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Symbiotic organs shaped by distinct modes of genome evolution in cephalopods

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Microbes have been critical drivers of evolutionary innovation in animals. To understand the processes that influence the origin of specialized symbiotic organs, we report the sequencing and analysis of the genome of *Euprymna scolopes*, a model cephalopod with richly characterized host–microbe interactions. We identified large-scale genomic reorganization shared between *E. scolopes* and *Octopus bimaculoides* and posit that this reorganization has contributed to the evolution of cephalopod complexity. To reveal genomic signatures of host–symbiont interactions, we focused on two specialized organs of *E. scolopes*: the light organ, which harbors a monoculture of *Vibrio fischeri*, and the accessory nidamental gland (ANG), a reproductive organ containing a bacterial consortium. Our findings suggest that the two symbiotic organs within *E. scolopes* originated by different evolutionary mechanisms. Transcripts expressed in these microbe-associated tissues displayed their own unique signatures in both coding sequences and the surrounding regulatory regions. Compared with other tissues, the light organ showed an abundance of genes associated with immunity and mediating light, whereas the ANG was enriched in orphan genes known only from *E. scolopes*. Together, these analyses provide evidence for different patterns of genomic evolution of symbiotic organs within a single host.

cephalopods | symbiosis | evolution | genomics | transcriptomics

Numerous organisms have specialized organs to house their microbiota, yet the evolutionary processes underlying the origin of these tissues are not well understood (1–4). The Hawaiian bobtail squid, *Euprymna scolopes*, is a tractable animal model that has proven invaluable for interrogating symbiotic relationships and revealing common mechanisms by which successful colonization of animal epithelia by bacteria is established and maintained (Fig. 1A) (5). In the light organ (LO) of *E. scolopes*, a monospecific association with the bioluminescent bacterium *Vibrio fischeri* provides camouflage for the host and triggers a coordinated developmental remodeling of the surrounding epithelial tissues (6). Additionally, *E. scolopes* harbors a complex microbial consortium in the accessory nidamental gland (ANG), a component of the female reproductive system of many squids and cuttlefish that is hypothesized to play a role in egg defense (Fig. 1B) (7, 8). *Octopus bimaculoides*, the other cephalopod for which a genome has been reported to date, has neither a light organ nor an ANG making it an ideal organism for comparison (Fig. 1C). Therefore, to define elements of genome structure and function that are critical for symbiosis and the evolution of cephalopods, we sequenced and characterized the *E. scolopes* genome, the first example from the superorder Decapodiformes.

Results and Discussion

Our sequencing efforts have resulted in the most comprehensive cephalopod genome assembly to date, revealing the expansive and highly repetitive nature of the *E. scolopes* genome. Using a hybrid approach of shotgun and long-range linkage sequencing methods, an assembly estimated to be 5.1 Gigabases (Gb) was generated with a total half of the assembly in scaffolds of 3.7 Mb or longer (N50). The genome annotation was guided by 31 RNA-seq (Illumina) and ISO-seq (PacBio) transcriptomic libraries prepared from diverse tissues and developmental stages (*SI Appendix*, Table S1). The annotation resulted in 29,259 expressed

Significance

Animal–microbe associations are critical drivers of evolutionary innovation, yet the origin of specialized symbiotic organs remains largely unexplored. We analyzed the genome of *Euprymna scolopes*, a model cephalopod, and observed large-scale genomic reorganizations compared with the ancestral bilaterian genome. We report distinct evolutionary signatures within the two symbiotic organs of *E. scolopes*, the light organ (LO) and the accessory nidamental gland (ANG). The LO evolved through subfunctionalization of genes expressed in the eye, indicating a deep evolutionary link between these organs. Alternatively, the ANG was enriched in novel, species-specific orphan genes suggesting these two tissues originated via different evolutionary strategies. These analyses represent the first genomic insights into the evolution of multiple symbiotic organs within a single animal host.

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The authors declare no conflict of interest.

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Data deposition: Genome and transcriptome sequencing reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive, <https://www.ncbi.nlm.nih.gov/sra> (BioProject PRJNA470951).

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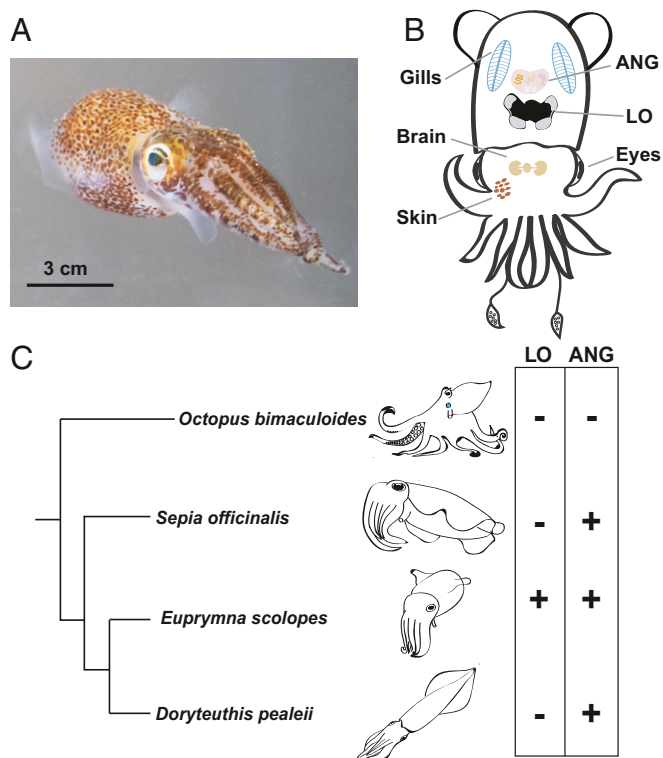


Fig. 1. The Hawaiian bobtail squid, *Euprymna scolopes*, a model host for microbiome research and cephalopod innovations. (A) The animal, shown here in the water column, is a nocturnal predator that uses the luminescence of the LO symbiont *Vibrio fischeri* for camouflage. Scale bar, 3 cm. Image courtesy of Elizabeth Ellenwood (photographer). (B) Overview of key symbiotic and nonsymbiotic organs within *E. scolopes*. Whereas males only have an LO symbiosis, the females have the additional symbiosis of the ANG, a reproductive organ housing a consortium of bacteria from predominantly two phyla (8). In addition to these symbiotic tissues, gene expression was also analyzed from the gills, brain, eye, and skin. (C) Distribution of LOs and ANGs that occur only in coleoid cephalopods. Branch lengths are derived from Tanner et al. (35).

protein-coding genes that yielded a BUSCO (Benchmarking Universal Single-Copy Orthologs) (9) score of 97% against the eukaryotic core set (*Materials and Methods*). A table summarizing the key statistics and a comparison with the *O. bimaculoides* genome is provided in *SI Appendix, Table S2*.

Genome analysis revealed differing histories of repeat element expansions between the bobtail squid and octopus, which may have contributed to the observed divergence in genome size (*SI Appendix, Fig. S1*). Although the estimated proportion of repetitive elements was >50% in both species, the abundance within individual repetitive element classes was strikingly different across the two species. Specifically, the abundance of long and short interspersed nuclear elements (LINEs and SINEs, respectively) differed; LINEs were the most abundant repeat class in *E. scolopes*, whereas SINEs dominated in *O. bimaculoides* (*SI Appendix, Fig. S1B*). Gene families in *E. scolopes* are similar to those reported in *O. bimaculoides* and also include tandem expansions in protocadherins and zinc finger transcription factors (*SI Appendix, Fig. S2*) (10). We infer that the last common coleoid cephalopod ancestor had 7,650 gene families, similar to the previously reported estimates for spiralian and bilaterian ancestors (10, 11). Because we do not find signatures of whole-genome duplication or evidence of extensive horizontal gene transfer in *E. scolopes*, the difference in the genome sizes between octopus and bobtail squid can be attributed to the increased LINE content in the *E. scolopes* genome. Future

sequencing of other Decapodiformes genomes should enable a more exact estimate of this repeat element expansion.

The *E. scolopes* genome revealed key genomic transitions toward cephalopod genomic architecture. We find that over half of the local gene linkages (i.e., microsynteny) conserved across many noncephalopod bilaterian genomes were disrupted in both *O. bimaculoides* and *E. scolopes*, indicating that a large genomic reorganization took place in the cephalopod ancestor (Fig. 2A). Additionally, we found numerous linkages shared between octopus and the bobtail squid that have not been previously identified in other animal genomes (Fig. 2B and C). Those linkages contain genes expressed in highly developed organ systems (e.g., the central nervous system) as well as in symbiotic organs from *E. scolopes* and testes of *O. bimaculoides* (Fig. 2C). In total, this taxon-specific microsynteny constituted 67% (126 out of 189) of all conserved syntenic linkages in both cephalopod genomes (*SI Appendix, Fig. S3* and *Table S2*).

The improved contiguity of the genome assembly also uncovered the presence of a partial *hox* cluster in *E. scolopes* with very large (1.5–2 Mb) intergenic distances that is in stark contrast to the previously reported atomized *hox* cluster in the octopus genome (10). These are to our knowledge the longest inter-*hox*-gene separations (Fig. 2D) known in animals and are consistent with the long, gene-free regions surrounding *hox* genes in *O. bimaculoides* (10) and the identified expansion of repetitive elements. Such large intergenic distances may be responsible for the evolution of unique regulatory mechanisms. This hypothesis is further supported by unconventional *hox* gene expression in *E. scolopes* (12).

To understand how this genome reorganization drove the evolution of symbiotic organs in *E. scolopes*, we studied genomic signatures of genes with tissue-specific expression in symbiotic versus nonsymbiotic organs. To identify possible candidate genes that evolved in symbiotic organs after a duplication event, we searched the *E. scolopes* genome for all paralogous pairs of genes in which one gene had tissue-specific expression and the second did not. Using these genes, we then calculated the number of synonymous substitutions (dS) between each gene pair (Fig. 3). We found that for the LO and ANG, the dS distance between tissue-specific genes and the respective closest non-LO and non-ANG paralog was relatively small [dS < 1, ~130 Mya, using a calibration point from Albertin et al. (10)]. These distances were in sharp contrast compared with genes specific to the eye and brain, which were much older (dS > 2, squid–octopus divergence, ~270 Mya) (10, 13). These paralog ages are consistent with a scenario in which the ANG and LO, which harbor dense populations of bacteria, underwent relatively recent innovations within the bobtail squid lineage through gene duplication, compared with the more ancient duplications of genes that are involved in development and function of cephalopod eyes and the nervous system.

The main mechanism behind gene duplication in *O. bimaculoides* is through tandem or segmental duplication (10). Genes exclusively expressed in either LO, eyes, or ANG were typically found in tandem clusters of paralogs located on single scaffolds. In particular, we found two gene clusters, the first composed of reflectins and the second of peroxidases that were expressed in the LO (see below, Fig. 4, and *SI Appendix, Figs. S4–S6*). In both cases, the tandem gene duplications forming those clusters were *E. scolopes*-specific and occurred after the octopus–squid split, consistent with the emergence of ANG and LO in the subsequent squid lineage. A large 2-Mb cluster of S-crystallins, which are predominantly expressed in the eyes (*SI Appendix, Fig. S7*), was expanded in tandem in *E. scolopes* but was missing from the octopus genome. The omega-crystallins, which are typically found in the LO and the eyes of *E. scolopes*, on the other hand, did not show signs of any expansion (*SI Appendix, Fig. S7*). The characteristic cluster in the ANG (~1 Mb)

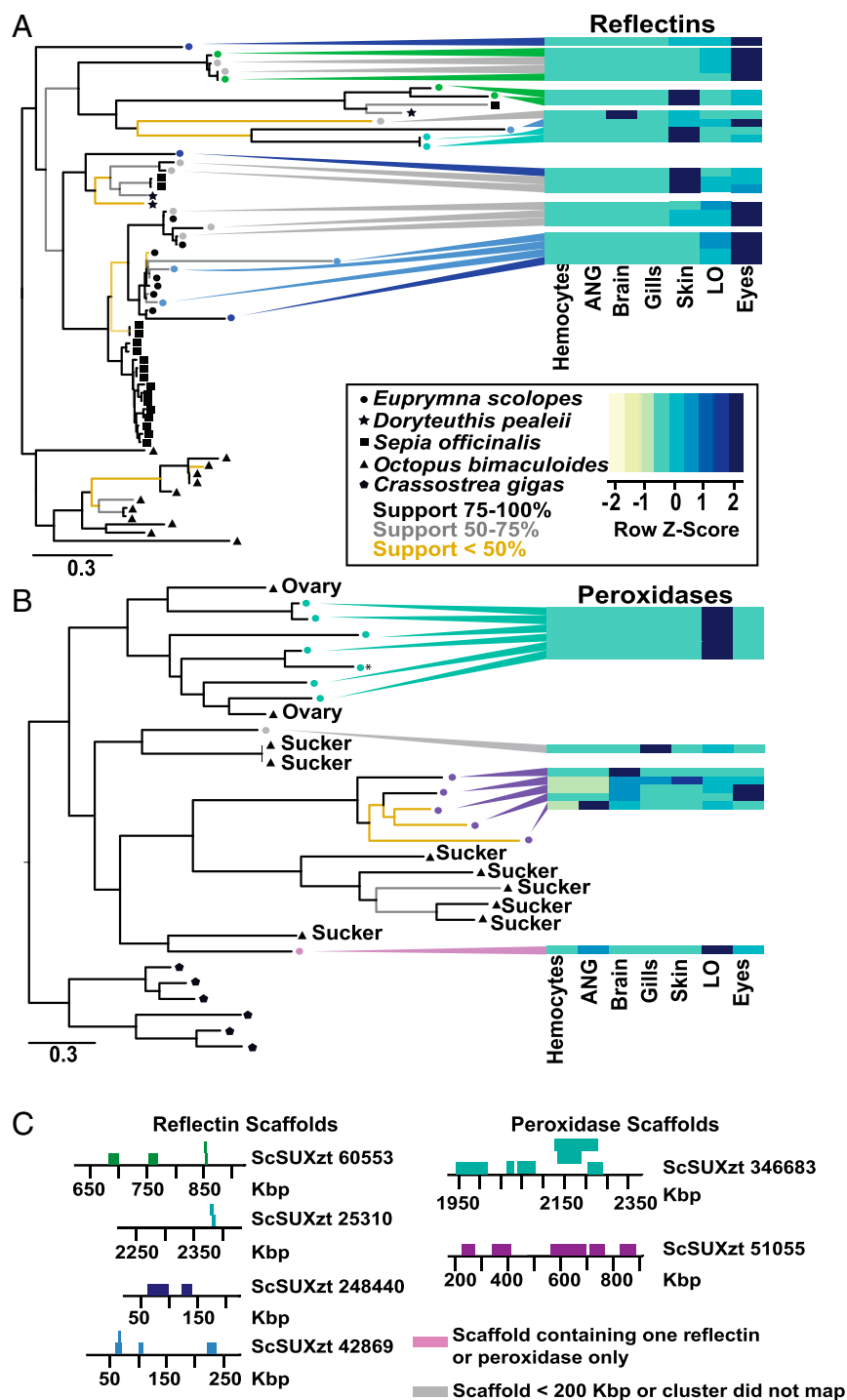


Fig. 4. Independent tandem gene cluster formation in squid and octopus and the origin of light organ-specific gene expression. Phylogenetic trees highlight highly specific expansion patterns that correlate with general shared expression between the light organ and the eyes for reflectins (A) or the appearance of the light organ-specific expression pattern for heme peroxidases (B). Heat maps indicate relative normalized expression levels for each tissue (Z scores). Scale bar underneath phylogenetic trees indicates amino acid substitutions per site. Color of the nodes (A and B) identifies genomically colocalized genes (shown in C). Those genes are clustered in tandem on several scaffolds with positions and gene identifiers labeled. (C) Tandem clusters of reflectin and peroxidase genes in the *E. scolopes* genome. Scaffold ID and approximate location (kbp) is shown for each gene (represented by rectangle). Colors correspond to the sequences on the trees in A for reflectins and (B) for peroxidases.

to be the major driver of innovation (33). Rapid gene evolution and/or gene loss in ancestral lineages may also have contributed to these unique signatures, especially in the ANG. Together, the presence of these two distinctive tissues within *E. scolopes* renders it a unique model organism to investigate the genetic mechanisms associated with the evolution of symbiotic organs (34).

Conclusions

Our analyses of the Hawaiian bobtail squid genome revealed large-scale genome reorganization, which preceded the coleoid cephalopod radiation, and provide evidence for two different

patterns of genomic evolution that contributed to functional novelty. First, the extensive structural reorganization through the loss of ancient bilaterian microsynteny and increase in genome size through repetitive element expansions resulted in a unique genomic architecture that may have contributed to the innovations in the general cephalopod body plan. Second, the coding and surrounding regulatory regions of microbe-associated tissues suggest distinctive evolutionary patterns, such as the expansion of genes associated with immunity and light production in the LO. In the ANG, the prevalence of taxon-specific, or orphan, gene expression suggests a highly derived organ that evolved by a different means than the LO, perhaps due to unique selection

pressures. Overall, our results set the stage for further functional analysis of genomic innovations that led to the evolution of symbiotic organs in a morphologically and behaviorally complex clade.

Materials and Methods

Data Access. Genome and transcriptome sequencing reads have been deposited in the Sequence Read Archive as Bioproject PRJNA470951.

Genome Sequencing and Assembly. Genomic DNA generated in this study was derived from a single adult male *E. scolopes* (SI Appendix). Illumina reads from several libraries (SI Appendix, Table S1) were assembled with Meraculous resulting in a preliminary assembly; scaffolds were generated using the in vitro chromatin conformation capture (Chicago) at Dovetail Genomics.

Gene Model Predictions. Gene prediction models were generated using Augustus training with transcriptomic data. The prediction was filtered to exclude sequences that overlap with masked repeats over at least 50% of the lengths. From the remaining models, those with support from transcript evidence and significant homology to metazoan protein sequences were included in the nonredundant database. This gene modeling set was combined with transcriptome mapping (SI Appendix, Table S2) resulting in 29,089 protein sequences that passed the filtering stage.

Transcriptome Sequencing. Thirty-one transcriptomes of *E. scolopes* were included in the reference transcriptome and sample metadata of the tissues and time points is provided in SI Appendix, Table S1. Different RNA extractions and sequencing platforms were used and are listed in SI Appendix, Table S1. For the tissue-specific transcriptomes, RNA was extracted from ANG, brain, eyes, gills, hemocytes, LO, and skin tissues, as well as juvenile head (white body, optic nerve, and brain), eyes, gills, and light organ. For the PacBio IsoSeq library, RNA was extracted, normalized, and pooled from

adult brain, eyes, white body, optical lobe, gills, LO, and skin as well as whole juvenile hatchling, 24-h aposymbiotic, and 24-h symbiotic animals (SI Appendix, Table S1).

Synteny Analysis. Using previously published methods of phylogeny-informed clustering, we have constructed the sets of orthologous gene families between the following species (11): *Capitella teleta*, *Helobdella robusta*, *Lottia gigantea*, *Octopus bimaculoides*, *Euprymna scolopes*, *Crassostrea gigas*, *Nematostella vectensis*, and *Branchiostoma floridae*. To account for differential gene loss that may impede our quantification of syntenic loss/gain we focused only on 3,547 clusters that have an ortholog from each of the species. We have implemented microsynteny detection algorithm, as described in Simakov et al. (11), and found, in accordance with previous results (10, 34), ~600 microsyntenic blocks (in 163 orthologous groups) that can be traced back to the bilaterian ancestor because they are shared between either both ingroups (protostome and deuterostome) or an ingroup and an outgroup species (*Nematostella*) (Dataset S1).

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