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Patrick Minx
Washington University School of Medicine in St. Louis

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Symbiotic organs shaped by distinct modes of genome evolution in cephalopods

Mahdi Belcaid\textsuperscript{a}, Giorgio Casaburi\textsuperscript{b}, Sarah J. McAnulty\textsuperscript{c}, Hannah Schmidbauer\textsuperscript{d}, Andrea M. Suria\textsuperscript{e}, Silvia Moriano-Gutierrez\textsuperscript{f}, M. Sabrina Pankey\textsuperscript{a}, Todd H. Oakley\textsuperscript{g}, Natacha Kremer\textsuperscript{h}, Eric J. Koch\textsuperscript{i}, Andrew J. Collins\textsuperscript{j}, Hoan Nguyen\textsuperscript{k}, Sai Lek\textsuperscript{l}, Irina Goncharenko-Foster\textsuperscript{m}, Patrick Minx\textsuperscript{n}, Erica Sodergren\textsuperscript{o}, George Weinstock\textsuperscript{p}, Daniel S. Rokhsar\textsuperscript{q,r,k}, Margaret McFall-Ngai\textsuperscript{a}, Oleg Simakov\textsuperscript{d,i,1,1}, Jamie S. Foster\textsuperscript{b,1,1} and Spencer V. Nyholm\textsuperscript{e,1}

\textsuperscript{a}Pacific Biosciences Research Center, University of Hawaii, Honolulu, HI 96822; \textsuperscript{b}Department of Microbiology and Cell Science, Space Life Science Lab, University of Florida, Merritt Island, FL 32953; \textsuperscript{c}Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269; \textsuperscript{d}Department of Molecular Evolution and Development, University of Vienna, 1090 Vienna, Austria; \textsuperscript{e}Ecology, Evolution, and Marine Biology Department, University of California, Santa Barbara, CA 93106; \textsuperscript{f}Laboratoire de Biométrie et Biologie Évolutive, Université de Lyon, Claude Bernard University Lyon 1, CNRS, UMR 5558, 69100 Villeurbanne, France; \textsuperscript{g}Jackson Laboratory for Genomic Medicine, Farmington, CT 06032; \textsuperscript{h}McDonnell Genome Institute, Washington University, St. Louis, MO 63108; \textsuperscript{i}Molecular Genetics Unit, Okinawa Institute of Science and Technology, Okinawa 904-0495, Japan; \textsuperscript{j}Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720; and \textsuperscript{k}Department of Energy, Joint Genome Institute, Walnut Creek, CA 94598

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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 	extit{Euprymna scolopes}, a model cephalopod with richly characterized host-microbe interactions. We identified large-scale genomic reorganization shared between 	extit{E. scolopes} and 	extit{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 	extit{E. scolopes}: the light organ, which harbors a monocolonure of 	extit{Vibrio fischeri}, and the accessory nidamental gland (ANG), a reproductive organ containing a bacterial consortium. Our findings suggest that the two symbiotic organs within 	extit{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 	extit{E. scolopes}. Together, these analyses provide evidence for different patterns of genomic evolution of symbiotic organs within a single host.

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, 	extit{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 epithelium by bacteria is established and maintained (Fig. 1A) (5). In the light organ (LO) of 	extit{E. scolopes}, a monospecific association with the bioluminescent bacterium 	extit{Vibrio fischeri} provides camouflage for the host and triggers a coordinated developmental remodeling of the surrounding epithelial tissues (6). Additionally, 	extit{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). 	extit{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 	extit{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 	extit{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 RNAseq (Illunma) and ISO-seq (PacBio) transcriptomic libraries prepared from diverse tissues and developmental stages (SI

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 	extit{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 	extit{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.


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/condb (BioProject PRJNA479051).

See Commentary on page 2799.

1To whom correspondence may be addressed. Email: oleg.simakov@univie.ac.at, jfoster@ufl.edu, or spencer.nyholm@uconn.edu.

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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 nonechelopod 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 *hox* gene-free regions surrounding the *hox* group 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 ANG paralogs 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*, Figs. S4–S6).
The characteristic cluster in the ANG (~1 Mb) contained both novel genes and transposable elements. Overall, there were significantly more tandemly duplicated genes expressed in the LO and eyes compared with the number of tandemly clustered genes specifically expressed in other tissues (Fisher’s exact test $P < 0.001$), which underscores the importance of gene duplication for those organ systems.

Our data also reveal that despite the broad phylogenetic distribution among cephalopods (14), the ANG of E. scolopes is highly derived and shows an elevated evolutionary accumulation in both its coding (novel gene formation) and noncoding (turnover of the regulatory sequence) complements. The ANG is a secretory organ within the female reproductive system containing a bacterial consortium that is deposited into the egg capsule and is believed to play a role in defense from fouling and/or pathogens during embryogenesis (7, 8, 15, 16). Taxonomically restricted (i.e., orphan) genes have contributed to the evolution of unique tissues and organs in a number of animals (17–19).

Within the two symbiotic organs of E. scolopes (LO and ANG), we profiled protein-coding transcripts that mapped to genome scaffolds from tissue-specific transcriptomes against the National Center for Biotechnology Information’s nonredundant database [using BLASTP (Basic Local Alignment Search Tool - Protein) $e$-value threshold of 1E-5; Fig. S4] and classified the transcripts as either E. scolopes-specific, cephalopod, molluscan, bilaterian, metazoan, or premetazoan (SI Appendix, Fig. S8).

The ANG had the highest proportion of E. scolopes-specific transcripts (>35%) among all tissues. Additionally, regions 20 kb up- and down-stream of genes specifically expressed in the ANG also showed a higher proportion of repetitive element content compared with genes expressed in any other tissue (Fig. 3B), suggesting a high evolutionary turnover in regulatory regions. These higher rates of genomic innovation, specific to the ANG, are also supported by our analysis of previously reported ANG transcriptomes from both E. scolopes and the (loliginid) swordtip squid, Uroteuthis edulis, the latter of...
A comparison of adult LO and eye transcriptomes (SI Appendix, Fig. S8) (20).

Within the LO of *E. scolopes*, there were several other genetic signatures contributing to the distinctive features of this symbiotic organ. First, the dominant transcripts within the light organ were reflectins (Fig. 4A), providing strong support for previous studies indicating that the anatomical features used to modulate light in the LO are similar to the eye, as are their physiology, biochemistry, molecular biology, and developmental induction (21–23). A comparison of adult LO and eye transcriptomes supports these previous studies (Figs. 3C and 4A). Identified reflectins (Fig. 4A) were almost exclusively (>99.99% of total expression) expressed in the LO, eyes, and skin. LO expression constituted around 19.5% of the total reflectin expression, whereas eyes and skin expressed 72.8% and 7.6%, respectively. Reflectins usually shared two or more expression domains and were rarely expressed in a single tissue. The majority of genes showed a distinctive LO and eyes expression domain, and no reflectins were expressed exclusively in both LO and skin (Fig. 4A and C). Evolutionary timing of the LO–eye reflectin clusters may thus hint at the origin of the LO. Molecular dating analysis using branch length and squid–octopus split as a calibration point (10, 13) revealed the age of those clusters at around 30 Mya or younger. Additionally, among the genomic expansion of 14 peroxidases unique to *E. scolopes* (Fig. 4B and C and SI Appendix, Fig. S6), six genes [including a previously reported halide peroxidase (24, 25)] formed a single genomic cluster expressed exclusively in the light organ. Unlike reflectins, this expansion did not form a monophyletic group, suggesting high gain or loss of genes within this family, or gene conversion. The host is known to produce a halide peroxidase that is expressed in the LO and generates hypohalous acid, a potent antimicrobial compound thought to contribute to specificity in the squid–vibrio association (26, 27). This peroxidase, along with several others, is expressed in the tissues directly in contact with *V. fischeri* (28, 29). The expansion of the peroxidases and the unique expression of a subset of these genes exclusively in the LO may reflect the development of a unique microenvironment that helps to maintain specificity with *V. fischeri* (SI Appendix, Fig. S6).

Analysis of protein families also revealed a number of genes that had multiple immune-associated domains, many of which were expressed in the LO, ANG, and skin (SI Appendix, Fig. S9). All three of these tissues interact directly with environmental and/or symbiotic microorganisms. A number of cellular and biochemical components of the innate immune system are known to play critical roles in mediating specificity in the LO symbiosis with *V. fischeri* (30–32). Previous observations of host hemocytes infiltrating the ANG (7), along with expression of immune-related genes reported here, suggest that the host’s immune system plays a critical role in this symbiotic organ as well.

Although bacterial LOs are restricted to just two families of cephalopods (sepiolids and loliginids), the ANG is a more broadly distributed organ, present in these and a number of other squid and cuttlefish species (Fig. 1C). Their distinct evolutionary histories and origins are supported by the different evolutionary patterns detected. The prevalence of functional novelty through gene duplication in the LO compared with taxon-specific genes in the ANG suggests two different patterns of genomic innovation underlying symbiotic organ evolution in *E. scolopes*. Because these two associations are not nutritionally coupled, the mechanisms for the evolution of these symbiotic organs may be different from what has been proposed for insect bacteriocytes where metabolic complementation appears
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 forces.

**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.
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 Mer-
cuculus 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 hatching, 24-h apysymbiotic, 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, Crostasstea gigas, Nematostella vectensis, and Branchiostoma floridae. To account for differential gene loss that may impede our quantification of synteny 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 microsynteny 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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