Independent Losses of the Hypoxia-Inducible Factor (HIF) Pathway within Crustacea

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Abstract

Metazoans respond to hypoxic stress via the hypoxia-inducible factor (HIF) pathway, a mechanism thought to be extremely conserved due to its importance in monitoring cellular oxygen levels and regulating responses to hypoxia. However, recent work revealed that key members of the HIF pathway have been lost in specific lineages (a tardigrade and a copepod), suggesting that this pathway is not as widespread in animals as previously assumed. Using genomic and transcriptomic data from 70 different species across 12 major crustacean groups, we assessed the degree to which the gene *HIFα*, the master regulator of the HIF pathway, was conserved. Mining of protein domains, followed by phylogenetic analyses of gene families, uncovered group-level losses of *HIFα*, including one across three orders within Cirripedia, and in three orders within Copepoda. For these groups, additional assessment showed losses of HIF repression machinery (EGLN and VHL). These results suggest the existence of alternative mechanisms for cellular response to low oxygen and highlight these taxa as models useful for probing these evolutionary outcomes.

Key words: **bHLH-PAS, Cirripedia, Copepoda, gene loss, transcription factor, oxygen sensing.**

Letter

Multicellular eukaryotes have evolved tight coordination of genes controlling specialized mechanisms that enhance O_2 uptake and distribution. These systems are capable of responding to changes in O_2 availability on local, organismal, and temporal levels. In general, one strategy for surviving in a dynamic environment is to maintain an array of oxygen responders that can regulate function across the spectrum of environmental changes which the organism might experience. These responses are mediated in part through the induction of hypoxia-inducible factors (HIF), composed of regulatory components HIFα and HIFβ/ARNT (Rytkönen et al. 2011; Graham and Presnell 2017). The mechanism controlling HIFα function is the primary cellular oxygen-sensing and responding pathway in animals. Under normoxia, the constitutively expressed HIFα subunit is hydroxylated by EGLN, a prolyl hydroxylase, which tags HIFα for proteasomal degradation by the von Hippel-Lindau (VHL) ubiquitination complex, thus preventing activation of the pathway. While under low oxygen tension, the O_2-dependent EGLN is disabled, allowing HIFα to form the transcription factor dimer with HIFβ/ARNT. The functional heterodimer binds to DNA regions called HREs (hypoxia response elements) located upstream of certain target genes, effectively manipulating their expression patterns and regulating a network for a system-wide response to low-oxygen (Wenger et al. 2005).

The HIF pathway appeared early in metazoan evolution (Loenarz et al. 2011; Mills et al. 2018), and was previously considered to be functionally and structurally conserved in animals, as it has been identified in every bilaterian as well as placozoan and cnidarian species in which it has been explicitly examined. Recent genomic analyses, however, have documented two losses of this gene, one in a tardigrade (Hashimoto et al. 2016) and one in a harpacticoid copepod (Graham and Barreto 2019). These findings suggest that the HIF pathway may not be as universally present as previously thought. Therefore, examining the distribution of HIFα, EGLN and VHL loss across animals may identify taxa in which alternative mechanisms have evolved and provide new lines of research for understanding cellular physiological response to hypoxic stress.

The presence of the HIF pathway (via the presence of *HIFα*) is well known in certain crustacean species, but those only represent a few higher-level taxonomic groups, primarily Decapoda and Branchiopoda (Gorr et al. 2004; Soñanez-Organis et al. 2009; Hardy et al. 2012). Crustaceans include many other animal groups that are dominant by biomass, distribution, and species number, especially in aquatic systems (Price et al. 2011), but genetic resources for this large subphylum as a whole are sparse compared with insects, a group nested within Crustacea. Major crustacean groups such as Amphipoda, Isopoda, Copepoda, and Cirripedia are commonly used for studies of environmental physiology, including organismal responses to hypoxia; the regulatory mechanisms underlying these phenotypes, however, are largely unexplored in these groups, and are likely assumed to be based on the HIF pathway. Based on the recent genomic findings mentioned above (Hashimoto et al. 2016; Graham and Barreto 2019), we can no longer assume that this pathway is conserved and functional in every group.

In this study, we mined publicly available genomic and transcriptomic resources of 70 species representing most of the major clades within Crustacea, and assayed them for the presence of the HIF pathway. We show that there have been multiple losses of these interacting sets of genes (potentially

independently), including specific subgroups of Copepoda (orders Harpacticoida, Cyclopoida, and Siphonostomatoida), as well as across three orders of barnacles (Cirripedia: Sessilia, Pedunculata, and Kentrogonida). These results open up exciting lines of research that involve characterizing novel underlying mechanisms associated with oxygen sensing and homeostasis in these groups.

Results

We used a combination of Hidden Markov Model (HMM) searches, BLAST, InterproScan, and phylogenetic analyses to screen for the three main elements of the HIF-pathway (HIFα, EGLN, VHL) across multiple crustacean taxa. We first searched for HIFα across all species. In groups with evidence for loss of *HIFα*, we also searched for its repression machinery (EGLN, VHL), as a way to assess whether the canonical oxygen-sensing and -responding HIF pathway was lost. These proteins were identified based on the presence of specific protein domain elements, including 1) PAS domains, to identify HIFα copies; 2) P4HC domains, in an effort to identify EGLN copies; and 3) the VHL-β domain to find VHL.

We used protein sequences from a combination of publicly available assembled genomes and transcriptomes, and several we assembled de novo from available raw data. In total, we analyzed data from 70 species across 12 higher-level taxonomic groups (supplementary table S1, Supplementary Material online). For some groups, we were able to include multiple members within multiple orders (e.g., Copepoda, Amphipoda, and Decapoda). For others, despite using all known transcriptomic/genomic resources, the coverage was relatively low, with only 1–4 representatives.

Because of the wide variation in quality and the necessarily incomplete nature of transcriptome and genome data available, our goal was not to assess gene loss at the individual species level. Therefore, we sampled multiple species within a group (when possible) in order to offset any possibility of individual transcriptome missing a *HIFα* for technical reasons. Ultimately, our determination of HIFα loss (and that of the EGLN or VHL) involved assessment at the group level—if one or more members of group showed evidence of a HIFα copy, the group was conservatively considered to have retained the gene (supplementary methods, Supplementary Material online).

Overall, BLASTP searches and phylogenetic analyses of bHLH-PAS-containing proteins were highly concordant in identifying HIFα members, but in a few cases where BLAST was unable to detect a HIFα protein, the phylogenetic analysis was more sensitive to identification (supplementary table S1, Supplementary Material online). For example, using BLAST, no sequences from either *G. chevreuxi* or *H. gigas* in the Amphipoda showed a significant hit to HIFα, but the phylogenetic groupings identified putative HIFα sequences in these species (supplementary fig. S1, Supplementary Material online) that were then confirmed by InterproScan assessment. Representation of the other bHLH-PAS members (NPAS4, NCOA, ARNT, SIM, ARNTL, AhR, NPAS2/CLOCK, NPAS1/3, and Met) was also largely consistent across all groups. However, NPAS4 shows the potential for having been lost in Isopoda, Amphipoda, and Decapoda, but we caution that we did not further scrutinized this pattern because our focus is on HIFα. In addition, sequences which were outliers in the resulting trees were queried further for identity using InterproScan—these were detected to belong to other gene families which contained PAS domains plus domains not associated with the bHLH-PAS-containing families. These included PAS-containing (though not bHLH-PAS) cyclic nucleotide phosphodiesterases, HAMP/histidine kinases, MAP/microtubule affinity regulating kinases, and potassium voltage-gated channel subfamilies, and therefore were not considered potential missing HIF members.

All eight members of four infraorders in Decapoda (Brachyura, Caridea, Dendrobranchiata, Pleocyemata) contained HIFα members (supplementary fig. S2, Supplementary Material online). Some, yet not all, of the species in Amphipoda (supplementary fig. S1, Supplementary Material online), Isopoda (supplementary fig. S3, Supplementary Material online), Mysida and Euphausiacea (supplementary fig. S4, Supplementary Material online), Remipedia (supplementary fig. S5, Supplementary Material online), and Ostracoda (supplementary fig. S6, Supplementary Material online) contained HIFα members—those for which a HIFα was not found include *Grandidierella japonica*, *Eulimnogammarus cruentus*, *Hyalella azteca* (Amphipoda), *Xibalbanus tulumensis* (Remipedia), *Armadillidium vulgare* (Isopoda), *Neomysis awatschensis* (Mysida), and *Conchoecia obtusata* and *Eusarsiella* sp. (Ostracoda). However, those examples are likely due to low-quality sequence/assembly, especially for *N. awatschensis*, *C. obtusata* and *Eusarsiella*, which had a low total number of PAS-containing proteins (supplementary table S1, Supplementary Material online). For *X. tulumensis*, although a HIFα was identified through BLASTP and InterproScan (Xiba_DN232543_c1_g1_i2; 354 aa), no HIFα grouped with the other remipede species and with anchor HIFα; that sequence instead grouped with NPAS2/CLOCK and suggests a divergent HIF sequence or a misassembled transcript. For *H. azteca*, a HIFα was identified through BLASTP and InterproScan (Hyaztec_GEHV01020431.1; 148 aa), yet the same sequence grouped instead with the Met gene family. In general, these "losses" likely are not biological, but instead represent absences due to issues in sequencing and transcriptome assembly, especially given other members of the same taxonomic group showed clear presence of HIFα. Therefore, our results suggest HIF pathway was retained in Amphipoda (supplementary fig. S1, Supplementary Material online), Isopoda (supplementary fig. S3, Supplementary Material online), Decapoda (supplementary fig. S2, Supplementary Material online), Mysida and Euphausiacea (supplementary fig. S4, Supplementary Material online), Remipedia (supplementary fig. S5, Supplementary Material online), and Branchiopoda, Cephalocarida, Stomatopoda, and Ostracoda (supplementary fig. S6, Supplementary Material online), because we observed that a HIFα family member was unambiguously present in at least

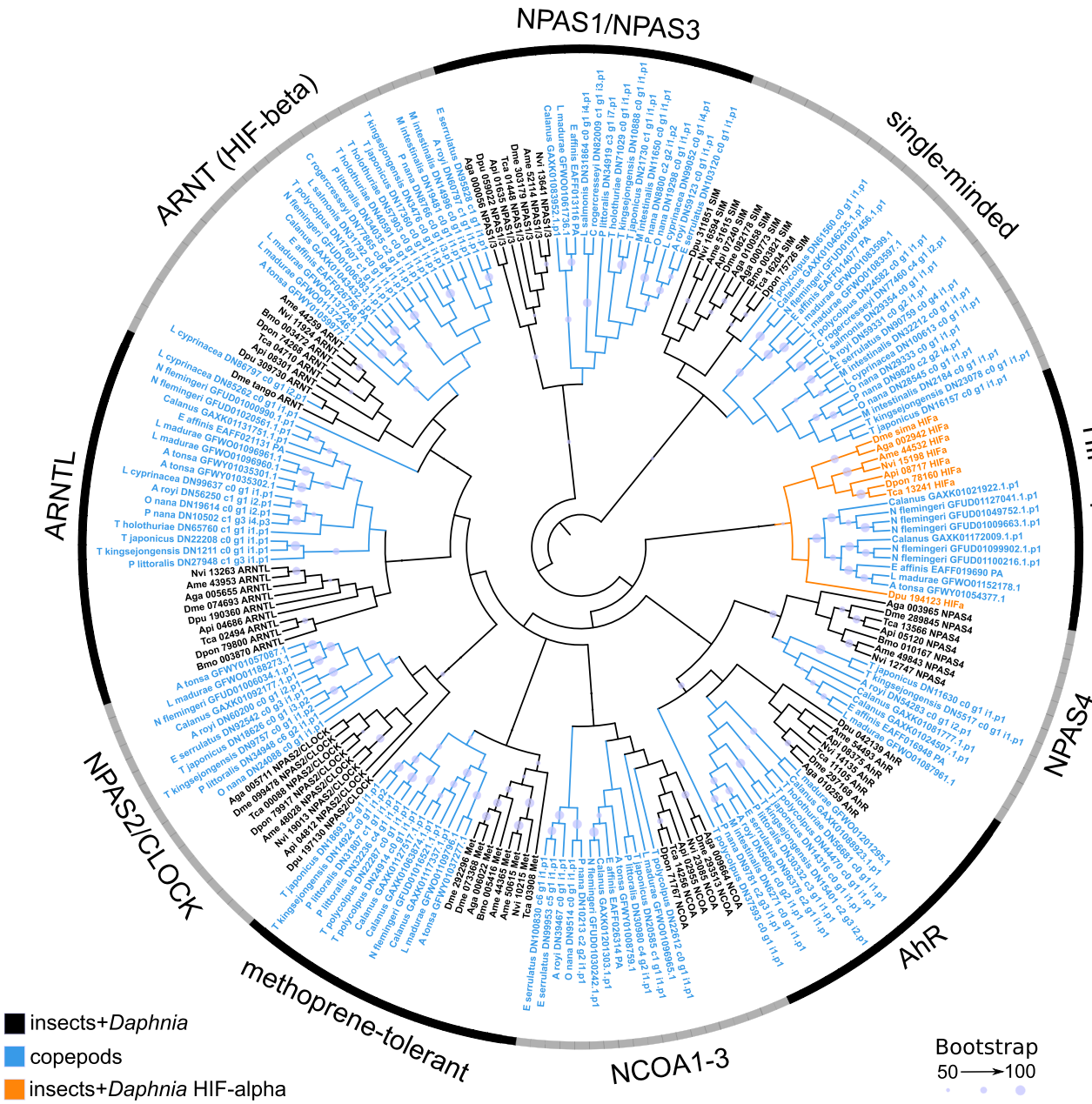

FIG. 1. Maximum-likelihood phylogenetic tree of bHLH-PAS proteins of Copepoda, containing 18 species (in blue) across four orders (RAxML; 200 bootstraps). Three orders within Copepoda showed a loss of *HIFα*, (Harpacticoida, Cyclopoida, Siphonostomatoida). See supplementary table S1, Supplementary Material online for the full species names and their classification. In orange are the "anchor" sequences of HIFα from insects and *Daphnia pulex* (Dpu)—including *Anophales gambii* (Aga), *Bombyx mori* (Bmo), *Apis mellifera* (Ame), *Acyrthosiphon pisum* (Api), *Dendroctonus ponderosae* (Dpon), *Drosophila melanogaster* (Dme), *Nasonia vitripennis* (Nvi), and *Tribolium castaneum* (Tca).

one species in each group (supplementary table S1, Supplementary Material online).

In the Copepoda, only 5 of the 18 species examined exhibited a copy of HIFα and EGLN. However, these five species are all contained within the order Calanoida; therefore, all species available and analyzed from orders Cyclopoida, Harpacticoida, and Siphonostomatoida showed no evidence of a canonical HIFα (fig. 1; supplementary table S1, Supplementary Material online) or EGLN (supplementary fig. S7, Supplementary Material online; supplementary table S2, Supplementary Material online) protein at any stage of the analyses. Initial screening found evidence for a HIFα and EGLN in the three species of Siphonostomatoida and in one Cyclopoid (*Lernaea cyprinacea*), but further BLAST analyses revealed that these sequences had >97% similarity and >90% coverage to fish GenBank accessions. Since these four species are obligate fish parasites, this pattern is consistent with contamination from host tissue during RNA isolation. We implemented a step to remove contaminant proteins from these assemblies through a BLAST search against a custom database that included a transcriptome from host fish species (supplementary methods, Supplementary Material online). After this step, we rescreened the proteomes and found no evidence of a HIFα or EGLN in those groups.

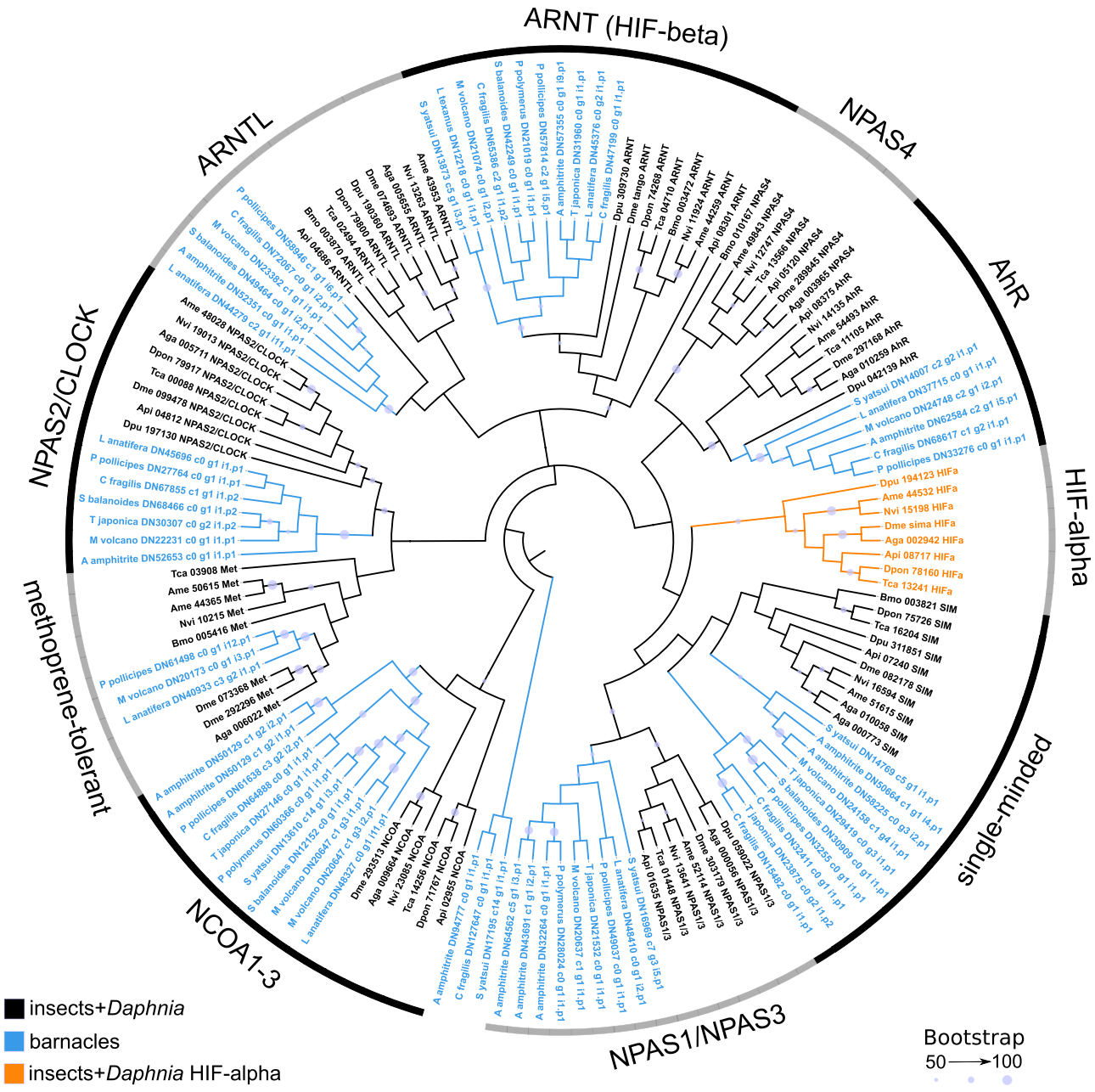

FIG. 2. Maximum-likelihood phylogenetic tree of bHLH-PAS proteins of Cirripedia containing 10 species (in blue) across three orders (RAxML; 200 bootstraps). See supplementary table S1, Supplementary Material online for the full species names and their classification. In orange are the "anchor" sequences of HIFα from insects and *Daphnia pulex* (Dpu)—including *Anophales gambii* (Aga), *Bombyx mori* (Bmo), *Apis mellifera* (Ame), *Acyrthosiphon pisum* (Api), *Dendroctonus ponderosae* (Dpon), *Drosophila melanogaster* (Dme), *Nasonia vitripennis* (Nvi), and *Tribolium castaneum* (Tca).

With regard to VHL, patterns of loss were spotty—most of Harpacticoida (except *Tigriopus californicus*), Calanoida (except *Eurytemora affinis*) and Cyclopoida (except *Paracyclopina nana* and *Oithona nana*) showed evidence for the presence of a VHL, whereas none was detected in Siphonostomatoida (figs. 3 and 4B; supplementary table S3, Supplementary Material online). This may suggest either VHL was lost independently in species-specific manner, or that it was simply not captured in the transcriptomic data.

In Cirripedia, all species surveyed across three orders (Sessilia, Pedunculata, Kentrogonida) showed no evidence for the presence of HIFα (fig. 2; supplementary table S1, Supplementary Material online), EGLN (supplementary fig. S8, Supplementary Material online; supplementary table S2, Supplementary Material online), or VHL members (figs. 3 and 4A; supplementary table S3, Supplementary Material online).

As a final check, we performed a reciprocal BLAST between a known decapod HIFα (GenBank accession ACU30154.1), the transcriptome assemblies of the copepod and barnacle taxa, and the Uniprot/Swiss-Prot database. This was done at the transcript stage of the assemblies, instead of the predicted proteins, so that even poorly assembled possible fragments of

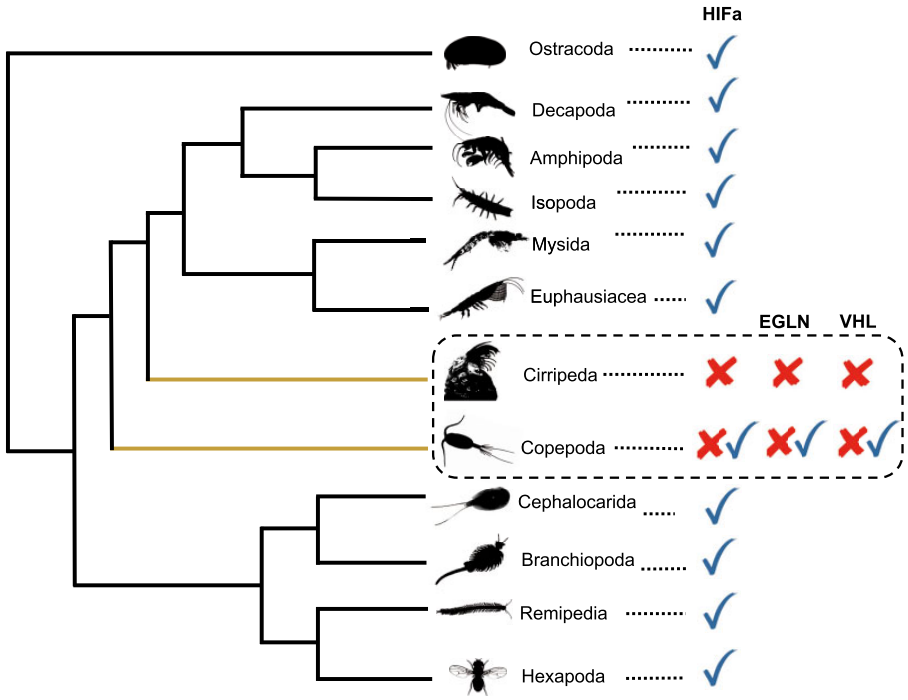

FIG. 3. Distribution of HIF pathway members HIFα, EGLN, and VHL across crustacean groups surveyed. Branch lengths in the cladogram are not to scale. General crustacean tree was created based on combination of prior phylogenetic work (Regier et al. 2010; von Reumont et al. 2011; Oakley et al. 2013; Schwentner et al. 2017, 2018). A check mark represents a confirmed presence, an "X" represents a confirmed absence. Silhouette images are from Phylopic (CC BY-SA 3.0).

the gene would not be missed (supplementary methods, Supplementary Material online). This analysis was entirely consistent with the full screen above (supplementary table S4, Supplementary Material online).

Discussion

Hypoxia is a critical physiological constraint, with a highly conserved molecular pathway that monitors and responds to periods of low oxygen. Many crustacean taxa are subjects of studies of hypoxia physiology and tolerance, but the regulatory mechanisms of hypoxia response are largely unexplored in most groups. The presence of *HIFα* is well known in many crustacean species, but those only represent a few higher-level taxonomic groups, primarily Decapoda and Branchiopoda (Gorr et al. 2004; Soñanez-Organis et al. 2009; Hardy et al. 2012). Our analyses in these two groups unsurprisingly showed clear evidence of this gene in all species, and the single target branchiopod examined (*Triops newberryi*) grouped with the model branchiopod *Daphnia pulex* which was included among "anchor" taxa. In the light of our results, presence of a HIF pathway can no longer be assumed, because it is clear that critical members of this pathway, including the main transcription factor (*HIFα*) and part of its regulatory machinery (*EGLN* and *VHL*), has been lost multiple times in Crustacea (fig. 3).

The framework for our understanding of the dynamics and importance of the HIF pathway is largely in the context of vertebrate species, where HIFα members of the pathway have been fully integrated into various elements of embryonic development. However, the physiology, and thus the oxygen requirements of a variety of invertebrates likely differ, with the utility of the HIF pathway differing as well (Harrison 2015; Harrison et al. 2018). This leaves open the potential for gene loss, or pseudogenization, as a result of unique evolutionary histories and lineage-specific requirements. In general, individual gene loss during speciation and macroevolution is pervasive, although which genes are being lost depends on their individual "dispensability," or their effect on fitness (Albalat and Cañestro 2016). Rewiring of regulatory networks influenced by transcription factors happens readily (Bhardwaj et al. 2010; Lynch et al. 2011; De Smet and Van de Peer 2012; Erkenbrack and Davidson 2015), but a loss of the main regulatory machinery likely means a loss of the pathway. Losses of such a fundamental eukaryotic pathway such as the HIF are largely undiscovered. Prior assessment of this pathway has included a large contingent of invertebrate members, but has remained biased toward terrestrial hexapods, resulting in a substantial gap of knowledge about aquatic crustaceans. Our results are an in-depth assessment of the HIF pathway by examining the presence/absence of its primary transcription factor and its regulatory elements, based on currently available genomic and transcriptomic resources available. Ultimately, we found several instances of independent *HIFα*, *EGLN*, and *VHL* loss, within Copepoda and Cirripedia, which were previously unknown.

As mentioned above, our analyses come with the general caveat of the possibility that incompleteness of genome or transcriptome assemblies creates artefactual gene "losses." Our approach to minimize false negative findings (i.e., false losses) was to examine multiple species within each group

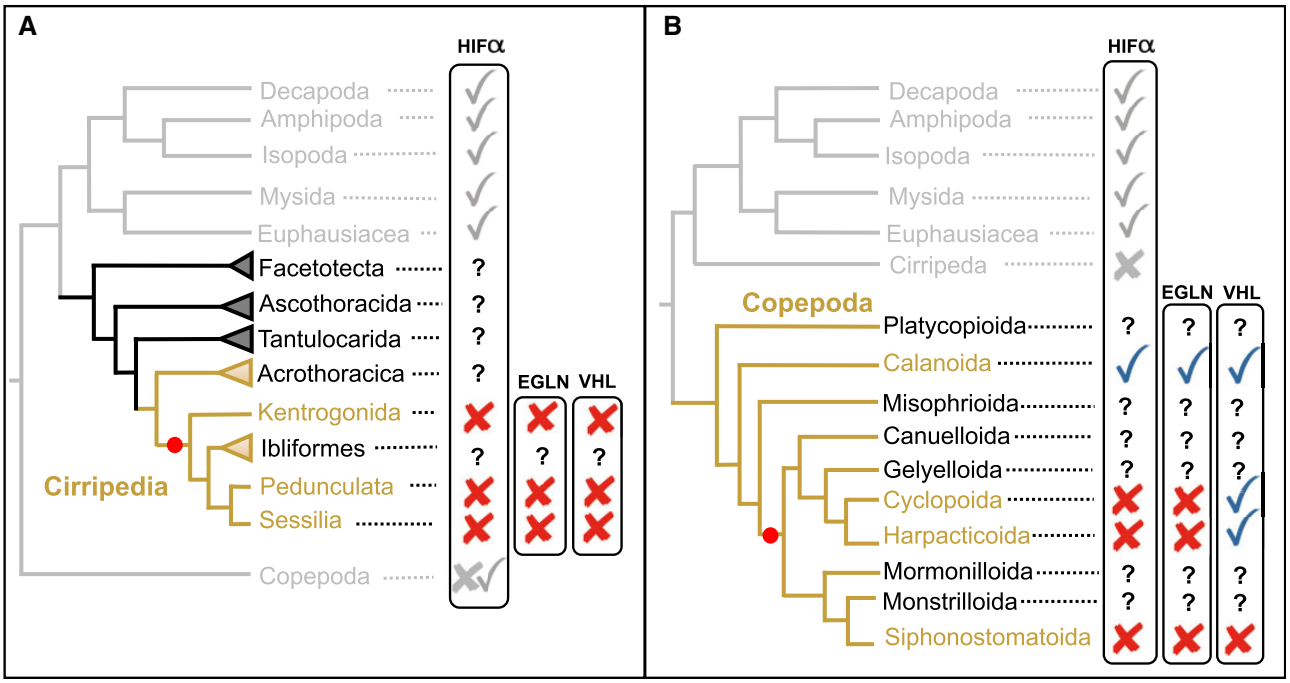

Fig. 4. Fine-scale distribution of HIF pathway members HIFα, EGLN, and VHL within the two groups which showed loss. (*A*) Cirripedia (gold lines) and allies (black lines); phylogenetic relationships within Cirripedia taken from Ewers-Saucedo et al. (2019) and Pérez-Losada et al. (2009). (*B*) Copepoda; phylogenetic relationships within Copepoda taken from Khodami et al. (2017). A check mark represents a confirmed presence, an "X" represents a confirmed absence, whereas a question mark represents unknown distribution due to a lack of available sequence data. Red circle in each tree represents our hypothesized point of loss under parsimony.

when possible, and to conservatively claim loss of a gene only when it was not detected in all species examined within the respective group. Also, although for most groups we examined nearly all species with resources available, these represent only a small fraction of total species in these taxa. Thus, our generalizations regarding loss (or maintenance) of HIF pathway genes in each group are to be interpreted cautiously and should be subject to re-examination when more taxa are sequenced. Finally, the transcriptome data from parasitic copepods species had a large amount of contamination from their fish hosts. We chose to retain these assemblies because in some cases (e.g., order Siphonostomatoida), all species examined are fish parasites, and we did not want to exclude a full taxonomic order. We computationally filtered likely contaminant sequences from these assemblies before performing our protein screening but results from these parasitic species should be interpreted with care.

Independent HIF Pathway Losses within Cirripedia and Copepoda

The phenotypic response to hypoxia in barnacles has been studied to some degree in adults and juveniles. The sessile adults are routinely out of the water during low tides in intertidal species (Davenport and Irwin 2003), and both adults and motile juveniles may encounter pelagic or benthic hypoxic zones (Gilbert et al. 2010). However, the underlying cellular response to hypoxia has been only sparsely documented (López et al. 2003; Desai and Prakash 2009; Campanati et al. 2016), with none describing the transcriptional landscape in any barnacle species. In addition, the current literature in barnacles does not contain work on the HIF pathway explicitly, or its role in mediating the response to low oxygen stress. Our results suggest a loss of *HIFα*, *EGLN*, and *VHL* across all species examined in the barnacle orders Sessilia, Pedunculata, and Kentrogonida (fig. 2; supplementary fig. S8, Supplementary Material online; supplementary tables S1–S3, Supplementary Material online). The three orders form a clade along with the order Ibliformes. Therefore, it is likely that only one loss of each gene occurred at the base of this clade. However, given their phylogenetic branching order (Pérez-Losada et al. 2008), and the lack of transcriptomic data for Ibliformes, the possibility of two or more independent losses cannot be excluded at this point (fig. 4*A*). In addition, other taxonomic orders within Cirripedia exist, but no transcriptome/genome data were available, so we cannot hypothesize the breadth of this pattern across this group.

As a group, copepods are one of the most successful metazoan taxa, occurring in nearly every aquatic habitat, both in freshwater and saltwater, planktonic and benthic divisions, from polar waters to hot springs, and in bodies of water of wide array of sizes such as swamps, ephemeral ponds, damp moss, and phytoelmata of plants (i.e., water filled recesses), sinkholes and caves, to even being obligate parasites (Boxshall 2000; Hamilton et al. 2000; Boxshall and Defaye 2007; Kiørboe 2011). The most studied forms are pelagic species inhabiting ocean plankton, where they can account for over 80% of total plankton abundance and form an essential trophic level of marine food webs. With such a wide array of environments where low oxygen stress may be encountered, retaining an important pathway would seem crucial; yet, three of the four

orders of Copepoda examined (fig. 1; supplementary fig. S7, Supplementary Material online) appear to have lost the use of the HIF pathway, at least in its canonical form. The specific phylogenetic relationship among the three orders examined is not resolved (Eyun 2017; Khodami et al. 2017), but our findings suggest a single loss at the most recent common ancestor of the three orders (fig. 4*B*). The three orders are diverse in number of species and in the range of environments they inhabit; hence, we cannot speculate on ecological commonalities that might explain their ability to thrive without this critical pathway.

Both barnacles and copepods have converged on the loss of two of the most important regulators of the HIF pathway (*HIFα* and *EGLN*). In addition, there is also potential losses of VHL in both groups, though the extent to which this is the case, seems linage specific; the fact that VHL has also not been fully lost in copepods may be partially driven by the fact it is known to have HIF-independent functions (Calzada et al. 2006; Berndt et al. 2009; Gossage et al. 2015; Nicholson et al. 2019), thus explaining why VHL seems to have been retained. Some recent work suggests that Copepoda and Thecostraca, which includes Cirripedia, are monophyletic and form the clade Hexanauplia (Oakley et al. 2013; Lozano-Fernandez et al. 2019). This does not change our observed pattern that the loss of the HIF pathway occurred independently for barnacles and copepods, although it may suggest that this pathway is more prone to loss within this particular lineage compared with other lineages sampled.

The independent losses of the HIF pathway across two abundant groups of crustaceans raise numerous questions about how these animals are able to sense and regulate oxygen tension on a continuous basis. Alternative regulatory mechanisms of hypoxia response have never been proposed, because our current understanding of this cellular process is based on the regulation by a HIF heterodimer involving a HIFα and a HIFβ subunits, and their repression machinery (EGLN and VHL). The taxa identified in this study will serve as models for new lines of cellular, genetic, and physiological studies aimed at discovering such alternative mechanisms. We hypothesize that a different transcription factor may be involved in regulating a HIF-like pathway, and that this protein is either new (i.e., lineage-specific) or has been co-opted from other existing stress response pathways. Finally, another exciting line of questions include to what degree these lineages have converged upon certain mechanisms, from broad physiological to molecular and genetic scales.

Data and Code Availability

The genome and transcriptome sequencing reads are available at either NCBI Sequence Read Archive (SRA) or NCBI Transcriptome Shotgun Assembly Sequence Database (TSA) (supplementary table S1, Supplementary Material online). Protein files from the de novo assembled transcriptome assemblies, consensus newick trees, and alignments are available on Dryad (doi:10.5061/dryad.8cz8w9gkq).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

A.M.G. and F.S.B. designed the research, analyzed the data, and wrote the manuscript.

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