Diversification of leech proboscis structure according to prey ingestion behavior

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Research

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Abstract

Background Adaptive radiation is a phenomenon in which various organs are diversified morphologically or functionally as animals adapt to environmental inputs such as diet and circumstance. Although previous studies have addressed changes caused by various external pressures, the evidence for variation in invertebrates is not well known. Leeches comprise a carnivorous or ectoparasitic group of animals that feed on a wide range of prey. They exhibit a corresponding variety of ingestion behaviors and morphological diversity of mouthparts and gut specializations. However, research on the diversity of ingestion behaviors and the internal structure of feeding organs in leeches is little known. In this study, we use histological analyses, fluorescent labeling and immunohistochemistry to reveal the detailed proboscis structure in the family Glossiphoniidae, while also suggesting the diversification of proboscises.

Results We identified the feeding behavior of rhynchobdellid leeches, which have the proboscises. *Alboglossiphonia* sp. swallows prey whole using its proboscis, whereas other leeches exhibit typical fluid-sucking behavior. Glossiphoniid leeches exhibit fluid ingestion behavior along with clear arrangement of longitudinal muscles, circular muscles surrounding the lumen, and radial muscles, while *Alboglossiphonia* sp., which displays macrophagous ingestion like salifid *Barbronia* sp., has a partial circular muscle distribution and spacious lumen that extends to longitudinal muscle layer. To address whether the different feeding behaviors are intrinsic, we investigated the behavioral patterns and muscle arrangements in the earlier developmental stage of glossiphoniid leeches. Juvenile Glossiphoniidae including the *Alboglossiphonia* sp. exhibit the fluid ingestion behavior and have the proboscis with the compartmentalized muscle layers.

Conclusions Genetic, morphological and behavioral differences between juvenile and adult stages of *Alboglossiphonia* sp. suggest their adult feeding biology has diverged from ancestral glossiphoniid leeches, while retaining developmental vestiges of the typical juvenile feeding morphology currently observed across Glossiphoniidae. This study provides the characteristics of leeches with specific ingestion behaviors, and a comparison of structural differences that serves as the first evidence of the proboscis diversification.

Background

Diverse animals have evolved a great variety of ways to obtain the energy needed for survival. As ingestion methods have diversified and evolved across species, they have the functional morphology of the ingestion tube. A few invertebrates and vertebrates use potent jaws to swallow the entire prey (macrophagous) [1-5], while others use various other organs such as proboscises and stylets to penetrate the body wall of the prey and suck out fluid (fluid ingestion) [6-11]. Leeches (Phylum Annelida), are included in the class Clitellata, superorder Euhirndinea. The group of Clitellata consists of Euhirudinea, Acanthobdellida, Branchiobdellida, and Oligochaeta, all of which are hermaphroditic and deposit cocoons through a special organ called clitellum [12]. Leeches comprise a carnivorous or ectoparasitic group of

animals that feed on a wide range of prey. Accordingly, they exhibit a corresponding wide variety of ingestion behaviors and morphological diversity of mouthparts and gut specializations. Various studies have partly classified leeches based on the differences in their feeding structures (e.g., Rhynchobdellida, and Arhynchobdellida) [13, 14], and these groupings have been supported by modern molecular phylogenies. Representative classification of Hirudinea is as follows: Hirudiniformes comprise jawed leeches, which use jaws with teeth to injure the host's body wall and ingest body fluids; Rhynchobdellida consists of jawless leeches, which use the proboscis to penetrate the host's body wall and suck soft body fluids; and Erpobdelliformes includes jawless leeches, which swallow organic material [13, 15-17]. The food ingestion behaviors of leeches have previously been identified; however, studies investigating differences in internal organ structure according to behavioral patterns are limited to selected species.

Among the fluid ingestion leeches, the glossiphoniid leeches display diverse trophic levels in the ecosystem [6, 13, 17-21]. Their food consumption behavior shows a consistent and stereotyped pattern involving a structure called proboscis used to penetrate the body wall and ingest body fluids of hosts. In the well-studied leech model *Helobdella*, a clitellate annelid, proteoblast DM" (labeled as the 4d cell in Spirallian nomenclature) contributes to development of an unsegmented prostomium which develops into a proboscis during organogenesis [22, 23]. The proboscis is comprised of longitudinal, radial and circular muscles. It extends forward and backward to penetrate the body wall of the host and ingest body fluids [13]. In the Lophotrochozoans, the striated muscle specific gene *st-mhc* and Troponin complex are known to be involved in the development of foregut muscles as well as somatic muscles [24, 25]. These previous studies suggest the possibility of the presence of foregut specific factors in the leech model, and imply that the corresponding factors can be visualized in the foregut muscle structure. Also, it suggests that these foregut-specific muscle complexes may be regulated by innervation [24, 26]. Thus, we confirmed the presence of fluorescence-labeled muscle and nerve fibers by F-actin marker phalloidin and nerve fiber marker anti-acetylated tubulin with aforementioned markers to characterize the muscle arrangement in the foregut region.

Glossiphoniid leeches are known for their typical dietary feeding behaviors that rely on their whip-shaped proboscises [12, 13]. *Theromyzon tessulatum*, parasitic in the nasal passages of aquatic birds, and *Placobdella costata*, ectoparasitic in freshwater turtles, have proboscises that consist of outer longitudinal muscle, circular muscle which circumscribe the lumen, and radial muscle fibers [13]. However, the relationship between internal structure and ingestion behavior is not well understood. Here, we investigated the internal structure of proboscis in the family Glossiphoniidae. Interestingly, despite *Alboglossiphonia* sp. representing the distinct morphological and molecular phylogenetic features of genus *Alboglossiphonia*, which belongs to the family Glossiphoniidae, it shows the macrophagous behavior by surrounding and swallowing a prey like a salifid leech *Barbronia* sp.

In this study, we demonstrate the characteristics of leeches with different types of ingestion behavior, and compare structural differences that provide the first evidence of proboscis diversification.

Results And Discussion

External morphological features and phylogenetic status of leeches

The present molecular phylogenetic analysis shows a clear separation of the four main clades of leeches, Erpobdelliformes, Hirudiniformes, Glossiphoniidae, and Piscicolidae with strong branch-support values (Fig. 1B). This result is generally congruent with the conventional classification based on their morphological characteristics (Fig. 1 and Additional file1: Fig. S1) [27]. All species of *Alboglossiphonia* with a proboscis are placed within Glossiphoniidae. Though placement within a jawless (fluid-ingestion leech group), *Alboglossiphonia* sp. has an extraordinary behavior on feeding, i.e., swallowing preys using the proboscis (Additional file2: Video S1). Macrophagy is one of the characteristics of the members of Erpobdelliformes [15]. The phylogenetic relation between the erpobdelliformes group and *Alboglossiphonia* sp. is, however, unsupportable with the current molecular result due to their separation at an earlier node. The genus *Alboglossiphonia* forms a monophyletic group within Glossiphoniidae with relatively high branch supporting values (BS=100%, and PP=1.00) but the origin of the ingestion behavior, which differs from that of the close congener *Alboglossiphonia lata* reamains unclear. In other words, it is difficult to explain the behavioral features based on external and molecular phylogenetic characteristics. From another perspective, these issues question the unique changes that may occur within the same family. Also, the findings suggest that the ingestion characteristics of *Alboglossiphonia* sp. arise from changes in the internal structure of esophagus.

Comparative ingestion behavior of leeches with different food sources

Systematically different leech species exhibit overlapping or unique trophic niche in the fauna and display diverse feeding behaviors for target food sources [6, 13, 20, 28]. In order to observe the exact feeding behavior patterns of different species, behavioral experiments involving various feeding conditions are required. Nevertheless, most reported analyses have focused on quantitative evaluation or positive reaction based on serological tests [13, 28-30]. Therefore, we first investigated the food preferences of leeches and their behavioral differences during ingestion using various preys have been reported [3, 6, 13, 19]. We tested the following prey species in the present study to determine the specific ingestion behavior of leeches: *Limnodrilus hoffmeisteri*, a vermiform freshwater oligochaete, which can be swallowed whole; *Biomphalaria* sp. and *Physella* sp., freshwater snails that cannot be swallowed by leeches; and *Chironomus* sp., an insect larva that is unswallowable due to its large size and/or presence of cuticle (Fig. 2). *H. austinensis* ingests body fluids of bloodworms and snails by inserting its proboscis into the host's body wall thereby sucking out the body fluids as reported previously [6]. A second glossiphoniid species, *A. lata*, exhibits a feeding behavior pattern very similar to *H. austinensis*. It attacks only snails, and does so by inserting its anterior end into the snail's shell and sucking the body fluids through the inserted proboscis (Fig. 2C, Additional files: Video S2-3). Unlike other glossiphoniid leeches, *Alboglossiphonia* sp. intakes only freshwater earthworms and exhibits macrophagy by wrapping the prey and eating it as a whole, similar to *Barbronia* sp. (Fig. 2C, Additional files: Video S1 and S4). Through

food preferences, we identified the unique trophic niche under non-competitive conditions of the sympatric leeches *Alboglossiphonia* sp. and *A. lata*. They can be assumed that, despite the same habitat environment, due to the trophic niche partitioning, they show coexistence without competition [31-33]. Also, unique ingestion behaviors support the contention that the structure of feeding organ in *Alboglossiphonia* sp. is different from that of the other glossiphoniid leeches and resemble that of macrophagous leeches.

Comparative structural morphology of leech feeding organs

Species in different ecosystems have evolved in habitats by undergoing changes in their external and internal structure, and manifested behavioral variations to survive against various external pressures [34-36]. These changes resulted in behavioral convergence despite differences across the species (e.g., Rodent turbinate; Arachnid web architectures) [37, 38]. Among various environmental factors, the specific behavioral convergence about food that is essential for survival manifests in various aspects (e.g., Ultrasonic predator whale and bat) [39, 40]. These behavioral convergences beyond species cannot be explained phylogenetically, suggesting that behavioral convergence is the result of evolutionary convergence depending on the choice of similar food sources via speciation. Thus, similar patterns of ingestion behavior among *Alboglossiphonia* sp. and *Barbronia* sp., suggest the presence of possible differences in the proboscis of *Alboglossiphonia* sp. despite belonging to Glossiphoniidae. In the present study, the structure of the ingestion tube was elucidated via histological analysis and molecular methods in order to identify the structural similarities and differences between these leeches.

The proboscis of glossiphoniid leeches such as *H. austinensis* and *A. lata* is a muscularized tube-like organ specialized for penetration into the prey and ingestion of the prey's blood or other body fluids and soft tissues. Developmentally, the proboscis arises primarily from mesodermal precursor cells known as M teloblasts. Structurally, it is characterized by a sharply defined complement of longitudinal, radial, and circumferential muscles [22]. Longitudinal muscles form the outer edge of the proboscis and radial muscles span the thick wall of the proboscis from just within the longitudinal muscles to its three-fold symmetric lumen in cross-section (Figs. 3A-B). The lumen assumes a narrow three-pronged stellate shape when the radial muscles are relaxed and expands to an approximately triangular form when the radial muscles contract [41, 42]. Finally, prominent circular muscles lie roughly halfway between the center and the edge of the proboscis, thereby forming a circular band defined by the three tips of the lumen [13, 22]. This compartmentalized structure may facilitate independent movement of proboscis from the anterior to the posterior direction and suggests that it is associated with fluid ingestion (Fig. 3 and Additional file 1: Fig. S2A) [43, 44].

Compared with the well-defined proboscis of the fluid-feeding species, the feeding organ of the macrophagous leech *Barbronia* sp. shows differences. *Barbronia* sp. does not have a proboscis, instead, it has an integrated esophageal structure connected to buccal cavity with a band of circular muscles that circumscribes a tri-radiate and spacious lumen (Fig. S2B) [45]. Intriguingly, the circular muscle of

Alboglossiphonia sp. in proboscis is partially distributed, and the tri-radiate tips of its lumen also extend further radially towards the outer band of longitudinal muscles, causing spacious (Figs. 3C-D). We speculate that this morphological difference in macrophagous species compared with fluid-ingesting species is an evidence of speciation and evolutionary adaptation [46, 47], in that facilitates further expansion of the lumen for ingestion of intact prey (Fig. 3 and additional file 1: Fig. S2).

To delineate the muscular arrangement of the proboscis, we carried out molecular analyses to assess differences in the expression of a common muscle-patterning gene, and molecular markers of muscle anatomy, among four leech species. Previous studies reported on muscular differentiation in lophotrochozoan animals. In *Platynereis dumerillii*, visceral muscles in the foregut consist of striated and smooth muscles. Within this region, troponin T proteins and myosin heavy chain (MHC) genes are involved in muscle cells and are known as annelid foregut striated muscle markers [24]. Instead of visualizing the specific *st-mhc* ortholog in each species, we tried to express the striated muscle in the esophagus in each species based on the similarity between the orthologs of *Hirudo nipponia* (Hirudinidae) and *Helobdella austinensis* (Glossiphoniidae), which are systematically distant. The two sequences showed a high level of similarity (81% identity at the nucleotide level and 93% identity at the amino acid level), suggesting that the esophagus muscle layer can be indirectly visualized using *st-mhc* orthologs of two species (Additional file 1: Fig. S3). In addition, nerves are distributed in the foregut region, and it can be assumed that muscle movement, which is controlled by the innervation pattern and the detailed arrangement of muscles in the foregut, can be confirmed by co-visualizing the nerve and muscle fibers [26]. Various muscle markers, *st-mhc* transcript, and the innervation marker acetylated tubulin show detailed intra-structural and nerve distribution according to muscle fibers in each species. Within 4 species, we confirmed that the nerve fibers are distributed along the arrangement of muscles in the esophagus (Fig. 3). Innervation of the muscle suggests that the muscles in the esophagus are regulated by neuronal stimulation, and the spatial expressions of *st-mhc* orthologs reveal the possibility of existence of those *st-mhc* orthologs and conservation of foregut muscle components in different leech species (Fig. 3 and Additional file1: Fig. S4). Fluid-ingesting leeches have a distinct longitudinal, circular, and radial muscle arrangement, and the lumen extends to the circular muscle layer (Figs. 3A-B, and Additional file1: Fig. S4). The configuration of muscles in the leech proboscis exhibits structural similarity to that of vertebrate iris muscles consisting of circular sphincter and radial muscle [13, 48]. In the case of *Alboglossiphonia* sp., the circular muscle layer is partial, with more spacious lumen that extended to the longitudinal muscle layer than in *H. austinensis* and *A. lata*, which present clear muscle layers (Figs. 3A-C). Due to these structural differences, the tip of the proboscis of fluid-sucking leeches shows condensed apical structure, while *Alboglossiphonia* sp. exhibits an uncondensable cylindrical tip with expanded proboscis pore (Fig. 4A). These features are likely related to the limited ability to condense the proboscis tip given the partial distribution of circular muscle, suggesting possible macrophagy via loose internal space construction (Figs. 3C-D, 4B). Also, numerous cilia bundles are clearly visible at the tip of the proboscis of *Alboglossiphoia* sp. (Fig. 4A). It is assumed that the cilia bundles are related to the recognition of prey as sensory cilia (sensilla) and the use of the proboscis for macrophagy [49, 50].

These results provide the first evidence suggesting that muscular organization, including differences in muscle-type composition within the proboscis, may facilitate macrophagous feeding behavior in fresh water leeches. *Barbronia* sp. is a macrophagous leech with radial musculature of the esophagus extending outwardly beyond longitudinal musculature, as seen in macrophagous oligochaetes [51], without forming a proboscis organ (Fig. 3D, Additional file 1: Figs. S2B and S4). This feeding organ is not an isolated proboscis, and cannot elongate the structure or penetrate the prey, resulting in altered feeding behavior. Esophageal intramuscular complexes generate a strong suction force, resulting in a unique pattern of ingestion behavior such as swallowing of organic matter or popping of soft parts (Additional file 5: Video S4). The peristaltic movement of the esophagus in vertebrates suggests that the action of extending food in the aboral direction via complex interaction between circular and longitudinal muscles results in radial contraction [52]. In *Barbronia* sp., the muscular arrangement of the feeding organ is similar to that of vertebrates, thereby suggesting macrophagy via peristalsis of longitudinal and circular muscles in the esophagus (Fig 3D and Additional file 1: Fig. S2C). In addition, it is speculated that the dense longitudinal muscles located outside the esophagus may be related to the locomotion of *Barbronia* sp. (Figs. 3D and 4B). In summary, *Alboglossiphonia* sp. exhibits and alternative distribution of circular musculature, along with an expanded luminal space, designed in combination for feeding behavior that is intermediate between fluid-sucking and macrophagous leeches. Furthermore, this structural organization is hypothesized to facilitate a pattern of ingestion behavior similar to that of *Barbronia* sp.

Conserved fluid ingestion behavior and compartmentalized foregut musculature in glossiphoniid juveniles

Glossiphoniid leeches bearing a cocoon with a thin membrane and without the hardened shell similar to erpobdelliformes have the embryos attached to the abdomen until they grow to a sufficient size. These growing embryos can parasitize the host and ingest body fluids. After receiving parental care, the individuals exhibit a parasitic life on the host and use their developed proboscis to ingest body fluids [3, 17, 53-55]. However, the fluid-ingestion behavior of *Alboglossiphonia* sp. larvae is unknown apart from leeches belonging to glossiphoniid known as fluid-ingesting leeches. To investigate their ingestion behavior, we first analyzed the feeding behavioral patterns of different leech species in the juvenile stages (Fig. 5A). *Alboglossiphonia* sp. adults do exhibit macrophagous feeding behavior. However, the juveniles of this species exhibit fluid-sucking behavior similar to *A. lata* and *H. austinensis* (Fig. 5A and Additional file 6: Video S5). The differences in behavioral patterns are thought to be due to variation in the proboscis structure within the foregut. Thus, we conducted immunofluorescence staining to analyze the proboscis structures in juvenile stages of three species that exhibit food-ingestion activity. Our analyses of fluorescent muscle markers revealed the presence of a well-developed independent proboscis in the foregut, even at the juvenile stage of food intake (Fig. 5B). Cross-sectional analyses of the juvenile proboscis showed a well-partitioned musculature in all three species, although *Alboglossiphonia* sp. showed differences compared with its adult form. The arrangement of circular muscle in the adult proboscis was observed as comparatively less structured than the other leech species, while the circular

muscle layer in the juvenile stage exhibited a well-defined partition, as in *H. austinensis* and *A. lata* (Figs. 3A-C, and 5B). These results indicate that *Alboglossiphonia* sp. manifests fluid-sucking behavior using well-developed muscles in the juvenile stage. Subsequently, *Alboglossiphonia* sp. undergoes gradual changes in the structural arrangement of muscles in the proboscis along with the ingestion pattern changing to macrophagy. These findings explain the presence of an intermediate proboscis structure in *Alboglossiphonia* sp. compared with fluid-sucking and macrophagous structure seen in other leeches. Within glossiphoniid leeches, specific food preferences vary widely across species. For example, the Amazon leech *Haementeria ghilianii* is a large rhynchobdellid species adapted to feeding on mammalian blood [13, 21], and *Helobdella stagnalis* consumes diverse foods, whereas *Glossiphonia complanata* known as a specialist leech prefers gastropods [13, 20]. Similarly, *A. lata* and *Alboglossiphonia* sp., which belong to the same genus, have different food niches in the same habitat (Figs. 2C and 5A). These diverse food preferences suggest that ancestral glossiphoniid leeches may have ingested different prey. Subsequent divergence may have arisen from a combination of differences in morphological development that were associated with preferences for specific prey items. Furthermore, ingestion of selective prey may alter the structure of the feeding organ, and accordingly, results in differences in feeding behavior [56]. As representative examples, *H. austinensis* and *A. lata* show similar feeding behavior in the larval and adult stages, and the proboscis exhibits similar muscle structure. However, in the case of *Alboglossiphonia* sp., the larval stages ingest fluids with their proboscis, while the adults show macrophagous behavior, attributable to the differences in the arrangement of muscle layers. Therefore, within Glossiphoniidae, it appears that juvenile structural morphology facilitates ingestion of body fluids by two of the leech species we investigated, while the particular proboscis structure and feeding behavior in juvenile of *Alboglossiphonia* sp. may persist as vestiges of the ancestral, or most common, pattern observed among glossiphoniid leeches [57, 58] (Fig. 5C).

Conclusions

The results of this investigation suggest that there is an observable correlation between internal morphological structure and ingestion behavior in the proboscis of leech annelids. The organization of tissue and musculature in the proboscis of macrophagous leeches enables ingestion of whole organisms, unlike fluid-ingestion mechanisms. *Alboglossiphonia* sp. exhibits an esophageal structure intermediate between macrophagous and fluid-feeding leeches and manifests similar fluid intake behavior during the juvenile stage as other proboscis leeches. This behavioral pattern suggests that the feeding behavior of leech is not intrinsic and may change to other patterns of ingestion depending on the development of feeding organ structure. Also, similar food preferences reveal structural and behavioral convergence among other species, despite the species diversity. Genetic, morphological and behavioral differences between juvenile and adult stages of *Alboglossiphonia* sp. suggest their adult feeding biology has diverged from ancestral glossiphoniid leeches, while retaining developmental vestiges of the typical juvenile feeding morphology currently observed across Glossiphoniidae.

Declarations

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AVAILABILITY OF DATA AND MATERIALS

Not applicable

AUTHOR CONTRIBUTION

H.-J.K. S.C.P., and S.-J.C. designed research; H.-J.K., J.-H.K., J.-Y.K., D.J., D.-H.L., and S.-I.E. performed research; H.-J.K., J.-H.K., and S.-J.C. analyzed data; H.-J.K., S.Y., S.C.P., J.K. and S.-J.C wrote the paper

ETHNICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

COMPETING INTERESTS

The authors declare that they have no competing interests.

Methods And Materials

Animals

Adult *Alboglossiphonia lata*, *Alboglossiphonia* sp., and *Barbronia* sp. specimens were collected by examining submerged plants, leaves, and plastic bags in selected localities of Bangjook reservoir in Cheongju, Chungcheongbuk-do (South Korea). Adult *Glossiphonia* sp. was collected in selected localities of Dal stream in Goesan-gun, Chungcheongbuk-do (South Korea). Adult *Hemiclepsis* sp. was collected in selected localities of Miho stream in Cheongju, Chungcheongbuk-do (South Korea). *Helobdella austinensis* was bred in the laboratory. All adult specimens except *Glossiphonia* sp. and *Hemiclepsis* sp., which cannot be incubated in artificial conditions, were incubated in a bowl containing artificial pond water. *Glossiphonia* sp. and *Hemiclepsis* sp. were fixed with 100% EtOH until used for the histological analysis. The specimens were cared for once daily by changing solution and the bowl was scrubbed manually to get rid of any residual waste. They were stored in a BOD incubator at 22 ℃.

CO1 gene cloning and sequencing

Total RNA was isolated from *Alboglossiphonia* sp. embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was purified from Total RNA with Oligo (dT) primer (Promega, Madison, WI, USA), and reverse transcribed into cDNA with a SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA USA). Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, NW, Germany). We amplified the *A. lata* CO1 gene sequences [54] and other leech-specific CO1 and 18S rRNA genes using universal primers [27]. We used the TaKaRa Ex Taq® kit (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions (predenaturation - 94℃, 5min; denaturation - 94℃, 30sec; annealing – variable, 30sec; extension - 72℃, 1min for COI or 1min 30sec for 18s rRNA sequence fragments; post extension - 72℃, 5min).

Phylogenetic analysis

Three partial nucleotide sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) (*Alboglossiphonia* sp., *A. lata*, and *Barbronia* sp., about 700 bp), and four partial sequences of 18S ribosomal RNA (18S rRNA, about 1.8 kb) from the same three species plus *Helobdella austinensis* were obtained in this study by PCR amplification. Additional sequences of both genes were obtained from the GenBank, and two alignments of 62 COI and 62 18S genes from the same group of species (see Additional file1: Table S1 for GenBank accession number) were prepared using ClustalW implemented in MEGA7 software (ver. 7.0.26) [59], and then concatenated. Phylogenetic tree hypotheses were prepared from the concatenated matrix using Maximum Likelihood (ML) and Bayesian Inference (BI). The best-fit model was searched based on the corrected Akaike Information Criterion (AICc) using IQ-TREE [60] web-server (http://www.iqtree.org). The ML and BI analyses were conducted using RAxML-NG software (v

0.9.0) [61] and MrBayes software (ver. 3.2.7a) [62] under the General Time Reversible model (GTR) with a proportion of invariable sites (I) and a gamma-shaped distribution rates (G4). The ML tree reconstruction was initially attempted by generating 3,000 bootstrap replicates with “autoMRE” command. The bootstrapping support values for branches were estimated under the transfer bootstrap expectation (TBE) [63]. Markov Chain Monte Carlo (MCMC) for the BI tree was run with 5,000,000 generations and the BI tree was constructed by discarding the first 25% generations. The trees were visualized with FigTree software (ver. 1.4.4).

Prey selection test and tracking analysis

In order to compare feeding behaviors of leeches, we conducted a survey in the laboratory environment using various food types: *Limnodrilus hoffmeisteri* (Clitellata, Annelida), swallowable and worm shape; *Biomphalaria* sp. and *Physella* sp. (Gastropoda, Mollusca), unswallowable and carrying a shell; and *Chironomus* sp. (Insecta, Arthropoda), unswallowable and exhibiting a worm shape. First, several individuals of each leech species were placed in the 55 mm petri-dish, and ingestion patterns were observed under mixed prey species (single leech species vs. multiple prey species) and each prey species (single leech species vs. single prey species) to confirm exact preferences and ingestion behavior. After observation, one or two prey organisms were provided to each leech. Each experimental dish was video-recorded using a DCR-SR200 camcorder (SONY, Minato, TYO, Japan) over 8 hr, or until the leeches completed feeding under room temperature. Ingestion behavior tests were performed on three biological replicates in the same condition as described above. Among the recorded videos, location analysis on ingestion behavior was conducted using one representative video for each species. To analyze the behavior of both leeches and the prey, the location of all individuals present in the petri dish was tracked every 3 min using EthoVision software (Noldus Information Technology, Wageningen, GE, Netherlands). When the predators were supplied with two species of prey, only the behavior of prey that was ingested was tracked. However, when *Barbronia* sp. was provided with *L. hoffmeisteri* or *Chironomus* sp., the individual location was tracked every 30 s due to their relatively rapid ingestion. Distances between leeches or preys and a reference point established on the 12 o’clock edge of the petri-dish were recorded.

Histological analyses

To visualize differentiation of proboscis muscle structure, adult leeches were treated with relaxation buffer (4.8 mM NaCl2, 1.2 mM KCl, 10 mM MgCl2, 8% EtOH) and fixed in 4% PFA (Electron Microscopy Sciences, Hatfield, PA, USA) in 1X phosphate buffered saline (PBS) overnight at 4°C. For H&E staining, leeches were dehydrated in EtOH series and cleared in Xylene (Central Drug House, New Delhi, DL, India) for 2 hr. The leeches were embedded in paraffin (Leica, Wetzlar, HE, Germany) and stored at –20°C. Paraffinized samples (10 μm thickness) were cut with a RM2235 microtome (Leica, Wetzlar, HE, Germany) and stained with Mayer’s Hematoxylin (Cancer Diagnostics, Durham, NC, USA) and Eosin

(Cancer Diagnostics, Durham, NC, USA). Samples were mounted on glass slides with an Organo Mount (ImmunoBioScience, Mukilteo, WA, USA) and dried overnight at room temperature. Sections were imaged with a LEICA DM6 B compound light microscope (Leica, Wetzlar, HE, Germany) and a LEICA DFC450 C camera (Leica, Wetzlar, HE, Germany). The obtained images were edited using Las X software (Leica, Wetzlar, HE, Germany) and Adobe Photoshop CS5 (Adobe, San Jose, CA, USA). The edited images were prepared as figure plates using Adobe Illustrator CS6 (Adobe, San Jose, CA, USA). To obtain cryo-sections, leeches were embedded in O.C.T. compound (VWR, Radnor, PA, USA) and rapidly frozen in liquified nitrogen. Cryo-sectioned samples (15 μm in thickness) were cut with a CM1520 cryostat (Leica, Wetzlar, HE, Germany) and stored at −70°C until use.

Scanning electron microscopy of proboscis feeding organs

For scanning electron microscopy, leech specimens were treated with 16% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) or relaxation solution (4.8 mM NaCl2, 1.2 mM KCl, 10 mM MgCl2, 8% EtOH) while feeding or relaxing. After treatment, the head region containing the proboscis was cut and fixed in 4% PFA at room temperature for overnight. The tissues were washed three times with PBT (1X PBS + 0.1% Tween-20) for 20 min at room temperature, and then fixed in 1% osmium tetroxide (Ted Pella Inc., Redding, CA, USA) in 1M PBS for 1 hr. Osmium tetroxide was removed by washing three times with PBT. Thereafter, the tissues were gradually dehydrated with ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95%, 100% in 1X PBS) for 20 min per step. Dehydrated tissues were treated with stepwise concentrated isopentyl acetate (Alfa Aesar, Ward Hill, MA, USA) (isopentyl acetate: EtOH = 1:3, 1:1, and 3:1) for 15 min per step, and then transferred to 100% isopentyl acetate. After the solution was removed, the samples were dried for 3 days in the hood. Dried samples were coated with gold particle and examined with an UltraPlus field emission scanning electron microscope (Carl Zeiss, Oberkochen, BW, Germany).

Fluorescent labeling and immunohistochemistry

Whole-mount immunostaining was performed according to previously published protocols [54], with the following details: The cross-sections were dried and washed in PBT (0.1% Tween-20 with 1X PBS) five times. The nerve and muscle fibers were visualized after double immunostaining as follows. After washing with PBT, the sections were incubated in diluted blocking solution (1:9 = 10X Roche Western Blocking Reagent : PBT) for 2 h. Samples were incubated with primary antibodies (anti-acetylated-α-Tubulin produced in mouse, Sigma Aldrich, T-7451; or anti-cardiac TroponinT produced in rabbit, Abcam, ab115134) in diluted blocking Solution (1:500) at 4 ℃ for 48 h. After five consecutive washes with PBT, the sections were incubated with a secondary antibody (goat anti-mouse IgG H&L Alexa Fluor 488, Abcam, ab150113; goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 568, Invitrogen, A11011) in diluted blocking Solution (1:1000) at 4 ℃ for 24 h. After checking the labeled

signal, the samples were washed five times with PBT, and then stained with Texas Red™-X Phalloidin (ThermoFisher, T7471) for 1 h to visualize F-actin. After checking the labeled signal, the samples were washed five times with PBT and labeled with DAPI in PBT (1:100) at room temperature in the dark overnight. After washing with PBT five times, the samples were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Fluorescence-stained embryos and slide samples were imaged using a LEICA DM6 B with a LEICA DFC450 C camera (Leica, Wetzlar, HE, Germany). The obtained images were edited using Las X software (Leica, Wetzlar, HE, Germany). To confirm the detailed muscle structure and innervation in the proboscis, slides co-labeled with F-actin and acetylated tubulin were imaged with a LSM 710 confocal microscope (Carl Zeiss, Oberkochen, BW, Germany). The obtained images were edited using ZEN software (L Carl Zeiss, Oberkochen, BW, Germany). The edited images were prepared as figure plates using Adobe Illustrator CS6 (Adobe, San Jose, CA, USA).

ST-MHC gene identification, probe synthesis and in situ hybridization

Total RNA was isolated from *H. austinensis* mixed-stage embryos and *Hirudo nipponia* head tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). We selected mRNA from total RNA using Oligo (dT) primer (Promega, Madison, WI, USA) and synthesized cDNA (SuperScript II First Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA, USA). To isolate the *H. austinesis* striated myosin heavy chain (ST-MHC) gene, a previously published sequence [64] was used and screened using a BLAST implemented in the whole draft-genome reference (http://genome.jgi.doe.gov/Helro1/Helro1.home.html). Two candidate genes (protein id 64397 and 129847) were screened, and the foregut specific *st-mhc* gene was isolated by confirming the foregut specific expression pattern (protein id: 129847) (Additional file 1: Fig S3B). In the *H. nipponia* transcriptome data, only a single striated myosin heavy chain transcript was found, which showed a high degree of similarity to the *H. austinensis* foregut specific *st-mhc* gene (nucleotide similarity: 81%, translated sequence similarity: 93%) (For nucleotide similarity, see Additional file 1: Fig S3C). The *st-mhc* specific primers were designed to amplify the consensus region of the two sequences producing similar length (product sizes about 850 nucleotides - protein id 129847, *Hau st-mhc* forward: 5'-GCCACCAAAGGTGAAGAG-3'; *Hau st-mhc* reverse: 5'-GTCCTCAACGAGCTGCAT-3'). *H. nippoinia st-mhc* transcript (*Hni st-mhc* forward: 5'- GCCACCAAGGGCGAAGAA-3'; *Hni st-mhc* reverse: 5'-TCCTCGACCAATTGCATTTCC-3'). These amplified fragments were cloned into pGEM T vector (Promega, Madison, WI, USA). RNAprobes labeled with digoxigenin were made using the MEGAscript kit (Ambion, Austin, TX, USA) and DIG RNA Labeling Mix (Roche, Basel, Switzerland), according to the manufa cturer's instructions. The synthesized RNA probes were applied to each sample at a final concentration of 2 ng/μl, and the probe labeled samples were incubated with an Anti-Digoxigenin-POD Fab fragments produced in sheep (Roche, Basel, Switzerland) in diluted blocking solution (1:1000). The detail procedure of *in situ* hybridization was followed using previously published methods [54, 65, 66]. After cryosection, stored samples were dried to remove residual moisture. Dried samples were treated with 0.2N HCl buffer to inhibit endogenous enzymes and rinsed three times with PBT. After this process, the following experiments were carried out using the same protocol as described above.

References

1. Binder WJ, Van Valkenburgh B: **Development of bite strength and feeding behaviour in juvenile spotted hyenas (Crocuta crocuta).** *Journal of Zoology* 2000, **252:**273-283.
2. RAUPP MJ: **Effects of leaf toughness on mandibular wear of the leaf beetle, Plagiodera versicolora.** *Ecological Entomology* 1985, **10:**73-79.
3. Kutschera U: **The feeding strategies of the leech Erpobdella octoculata (L.): a laboratory study.** *International Review of Hydrobiology: A Journal Covering all Aspects of Limnology and Marine Biology* 2003, **88:**94-101.
4. Staniczek A: **The mandible of silverfish (Insecta: Zygentoma) and mayflies (Ephemeroptera): its morphology and phylogenetic significance.** *Zoologischer Anzeiger* 2000, **239:**147-178.
5. Jensen KR: **Morphological adaptations and plasticity of radular teeth of the Sacoglossa (= Ascoglossa)(Mollusca: Opisthobranchia) in relation to their food plants.** *Biological Journal of the Linnean Society* 1993, **48:**135-155.
6. Kutschera U, Langguth H, Kuo DH, Weisblat D, Shankland M: **Description of a new leech species from North America, Helobdella austinensis n. sp.(Hirudinea: Glossiphoniidae), with observations on its feeding behaviour.** *Zoosystematics and Evolution* 2013, **89:**239-246.
7. Kutschera U: **The Golden Gate Leech Helobdella californica (Hirudinea: Glossiphoniidae): Occurrence and DNA-Based Taxonomy of a Species Restricted to San Francisco.** *International review of Hydrobiology* 2011, **96:**286-295.
8. Gardell S, Duong LT, Diehl RE, York JD, Hare TR, Register RB, Jacobs JW, Dixon R, Friedman PA: **Isolation, characterization, and cDNA cloning of a vampire bat salivary plasminogen activator.** *Journal of Biological Chemistry* 1989, **264:**17947-17952.
9. Saleuddin A, Wilbur KM: *The Mollusca: Physiology.* Academic Press; 2012.
10. James D, Prator CA, Martin GG, Schulz JR: **Morphology of sensory papillae on the feeding proboscis of cone snails (Mollusca, Gastropoda).** *Invertebrate biology* 2014, **133:**221-231.
11. Hawkey C: **Plasminogen activator in saliva of the vampire bat Desmodus rotundus.** *Nature* 1966, **211:**434.
12. Likens G, Benbow M, Burton T, Van Donk E, Downing J, Gulati R: **Encyclopedia of inland waters.** 2009.
13. Sawyer RT: *Leech biology and behaviour.* Clarendon Press Oxford; 1986.
14. Muller KJ, Nicholls JG, Stent GS: *Neurobiology of the Leech.* Cold Spring Harbor Laboratory; 1981.
15. Borda E, Siddall ME: **Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): phylogenetic relationships and evolution.** *Molecular phylogenetics and evolution* 2004, **30:**213-225.

16. Salas-Montiel R, Phillips AJ, De Leon GP-P, Oceguera-Figueroa A: **Description of a new leech species of Helobdella (Clitellata: Glossiphoniidae) from Mexico with a review of Mexican congeners and a taxonomic key.** *Zootaxa* 2014, **3900:**77-94.
17. El-Shimy N, Davies RW: **The life-cycle, ecology and host specificity of the freshwater leech Alboglossiphonia polypompholyx (Glossiphoniidae) in Egypt.** *Hydrobiologia* 1991, **222:**173-178.
18. Wrona F, Davies RW, Linton L, Wilkialis J: **Competition and coexistance between Glossiphonia complanata and Helobdella stagnalis (Glossiphoniidae: Hirudinoidea).** *Oecologia* 1981, **48:**133-137.
19. el-Shimy NA: **Description of a new species of Alboglossiphonia Lukin, 1976 (Hirudinea: Glossiphoniidae) from Egypt.** *Zoology in the Middle East* 1990, **4:**93-102.
20. Young J, Ironmonger J: **A laboratory study of the food of three species of leeches occurring in British lakes.** *Hydrobiologia* 1980, **68:**209-215.
21. Sawyer R, Casellas M, Munro R, Jones CP: **Secretion of hementin and other antihaemostatic factors in the salivary gland complex of the giant Amazon leech Haementeria ghilianii.** *Comparative Haematology International* 1991, **1:**35-41.
22. Gline SE, Nakamoto A, Cho S-J, Chi C, Weisblat DA: **Lineage analysis of micromere 4d, a super-phylotypic cell for Lophotrochozoa, in the leech Helobdella and the sludgeworm Tubifex.** *Developmental biology* 2011, **353:**120-133.
23. Weisblat DA, Kuo D-H: **Developmental biology of the leech Helobdella.** *The International journal of developmental biology* 2014, **58:**429.
24. Brunet T, Fischer AH, Steinmetz PR, Lauri A, Bertucci P, Arendt D: **The evolutionary origin of bilaterian smooth and striated myocytes.** *Elife* 2016, **5:**e19607.
25. Han Y-H, Ryu K-B, Medina Jiménez BI, Kim J, Lee H-Y, Cho S-J: **Muscular Development in Urechis unicinctus (Echiura, Annelida).** *International Journal of Molecular Sciences* 2020, **21:**2306.
26. Schmidbaur H, Schwaha T, Franzkoch R, Purschke G, Steiner G: **Within-family plasticity of nervous system architecture in Syllidae (Annelida, Errantia).** *Frontiers in zoology* 2020, **17:**1-44.
27. Apakupakul K, Siddall ME, Burreson EM: **Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences.** *Molecular Phylogenetics and Evolution* 1999, **12:**350-359.
28. Wrona FJ, Davies RW, Linton L: **Analysis of the food niche of Glossiphonia complanata (Hirudinoidea: Glossiphoniidae).** *Canadian Journal of Zoology* 1979, **57:**2136-2142.
29. Anholt B: **Prey selection by the predatory leech Nephelopsis obscura in relation to three alternative models of foraging.** *Canadian Journal of Zoology* 1986, **64:**649-655.
30. Blinn DW, Davies RW, Dehdashti B: **Specialized pelagic feeding by Erpobdella montezuma (Hirudinea).** *Ecography* 1987, **10:**235-240.
31. Schoener TW: **Resource partitioning in ecological communities.** *Science* 1974, **185:**27-39.
32. BERGALLO HG, ROCHA CFD: **Spatial and trophic niche differentiation in two sympatric lizards (Tropidurus torquatus and Cnemidophorus ocellifer) with different foraging tactics.** *australian*

Journal of ecology 1994, **19:**72-75.

33. Lanszki Z, Horváth GF, Bende Z, Lanszki J: **Differences in the diet and trophic niche of three sympatric carnivores in a marshland.** *Mammal Research* 2020, **65:**93-104.
34. Pearse AS: **The effects of environment on animals.** *The American Naturalist* 1922, **56:**144-158.
35. Gajardo GM, Beardmore JA: **The brine shrimp Artemia: adapted to critical life conditions.** *Frontiers in physiology* 2012, **3:**185.
36. Bosch TC, Adamska M, Augustin R, Domazet-Loso T, Foret S, Fraune S, Funayama N, Grasis J, Hamada M, Hatta M: **How do environmental factors influence life cycles and development? An experimental framework for early-diverging metazoans.** *Bioessays* 2014, **36:**1185-1194.
37. Martinez Q, Lebrun R, Achmadi AS, Esselstyn JA, Evans AR, Heaney LR, Miguez RP, Rowe KC, Fabre P-H: **Convergent evolution of an extreme dietary specialisation, the olfactory system of worm-eating rodents.** *Scientific reports* 2018, **8:**17806.
38. Blackledge TA, Gillespie RG: **Convergent evolution of behavior in an adaptive radiation of Hawaiian web-building spiders.** *Proceedings of the National Academy of Sciences* 2004, **101:**16228-16233.
39. Jackson RR, Deng C, Cross FR: **Convergence between a mosquito-eating predator's natural diet and its prey-choice behaviour.** *Royal Society Open Science* 2016, **3:**160584.
40. Wilson M, Wahlberg M, Surlykke A, Madsen PT: **Ultrasonic predator–prey interactions in water–convergent evolution with insects and bats in air?** *Frontiers in physiology* 2013, **4:**137.
41. Kang D, Huang F, Li D, Shankland M, Gaffield W, Weisblat DA: **A hedgehog homolog regulates gut formation in leech (Helobdella).** *Development* 2003, **130:**1645-1657.
42. Nielsen C: **The triradiate sucking pharynx in animal phylogeny.** *Invertebrate Biology* 2013, **132:**1-13.
43. Haswell W: **Memoirs: The Proboscis of the Syllidea: Part I. Structure.** *Journal of Cell Science* 1921, **2:**323-337.
44. Filippova A, Purschke G, Tzetlin AB, Müller MC: **Musculature in polychaetes: comparison of Myrianida prolifera (Syllidae) and Sphaerodoropsis sp.(Sphaerodoridae).** *Invertebrate Biology* 2010, **129:**184-198.
45. Govedich FR, Bain BA, Burd M, Davies RW: **Reproductive biology of the invasive Asian freshwater leech Barbronia weberi (Blanchard, 1897).** *Hydrobiologia* 2003, **510:**125-129.
46. Grant PR: *Ecology and evolution of Darwin's finches.* Princeton University Press; 1999.
47. Lamichhaney S, Berglund J, Almén MS, Maqbool K, Grabherr M, Martinez-Barrio A, Promerová M, Rubin C-J, Wang C, Zamani N: **Evolution of Darwin's finches and their beaks revealed by genome sequencing.** *Nature* 2015, **518:**371-375.
48. Langley JN, Anderson H: **On the mechanism of the movements of the iris.** *The Journal of physiology* 1892, **13:**554-597.
49. Derosa YS, Friesen WO: **Morphology of leech sensilla: observations with the scanning electron microscope.** *The Biological Bulletin* 1981, **160:**383-393.

50. Blinn DW, Wagner VT, Grim JN: **Surface sensilla on the predaceous fresh-water leech Erpobdella montezuma: possible importance in feeding.** *Transactions of the American Microscopical Society* 1986**:**21-30.
51. Brinkhurst R, McKey-Fender D: **The anatomy of the pharynx of two predatory aquatic oligochaetes.** *Canadian journal of zoology* 1991, **69:**669-675.
52. Paterson WG: **Esophageal peristalsis.** *GI Motility online* 2006.
53. Kutschera U: **Description of a new leech species, Erpobdella wuttkei nov. sp.(Hirudinea: Erpobdellidae).** *Lauterbornia* 2004, **52:**147-151.
54. Jiménez BIM, Kwak H-J, Park J-S, Kim J-W, Cho S-J: **Developmental biology and potential use of Alboglossiphonia lata (Annelida: Hirudinea) as an "Evo-Devo" model organism.** *Frontiers in zoology* 2017, **14:**60.
55. Gouda HA: **A new Alboglossiphonia species (Hirudinea: Glossiphoniidae) from Egypt: Description and life history data.** *Zootaxa* 2010, **2361:**46-56.
56. Skinner MK: **Environmental epigenetics and a unified theory of the molecular aspects of evolution: a neo-Lamarckian concept that facilitates neo-Darwinian evolution.** *Genome biology and evolution* 2015, **7:**1296-1302.
57. Werth AJ: **Vestiges of the natural history of development: historical holdovers reveal the dynamic interaction between ontogeny and phylogeny.** *Evolution: Education and Outreach* 2014, **7:**12.
58. Cupello C, Brito PM, Herbin M, Meunier FJ, Janvier P, Dutel H, Clément G: **Allometric growth in the extant coelacanth lung during ontogenetic development.** *Nature communications* 2015, **6:**1-5.
59. Kumar S, Stecher G, Tamura K: **MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets.** *Molecular biology and evolution* 2016, **33:**1870-1874.
60. Trifinopoulos J, Nguyen L-T, von Haeseler A, Minh BQ: **W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis.** *Nucleic acids research* 2016, **44:**W232-W235.
61. Kozlov A, Darriba D, Flouri T, Morel B, Stamatakis A: **RAxML-NG: A fast, scalable, and user-friendly tool for maximum likelihood phylogenetic inference.** *bioRxiv* 2019**:**447110.
62. Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP: **MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space.** *Systematic biology* 2012, **61:**539-542.
63. Lemoine F, Entfellner J-BD, Wilkinson E, Correia D, Felipe MD, De Oliveira T, Gascuel O: **Renewing Felsenstein's phylogenetic bootstrap in the era of big data.** *Nature* 2018, **556:**452-456.
64. Pfeifer K, Schaub C, Domsch K, Dorresteijn A, Wolfstetter G: **Maternal inheritance of twist and analysis of MAPK activation in embryos of the polychaete annelid Platynereis dumerilii.** *PloS one* 2014, **9:**e96702.
65. Cho S-J, Vallès Y, Giani Jr VC, Seaver EC, Weisblat DA: **Evolutionary dynamics of the wnt gene family: a lophotrochozoan perspective.** *Molecular biology and evolution* 2010, **27:**1645-1658.

66. Kim J-S, Jiménez BIM, Kwak H-J, Park SC, Xiao P, Weisblat DA, Cho S-J: **Spatiotemporal expression of a twist homolog in the leech Helobdella austinensis.** *Development genes and evolution* 2017, **227:**245-252.

Figures

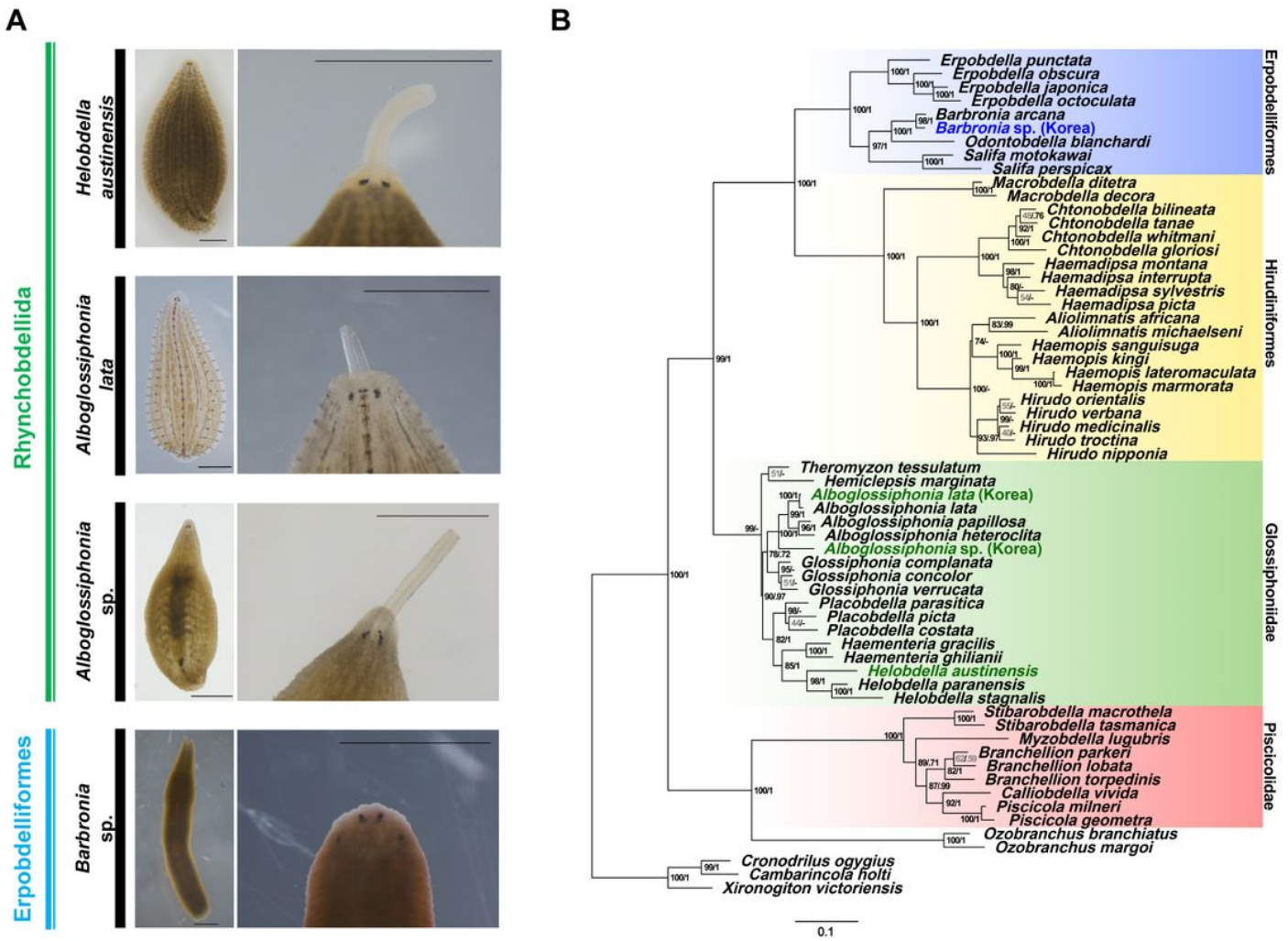

Figure 1

External morphological features and phylogenetic status of leeches. (A) Three glossiphoniid leeches (Helobdella austinensis, Alboglossiphonia lata, and Alboglossiphonia sp.) have a retractable proboscis that is characteristic of Rhynchobdellida in contrast to erpobdelliformes specimen Barbronia sp. (Scale bars 2mm). (B) Maximum likelihood (ML) phylogeny based upon the concatenated sequences of CO1 and 18S rRNA including three branchiobdellid taxa (Xironogiton victoriensis, Cronodrilus ogygius and Cambarincola holti) as outgroups. The ML tree was estimated under the GTR+I+G (4 gamma categories)

model with 3,000 bootstrap replicates. The numbers near branching points indicate the transfer bootstrap expectation (TBE) supports (BS, in percentage) and Bayesian Posterior Probabilities (PP, in probability) and are presented as "BS/PP". Dashes (-) after BS indicate PP that has not been applicable for the ML tree mainly due to the topological discrepancies between ML and Bayesian Inference (BI) trees. For BI topology, see Additional file 1: Fig. S1B.

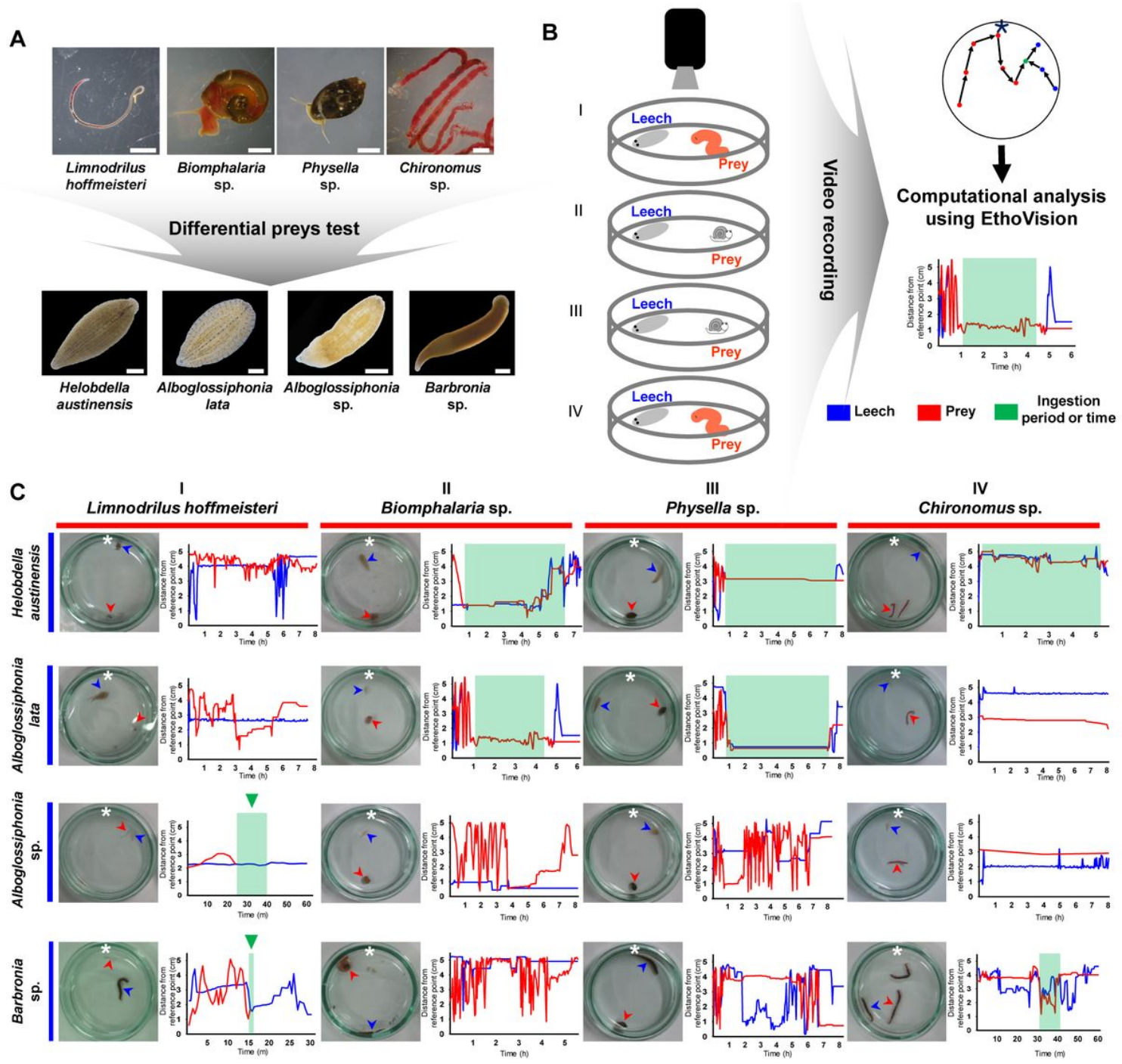

Figure 2

Comparative ingestion behavior of leeches with different food sources. (A) Representative leech species and prey types. See also Additional files: Video S1-4. Scale bars 2 mm. (B) Schematic procedure of

different ingestion tests depending on prey types. The recorded locomotion of a leech and its prey was analyzed using EthoVision, a target tracking program. The relative distance from a leech (shown in blue) or a prey (shown in red) to the reference point (asterisk) was measured. Ingestion period is indicated by green box. (C) Representative behaviors of leeches in the presence of specific prey. Each graph represents the distance between the leech (blue arrowhead) and the food (red arrowhead) from the reference point (white asterisk). When a fluid sucking leech adhered to food, its position was consistent (green box) for a period of time. After ingestion of food, the remaining prey that cannot be swallowed persisted. In the case of macrophagous leeches, only locations of the leech remained detectable (green box with green arrowhead) after ingestion of whole prey targets by the leech. Only Limnodrilus hoffmeisteri was fully ingestible by macrophagy.

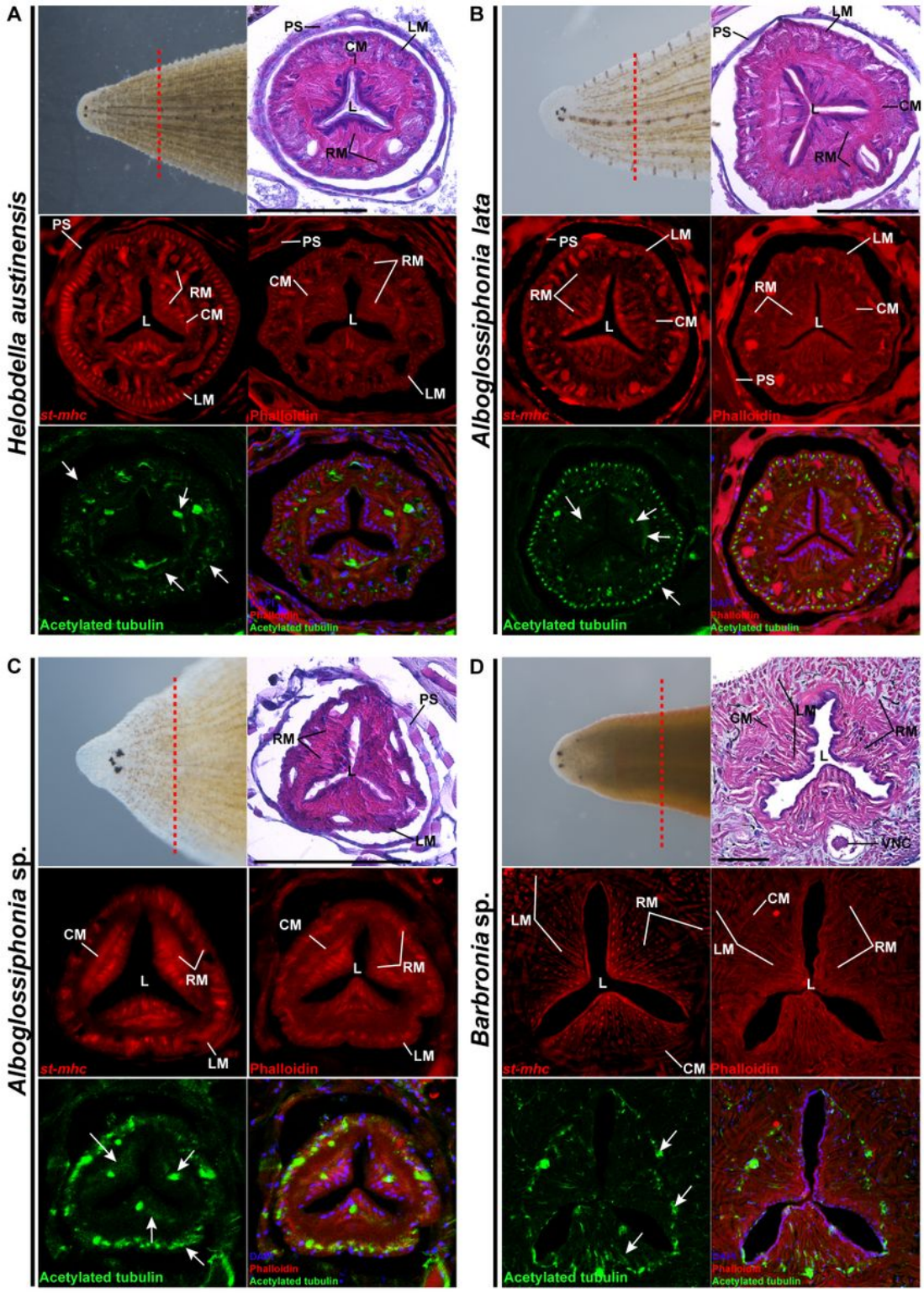

Figure 3

Comparative structural morphology of leech feeding organs. (A-D) Histological analyses of cross sections of the leech proboscis. The upper left corner of each set of cross-sectional images shows a dorsal-anterior view of the leech species and the sectional region (red dashed lines). Longitudinal, circular and radial muscle structures are labeled for sections with H&E staining (top right rows) and st-mhc gene expression patterns (middle left rows). Fluorescent labeling of neuronal (white arrow) and muscular

structures by anti-acetylated tubulin and phalloidin, respectively. DAPI staining was performed to visualize entire morphology of proboscis by nuclei labeling. H. austinensis and A. lata exhibit clear compartmentalization of the innervated muscle layers (A, B). Alboglossiphonia sp. has proboscis but does not have clear distribution of muscle layers in histological analysis; only the outer longitudinal muscle and the partial circular muscle layers are identified (C). Barbronia sp., which has esophagus, shows an extended lumen with a circular muscle, and the inner cavity is composed of a complex of radial and longitudinal muscles (D). Red dotted lines represent the top view of cross section. CM, circular muscle; L, lumen; LM, longitudinal muscle; PC, proboscis cavity; RM, radial muscle; PS, proboscis sheath; VNC, ventral nerve cord. See also Additional file 1: Fig S2B for structural details of histological analyses. Scale bars 150μm.

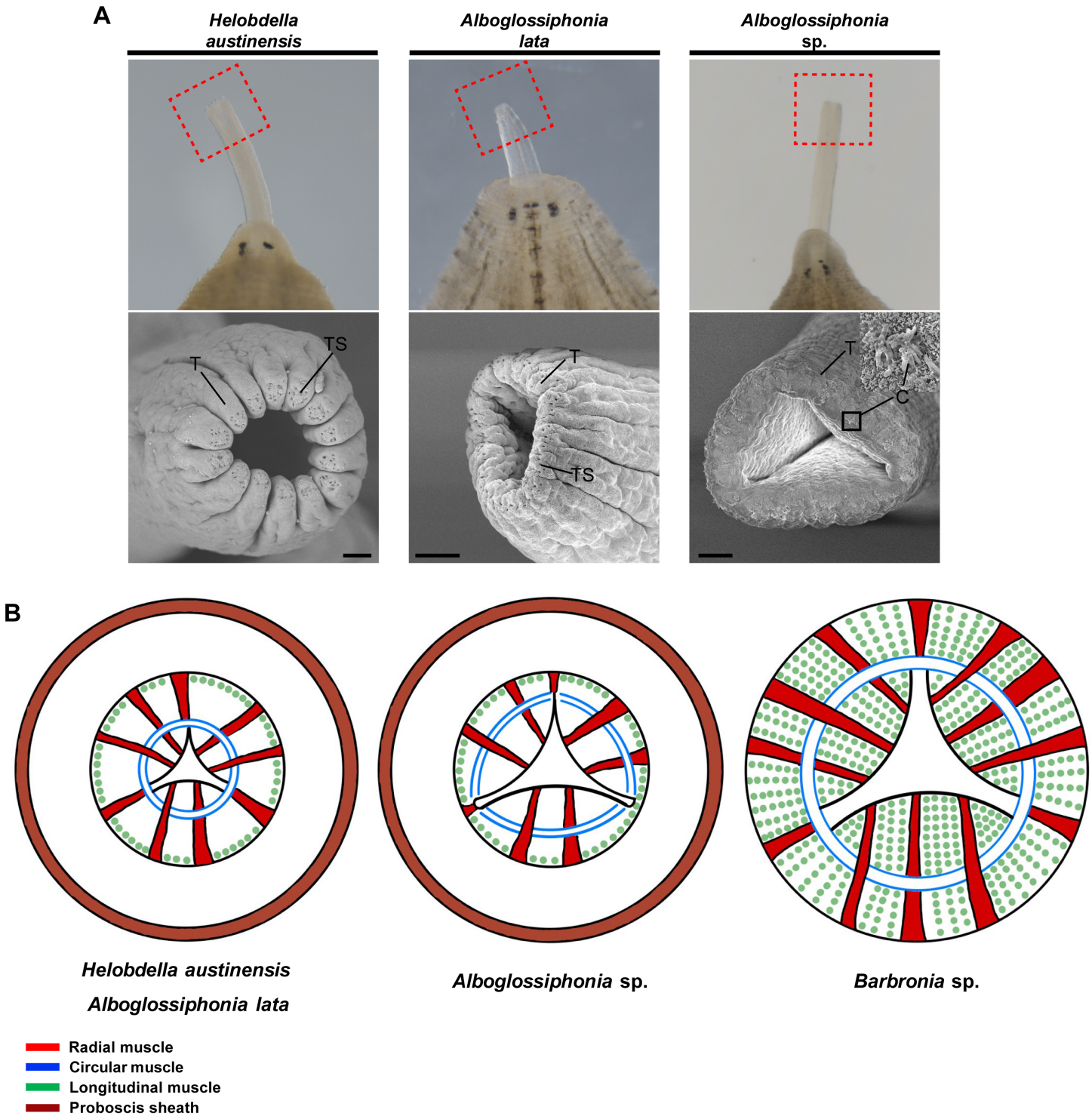

Figure 4

Apical structure of proboscis in glossiphoniid leeches and simplified schematics of muscle structure organization (A) SEM images of proboscis tips (red dotted square) show that tips of the proboscis of fluid-sucking species are contracted, whereas the tip of the proboscis of Alboglossiphonia sp. is broad and uncontracted. Numerous cilia bundles (black square) are visible at the tip of the proboscis in Alboglossiphonia sp. T, tip of proboscis; TS, secretion pore of proboscis tip; C, cilia. Scale bars 20μm. (B) Schematics of comparative muscle structure organization in leech feeding organs. Fluid ingestion

leeches have compartmentalized muscle layers, with a distinct ring of circular muscles surrounding the proboscis cavity and the radial muscles extending from inner to the outer region of the proboscis. In contrast, Alboglossiphonia sp. shows three sets of separate circular muscles, radial muscles, and an expanded lumen within the proboscis. The esophagus of Barbronia sp. is surrounded by circular muscles, radial muscles extend throughout the body, inner radial muscles are well developed, and the lumen expands to the circular muscle layer.

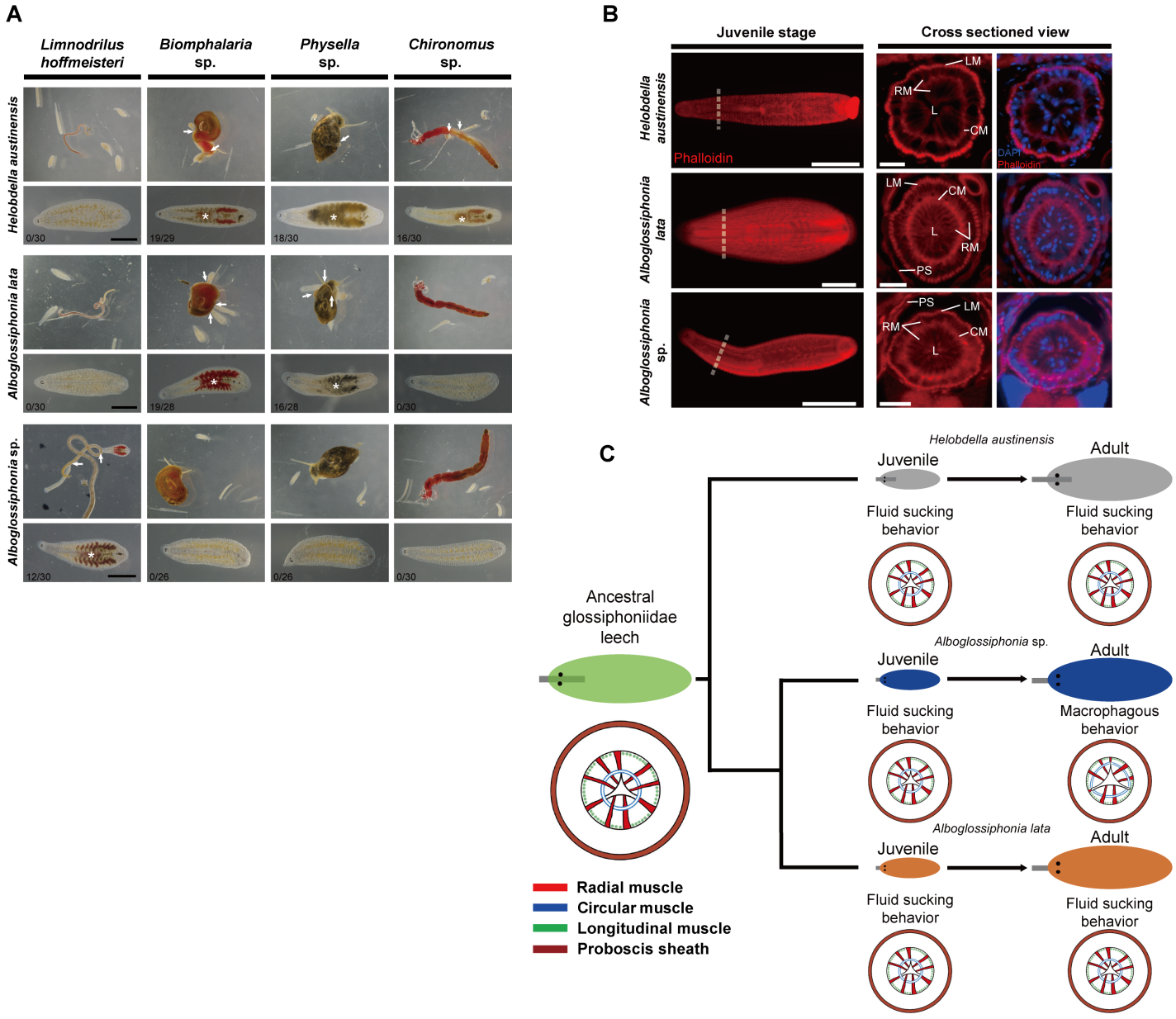

Figure 5

Juveniles of Alboglossiphonia sp. show fluid-sucking behavior and well-developed muscular structures. (A) Selective prey preferences among the juvenile stages of leeches. Four different prey organisms were introduced to the juvenile stages of three leech species. White arrows show the locations at which leeches fed on different species of prey. The juvenile stage of Alboglossiphonia sp. has the same ingestion behavior as H. austinensis and A. lata. Asterisks indicate contents in the intestine 48 h after the start of

test. The numbers of bottom left indicate the number of juveniles with filled guts compared to the total number. See also Additional file 6: Video S5 for ingestion behavior of Alboglossiphonia sp. Scale bars 500μm. (B) A comparison of proboscis musculature in the juvenile stage indicates that three species have well-developed and partitioned radial muscles, as well as circular muscles in the proboscis. F-actin (red, phalloidin) and nuclei (blue, DAPI) were stained to confirm the morphology and muscle arrangement of proboscis within juvenile leeches. White dashed lines in the first column indicate the sectioned region. CM, circular muscle; L, lumen; LM, longitudinal muscle; PC, proboscis cavity; PS, proboscis sheath; RM, radial muscle. Left column scale bars 500μm; middle columns scale bars 20μm. (C) Schematic of divergent proboscis structure according to prey preference in glossiphoniid leeches. The results of this study show that juveniles have conserved ingestion behavior and proboscis muscular structure. From a putative ancestral mechanism of feeding in glossiphoniid leeches, differences in prey preference may have influenced speciation events, and with them, changes in morphology associated with feeding behavior. Thus far, observations suggest a pattern of conservation in fluid-sucking ingestion among glossiphoniid juveniles, and at least one case of divergence to macrophagous feeding in the adult form of Alboglossiphonia sp.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- KwaketalAuthorresponse.docx
- Additionalfile6VideoS5.Alboglossiphoniasp.juvenile.avi
- Additionalfile5VideoS4.Barbroniasp.ingestion.avi
- Additionalfile4VideoS3A.lataingestion.avi
- Additionalfile3VideoS2H.austinensisingestion.avi
- Additionalfile2VideoS1.Alboglossiphoniasp.ingestion.avi
- Additionalfile1FigS1S2S3S4andTableS1.docx