Comprehensive Transcriptome Sequencing of Tanaidacea with Proteomic Evidences for Their Silk

Keiichi Kakui 1, James F. Fleming 2, Masaru Morii 2,3, Yoshihiro Fujiwara 4, and Kazuharu Arakawa 2,3,5,*

1Faculty of Science, Hokkaido University, Sapporo, Hokkaido, Japan
2Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata, Japan
3Graduate School of Media and Governance, Keio University, Fujisawa, Kanagawa, Japan
4Research Institute for Global Change, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Kanagawa, Japan
5Faculty of Environment and Information Studies, Keio University, Fujisawa, Kanagawa, Japan

*Corresponding author: E-mail: gaou@sfc.keio.ac.jp.
Accepted: 9 December 2021

Abstract
Tanaidaceans are small benthic crustaceans that mainly inhabit diverse marine environments, and they comprise one of the most diverse and abundant macrofaunal groups in the deep sea. Tanaidacea is one of the most thread-dependent taxa in the Crustacea, constructing tubes, spun with their silk, for shelter. In this work, we sequenced and assembled the comprehensive transcriptome of 23 tanaidaceans encompassing 14 families and 4 superfamilies of Tanaidacea, and performed silk proteomics of Zeuxo ezoensis to search for its silk genes. As a result, we identified two families of silk proteins that are conserved across the four superfamilies. The long and repetitive nature of these silk genes resembles that of other silk-producing organisms, and the two families of proteins are similar in composition to silkworm and caddisworm fibroins, respectively. Moreover, the amino acid composition of the repetitive motifs of tanaidacean silk tends to be more hydrophilic, and therefore could be a useful resource in studying their unique adaptation of silk use in a marine environment. The availability of comprehensive transcriptome data in these taxa, coupled with proteomic evidence of their silk genes, will facilitate evolutionary and ecological studies.

Key words: tanaidaceans, transcriptome, silk, proteome, phylogenomics.

Significance
Here we report the transcriptome sequencing and assembly of 23 tanaidaceans, a group of small benthic crustaceans, encompassing 14 families and 4 superfamilies of Tanaidacea. This is the largest data set of this underrepresented crustacean order to date. Comprehensive transcriptomic data will accelerate tanaidacean studies in evo-devo contexts, an otherwise untouched field. Tanaidaceans are unique in their use of silk to create tube-shaped habitat, and we combined the transcriptome assembly with proteomic analysis of the silk protein to characterize two families of tanaidacean silk genes. The knowledge of the molecular compositions of tanaidacean silks spun in sea water would substantially contribute to the study of water-resistant protein materials, taking advantage of the renewable, biodegradable nature of these silks.

Introduction
The order Tanaidacea is a group of benthic crustaceans with about 1,500 described species (Anderson 2020). Tanaidaceans are small, typically a few millimeters long, and mainly inhabit marine environments ranging in depth from intertidal to hadal zones. They often occur in shallow and deep waters at high densities (e.g., more than 10,000 individuals/m²; Larsen 2005) and comprise one of the most
proteomic evidence, in the characterization of at least two underrepresented crustacean order. Moreover, we provide to the best of our knowledge, is the largest data set of this passing 14 families and 4 superfamilies of Tanaidacea, which, tomes sequencing and assembly of 23 tanaidaceans encom-
substantially contribute to the study of water-resistant protein compositions of tanaidacean silks spun in sea water would
(Insecta: Trichoptera) (Lane et al. 2015; Wang et al. 2015; spun in water have been extensively studied in caddisfly larvae
distinguished mechanical properties. Water and moisture sensitivity is
materials in industrial applications (Abascal and Regan 2018; Numata 2020), which have been intensively studied within
accessory, biodegradable, and thus sustainable nature, as well as their extraordinary mechanical properties. Water and moisture sensitivity is a key issue in such applications, and thus the properties of silks spun in water have been extensively studied in caddisfly larvae (Insecta: Trichoptera) (Lane et al. 2015; Wang et al. 2015; Ashton et al. 2016; Ashton and Stewart 2019). Alongside freshwater caddisworm silk, the knowledge of the molecular compositions of tanaidacean silks spun in sea water would substantially contribute to the study of water-resistant protein materials.
To this end, here we report the comprehensive transcriptome sequencing and assembly of 23 tanaidaceans encompassing 14 families and 4 superfamilies of Tanaidacea, which, to the best of our knowledge, is the largest data set of this underrepresented crustacean order. Moreover, we provide proteomic evidence, in the characterization of at least two families of silk proteins.

Results
A high-quality transcriptome assembly of 22 tanaid taxa and one outgroup (R07, Asellus hilgendorfii, Isopoda) was successful-fully obtained, mostly with BUSCO completeness above 90% (table 1). One sample had low amounts of extracted RNA which resulted in incomplete transcriptome assembly (66.2%, R05 Apseudomorpha sp.), and this sample was omitted from subsequent analyses. These samples encompass 14 families and 4 superfamilies of Tanaidacea.

Our BUSCO phylogenetic analyses of Tanaidacea produced interesting and controversial results. Both the partitioned and unpartitioned maximum likelihood and the Bayesian analyses recovered the same topology with a very high level of support, suggesting a strong and reliable signal from the conserved BUSCO genes (fig. 1, supplementary figs. S1–S3, Supplementary Material online). Prior 18S analyses of the clade have previously recovered similar high-level clade structures (Kakui et al. 2011), with the Tanaidoidea and Neotanaoidea as sister taxa to the exclusion of Paratanatoidea, with Apseudoidae as the sister group to all other tanaidaceans. However, this is in contrast to a more classical understanding of the clade (Lang 1956; Lauterbach 1970; Gardiner 1975). This can be seen as a corroboration of our current molecular understanding of the order. However, the organization of taxa within the superfamilies differs from prior work. Notably, we find a monophyletic Apseudoidae inclusive of Kalliapseudidae under both maximum likelihood and Bayesian frameworks, which was inconsistently recovered in prior assessment of 18S genes, where it was not recovered under Parsimony or Minimum-Evolution (Kakui et al. 2011). Within the Paratanatoidea, our most noteworthy find is that, with increased sampling from within the clade, we do not recover the Akanthophoreidae as monophyletic, instead finding Parakanthophoreus in a monophyletic clade with Tanaella kommitzii and Siphonolabrum sp. to the exclusion of the two other aphanthophoreids in the data set, Akanthophoreus sp. and Chauliopelea cf. sinusa. This discovery may prompt a review of the defining characteristics of the family in the future, though further genomic data from within Paratanatoidea is certainly required to explore these questions further.

Detailed microscope observation of the silk tube of Zeus ezoensis showed a complex organization of the tube (fig. 2). Nano-scale fibers form fine meshes, and were sometimes bundled into thick stems (fig. 2D, arrowhead). Moreover, the holes of fine meshes were often padded with sheet-like components (fig. 2D, arrow), which could be contaminants but could also suggest the use of nonsilk materials as a composite.

Proteomic analysis of the Z. ezoensis silk tube detected 60 proteins consistently in the five replicates, of which 41 did not match any of the proteins in the UniProt database. Excluding proteins with only one peptide support, high variability in abundance among replicates, and proteins without repetitive
| No.  | Taxon                          | Family                | Superfamily or Higher | Sex                   | Thread Use                  | Number of Transcripts | Longest Transcript | N50 Length (bp) | BUSCO Completeness (Longest informer) |
|------|--------------------------------|-----------------------|-----------------------|-----------------------|-----------------------------|-----------------------|-------------------|------------------|---------------------------------------|
| R02  | Zeuxo ezoensis                 | Tanaidaeae            | Tanaidomorpha         | Not checked           | Observed by KK (thoracic-gland system) | 41,001                | 30,563            | 3,040            | C: 92.5% | [S: 40.0%, D: 52.5%, F: 1.1%, M: 64%, n: 1,103] |
| R05  | Sinelobus sp.                  | Tanaidaeae            | Tanaidomorpha         | Male                  | Observed by KK (thoracic-gland system) | 38,989                | 27,117            | 2,886            | C: 92.3% | [S: 33.4%, D: 58.9%, F: 1.2%, M: 75.5%, n: 1,103] |
| R06  | Apsyaeomorpha sp.              | Metapneuidea          | Tanaidomorpha         | Female                | Not observed by KK (epibenthi) | 35,946                | 14,971            | 1,370            | C: 66.2% | [S: 45.5%, D: 20.7%, F: 1.1%, M: 22.0%, n: 1,103] |
| R07  | Aezilus hilgendorfi            | Isopoda               | Tanaidomorpha         | Male                  | Not observed by KK (epibenthi) | 43,376                | 28,641            | 3,220            | C: 93.0% | [S: 48.4%, D: 44.6%, F: 1.5%, M: 55.5%, n: 1,103] |
| R08  | Aepyaeus sp.                   | Apsyaeomorpha         | Tanaidomorpha         | Female                | Not observed by KK (thoracic-gland system) | 35,766                | 28,355            | 2,871            | C: 93.9% | [S: 75.2%, D: 186%, F: 1.3%, M: 48%, n: 1,103] |
| R10  | Hexapleomea asuke              | Tanaidaeae            | Tanaidomorpha         | Female                | Observed by KK (thoracic-gland system) | 39,120                | 24,047            | 2,626            | C: 92.5% | [S: 49.4%, D: 43.1%, F: 6.0%, M: 6.5%, n: 1,103] |
| R11  | Phorakallia pongledes          | Kalliaepyaeomorpha    | Tanaidomorpha         | Female                | Observed by KK (peripodal-gland system) | 40,894                | 37,262            | 2,951            | C: 93.6% | [S: 62.4%, D: 30.2%, F: 0.8%, M: 56%, n: 1,103] |
| R12  | Carpoapsaeus sp.               | Apsyaeomorpha         | Tanaidomorpha         | Female                | Not observed by KK (thoracic-gland system) | 42,133                | 27,246            | 2,472            | C: 89.1% | [S: 64.3%, D: 248%, F: 3.1%, M: 78%, n: 1,103] |
| R14  | Pseudaphyapus sp.              | Sphyrapodiidae        | Tanaidomorpha         | Not checked           | Not observed in one conspecific species by KK (thoracic-gland system) | 41,538                | 29,339            | 2,414            | C: 94.1% | [S: 58.1%, D: 36.0%, F: 5.0%, M: 5.4%, n: 1,103] |
| R15  | Pakistanapsaeus sp.            | Parapsaeididae        | Tanaidomorpha         | Female                | nd                          | 28,137                | 28,137            | 2,414            | C: 94.2% | [S: 52.3%, D: 419%, F: 1.6%, M: 4.2%, n: 1,103] |
| R18  | Acr. tanaii alakeiini          | Tanaidaeae            | Tanaidomorpha         | Not checked           | Observed by KK (thoracic-gland system) | 36,930                | 26,503            | 2,412            | C: 89.9% | [S: 62.0%, D: 279%, F: 2.0%, M: 81%, n: 1,103] |
| R19  | Neotanais cf. kunshio          | Neotanaiidae          | Tanaidomorpha         | Female                | nd                          | 36,954                | 24,195            | 1,995            | C: 79.9% | [S: 66.3%, D: 132%, F: 6.6%, M: 17%, n: 1,103] |
| R20  | Chauiopleona cf. sinusa        | Parataanaiidae        | Tanaidomorpha         | Not checked           | nd                          | 42,339                | 26,666            | 3,028            | C: 92.0% | [S: 48.6%, D: 43.4%, F: 1.8%, M: 66%, n: 1,103] |
| R21  | Agasthanais misakierseni       | Agasthanaiidae        | Tanaidomorpha         | Not checked           | One conspecific species in a tube was reported once | 37,541                | 26,257            | 2,788            | C: 91.6% | [S: 71.8%, D: 138%, F: 1.0%, M: 68%, n: 1,103] |
| R22  | Tanaella kommitrata            | Tanaidaeae            | Tanaidomorpha         | Not checked           | Individuals in a tube were observed by KK (thoracic-gland system) | 36,514                | 26,731            | 2,619            | C: 91.7% | [S: 63.6%, D: 281%, F: 1.0%, M: 73%, n: 1,103] |
| R23  | Parapsaeus algiolela           | Parapsaeididae        | Tanaidomorpha         | Not checked           | Observed by KK (pleopodal-gland system) | 37,032                | 28,519            | 3,000            | C: 94.2% | [S: 75.1%, D: 94.1%, F: 0.8%, M: 50%, n: 1,103] |
| R24  | Heterotanaiidae sp.            | Heterotanaiidae       | Tanaidomorpha         | Female                | nd                          | 42,874                | 43,880            | 2,295            | C: 88.6% | [S: 79.6%, D: 80.8%, F: 5.2%, M: 111%, n: 1,103] |
| R27  | Chondrochelidae sp.            | Leptochelidae         | Tanaidomorpha         | Female                | Congeneric species in a tube were reported | 38,274                | 27,116            | 2,048            | C: 85.0% | [S: 72.2%, D: 134%, F: 4.8%, M: 96%, n: 1,103] |
| R32  | Siphonolabrum sp.              | Anarthuridae          | Tanaidomorpha         | Female                | Conspecific species in a tube were reported | 40,128                | 24,313            | 3,118            | C: 91.1% | [S: 54.9%, D: 36.2%, F: 1.8%, M: 71%, n: 1,103] |
| R33  | Tanaopsids sp.                 | Tanaidaeae            | Tanaidomorpha         | Female                | One conspecific species in a tube was reported once | 36,398                | 27,345            | 2,526            | C: 91.0% | [S: 69.9%, D: 211%, F: 2.0%, M: 70%, n: 1,103] |
| R34  | Parathanthae sp.               | Agasthanaiidae        | Tanaidomorpha         | Female                | One conspecific species in a tube was reported once | 43,123                | 24,746            | 2,928            | C: 92.3% | [S: 46.0%, D: 46.3%, F: 1.0%, M: 17%, n: 1,103] |
| R35  | Parakanthhoresp.               | Akanthhorespidae      | Tanaidomorpha         | Not checked           | nd                          | 41,697                | 24,350            | 3,040            | C: 91.2% | [S: 45.2%, D: 46.0%, F: 1.9%, M: 63%, n: 1,103] |
| R36  | Akanthhoresp. sp.              | Akanthhorespidae      | Tanaidomorpha         | Female                | nd                          | 41,168                | 25,306            | 2,888            | C: 91.8% | [S: 53.2%, D: 35.6%, F: 1.9%, M: 63%, n: 1,103] |
motifs, 21 transcripts (including isoforms) remained, that were further clustered into six proteins based on sequence identity (fig. 3). Screening and error correction of the nanopore long reads of cDNA successfully recovered the full-length sequence of five proteins, and N-terminal and C-terminal fragments of one protein (comp408). Of these 6 proteins, comp9210 was the most abundant protein (fig. 3A), and in fact it is the most abundant protein among the all 60 proteins detected, suggesting this protein to be the main component of the Zeuxo ezoensis silk. Interestingly, all six of these proteins were large proteins (633–1,909 amino acids), and were mostly comprised characteristic repeats, which strongly suggests that all of these are structural proteins (fig. 3B). Comp9210 contains abundant (GA)n and GAGAGS resembling the fibroin of the silkworm Bombyx mori, GPG/GPY linker motif resembling the amorphous region of major ampullate spidroin (MaSp) of spiders, and another characteristic linker motif SGVRQQTYSF. N/C-terminal sequences do not show similarity to B. mori fibroin or MaSp, suggesting that these genes, whilst similar to the repetitive motifs, are not homologous. The repetitive motif of GPX of comp235, but not the terminal sequences, matches vertebrate collagen alpha-1 (e-value = 3e-57, XP_038241255), and likewise, the repetitive motif of CC of comp856 weakly matches keratin-associated protein (e-value = 8e-16, XP_018008435). The remaining three proteins (comp1, comp300, comp408) do not have significant matches, but all contain Serine-enriched motifs, which resemble the SXSXSXSX motif of caddisworm fibroin. Interestingly, although the repetitive motifs of these three proteins are similar but distinct, the N-terminal sequences are conserved (fig. 3C), suggesting that these three genes are orthologous. All of these six proteins show lower Kyle–Doolittle hydropathy values compared with the similar proteins, suggesting adaptation to underwater spinning of these silks. Searching through the 23 transcriptome assembly, the conservation and gene expression abundance of four classes of proteins (collagen-like, keratin-associated protein-like, silkworm fibroin-like, and caddisworm fibroin-like) were assessed (fig. 4). All of these proteins were mostly conserved throughout Tanaidacea, and collagen-like and keratin associated protein-like proteins (comp235 and comp856) were even conserved outside of Tanaidacea, in the outgroup Isopoda.
A. hilgendorfii which does not utilize silk. Therefore, these proteins are likely contaminants from the mucus as abundant structural proteins, and the main silk constituents are most likely to be the silkworm fibroin-like protein and caddisworm fibroin-like proteins, mirroring detected protein abundance as well as the characteristic motifs. Conservation of silkworm fibroin-like protein is limited in Apseudoidea, and only one of three caddisworm fibroin-like proteins (comp408) is conserved in this superfamily.

**Discussion**

Here we present the comprehensive transcriptome sequencing and assembly of 23 tanaidaceans encompassing 14 families and 4 superfamilies of Tanaidacea. This is the largest data set of this underrepresented crustacean order to date. Comprehensive transcriptomic data will accelerate tanaidacean studies in evo-devo contexts, an otherwise untouched field. Here we focused on the silk use, but Tanaidacea harbors many other interesting topics. One example can be found in the convergent evolution of hermit-crab-like shape in Crustacea. One tanaidacean group shows striking morphological adaptations for gastropod-shell use (e.g., a twisted body) and utilizes empty gastropod shells as mobile shelters in the same fashion that phylogenetically distinct decapods (= hermit crabs) do (Bird and Webber 2015; Kakui 2019), providing us with the opportunity to investigate which changes are general or group specific in the evolution of shell-inhabiting lifestyles in crustaceans. Another is the high diversity of sexual systems in Tanaidacea. Tanaidacea is the only taxon in Malacostraca, the most species-rich class in Crustacea, that contains simultaneous hermaphroditic species having separate male and female reproductive gonads (not forming an ovotestes; cf. Bauer and Holt 1998; Kakui and Hiruta 2013), which will give many hints toward understanding how the simultaneous hermaphroditic condition evolved and is maintained in Crustacea. Tanaidaceans also show variable morphology of eyes, and this feature was confirmed to also possess a molecular component, through the lack of short wavelength opsins in pigment-less and eye-less species (supplementary text, Supplementary Material online).

Through comprehensive phylogenetic analysis, all four superfamilies were recovered as fully supported monophyletic groups in the present tree, although the monophyletic condition of Apseudoidea was questioned relative to prior 18S-rRNA based analyses (Kakui et al. 2011). The relationships amongst the four superfamilies were identical to those in Kakui et al. (2011). Among five families containing two or more species used in this analysis, Akanthophoreidae was not recovered as a strongly supported monophyletic group. The nodal support for the clade comprised three aphanthophoreid, one tanaellid, and one anarthrurid species was low (bootstrap value = 0.70; partitioned bootstrap value = 0.70; posterior probability = 1), suggesting the need for a more comprehensive analysis using more paratanaisoidean groups and the reassessment of the family-level framework.

The use of silk distinguishes tanaidaceans from most other crustaceans, and through a multiomics approach combining
comprehensive transcriptomics as well as silk proteomics from Z. ezoensis, we have identified two families of tanaidacean silks. The most abundant protein in the silk, comp9210, contained repetitive motifs similar to silkworm silk, whereas the second most abundant group (comp1, comp300, and comp408) shared conserved N-terminal sequences suggesting a homologous origin, and contained repetitive motifs resembling those of caddisworm silks. On the other hand, the N/C-terminal sequences showed no similarity to the fibroin genes of other species, suggesting an independent evolution of these silk genes in tanaidaceans. Although the repetitive motifs are similar to silkworm and caddisworm silks, tanaidoidean silk proteins tend to be more hydrophilic compared with their analogs, representative of their unique adaptation to a marine environment. The tanaidoidean thoracic gland system harbors two secretory glands (tg1 and tg2), where tg1 is comparably larger than tg2 (Kaji et al. 2016). The existence of a pair of glands coincides with the two families of proteins identified, and the higher protein abundance of comp9210 is suggestive of its relationship with the larger tg1 gland, but a more detailed gland-specific expression analysis is necessary to fully determine such localizations.

These genes were found to be conserved throughout tanaidaceans, though some species lack expression. Among seven paratanatoidean families included in this study, Akanthophoreidae and Heterotanoididae lack members with previous records of tube-use (Kakui 2021). In all species in the two families (Akanthophoreus sp., Chauliopleona cf. sinusa, and Parakanthophoreus sp.; Heterotanoides sp.), the gene expression of the caddisworm fibroin-like proteins was not detected and that of the silkworm fibroin-like protein was low level or not detected, which may imply that they have actually adopted a silk-free mode of life, such as burrowing or subsistence in the epibenthos. However, even in the well-known tube-dwelling genus Tanaella (cf. Kakui 2021), whilst the gene expression of silkworm fibroin-like silk is moderately high, expression of caddisworm fibroin-like proteins was not detected. Likewise, Neotanais, which is epifaunal and lacks tube use, shows low, but detectable expression of comp9210 and comp408. More detailed study of inner morphology and

**Fig. 3.**—Proteomic analysis of Z. ezoensis silk. (A) Proteome and transcriptome abundances of six proteins detected in the proteome analysis. Comp9210 is the most abundant constituent of the silk tube. (B) Repetitive motifs of the six proteins. Comp9210 contains GAGAGS motif resembling the silkworm (GAl)n. Comp235 has GPX motif resembling collagen, comp856 has CC motif resembling keratin-associated protein, and comp1, comp300, and comp408 resembles in the abundance of S of caddisworm fibroins. (C) Alignment of N-terminus of comp1, comp300, and comp408. High sequence conservation suggests the homology of these three proteins. (D) Kyte–Doolittle hydrophathy plot of N-terminal 1,000 amino acids of the six proteins as well as their analogs. Positive value represents hydrophobicity.
gland-specific expression analyses of these species are re-quired to fully understand these exceptions.

Three currently known gland systems related to tube con-structions in Tanaidacea, namely thoracic-gland, pereopodal-gland, and pleotelsonal-gland systems (hereafter, Th-, Pe-, and Pl-systems), differ in various ways. In the Th-system found in Paratanaoidea and Tanaidoidea, silk is made by mixing secretions from two types of glands (Kaji et al. 2016); in the Pe-system in Phoxokalliapseudes tomiokaensis (Apseudoidea: Kalliapseudidae) and the Pl-system in Parapseudes algicola (Apseudoidea: Parapseudidae), there is no evidence of mixing secretions from multiple types of glands to make silk (Kakui and Hiruta 2014, 2017). Tubes made by the Th- and Pe-systems are stout enough to be picked up by forceps whereas those made by the Pl-system are not, indicating that the components of the tubes are different among systems. The gene expression patterns in Pe- and Pl-systems are similar to Carpoapseudes spinigena, a burrower (Kakui et al. 2020), indicating that the silk-related genes in tube dwellers bearing Pe- and Pl-systems may not be detected in our analysis and may greatly differ from those in tube dwellers bearing the Th-system. Of the three paralogs of caddisworm fibroin-like proteins, only comp408 is conserved in Apseudoidea, suggesting duplication of these genes in the common ancestor of Paratanaoidea and Tanaidoidea, following the divergence of Apseudoidea. Amongst four tanaidids, only Arctotanais alascensis lacks the expression of comp300. We observed that this species...
constructs stouter tubes than those of the other tanaidids (data not shown). The lack of comp300 in A. alascensis may therefore be related to the above difference in the tube feature.

**Materials and Methods**

**Sampling of Tanaidaceans, Mode of Life Investigation, and Silk Collection**

We collected 23 tanaidacean and one isopod species for transcriptomic analyses at various localities presented in supplementary table S1, Supplementary Material online. Fresh animals were fixed in RNAlater Stabilization Solution (Invitrogen) at 4 or −20 °C for 1 day, then kept at −20 °C for several days (only in some specimens due to facility limitation), and finally stored at −80 °C. Species identification of each specimen was made by observation of the specimen before fixation and of other specimens collected at the same locality and judged as conspecific (if available).

To check their mode of life, living individuals of nine tanaidacean species (Apsuedes sp., Paradoxapuses litoralis, Phoxokalliaspeudes toniokaensis, Apsuedomorpha sp., Parapseudes aligcola, and four tanaidid species) and Asselius hilgendorfi were reared at 4, 21, or 25 °C for weeks. The mode of Carpoapuses spinigena was presented by Kakui et al. (2020). The mode of Pseudopharyrus quintolongus was inferred based on the observed mode in the congener, P. malyutinae (Kakui K. unpublished data). The mode of the other species was inferred based on the literature regarding confamilial or congeneric species (cf. Kakui, 2021).

Silk of Z. ezoensis was collected as follows. Around 80 individuals and several pieces of mesh (425-μm mesh opening) were put in a petri dish filled with 0.20-μm filtered seawater in a dark condition at 14 °C. The animals were fed every 3 days with porphyrized dry feed for crayfish (JAN code 1363 8000 0371 00902; Kyorin). The day after feeding, all individuals were transferred to a new dish filled with 0.45-μm filtered seawater twice, and finally to a new dish filled with 0.20-μm mesh open pieces were put in the dish. The day after cleaning, tubes made by tanaidaceans were picked up with fine for-

Transcriptome Sequencing and Assembly

Sample preservation, RNA extraction, sequencing, and assembly were conducted based on methods previously described for spiders (Kono et al. 2016), with some modifications. Briefly, a single specimen of each tanaidacean was preserved in RNAlater Stabilization Solution, then stored at −80 °C. RNA was extracted using a Direct-zol RNA MicroPrep Kit (Zymo Research). The Illumina library was prepared using the KAPA RNA HyperPrep Kit (KAPA Biosystems) targeting 300-bp fragments, but in order to amplify larger silk-related transcripts, PCR extension time was increased to 3 min per cycle. Extracted RNA was below minimum requirement for KAPA RNA HyperPrep Kit for samples R05 Apsuedomorpha sp., R06 Paradoxapuses litoralis, and R24 Heterotanoides sp., and therefore these samples were prepared using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech) also with increased PCR extension time, followed by KAPA HyperPlus Kit (KAPA Biosystems) fragmentation and cDNA library preparation. The sequence library was then sequenced on a NextSeq 500 (Illumina) with 300 cycles of high-output mode as paired-end reads. Sequences were base called and demultiplexed, and adaptor sequences were removed with bcIs2fastq v.2 software (Illumina).

A total of 20 ng of Z. ezoensis extracted RNA was further utilized for long read sequencing. The mRNAs were amplified using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing for 12 PCR cycles, and following purification with 2X AMPure XP (Beckman Coulter), the cDNA was further amplified using ISPCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') for 12 cycles with SeqAmp DNA polymerase (Takara Bio) to eliminate blocked DNA ends. After 0.45X AMPure XP purification to filter out short transcripts, the cDNA sequencing library was prepared using Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies) with Short Fragment Buffer protocol. Resulting cDNA library was sequenced with R9.4.1 flow cell (FLO-MINT06) on a GridION device (Oxford Nanopore Technologies). Basecalling was performed using MinKNOW and the high accuracy mode of Guppy basecaller (Oxford Nanopore Technologies).

Transcriptome assembly was performed using Bridger with default parameters using Illumina reads (Chang et al. 2015). In order to eliminate possible cross contaminations, transcripts with TPM (transcripts per million) value less than 1 and transcript ID over 30,000 were discarded from the following analyses. Assembly completeness was assessed using BUSCO v.3 (Seppey et al. 2019) with the Arthropoda data set through the gVolante server (Nishimura et al. 2017). One SMART-Seq amplified sample R05 Apsuedomorpha sp. resulted in very low BUSCO completeness (66.2%), and this sample was omitted in further analyses.

**Phylogenetic Analysis**

A BUSCO analysis using Drosophilida as the model species was conducted on each tanaidacean genome (Simao et al. 2015). As the Apsuedomorpha sp. showed less than 75% completeness, it was removed from further phylogenetic analysis. Then, complete, single-copy BUSCO sequences (as determined vs. the reference species Drosophila) that were present in 20 out of the 23 remaining taxa (in over 85% of the genomes), were then selected for use in constructing the phylogeny. This generated a multiple sequence alignment of 28 BUSCO genes. Each gene was aligned separately in
MUSCLE (Edgar 2004), and the resultant alignments were concatenated using SequenceMatrix (Vaidya et al. 2011) to produce the analysis data set. A preliminary maximum likelihood phylogenetic tree was constructed using the WAG model in PhyML (Whelan and Goldman 2001; Guindon et al. 2010), and a final maximum likelihood analysis was then undertaken in IQTree under the model recommended by ModelFinder, LG + I + G4 (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017), with 1,000 bootstrap replicates. Following this, an additional Partitioned maximum likelihood analysis was undertaken in order to account for variation in evolutionary rates amongst genes (Lanfear et al. 2012). Partition files were generated using catsequences 1.3 (Creevey and Weeks 2021) and partition models were determined using the greedy partition finding algorithm present in IQTree (Chernomor et al. 2016). Finally, a Bayesian phylogenetic tree was constructed using the GTR + G model in Phylobayes 4.1 (Lartillot and Philippe 2004, 2006; Lartillot et al. 2012). A burnin of 50% of the maximum discrepancies observed over the bipartitions and effective sample size in bpcomp and tracecomp. For all analyses, two independent chains were run. A burnin of 50% of the sample size was used for all analyses, sampling every 50th tree following the burnin period.

Proteome Analysis of the Z. ezoensis Silk

PBS-washed silk samples were immersed in 6 M guanidine-HCl buffer (pH 8.5) and protein extraction was performed by homogenization with BioMasher II (Nippi) and sonication with Bioruptor II (BM Equipment). The extracts containing 10 μg of protein were reacted with dithiotreitol for 30 min at 37 °C followed by iodoacetamide for 30 min at 37 °C in the dark. After 5 times dilution by 50 mM ammonium carbonate in distilled water, the samples were reacted with Lys-C for 3 h at 37 °C followed by trypsin for 16 h at 37 °C to digest proteins. The reaction was quenched by acidification with trifluoroacetic acid and the digested samples were desalted using SDB-XC-StageTips (Rappsilber et al. 2003) followed by drying under reduced pressure.

Each sample was dissolved with 0.1% formic acid (FA) and 2% acetonitrile (ACN) and analyzed with a nanoFlow, and a timsTOF Pro (Bruker Daltonics). Two hundred nanograms of the digest was injected into a column, Aurora UHPLC column C18 1.6 μm (75 μm ID × 250 mm, IonOpticks), and separated by linear gradient elution with two mobile phases: (A) 0.1% FA in water and (B) 0.1% FA in ACN, at the flow rate of 400 nl/ min under the temperature at 50 °C. The mobile phase composition was changed as follows: (A) + (B) = 100%, (B) 2–17% (0–60 min), 17–25% (60–90 min), 25–37% (90–100 min), 37–80% (100–110 min), and 80% (110–120 min). Separated peptide was ionized at 1,600 V and analyzed by PASEF (Meier et al. 2018) scan. Briefly, the PASEF scan was performed at the ion mobility coefficients (1/K0) range from 0.6 to 1.6 V s/cm² within the ramp time of 100 ms keeping the duty cycle at 100%. A MS scan was performed at the mass range from m/z 100 to 1,700, followed by 10 PASEF-MS/MS scans per cycle. Precursor ions was selected from top 12 intense ions in a TIMS-MS survey scan (precursor ion charge: 0–5, intensity threshold: 2,500, target intensity: 20,000). In addition, a polygon filter was applied to the m/z and ion mobility plane to select most likely representing peptide precursors without singly charged ions. Collision-induced dissociation was performed by the default settings (isolation width: 2 Th at m/z 700 and 3 Th at m/z 800, collision energy: 20 eV at 1/k0 0.6 V s/cm² and 59 eV at 1/k0 1.6 V s/cm²).

LC-MS data were analyzed using PEAKS studio X+ (Bioinformatics Solutions) (Zhang et al. 2012) with the following conditions. Briefly, de novo sequencing and database search were performed with the error tolerance of 20 ppm for precursor ions and 0.05 Da for fragment ions. Enzyme was set to trypsin, and up to two missed cleavages was allowed. Carbamidomethylation at cysteine residue was set as a fixed modification. N-acetylation at protein N-term and oxidation at methionine residue were set as variable modifications allowing for up to three positions per peptide. Protein sequence database (383,892 entries) was created from transcriptome assembly data and used for identification. MaxQuant (version 1.6.10.43) (Cox and Mann 2008) contaminants database (245 entries, major experimental contaminants) was used as a contaminant database. Criteria of identification was set to less than 1% FDR at the peptide-spectrum match level. Feature area of each identified peptide ion was calculated automatically with PEAKS software algorithm. The intensity-based absolute quantification value (Schwanhausser et al. 2011) of each identified protein was calculated from the feature area values.

Screening of Silk Genes and Their Conservation Analysis

PEAKS searches did not immediately result in silk-related genes, so the silk genes were screened from the proteome analysis with the following criteria: 1) lack of similarity to proteins in UniProt (BlastP e-value < 1e-15), 2) more than two peptide fragments identified from the protein, 3) consistent detection among replicates (normalized intensities have normalized standard deviation of Z < 1), and 4) repetitive units comprise at least 10% of the sequence as identified by the XSTREAM web service (Newman and Cooper 2007). Nanopore reads were searched using the putative silk transcripts, and the longest reads with the highest sequence identity within the Illumina transcriptome assembly were used to obtain the full-length sequence. Extracted nanopore reads corresponding to the putative silk transcripts were error corrected using proovread with the Illumina reads (Hackl et al. 2014). Using the full-length gene sequences as well as their translated amino acid sequences, conservation was analyzed
Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

Acknowledgments

The authors are grateful to Yuki Takai (Keio University, KU) for experimental support; Ken Fujimoto (National Research Institute of Fisheries Science, NRIFS), Jun Hashimoto (Nagasaki University, NU), Hideki Kaeriyama (NRIFS), Hisanori Kohtsuka (The University of Tokyo, UT), Hiroaki Nakano (University of Tsukuba), Shouzo Ogiso (Kanazawa University, KaU), and Michitaka Shimomura (Kyoto University, KyU) for the opportunity to join research cruises and joint surveys; the captains and crews of R/V Aosagi (KyU), R/V Mirai (JAMSTEC), TRV Nagasaki-maru (NU), RV Rinkai-maru (UT), TR/V Seisui-maru (Mie University), RV Soyo-maru (NRIFS), and RV Tansei-maru (JAMSTEC), and technicians and researchers on board for support during cruises; Shinta Fujimoto (Tohoku University), Futoshii Kakizoe (Port of Nagoya Public Aquarium, PNPA), Hinako Katsushima (Hokkaido University, HU), Hisanori Kohtsuka (UT), Tsuyoshi Matsuda (PNPA), Akira Ogushi (PNPA), Susumu Ohtsuka (Hirosima University), Koji Shibazaki (HU), Takako Suzuki (Yokohama Hakkeijima Sea Paradise), and the Japanese Association for Marine Biology (JAMBO) Coastal Organism Joint Survey for contributing specimens; Hinako Katsushima (HU), Nobuya Okamoto (HU), Akari Sato (HU), and Michitaka Shimomura (KyU) for help in identification of some specimens; and Atsushi C Suzuki (HU) for inspiring and encouraging this project. This study is an outcome of the following project directed by Y.F.: “Aleutian Magic” observations in the south-eastern Bering Sea. This research was supported by research funds from the Yamagata Prefectural Government and Tsuruoka City, KAKENHI grants JP100J119, JP16K18597, JP16H04611, and JP19K06800 from the Japan Society for the Promotion of Science (JSPS), research funds from JAMBO, and research funds from the Research Institute of Marine Invertebrates Foundation.

Data Availability

The transcriptome sequence data have been deposited in the Sequence Read Archive under BioProject ID PRJNA718396, and the mass spectrometry proteomics data have been deposited as PXD024972 for ProteomeXchange and JPST001114 for jPOST. Transcriptome assembly is available through NCBI/TSA under accession numbers GICB00000000, GICC00000000, GICD 00000000, GICE00000000, GICF00000000, GICG000000 0, GICHH00000000, GICIO00000000, GICJ00000000, GICK 00000000, GICLO00000000, GICM00000000, GICN000000 0, GICOO00000000, GICP00000000, GICQ00000000, GIC R00000000, GICS00000000, GICT00000000, GICU00000 00, GICV00000000, and GJCV00000000. Nucleotide and amino acid sequences of fibrinos are deposited in GenBank under accession numbers OK382955–OK382960. All of the alignments and tree files used are deposited at FigShare https://doi.org/10.6084/m9.figshare.16750393.v1.

Literature Cited

Abascon NC, Regan L. 2018. The past, present and future of protein-based materials. Open Biol. 8(10):180113.
Anderson G. 2020. Tanaidacea—forty years of scholarship, version 3.0. [Internet]. Available from: https://aquila.usm.edu/tanaisd305/.
Ashton NN, Pan H, Stewart RJ. 2016. Connecting caddisworm silk structure and mechanical properties: combined infrared spectroscopy and mechanical analysis. Open Biol. 6(6):160067.
Ashton NN, Stewart RJ. 2019. Aquatic caddisworm silk is solidified by environmental metal ions during the natural fiber-spinning process. FASEB J. 33(1):572–582.
Bauer R, Holt G. 1998. Simultaneous hermaphroditism in the marine shrimp Lysmata wurdemanni (Caridea: Hippolytidae): an undescribed sexual system in the decapod Crustacea. Mar Biol. 132(2):223–225.
Bird GI, Webber WR. 2015. The first pagurapseudid species (Peracarida: Tanaidacea) from New Zealand with remarks on paguridean (Decapoda: Paguridae) and pagurapseudid convergence and competition. Zootaxa 3995:149–168.
Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 34(5):525–527.
Chang Z, et al. 2015. BridgeR: a new framework for de novo transcriptome assembly using RNA-seq data. Genome Biol. 16:30.
Chenomor O, von Haeseler A, Minh BQ. 2016. Terrace aware data structure for phylogenomic inference from supermatrices. Syst Biol. 65(6):997–1008.
Cox J, Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 34(5):525–527.
Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5):1792–1797.
Gardiner LF. 1975. The systematics, postmarsupial development, and ecology of the deep-sea family Neotanaiidae (Crustacea: Tanaidacea). Smithson Contrib Zool. 170:1–265.
Gasteiger E, et al. 2005. Protein identification and analysis tools on the ExPASy server. In: Walker JM, editor. The proteomics protocols handbook. Totowa (NJ): Humana Press. p. 571–607.
Guindon S, et al. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 59(3):307–321.
Hackl T, Hedrich R, Schulz J, Forster F. 2014. proovread: large-scale high-accuracy PacBio correction through iterative short read consensus. Bioinformatics 30(21):3004–3011.
Johnson S, Attamadad Y. 1982a. Reproductive behaviour and larval development of Tanais cavolinii (Crustacea: Tanaidacea). Mar Biol. 71(1):11–16.

Johnson S, Attamadad Y. 1982b. A functional-morphological model of Tanais cavolinii Milne-Edwards (Crustacea: Tanaidacea) adapted to a tubiculous life-strategy. Senisja 67(1):29–42.

Kaji T, Kakui K, Miyazaki N, Murata K, Palmer AR. 2016. Evaluation of Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: a fast model selection for accurate phylogenetic estimates. Mol Biol Evol. 34(10):2725–2739.

Kakui K. 2016. Review of the taxonomy, diversity, ecology, and other biological aspects of order Tanaidacea from Japan and surrounding waters. In: Motokawa M, Kajihara H, editors. Species diversity of animals in Japan. Berlin (Germany): Springer. p. 603–627.

Kakui K. 2019. Shell-exchange behavior in a hermit-crab-like tanaidacean (Crustacea: Malacostraca). Zool Sci. 36(6):468–470.

Kakui K. 2021. Tube-constructing paratanaoidean tanaidaceans (Crustacea: Peracarida): a brief review. Aquat Anim. 2021:AA2021–2024.

Kakui K, Hiruta C. 2014. Diverse perepodal secretory systems implicated in thread production in an apseudomorph tanaidacean crustacean. J Morphol. 275(9):1041–1052.

Kakui K, Hiruta C. 2017. Tube construction by a tanaidacean crustacean using a novel mucus secretion system involving the anal opening. Zool Lett. 3:20.

Kakui K, Katoh T, Hiruta SF, Kobayashi N, Kajihara H. 2011. Molecular systematic of Tanaidacea (Crustacea: Peracarida) based on 18S sequence data, with an amendment of suborder/superfamily-level classification. Zool Sci. 28(10):749–757.

Kakui K, Nomaki H, Komatsu H, Fujivara Y. 2020. Unexpected low genetic differentiation between Japan and Bering Sea populations of a deep-sea benthic crustacean lacking a planktonic larval stage (Peracarida: Tanaidacea). Biol J Linn Soc. 131(3):566–574.

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods. 14(6):587–589.

Larsen K, G ¸u M, Sieg J. 2015. Toughened hydrogels inspired by aquatic caddisworm silk. Soft Matter. 11(35):6981–6990.

Larsson C, Calcott B, Ho SY, Guindon S. 2012. Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol. 29(6):1695–1701.

Lang K. 1956. Neotanaidae nov. fam., with some remarks on the phylogeny of the Tanaidacea. Arkiv För Zoologi. 9:469–475.

Larsen K. 2005. Deep-sea Tanaidacea (Peracarida) from the Gulf of Mexico. Leiden (The Netherlands): Brill.

Larsen K, Gutu M, Sieg J. 2015. Order Tanaidacea Dana, 1849. In: von Vaupel Klein J, Charmantier-Daures M, Schram F, editors. The Crustacea. Revised and updated, as well as extended from the Traité de Zoologie S. Leiden (The Netherlands): Brill. p. 249–329.

Lartillot N, Brinkmann H, Philippe H. 2007. Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. BMC Evol Biol. 7(Suppl 1):54.

Lartillot N, Philippe H. 2004. A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. Mol Biol Evol. 21(6):1095–1109.

Lartillot N, Philippe H. 2006. Computing Bayes factors using thermodynamic integration. Syst Biol. 55(2):195–207.

Lauterbach KE. 1970. Der Cephalothorax von Tanais cavolinii Milne Edwards (Crustacea-Malacostraca). Ein Beitrag zur vergleichenden Anatomie und Phylogenie der Tanaidacea. Zool Jahrb Abt Anat Ontog Tiere. 87:94–204.

Meier F, et al. 2018. Online parallel accumulation-serial fragmentation (PASEF) with a novel trapped ion mobility mass spectrometer. Mol Cell Proteomics. 17(12):2534–2545.

Newman AM, Cooper JB. 2007. XSTREAM: a practical algorithm for identification and architecture modeling of tandem repeats in protein sequences. Bioinformatics. 33(22):3635–3637.

Numata K. 2020. How to define and study structural proteins as biopolymer materials. Polym J. 52(9):1043–1056.

Rappsilber J, Shihiama Y, Mann M. 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem. 75(3):663–670.

Schwanhauser B, et al. 2011. Global quantification of mammalian gene expression control. Nature 473(7347):337–342.

Seppey M, Manni M, Zdobnov EM. 2019. BUSCO: assessing genome assembly and annotation completeness. Methods Mol Biol. 1962:227–245.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31(19):3210–3212.

Sutherland TD, Young JH, Weissman S, Hayashi CY, Merritt DJ. 2010. Insect silk: one name, many materials. Annu Rev Entomol. 55:171–188.

Tanais cavolinii Milne-Edwards (Crustacea, Tanaidacea) adapted to a heterogeneities in the amino-acid replacement process. Mol Biol Evol. 2(16):1095–1109.

Vaidya G, Lohman DJ, Meier R. 2011. SequenceMatrix: concatenation and architecture modeling of tandem repeats in protein sequences. BMC Bioinformatics. 8:382.

Wang CS, Pan H, Weerasekare GM, Stewart RJ. 2015. Peroxidase-catalyzed interfacial adhesion of aquatic caddisworm silk. J R Soc Interface. 12:20150710.

Whelan S, Goldman N. 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol Biol Evol. 18(5):691–699.

Zhang J, et al. 2012. PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. Mol Cell Proteomics. 11(4):M111.010587.