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Cambrian lobopodians shed light on the origin of the tardigrade body plan

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Phylum Tardigrada (water bears), well known for their cryptobiosis, includes small invertebrates with four paired limbs and is divided into two classes: Eutardigrada and Heterotardigrada. The evolutionary origin of Tardigrada is known to lie within the lobopodians, which are extinct soft-bodied worms with lobopodous limbs mostly discovered at sites of exceptionally well-preserved fossils. Contrary to their closest relatives, onychophorans and euarthropods, the origin of morphological characters of tardigrades remains unclear, and detailed comparison with the lobopodians has not been well explored. Here, we present detailed morphological comparison between tardigrades and Cambrian lobopodians, with a phylogenetic analysis encompassing most of the lobopodians and three panarthropod phyla. The results indicate that the ancestral tardigrades likely had a Cambrian lobopodian–like morphology and shared most recent ancestry with the luolishaniids. Internal relationships within Tardigrada indicate that the ancestral tardigrade had a vermiform body shape without segmental plates, but possessed cuticular structures surrounding the mouth opening, and lobopodous legs terminating with claws, but without digits. This finding is in contrast to the long-standing stygarctid-like ancestor hypothesis. The highly compact and miniaturized body plan of tardigrades evolved after the tardigrade lineage diverged from an ancient shared ancestor with the luolishaniids.

Cambrian explosion | Panarthropoda | Tardigrada | lobopodia | morphological evolution

Significance

Panarthropoda, the most speciose animal group, consists of three phyla (Euarthropoda, Onychophora, and Tardigrada), all of which are considered to have originated from Cambrian lobopodians. Numerous investigations of the evolutionary origin of euarthropods and onychophorans have been conducted, but the origin of tardigrades (water bears) remains largely underexplored. Here, we present an integrative morphological comparison between tardigrades and lobopodians with a phylogeny of panarthropods including lobopodians and major tardigrade lineages. The results provide insights into how tardigrades evolved their current morphology from the Cambrian lobopodian bodyplan.

Tardigrades (water bears) are microscopic metazoans well known for their cryptobiotic abilities (1). They have four paired limbs generally terminating with claws or digits and have a bucco-pharyngeal apparatus lined by a cuticle as a foregut. They are an important part of the meiofaunal ecosystem, feeding on algae, moss cells, detritus, bacteria, fungi, protists, or smaller invertebrates (2). The phylum Tardigrada comprises two classes and four orders: the Eutardigrada (exclusively terrestrial apochelans and mostly terrestrial parachelans) and the Heterotardigrada (predominantly marine arthrotardigrades and mostly terrestrial echiniscoideans) (Fig. 1 *A* and *B*). Together with the other panarthropod phyla, Onychophora and Euarthropoda, Tardigrada is known to have originated from the lobopodians, which were extinct soft-bodied worms with lobopodous limbs that thrived during the Cambrian Period (3) (Fig. 1*C*). Due to the lack of a hard exoskeleton, most Cambrian lobopodian species have been only recovered from sites of exceptionally well-preserved fossils. Contrary to onychophorans and euarthropods, the origin of tardigrade morphology remains unclear, and detailed comparison with the lobopodian morphology has not been explored. The scarce fossil record of the tardigrade lineage has obstructed understanding of the early evolution of tardigrades. To date, only one stem-group and three crown-group tardigrade species have been reported. The "Orsten-type" fossil, discovered from the Middle Cambrian Kuonamka Formation of Siberia, was reported to be a stem-group tardigrade, which has only three pairs of limbs (4). However, it shows a gross morphology for a parasitic life mode, such as an anteroventral, pit-shaped mouth; sucking discs on the ventral body; lateral, rather than ventral, limbs with forwardly tilting; and outward-facing claws, implying that this taxon, if truly a stem-group tardigrade, likely lost plesiomorphic features. A comparable morphology can be seen in the parasitic extant tardigrade *Tetrakentron synaptae*, probably due to convergence (4, 5). Three crown-group tardigrade fossils, *Milnesium swolenskyi*, *Beorn leggi*, and *Paradoryphoribius chronocaribbeus*, embedded in Cretaceous and Miocene ambers, are all eutardigrades (6). The overall morphology of these amber fossils significantly resembles extant tardigrades.

Since the zoologist Simonetta compared the Cambrian lobopodian *Aysheaia* (*SI Appendix*, Fig. S1*A*) from the Burgess Shale (the only known lobopodian taxon, except for *Xenusion* at that time) to the marine arthrotardigrade genus *Parastygarctus*

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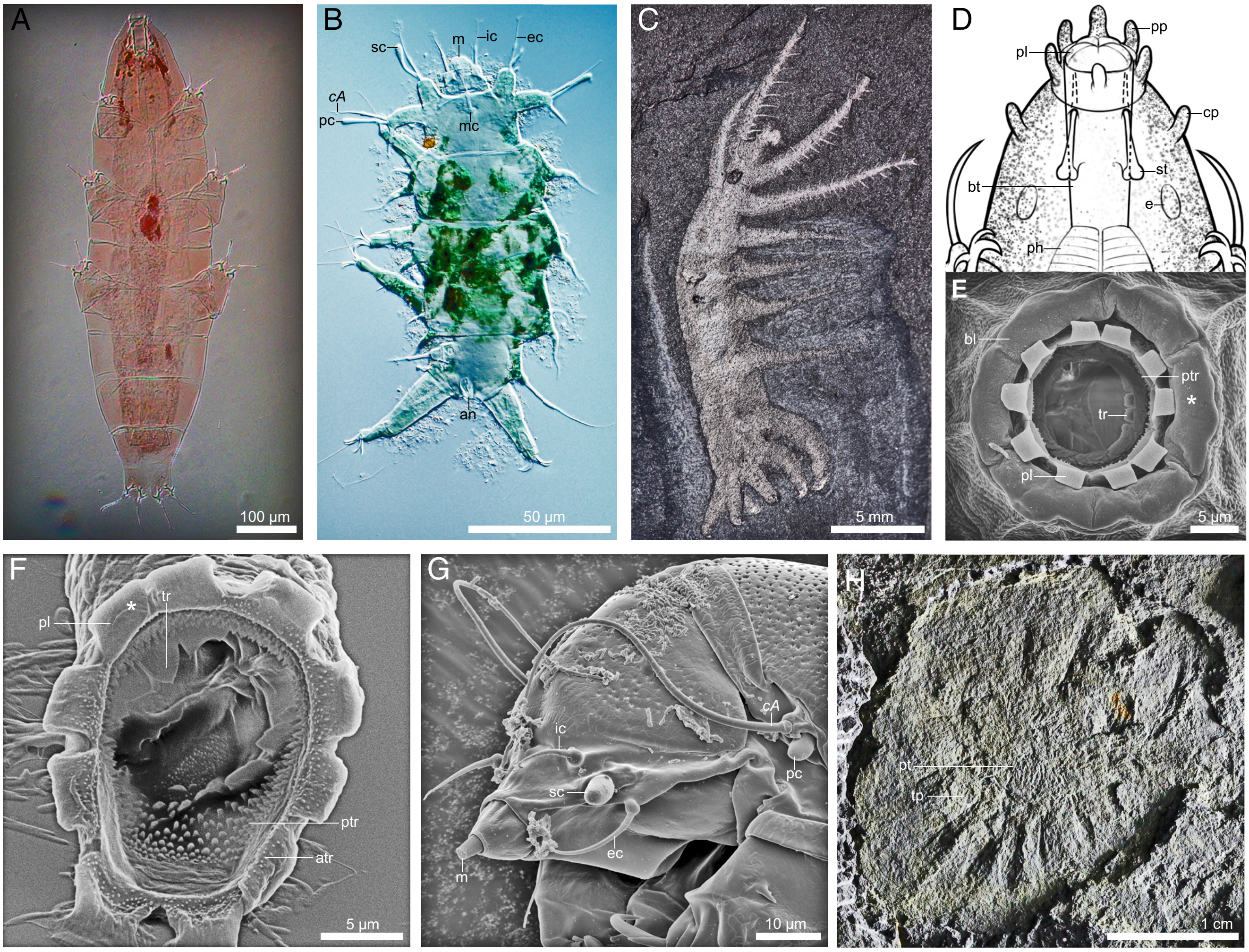

Fig. 1. Images of tardigrades and lobopodians. (*A*) Apochelan *Milnesium* sp., DIC image. (*B*) Arthrotardigrade *Parastygarctus* sp., DIC image (image courtesy of Shinta Fujimoto). (*C*) ROM 52707, Cambrian lobopodian *Ovatiovermis cribratus* (image courtesy of Jean-Bernard Caron). (*D*) Schematic drawing of the anterior part of *Milnesium.* (*E*) Mouth and COS structures of *Macrobiotus* sp., SEM image. (*F*) Extracted bucco-pharyngeal apparatus of parachelan tardigrade *Dactylobiotus ovimutans*, SEM image. (*G*) Echiniscoidean tardigrade *Echiniscus testudo*, SEM image. (*H*) SP-2018-43, mouth of Cambrian lobopodian *Pambdelurion whittingtoni*, PTM image. Abbreviations: an, anus; atr, anterior tooth row; bl, buccal lobe; bt, buccal tube; cA, cirrus A; cp, cephalic papilla; e, eye; ec, external cirrus; ic, internal cirrus; m, mouth; mc, median cirrus; pc, primary clava; ph, pharynx; pl, peribuccal lamella; pp, peribuccal papilla; pt, pharyngeal teeth; ptr, posterior tooth row; tp, triangular plate; tr, transverse ridge; sc, secondary clava; and st, stylet. Asterisk indicates the dorsal peribuccal lamella.

(Fig. 1*B*), stygarctid-like marine heterotardigrades, rather than eutardigrades, have been considered to retain primitive attributes (7–9). Subsequently, another marine heterotardigrade *Neostygarctus* was suggested to be a basal taxon which retains the most primitive body plan of tardigrades (10). Due to these suggestions, the morphological characters of *Parastygarctus* or *Neostygarctus,* such as segmentally arranged dorsal/ventral plates (11), middorsal spines (11), lateral processes of segmental plates (8), and digits on the tip of the limbs (10), have been considered possible plesiomorphic traits inherited from the tardigrade last common ancestor. However, the consideration of stygarctid-like features as primitive was not based on shared morphological characters, but on cursory aspects, such as remarkable morphological diversity, occurring in a marine interstitial biotope, and cosmopolitan distribution (7).

Since then, several Cambrian *Konservat-Lagerstätten*, like the Burgess Shale, Chengjiang Biota, Sirius Passet, and the Emu Bay Shale (EBS) have produced more than thirty lobopodian taxa, providing various morphological data for analysis (12–16). This has led to several studies on the phylogenetic relationships within panarthropods, the main goal of which was to understand the morphological origination of the crown groups. For example, the Cambrian lobopodians, *Kerygmachela kierkegaardi*, *Pambdelurion whittingtoni*, and radiodontans were interpreted as stem groups of Euarthropoda, based on a pair of frontal appendages on the head and the paired gut-diverticula (17, 18), while *Hallucigenia sparsa* was considered to be a stem-onychophoran based on the presence of stacked elements in sclerites of claws and dorsal spines (19). However, tardigrades have received little attention in studies of panarthropod phylogeny. Recently, several morphology-based phylogenetic studies included a few tardigrade taxa (*Batillipes pennaki*, *Echiniscus testudo*, *Actinarctus doryphorus*, *Macrobiotus* cf. *harmsworthi*, and *Hypsibius exemplaris*), but the characters for the analyses were not particularly focused on tardigrade morphology (20–22).

Here, we present detailed morphological comparison between the Cambrian lobopodians and tardigrades, with a phylogenetic analysis encompassing 40 tardigrade species (including three amber fossil species) belonging to 24 families, all available Cambrian lobopodian species, representative onychophorans, and euarthropods. This comparison will not only provide a glimpse into the morphological origin of tardigrades, but also help elucidate the relationship of tardigrades with other panarthropods.

Results

Morphological Comparison.

Circumoral elements. In many ecdysozoan taxa, including tardigrades and lobopodians, the mouth shows radially arranged circumoral elements, and this structure is considered a shared character inherited from the common ecdysozoan ancestor (20). While heterotardigrades have only a simple ring structure at the mouth opening (Fig. 1 *B* and *G*), most eutardigrades (all apochelans and many parachelans) possess peribuccal lamellae or papulae supported by a buccal ring as circumoral elements (23) (Fig. 1 *D*–*F*). Most apochelans have six lamellae, and several parachelans show six to more than thirty lamellae or papulae depending on the genus. Although detailed mouth structures are not well preserved in most lobopodians fossils, *H. sparsa*, *Pambdelurion*, *Jianshanopodia*, and radiodonts show lamella-like or plate-like circumoral elements (Fig. 1*H*) (20, 24, 25).

During or after death, by some currently unknown causes, the bucco-pharyngeal apparatus of tardigrades (the rigid cuticular foregut structure from the mouth opening to the pharynx) sometimes retracts backward, forming a cavity near the original mouth opening (Fig. 2 *A* and *B*). Interestingly, this structure evokes the buccal cavity (buccal chamber in ref. 21) and circumoral elements of *H. sparsa* (20). While the circumoral elements of other lobopodians occur at the mouth opening, the circumoral elements of *H. sparsa* are positioned inside the buccal cavity, posterior to the mouth opening, being reminiscent of the retracted bucco-pharyngeal apparatus of dead tardigrades. We propose the possibility that the circumoral element of *H. sparsa* may have been located at the mouth opening in life, like those of other lobopodians. Because the presence of the buccal cavity has been considered one of the important links between *H. sparsa* and onychophorans, our comparison may be worth considering in terms of the affinity issue between *H. sparsa* and onychophorans.

Pharyngeal teeth. The pharyngeal teeth, the sclerotized spinose structures lining the pharynx, have been considered as a shared character throughout ecdysozoan animals including tardigrades (20). However, tardigrades lack teeth structures in their pharynx (Fig. 2*B*). Instead, parachelan tardigrades possess a maximum of three rows of teeth: i.e., the anterior teeth row, the posterior teeth row, and the transverse ridge between the mouth opening and the buccal tube (Fig. 1*F*). The pharynx is separated from the mouth opening by the buccal tube. Therefore, tardigrades have oral teeth (26) rather than pharyngeal teeth. The anterior tooth row and the posterior tooth row consist of radially arranged small mucrones, and the transverse ridge comprises three or four crests with multiple cusps dorsally and ventrally. The anterior tooth row occurs on the base of circumoral elements (peribuccal lamellae), which is similar to the nodes on the surface of radiodontan plate–like circumoral elements (25). Both the anterior tooth row of tardigrades and the nodes of radiodontan plates project toward the mouth opening. The proboscis of the Cambrian lobopodian *Ovatiovermis* has numerous tooth-like elements (27), the position

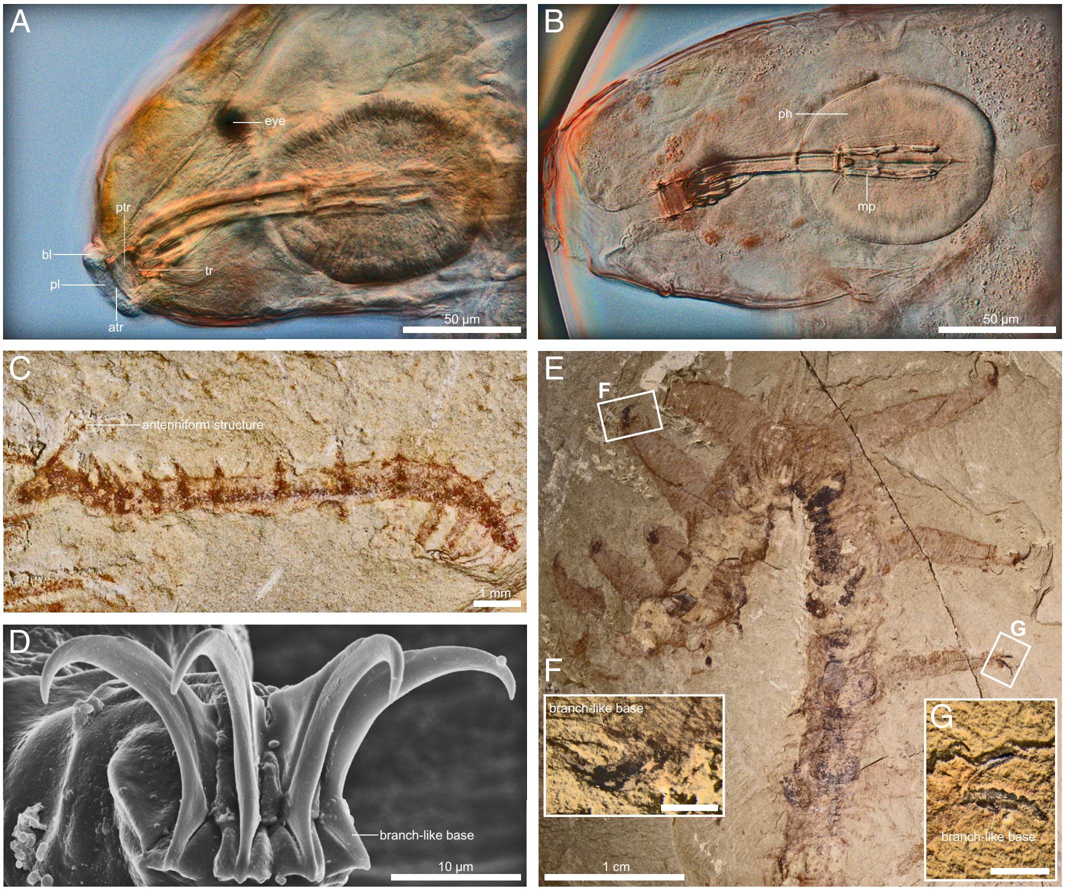

Fig. 2. The foregut, dorsolateral paired structures, and claws of tardigrades and lobopodians. (*A*) Bucco-pharyngeal apparatus in place of parachelan tardigrade *Dactylobiotus ovimutans*, DIC image. (*B*) Backwardly retracted bucco-pharyngeal apparatus of *D. ovimutans*, DIC image. (*C*) ML0020A-2, Cambrian lobopodian *Luolishania longicruris*. (*D*) Claw of echiniscoidean tardigrade *Cornechiniscus holmeni*, SEM image. (*E*–*G*) JS0001A, Cambrian lobopodian *Onychodictyon ferox* and its claws, digital camera images. The scale bars in (*F*) and (*G*) are 0.5 mm in length. Abbreviations: atr, anterior tooth row; bl, buccal lobe; bt, buccal tube; mp, macroplacoid; ph, pharynx; pl, peribuccal lamella; ptr, posterior tooth row; and tr, transverse ridge.

of which is similar to that of the posterior tooth row of parachelan tardigrades (ptr in Fig. 1*F*). However, the tooth-like elements of *Ovatiovermis* are needle-like structures and are unlikely to be arranged radially.

Cuticular structures surrounding the mouth opening. Tardigrades have a sensory field surrounding the mouth, the circumoral sensory field (COS) (28, 29). Some tardigrade groups possess specialized cuticular structures on the COS: six peribuccal papillae and their base in apochelans (Fig. 1*D*) and buccal lobes or papulae in several parachelans (six lobes in many cases) (Fig. 1*E*). Interestingly, similar structures are observed in Cambrian lobopodians. *Ovatiovermis* shows a bulbous proboscis surrounding the mouth opening (27), which evokes the cuticular structures on the COS of eutardigrades (bl in Figs. 1*E* and 2*A*). *Pambdelurion* has ovate plates surrounding the mouth opening (24). Based on their position and the morphology, the ovate plates of *Pambdelurion* are considered to be homologous to the scalids of priapulids (24). Interestingly, the COS of tardigrades and buccal scalids of priapulid larvae share position and function, i.e., they surround the mouth opening, and function as sensory organs.

Rostral spines and stylets. The stylets (Fig. 1*D*), a pair of spines within the mouth, are characteristic feeding organs of tardigrades and have been considered to be an internalized pair of frontal appendages in the mouth (30). The lower stem-group euarthropod *Kerygmachela* has a pair of spine-like structures flanking the mouth, which were interpreted as anterior paired projections homologous to those present at the anterior margin of the head of *Pambdelurion* and *Canadaspis* (31). While the circular structure at the posterior end of the paired projections was previously identified as eyes (14), recently, they were reinterpreted as apodemes (15). This implies that the spine-like structures of *Kerygmachela* were indeed a pair of spines, not sensory organs, being distinct from the anterior paired projections of *Pambdelurion*, *Canadaspis,* or *Tanazios* (31). Based on the similar morphology and location, the rostral spines of *Kerygmachela* are comparable to the stylets of tardigrades. If so, the stylets of tardigrades are not internalized frontal appendages, because *Kerygmachela* has both raptorial frontal appendages and a pair of rostral spines. The distant relationship between *Kerygmachela* and tardigrades in the phylogenetic trees (ref. 20 and Fig. 3) suggests that rostral spine–like structures may be an ancestral characteristic of most lobopodians. However, this structure has not been observed in other panarthropods so far, leaving a possibility of convergent evolution.

Dorsolateral paired structures on the midhead. Heterotardigrades have special sensory organs on the head which are innervated by the brain: i.e., three pairs of cirri [internal, external, and cirrus A (*cA*)] and an unpaired median cirrus (32). Ultrastructural analysis has revealed similarities between the cirri of heterotardigrades and scolopidia of euarthropods (33), which may imply a common origin of sensory organs in both groups. While *cA* of several arthrotardigrades occurs at the dorsolateral part of the head near the eyes (if eyes are present) (e.g., *Archechiniscus bahamensis* and *Neostygarctus grossmeteori*) or at the middle of the head (e.g., *Wingstrandarctus unsculptus*, *Actinarctus neretinus*, and *Parastygarctus renaudae*) (Fig. 1*B*), echiniscoidean *cA* tends to occur at the posterior part of the head segment (34, 35) (Fig. 1*G*). Although eutardigrades lack cuticular sensory structures corresponding to *cA* of heterotardigrades, they have sensory fields near or behind the eyes where, in arthrotardigrades, *cA* occurs. Hence, they are considered to have rudiments of the *cA* (28, 36). Some lobopodians have a pair of cirri-like antenniform structures on the middle of the head, which do not have annulations. These nonappendicular antenniform structures occur at a position that is similar to where the *cA* of arthrotardigrades occur: e.g., a pair of antenniform structures of *Luolishania* (Fig. 2*C*) occurs immediately behind the eye (37). Other lobopodian taxa with clear antenniform structures are *Collinsium* and *Collinsovermis* (38, 39). *Facivermis* also has a vague pair of antenniform structures (40). All these lobopodian taxa belong to the order Luolishaniida (38). Antenniform frontal appendage–like structures of other lobopodians, such as *Onychodictyon* (41) and *Antennacanthopodia* (42), have annulations on the surface of the appendage-like structures, which are as thick as half of the limb width; these features are similar to the antenna of onychophorans. However, the antenniform structures of luolishaniids lack annulations, and the width of the antenna is significantly thinner than that of the limbs, being reminiscent of the *cA* of tardigrades.

Epidermal specializations as muscle attachment sites. Some specialized structures in the epidermis of Cambrian ecdysozoans, including panarthropods, have been interpreted as sites for muscle attachment. These structures occur as scleritomes in palaeoscolecids; spines in hallucigeniids and luolishaniids (Fig. 2*C*); plates in *Microdictyon*, *Onychodictyon ferox* (Fig. 2*E*), and *O. gracilis*; and paired nodes in *Xenusion*, *Hadranax,* and *Kerygmachela* (20). Since they are widespread in paleoscolecids and lobopodians, their presence is considered an ancestral character of ecdysozoans (9, 20, 27). In tardigrades, muscle attachment sites often exhibit cuticular cribriform structures (43). The cribriform structures possess numerous tiny pseudopores and sometimes have a thick cuticular rim (*SI Appendix*, Fig. S1*B*). These closed pseudopores are associated with muscle filaments (44).

Differentiation of lobopodous trunk limbs into two types. The anterior limbs of luolishaniid lobopodians are differentiated from the posterior limb pairs. Luolishaniids exhibit a chevron-shaped pattern of spinules on the anterior limbs only (Fig. 1*C* and *SI Appendix*, Fig. S1*C*) (38). Although less prominent, tardigrades also show differences between the anterior three limb pairs and the posteriormost limb pair. In eutardigrades, claw shape/size is different between the anterior three limb pairs and the last limb pair, while in heterotardigrades, in addition to differences in claw shape/size, the aspect of sensory organ distribution is different (*SI Appendix*, Fig. S1 *D* and *E*). The limb musculature of the anterior three limb pairs is also more similar to each other than that of the posteriormost limb pair in tardigrades (44).

Claws. Unlike other tardigrades with directly inserted claws on each limb, several marine arthrotardigrades have digits (45) (*SI Appendix*, Fig. S1*D*), and the toe-like digits of those arthrotardigrades have a claw or a sucking disc on each tip (46). There is no other group in panarthropods that shows digits on the tip of a limb.

Several heterotardigrades, including most terrestrial echiniscoideans, have a branch-like base of the claw (e.g., *Coronarctus* and *Cornechiniscus holmeni*) (Fig. 2*D* and *SI Appendix*, Fig. S1*E*), and the lobopodian *Onychodictyon* has a similar base (Fig. 2 *E*–*G*). Additionally, *Onychodictyon* has a much smaller accessory claw adjacent to the main claw (47). Similarly, several tardigrades have both larger and smaller claws on a limb (e.g., smaller external and larger internal claws of arthrotardigrades and vice versa in eutardigrades) (*SI Appendix*, Fig. S1*F*).

Phylogenetic Analysis. The presence of comparable anatomical features in tardigrades and lobopodians provides a platform for a phylogenetic analysis, and included for this study are representatives of most tardigrade families; all available lobopodians; and representatives of Onychophora, Euarthropoda, and their stem-groups. We have run parsimony, maximum likelihood, and Bayesian phylogenetic inference using 121 characters from 79

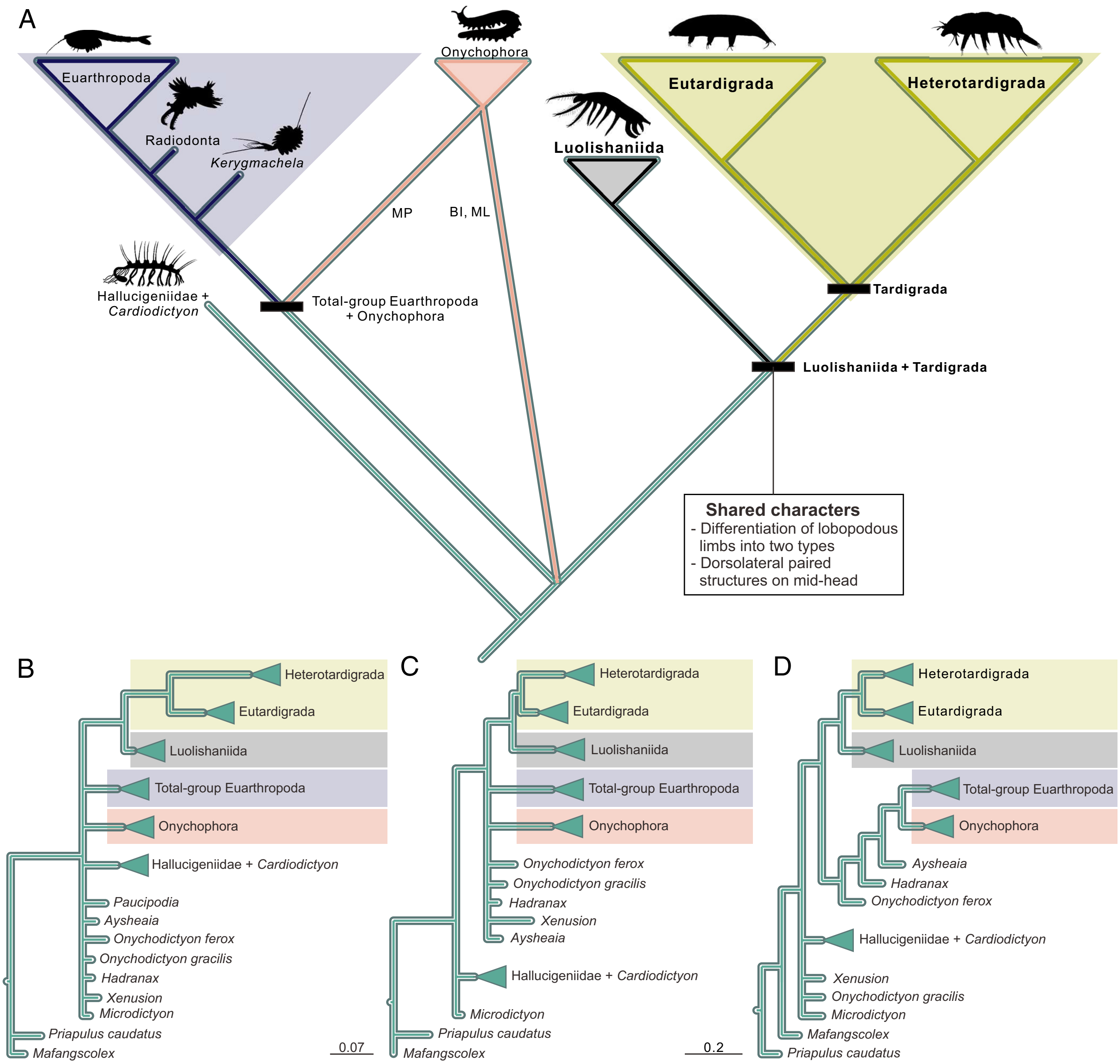

Fig. 3. Panarthropod phylogeny. (*A*) Summary tree showing relationships inferred under Bayesian, maximum likelihood, and maximum parsimony methods. (*B*) A simplified tree obtained from the Bayesian inference (BI). (*C*) A simplified tree obtained from the maximum likelihood analysis (ML). (*D*) A simplified tree obtained from the maximum parsimony strict consensus (MP). See *SI Appendix*, Figs. S3 and S4 for full tree topologies.

taxa (Fig. 3). All obtained trees show that the genera *Milnesium* and *Coronarctus* are the most basal groups of the Eutardigrada and the Heterotardigrada, respectively. The phylum Tardigrada is invariably recovered as the sister group of the Luolishaniida in all obtained trees from the maximum parsimony, Bayesian inference, and maximum likelihood (Fig. 3 and *SI Appendix*, Figs. S3 and S4). The Tardigrada + Luolishaniida clade does not fall closer to the total-group Euarthropoda than the Onychophora, thus not supporting the Tactopoda hypothesis (Tardigrada + Euarthropoda) (9, 20).

Discussion

Despite the possible loss of characters during miniaturization of tardigrades and the incomplete preservation of fossil lobopodians, detailed morphological comparison in this study reveals many shared characters between tardigrades and lobopodians, such as cuticular sensory structures surrounding the mouth opening (the circumoral sensory field, COS), dorsolateral paired structures on the midhead, differentiation of lobopodous limbs into two types, muscle attachment sites, and claws. Rostral spines may also be homologous characters. This indicates that tardigrades actually inherited many of their morphological features from their lobopodian-like ancestors. In contrast to the long-standing previous hypotheses in which stygarctid-like arthrotardigrades are considered to retain the plesiomorphic characters of tardigrades, the phylogenetic result in this study suggests that *Milnesium* and *Coronarctus* are the basal groups in Eutardigrada and Heterotardigrada, respectively. These groups share several characters that are lobopodian like: vermiform body shape without dorsal and ventral segmental plates, but possessed terminal mouth, cuticular structures surrounding the mouth opening, lobopodous limbs, and claws that are inserted directly without digits.

The phylogenetic trees suggest that tardigrades are closely related to luolishaniids. Shared characters of a tardigrade-luolishaniid lineage may include the presence of two different types of lobopodous limbs and dorsolateral paired structures on the mid-head. One notable difference between morphological traits is the relative length of anterior limbs, which is much shorter in tardigrades than that in luolishaniids (Fig. 4). This difference may be explained by loss of the *dachshund* (*dac*) gene in tardigrades (48). While other leg gap genes, *Distal-less* (*Dll*), *homothorax* (*hth*), and *extradenticle* (*exd*), were found in the tardigrade genome, and expression of those genes was observed during embryonic leg development, *dac* was not found (48). Because *dac* regulates the development of the intermediate region of euarthropod and onychophoran limbs, the loss of *dac* may have resulted in the loss of an intermediate region in tardigrade limbs. *Dac* knockout experiments conducted in amphipods and fruit flies have shown the loss of the intermediate region of mutant limbs, leading to shortened limbs (49, 50). These results could provide evidence to support the link between the relatively short limbs and the loss of *dac* in tardigrades. In addition, in tardigrades, the leg gap gene expression pattern in the posteriormost limbs is slightly different than the pattern in the anterior limbs (48); only *Dll* is expressed in the posteriormost limbs during embryonic development.

Another different morphological trait between tardigrades and luolishaniids is the number of trunk segments (Fig. 4). While tardigrades have four trunk segments, luolishaniids show 9 to 16 trunk segments, depending on the species (except EBS Collins monster and *Facivermis*, for which the number of trunk segments is unclear). According to the expression patterns of several anteroposterior patterning genes (51, 52), the anterior four segments of tardigrades (the head segment + the three trunk segments) correspond to the anterior four segments of euarthropods and onychophorans. The *Abdominal-B* gene (*Abd-B*), which is expressed at the posterior end of euarthropods and onychophorans, is expressed in the posterior part of the fifth (posteriormost) segment of tardigrades, indicating that the posteriormost region of tardigrades is homologous to the posteriormost region of other panarthropods. However, tardigrades lack several central class Hox genes that specify the midtrunk region of other panarthropods. Taken together, these results indicate that tardigrades have lost an intermediate region of the body which is homologous to the whole thorax and most of the abdomen of insects (51). This loss may be related to the loss of terminal addition from a posterior growth zone in tardigrade embryonic development, which might be involved in the miniaturization of tardigrades (51). The loss of the intermediate trunk region in tardigrades may explain the difference in the trunk segment number between tardigrades and luolishaniids. The pattern of Hox gene expression in tardigrades may also explain the differentiation of limbs in tardigrades. *Abd-B* is a Hox gene, and Hox genes are known to regulate the expression of leg gap genes in euarthropods (48). Therefore, *Abd-B* might be regulating the differences that are seen in the posteriormost legs compared to the more anterior leg pairs in terms of leg gap gene expression patterns in tardigrades. Additionally, it is possible that the central class and/or posterior Hox genes were also involved in the formation of the posterior batch of lobopodous limbs in luolishaniids, which are markedly different from the anterior limb batch.

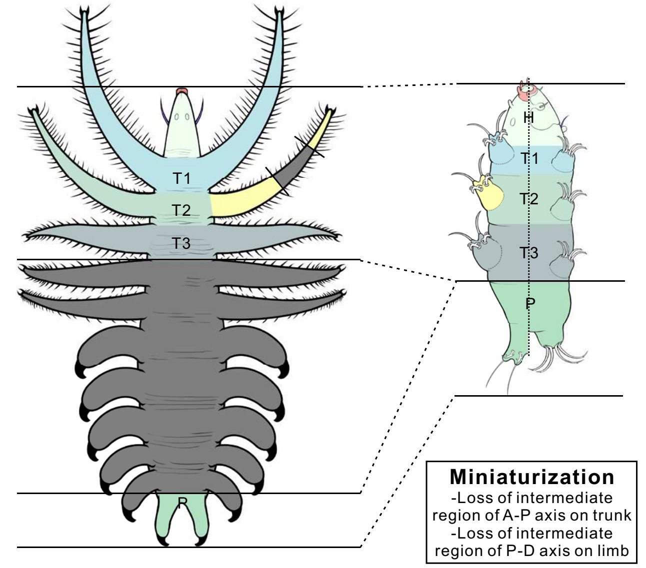

Fig. 4. Comparison of general body plan between luolishaniid lobopodians and tardigrades. A hypothetical luolishaniid lobopodian exhibiting five anterior limb pairs and six posterior limb pairs. A hypothetical tardigrade combining eutardigrade *Milnesium* (left side) and heterotardigrade *Coronarctus* (right side). Dark colored parts in the trunk and limb of the luolishaniid lobopodian (*Left*) are absent in the tardigrade (*Right*). The anterior regions in the red color represent COS (circumoral sensory field) (see text).

To sum up, the detailed morphological comparison and the phylogenetic analysis show that tardigrades have a close relationship with luolishaniids. This result suggests that the most primitive morphological characters of tardigrades are lobopodian-like characters, originating from the last common ancestor of tardigrades and luolishaniids, not stygarctid arthrotardigrade–like characters as in the long-standing previous hypothesis. The recent gene expression could explain key morphological differences between tardigrades and luolishaniids: Tardigrades appear to have lost an intermediate region of both anteroposterior axis and the proximodistal axis, potentially related to miniaturization in the tardigrade lineage.

Materials and Methods

Fossil Material. An *Aysheaia pedunculata* fossil from the Burgess Shale was observed for this study and is deposited in the Smithsonian Institution, Washington (USA), prefixed with United States National Museum (USNM). *Ovatiovermis cribratus* from Burgess Shale is deposited in the Royal Ontario Museum, Canada, prefixed with ROM. *Onychodictyon ferox* and *Luolishania longicruris* from Chengjiang Fauna, Yunnan Province, China, are deposited in Northwest University, Xi'an, China, prefixed with JS and ML, respectively. *Pambdelurion whittingtoni* from Sirius Passet, Nansen Land, North Greenland, is deposited in the Geological Museum, Natural History Museum of Denmark, University of Copenhagen, prefixed with MGUH.

Tardigrade Material. Observed limno-terrestrial tardigrade specimens of *Echiniscus testudo, Cornechiniscus holmeni, Milnesium* sp*.,* and *Macrobiotus* sp. are from Sirius Passet of North Greenland (82° 47′ 36.0″N, 42° 17′ 52.5″W), and Ella Island, East Greenland (72° 56′ 6.1″N, 25° 9′ 10.5″W), and are housed at the Korea Polar Research Institute (KOPRI). The specimens of *Dactylobiotus ovimutans* were extracted from the lake sediment samples collected from King George Island of Antarctica (62° 14′ 24.1″S, 42° 44′ 36.6″W) and are also housed at the KOPRI, prefixed with Antarctic Tardigrade Name of Specimen (ATNS). The specimens of marine arthrotardigrades (*Parastygarctus* sp. and *Coronarctus* sp. sensu ref. 53) were deposited in Shinta Fujimoto's personal collection of Japanese marine tardigrades.

Specimen Microscopy and Photography. Fossil photographs were taken using a Canon camera EOS 6D with the Canon EF 100 mm macro lens. The mouth image of *Pambdelurion whittingtoni* was taken with polynomial texture mapping (PTM), at the KOPRI. Tardigrade SEM images were taken using a Field Emission SEM JSM-7200F at the KOPRI. Tardigrade differential interference contrast (DIC) images were taken using Carl Zeiss Axio Imager 2, with an AxioCam HRc camera.

Phylogenetic Analysis. The phylogenetic data matrix of this study was based on a previous panarthropod character matrix (38), with several references (20, 21, 39, 54–56), and additional characters were added for tardigrade taxa. One or two tardigrade species which were reported with detailed description and clear images were chosen randomly from each family, except Carphaniidae, the images of which were unavailable. The Orsten-type stem-group tardigrade was also excluded. The final matrix contained 79 taxa and 121 characters (Dataset S1). Phylogenetic analyses were performed using Bayesian analyses, maximum likelihood, and maximum parsimony. Bayesian inference was performed by MrBayes 3.2.6. (57) using the Mkv + gamma model. We conducted two independent runs for 20 million generations each with sampling every 1,000th generations and discarded the initial 25% trees as burn-in. Convergence was assessed by checking the standard MrBayes convergence diagnostics (the estimated sample size scores >> 200; the average SD of split frequencies values < 0.01; potential scale reduction factor values ~1.00 across all parameters). Tree samples were summarized as a majority rule consensus. The maximum likelihood tree search was conducted in IQ-TREE (58) using the MK model (Jukes-Cantor type model for morphological data), and support was assessed using the ultrafast phylogenetic bootstrap replication method from 10,000 replicates (59). Branches with node values of 70 or less were collapsed. The maximum likelihood tree and the Bayesian tree were visualized in FigTree 1.4.4 (60). A maximum parsimony analysis was conducted using the Traditional search in Tree analysis using New Technology (TNT) 1.5 (61), under equal character weighting with 100 random seeds, using 1,000 replicates (producing a strict consensus of 20 trees). The obtained parsimony trees were visualized in Mesquite 3.7 (62). Although a recent paper (63) emphasized the critical importance of molecular data in resolving panarthropod phylogeny, the focus of this research lies on the relationship with the lobopodians known only from fossils, and the extant tardigrades, and thus morphology-based phylogenetic studies are required.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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