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, depending on their diet and circumstance, are diversified morphologically or functionally as animals adapt to the environment. Although previous studies on changes caused by various external pressures have been well studied, the evidence for variation in invertebrates is not well known. We used freshwater leeches as an invertebrate model to observe their specific trophic niche and diversity of ingestion organ. Our results show convergent evolution according to structural changes through a representative species *Alboglossiphonia* sp., and the origin from common ancestor due to the remaining fluid ingestion behavior of the larval stages as a vestige.

Results

We identified the feeding behavior of rhynchobdellid leeches, which have the proboscis. *Alboglossiphonia* sp. swallows the entire prey using its proboscis, whereas proboscis leeches exhibit typical fluid-sucking behavior. We observed that proboscis of fluid-sucking leeches encompasses compartmentalized and dense muscle layers. In contrast, macrophagous leeches have relatively simple esophagus structures. To address whether the different feeding behaviors were intrinsic, we investigated the behavioral pattern and muscle arrangement in the earlier developmental stage of rhynchobdellid leeches. Interestingly, juveniles of the macrophagous leech as well as fluid-sucking leeches have the proboscis with the compartmentalized muscle layers and exhibit fluid-sucking behaviors

Conclusions

Animals have adapted various ways to obtain the energy needed for their survival. Diversification and evolution of ingestion methods across species further exhibit the functional morphology of the ingestion organ. However, information on ingestion behavior and internal structure is still lack and unclear, especially in invertebrate models. Our results suggest that the proboscis leeches have originated from the common fluid-sucking glossiphoniid ancestor and species diversification has led to modifications in the structure of the feeding tube. Together, leeches represent a comparative model for esophagus development according to the ingestion pattern based on diverse muscular arrangement in proboscis.

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 the fluid (fluid ingestion)[6-11]. Leeches (Hirudinea, phylum Annelida) comprise a carnivorous or ectoparasitic group of animals that feed on a wide range of prey. Accordingly,

they exhibit a corresponding 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., Gnathobdellida, Rhynchobdellida, and Pharynchobdellida)[12, 13], and these groupings have been supported by modern molecular phylogenies. Representative classification of Hirudinea is as follows: Hirudiniformes comprising jawed leeches, using jaws with teeth to injure the host body wall and ingest body fluids; Rhynchobdellida consisting of jawless leeches, using the proboscis to penetrate the host body wall and suck soft body fluids; and Erpobdelliformes including jawless leeches, swallowing organic material[12, 14-16]. The food ingestion behaviors of leech 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, 12, 16-20]. 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. The whip-like 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[12]. Interestingly, despite *Alboglossiphonia* sp. represents the distinct morphological and molecular phylogenetic features of genus Alboglossiphonia, which grouped in glossiphoniid, with high bootstrap values (MLB = 100%, and BPP = 1.00) (Fig. 1 and Additional file 1: Fig. S1), it shows the macrophagous behavior by surrounding and swallowing a prey like a salifid leech (Additional file 4:Video S3). In other words, it is difficult to explain the behavioral features based on external and genetic 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 this species arise from changes in the internal structure of esophagus.

In this study, our results show the characteristics of leeches with each specific ingestion behavior, and the comparison of structural differences would provide the first evidence of the proboscis diversification.

Result And Discussion

Systematically different leech species exhibit an overlapping or unique trophic niche in the fauna and display diverse feeding behaviors for each target food source[6, 12, 19, 21]. In order to observe the exact feeding behavior patterns of the species, behavioral experiments involving various feeding conditions are required. Nevertheless, most of the reported analyses focused on quantitative evaluation or positive reaction based on serological tests[12, 21-23].Therefore, we first investigated the food preferences of leeches and their behavioral differences during ingestion using various preys have been reported[3, 6, 12, 18]. The following prey species were tested in the present study to determine the specific ingestion behavior of leeches: *Limnodrilushoffmeisteri*, a vermiform freshwater oligochaete, that 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 bloodworms and snails by inserting its proboscis into the host body wall

thereby suckingthe 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 by inserting their anterior ends into the shell and sucking the body fluids with the inserted proboscis (Fig. 2C, Additional files:Video S1-2). 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 the *Barbronia* sp.(Fig 2C, Additional files:Video S3-4).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 coexistencewithout competition[24-26]. Also, unique ingestion behaviors support the contention whether the structure of esophagus in *Alboglossiphonia* sp. is different from that of the other glossiphonid leeches and resemble that of macrophagous leeches.

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. These changes resulted in behavioral convergence despite differences across the species [27, 28]. Among various environmental factors, the specific behavioral convergence about food that is essential for survival manifests in various aspects [29, 30]. These behavioral convergences beyond species cannot be explained phylogenetically, suggesting that convergence is due to morphological similarity. Thus, similar patterns of ingestion behavior among*Alboglossiphonia* sp. and *Barbronia* sp., not only suggest origin in similar structures but also indicate presence of possible differences in the proboscis of *Alboglossiphonia* sp. despite belonging to rhynchobdellid. Apparently, in the present study, the structure of the ingestion tube was elucidated via histological analysis and molecular methods to confirm the structural similarities and differences between the leeches.

The proboscis of glossiphoniid leeches such as *H. austinensis* and *A. lata* is a tubular muscle 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 the mesodermal precursor cells known as M teloblasts and is structurally characterized by a sharply defined complement of longitudinal, radial, and circumferential muscles. 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. 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. Finally, prominent circumferential muscles lie roughly half way between the center and the edge of the proboscis, thereby forming a circular band defined by the three tips of the lumen[12, 31]. This compartmentalized structure ensures independent movement of proboscis from the anterior to the posterior direction (Fig. 3 and Additional file1: Fig. S2A).

Compared with the well-defined proboscis of the fluid-feeding species, the esophagus of the macrophagous leech *Barbronia* sp. differs in two ways. First, *Barbronia* sp. does not have independent proboscis, instead has an integrated esophageal structure with the band of circumferential muscles that circumscribe the tri-radiate tips of the lumen. Second, perhaps as a consequence of the first, the tips of

the lumen extend all the way to the layer of circumferential muscle presenting a spacious lumen. Intriguingly, the proboscis of *Alboglossiphonia* sp. lacks circumferential muscles, and the tri-radiate tips of its lumen also extend further radially towards the outer band of longitudinal muscles as in *Barbronia* sp. (Fig. 3 and Additional file1: Fig. S2). We speculate that this morphological difference in macrophagous species compared with fluid-sucking species is an evidence of speciation and evolutionary adaptation[32, 33], which facilitates further expansion of the lumen for ingestion of intact prey.

To delineate the muscular arrangement of the proboscis, we carried out the molecular analysis and confirmed the expression of muscle markers. Previous studies have reportedmuscular differentiation in lophotrochozoan animals. In *Platynereisdumerilli*, visceral muscles in the foregut consist of striated and smooth muscles. Within this region, troponin T and myosin heavy chain (MHC) involved in muscle contraction are expressed in muscle cells and are known as annelid foregut striated muscle markers[34]. Consequently, we stained troponin T protein using immunofluorescence (Additional file1:Fig. S2C), identified foregut-specific *MHC* gene in the genome of *H. robusta*, and confirmed foregut striated expression characterized by inconsistent expression of two *MHC* transcripts using in situ hybridization (Additional file1:Fig. S2B). The configuration of muscles in the esophagus of proboscis leech exhibits structural similarity to that of vertebrate iris muscles consisting of circular sphincter and dilator radial muscle[12, 35]. In the case of *Alboglossiphonia* sp., the circular muscle layer is partial, with less clear outer radial muscle array than in *H. austinensis* and *A. lata* (Fig. 3C), which present clear circular and outer radial muscles outside the proboscis cavity (Figs. 3A and 3B). Also, the lumen in the innermost segment of the proboscis extends to the longitudinal muscle layer like *Barbronia* sp. Due to these structural differences, the tip structure of the proboscis of fluid sucking leeches shows condensed apical structure, while the *Alboglossiphonia* sp. exhibits a monotonous cylindrical tip and expanded proboscis pore (Fig. 3E). These features, are speculated as limited proboscis condensation, suggesting possible macrophagy via loose internal space construction.

These results provide the first evidence suggesting that lack of musculature can constrain the condensation of the proboscis, leading to macrophagous feeding behavior. *Barbronia* sp. is a macrophagous leech with the radial muscle vigorously stretched to the outer body walls with the embedded longitudinal muscle in the esophagus without forming the proboscis (Fig 3D, Additional file1:Figs. S2A and S2C). This monolithic esophagus 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 files: Video S2-4). 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 [36]. In *Barbronia* sp., the muscular arrangement of the esophagus is similar to that of vertebrates, thereby suggesting macrophagy via peristalsis of longitudinal and circular muscles in the esophagus (Fig 3D and Additional file1: Fig. S2C). In summary, *Alboglossiphonia* sp. exhibits the partial distribution of muscles and a loose lumen structure, designed for feeding behavior that is intermediate between fluid-

sucking and macrophagous leeches. Furthermore, the structure is hypothesized to facilitate a pattern of ingestion behavior similar to that of *Barbronia* sp.

Glossiphoniidae leeches bearing a cocoon with a thin membrane and without the hardened shell similar to erpobdelliformeshave 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, 16, 37-39]. However, the ingestion behavior of *Alboglossiphonia* sp. larvae is unknown except that leeches belonging to glossiphoniid are known as fluid-ingesting leeches. To investigate their ingestion behavior, we first analyzed the behavioral pattern in the juvenile stage (Fig. 4A). *Alboglossiphonia* sp. juveniles, exhibit macrophagousfeeding behavior in adulthood, with fluid-sucking behavior similar to *A. lata* and *H. austinensis* (Fig. 4A 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 of three species at the commencement state of food intake. The analysis of muscle markers revealed the presence of a well-developed independent proboscis in the foregut, even at the juvenile stage of food intake (Fig. 4B). Cross-sectional analysis of proboscis showed a well-partitioned musculature in the three species, although *Alboglossiphonia* sp. showed differences compared with the adult forms. The arrangement of circular and radial muscles in the adult proboscis was observed as partial compared with other leech species while the two muscle layers in the juvenile stage exhibited a well-defined partition, as in *H. austinensis* and *A. lata*. These results indicate that *Alboglossiphonia* sp. manifests fluid-sucking behavior using well-developed muscles in the juvenile stage. Subsequently, the species undergoes gradual changes in the structural arrangement in 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 in other leeches. Within the glossiphoniid leeches, the specific food preferences vary widely across species. For example, the Amazon leech *Haementeriaghilianii* is a large rhynchobdellid species adapted to feeding on mammalian blood[12, 20], and *Helobdellastagnalis*, a leech consumes diverse foods, whereas *Glossiphoniacomplanata* known as specialist leech prefers gastropods [12, 19]. Similarly, *A. lata* and *Alboglossiphonia* sp., which belong to the same genus, have different food niches in the same habitat (Figs. 2C and 4A). The diverse food preferences suggest that ancestral glossiphoniid leeches may have ingested diverse prey with their divergence and might have developed a preference for specific preys. Furthermore, ingestion of selective prey alters the structure of the feeding organ, and accordingly, results in differences in feeding behavior. As representative examples, *H. austinensis* and *A. lata* show similar feeding behavior in larval and adult stages, and the proboscis exhibits similar muscle structure. However, in the case of *Alboglossiphonia* sp., the larval stages feed with proboscis, while the adults show macrophagous behavior, attributable to the differences in the arrangement of muscle layers. Therefore, it appears that the intermediate form facilitates ingestion of body fluids, and the proboscis structure in the juvenile stages of *Alboglossiphonia* sp. persists as a vestige in the common glossiphoniid leeches [40, 41](Fig. 4C).

Animals

Adult *A. 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). *H. austinensis* was bred in the laboratory. All adult specimens were incubated in a bowl containing artificial pond water. The specimens were cared once daily by changing solution and scrubbed manually to get rid of any residual waste. They were stored in a BOD incubator at 22°C.

CO1 gene cloning and sequencing

Total RNA was isolated from *Alboglossiphonia* sp. embryos using TRIzol (Invitrogen, Carlsbad, CA, USA). The mRNA was selected using Oligo (dT) primer (Promega, Madison, WI, USA), and reverse transcribed into cDNA (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[38]and other leech-specific CO1 and 18S rRNA genes using universal primers[42]. We followed the TaKaRa protocol for PCR according to standard procedure.

Phylogenetic analysis

Three partial nucleotide sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) from *Alboglossiphonia* sp., *A. lata*, and *Barbronia* sp. and *Barbronia* sp., and four partial sequences of 18S ribosomal RNA (18S rRNA) from the same three species plus *Helobdellaaustinensis* were determined in this study.Additional sequences of both genes were obtained from the GenBank, and two data sets consisting each of the same 62 leech species were eventually prepared for the analysis (see Additional file1:Table S1 for GenBank accession number). Each of CO1 and 18S rRNA data sets was individually aligned using ClustalW implemented in MEGA7 software(ver. 7.0.26) [43].and then both were concatenated. Phylogenetic tree reconstructions using Maximum Likelihood (ML) and Bayesian Inference (BI) were carried out. The best-fit model was searched based on the corrected Akaike Information Criterion (AICc) using IQ-TREE[44] web-server (http://www.iqtree.org). The ML and BI analyses were conducted using RAxML-NG software (v 0.9.0) [45]and MrBayes software (ver. 3.2.7a)[46]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)[47]. 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: *L. hoffmeisteri*, swallowable and worm shape; *Biomphalaria* sp., unswallowable and carrying a shell; and *Chironomus* sp., unswallowable and exhibiting a worm shape. First, several individuals were placed in the 55 mm petri-dish, and ingestion patterns were observed under various feeding conditions (data not shown). After observation, one or two preys were provided to each leech. Each experimental arena 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 in triplicate on three biological replicates (data not shown). 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 analysis

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 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 RT. Sections were imaged with LEICA DM6 B (Leica, Wetzlar, HE, Germany). 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

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 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 removing the solution, the samples were dried for 3 days in the hood. Dried samples were coated with gold particle and examined with UltraPlus field emission scanning electron microscope (Carl Zeiss, Oberkochen, BW, Germany).

Fluorescent immunostaining

Whole-mount immunostaining was performed according to previously published protocols[38]. Antibodies against anti-acetylated Tubulin (Sigma Aldrich, Saint Louis, MO, USA) and Alexa-488-conjugated antibody were purchased (Abcam, Cambridge, ENG, UK). The immunostaining protocol was as follows: 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 Block solution (1:9 10X Roche Western Blocking Reagent in PBT) for 2 h. Samples were incubated with primary antibodies (anti-acetylated-α-Tubulin, Sigma Aldrich, T-7451; or anti-cardiac TroponinT, Abcam, ab115134) in 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 Blocking Solution (1:1000) at 4℃ for 24 h. After washing with PBT five times, samples were stained with phalloidin to visualize F-actin (ThermoFisher, Waltham, MA, USA) for 1 h. Consequently, the samples were washed five times with PBT and dyed 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 by LEICA DM6 B (Leica, Wetzlar, HE, Germany).

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

Total RNA was isolated from *H. austinensis* mixed-stage embryos and *Hirudonipponia* head tissue using TRIzol (Invitrogen, Carlsbad, CA, USA). We selected mRNA from 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 *H. austinesisST-MHC* genes, two candidate genes (protein id 64397 and 129847) were screened using a previously published sequence[48]and whole draft-genome references (http://genome.jgi.doe.gov/Helro1/Helro1.home.html). Subsequently, the foregut-specific expression of the*ST-MHC* transcript was demonstrated (Protein id 129847, *Hau ST-MHC*forward: 5'-GCCACCAAAGGTGAAGAG-3'; *Hau ST-MHC* reverse: 5'-GTCCTCAACGAGCTGCAT-3'). *H. nippoiniaST-MHC*transcript (*Hni ST-MHC* forward: 5'- GCCACCAAGGGCGAAGAA-3'; *Hni ST-MHC* reverse: 5'-TCCTCGACCAATTGCATTTCC-3') was identified based on sequenced RNA database available in our

Laboratory of Cellular and Developmental Biology (LCDB). These amplified fragments were cloned into pGEM T vector (Promega, Madison, WI, USA). Dioxigenin-labeled RNA probes were synthesized from the cloned fragments. The in situ hybridization of sections was performed using previously published methods[38, 49, 50]. 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. The following experiments were carried out using the same method as described above.

Conclusions

Taken together, the comparison of feeding behaviors and proboscis structure of leeches has facilitated the correlation between ingestive behaviors and the internal structure of the proboscis. The loose structure of proboscis in macrophagous leeches allows the swallowing of the whole food by retaining enough space. *Alboglossiphonia* sp. exhibits an esophageal structure intermediate between macrophagous and fluid-feeding leeches and manifests similar fluid intake behavior at the juvenile stage as in 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 esophagus structure. Also, similar food preferences reveal structural and behavioral convergence among other species, despite the species diversity. The behavioral differences in the juvenile stages of *Alboglossiphonia* sp. indicate its origin from a common ancestral glossiphoniid and that the behavioral patterns in the juvenile stage remain as vestigial traits.

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.

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Figures

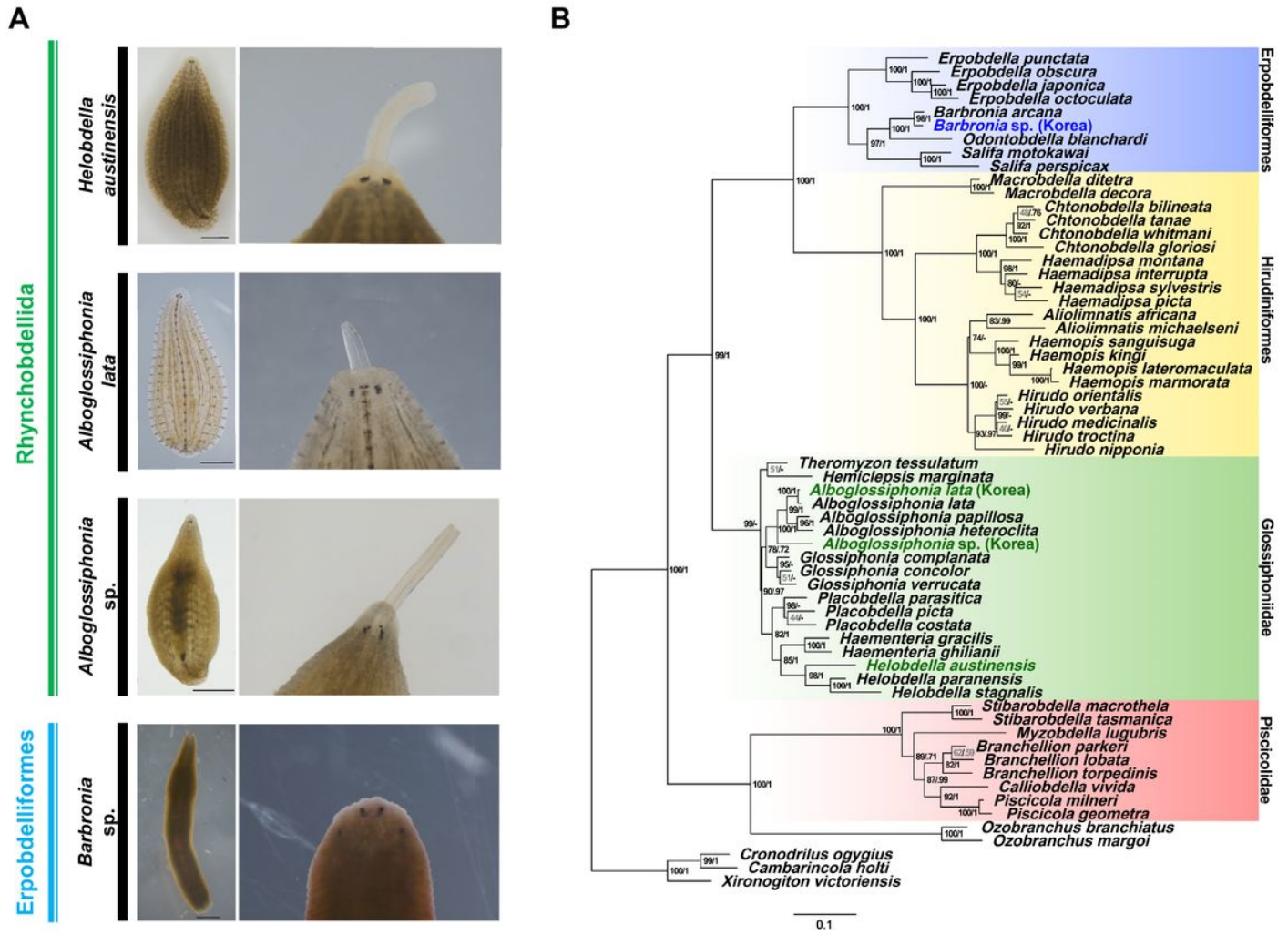

Figure 1

External morphological features and phylogenetic status of leeches. (A) Three glossiphoniid leeches (H. austinensis, A. lata, and Alboglossiphonia sp.) have a retractable proboscis that is characteristic of rhynchobdellidin contrast to erpobdelliformes specimen Babronia sp. (Scale bars 2mm). (B) Maximum likelihood (ML) phylogenetic tree reconstructed based on the concatenated sequences of CO1 and 18S rRNA including three branchiobdellid taxa (Xironogitonvictoriensis, Cronodrilusogygius and Cambarincolaholti) 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 TBE bootstrap supports (BP) and Bayesian Posterior Probabilities (BPP) and are presented in order of “BP (in percentage) / BPP (in probability)”. BP and BPP below 70% (or 0.70) are presented in grey-color and dashes (-) following BP indicate BPP 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 file1: Fig S1B

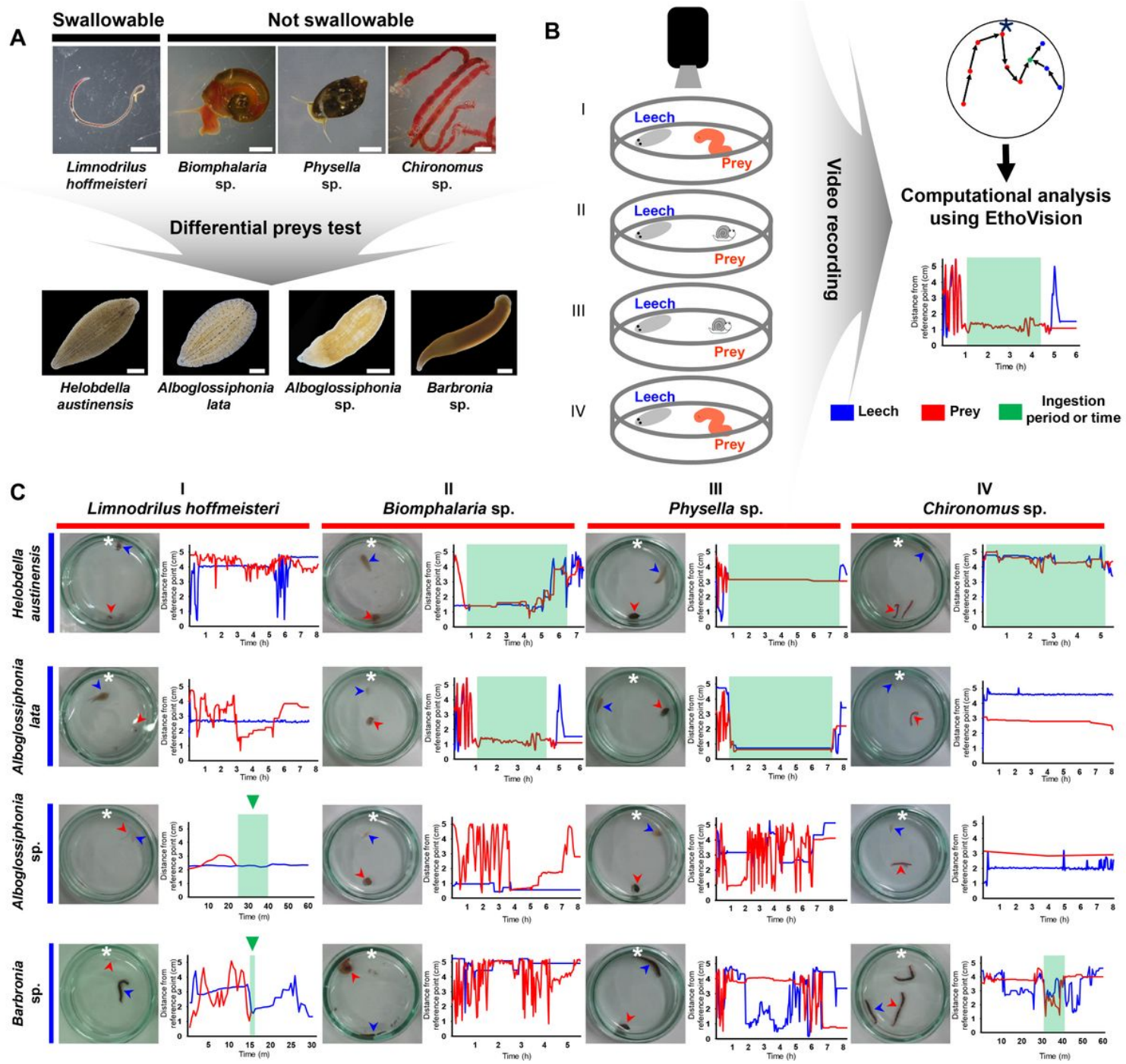

Figure 2

Ingestion behavior analyses using 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. Recorded locomotion of a leech and a prey is analyzed using EthoVision, a target tracking program. Relative distance from a leech (shown in blue) or a prey (shown in red) to the reference point (asterisk) is measured. Ingestion period is indicated by green box. (C) Representative behaviors of leeches to specific prey. Each graph represents the distance between the leech (blue arrowhead) and the food (red arrowhead) from the reference point (white asterisk). When 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 case of macrophagous leech (green box with green arrowhead), only the locations of the leech remained without prey graphs when swallowable food was ingested.

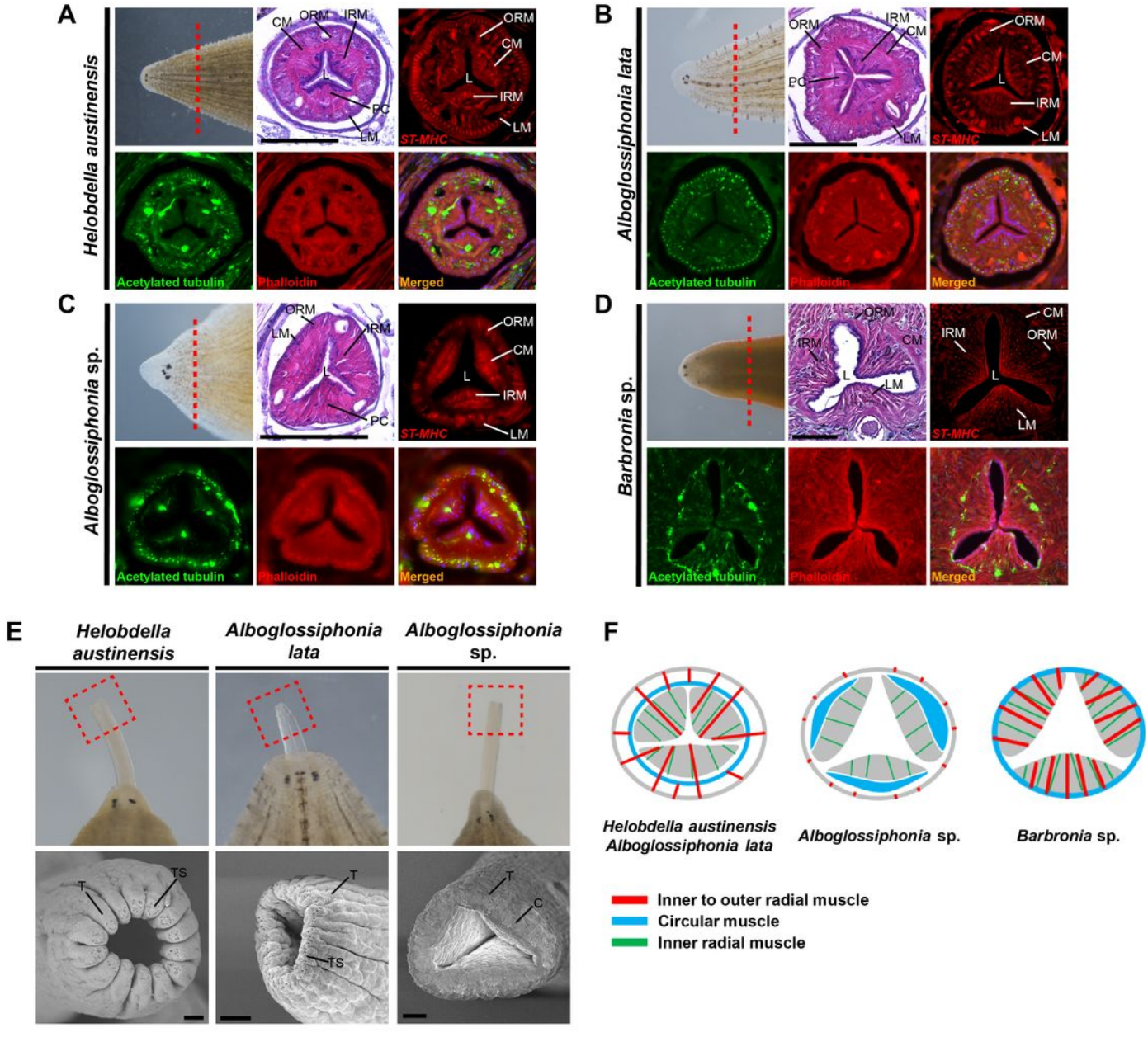

Figure 3

Alboglossiphonia sp. shows distinct proboscis structures from the esophagus of other leeches. (A-D) Histological analysis of a cross section of leech proboscis. Using H&E staining and ST-MHC images, structures of longitudinal, circular, and radial muscle layers in the proboscis are analyzed. Anti-acetylated tubulin staining and Phalloidin staining show neuronal and muscular structures, respectively. H. austinensis and A. lata exhibit clear compartmentalization of the muscle layers (A, B). Alboglossiphonia sp. has isolated proboscis but does not have clear distribution of muscle layers in histological analysis

and only the outer longitudinal muscle and the partial radial and circular muscle layers are identified (C). Barbronia sp., which has monolithic esophagus, shows the extended lumen to the 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. PS, proboscis sheath; VNC, ventral nerve cord; IRM, inner radial muscle; ORM, outer radial muscle; CM, circular muscle; LM, longitudinal muscle. See also Additional file 1: Fig S2 for structural details. Scale bars 150μm. (E) SEM images of proboscis tips (red dotted square) show that the fluid sucking leeches can condense at the tip of the proboscis, whereas Alboglossiphonia sp. cannot condense, and the proboscis pore space is wide open. T, tip of proboscis; TS, secretion pore of proboscis tip; C, cilia. Scale bars 20μm. (F) Characteristics of leech esophagus and proboscis structure. Fluid ingestion leeches have compartmentalized muscle layers, with a distinction of circular muscle layers surrounding the proboscis cavity and the radial muscles extending from inner to the outer region of proboscis. In contrast, Alboglossiphonia sp. has partial circular muscles, confined outer radial muscles, and expanded lumen in proboscis. The esophagus of Barbornia sp. is surrounded by circular muscle layer. The radial muscle that extends throughout the body and the inner radial muscles are well developed, and the lumen expands to the circular muscle layer.

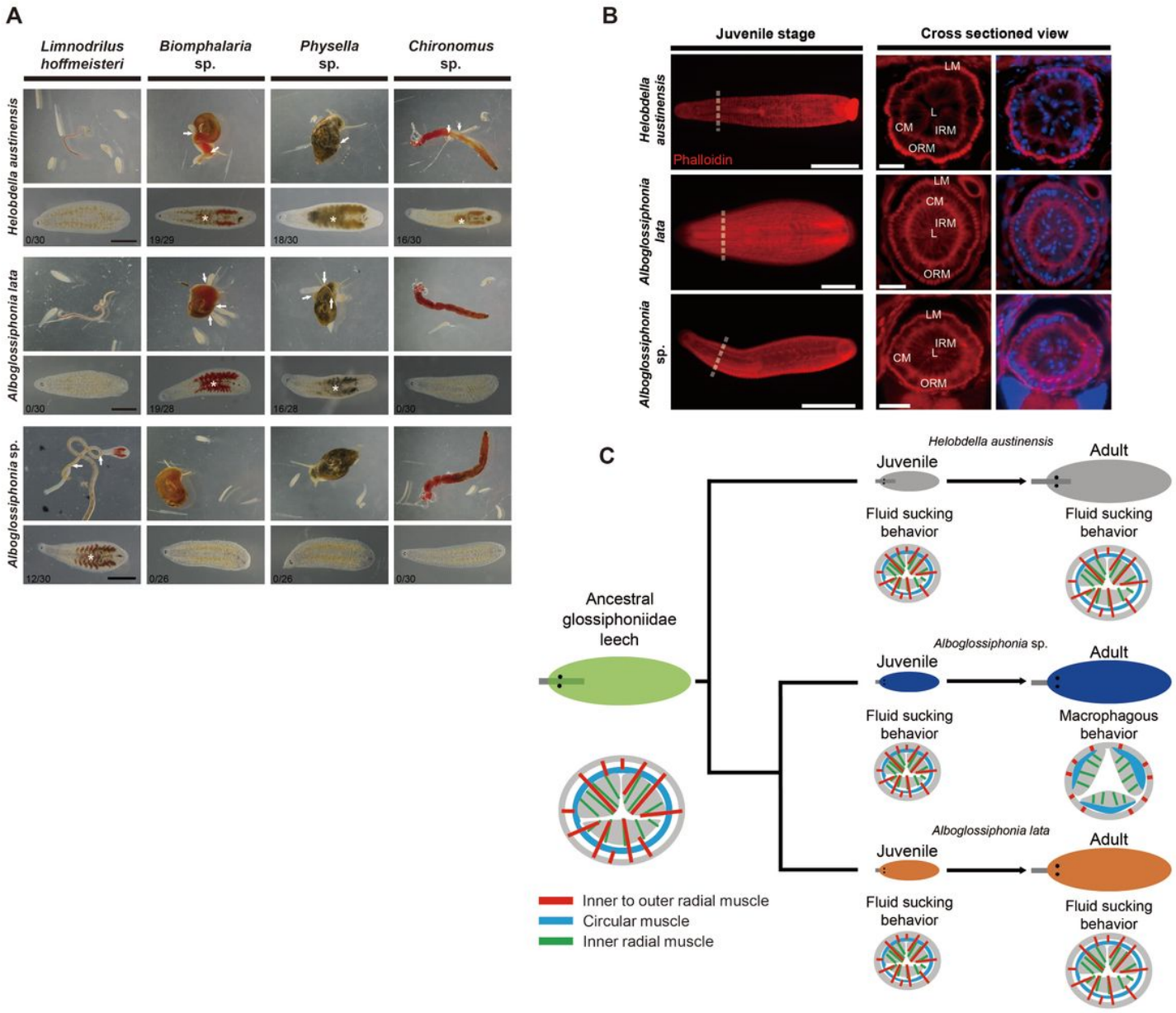

Figure 4

Juveniles of Alboglossiphonia sp. show fluid-sucking behavior and well-developed muscular structures. (A) Selective prey preference in the juvenile stage of leeches. We observed four different types of prey to confirm the prey preference at the juvenile stage. Three species show different prey attraction (white arrow). 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. 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 dilator radial muscle, and circular sphincter muscle in proboscis. C, circular muscle; IRM, inner radial muscle; L, lumen; LM, longitudinal muscle; ORM, outer 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 show that juveniles have conserved ingestion

behavior and proboscis muscular structure. Common ancestral glossiphoniid leeches exhibit a variety of food intake behaviors, and the preferred prey changed with species divergence. Because of the diversification from ancestors with similar ingestion behavior, the same proboscis structure and feeding behavior persisted as the vestigial traits in the juvenile stage and altered the specific ingestion behavior during the individual growth.

Supplementary Files

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

- Additionalfile1FigS1S2TableS1.docx
- Additionalfile3VideoS2A.lataingestion.avi
- Additionalfile4VideoS3.Alboglossiphoniasp.ingestion1.avi
- Additionalfile6VideoS5.Alboglossiphoniasp.juvenile.avi
- Additionalfile2VideoS1H.austinensisingestion.avi
- Additionalfile5VideoS4.Barbroniasp.ingestion.avi