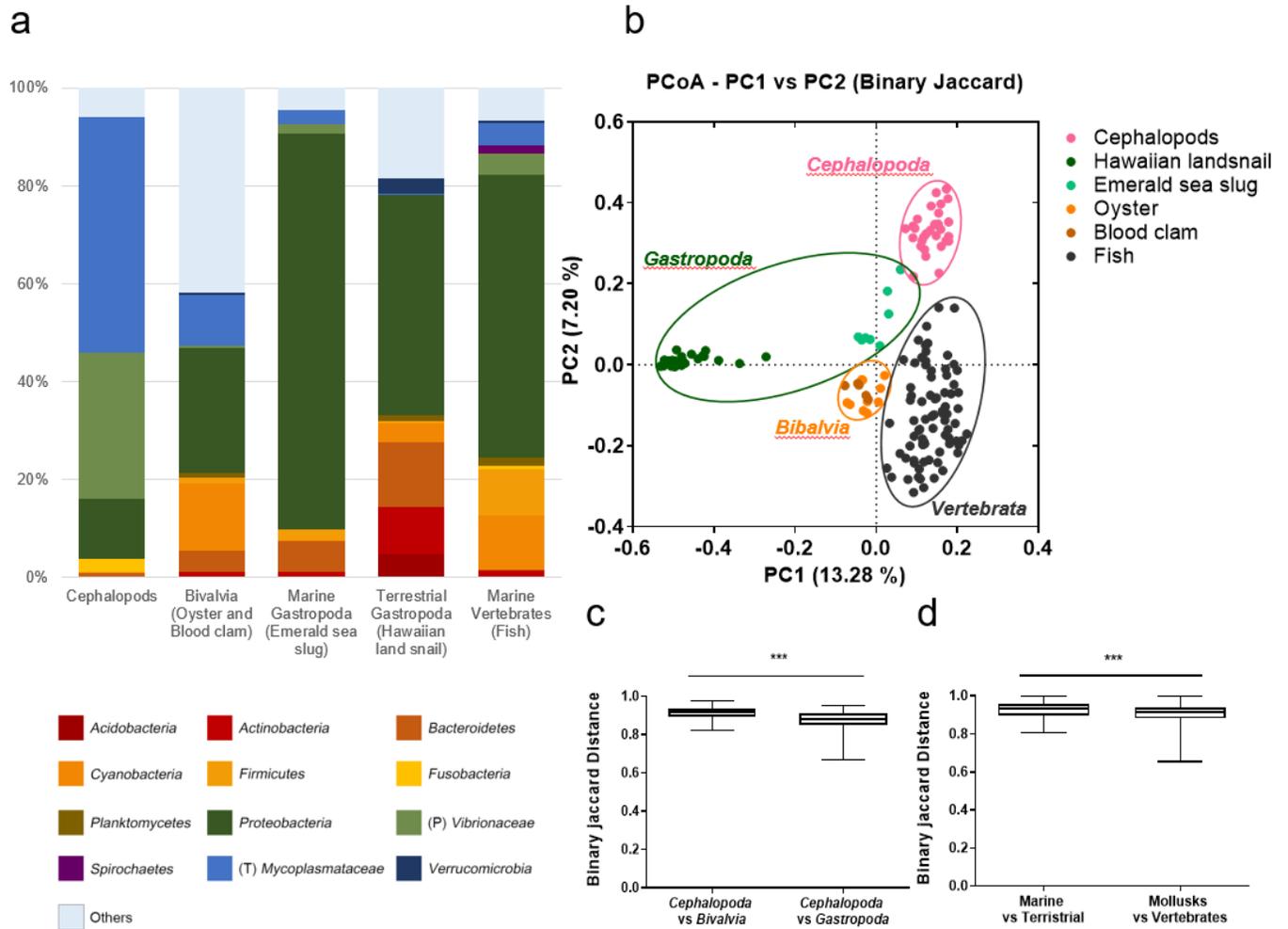


Figure 4

Ecological characteristics of global mollusk gut microbiomes. (a) Bar charts of the relative abundance of bacterial phyla and selected core families, Vibrionaceae and Mycoplasmataceae in various mollusks and marine vertebrates. (b) PCoA of the binary Jaccard indices of the gut microbiota from various mollusks (cephalopods, bivalves, and marine and terrestrial gastropods) and marine vertebrates (fishes). Colors of dots and ellipses in PCoA represent the host species and their classes (ANOSIM, $R = 0.84$, $p < 0.001$; adonis, $R^2 = 0.11$, $p < 0.001$). (c–d) Comparisons of microbial similarities of cephalopods with different mollusk classes (c; bivalves and gastropods) and difference between habitats (water vs. land) and between invertebrates and vertebrates (d), calculated based on binary Jaccard distances. Asterisks indicate significant differences between the binary Jaccard distances according to two-tailed Mann–Whitney U-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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