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# Molecular Phylogeny of the Family Capitellidae (Annelida)

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Capitellids have emerged as monophyletic in most but not all recent molecular phylogenies, indicating that more extensive taxon sampling is necessary. In addition, monophyly of most or all capitellid genera was questionable, as some diagnostic characters vary ontogenetically within individuals. We tested the monophyly of Capitellidae and eight capitellid genera using phylogenetic analyses of combined 18S, 28S, H3, and COI gene sequences from 36 putative capitellid species. In our trees, Capitellidae formed a monophyletic sister group to Echiura, and *Capitella* was also monophyletic, separated by a long branch from other capitellids. Well-supported clades each containing representatives of different genera, or containing a subset of species within a genus, indicated that *Barantolla*, *Heteromastus*, and *Notomastus* are likely not monophyletic. We mapped three morphological characters traditionally used to define capitellid genera (head width relative to width of first segment, number of thoracic segments, and number of segments with capillary chaetae) onto our tree. While *Capitella* showed unique character states, states in the other genera were decidedly not phylogenetically informative. Morphology-based capitellid taxonomy will require a fine-scale reevaluation of character states and detection of new characters.

**Key words:** Capitellida, capitellids, Scolecida, Sedentaria, systematics

## INTRODUCTION

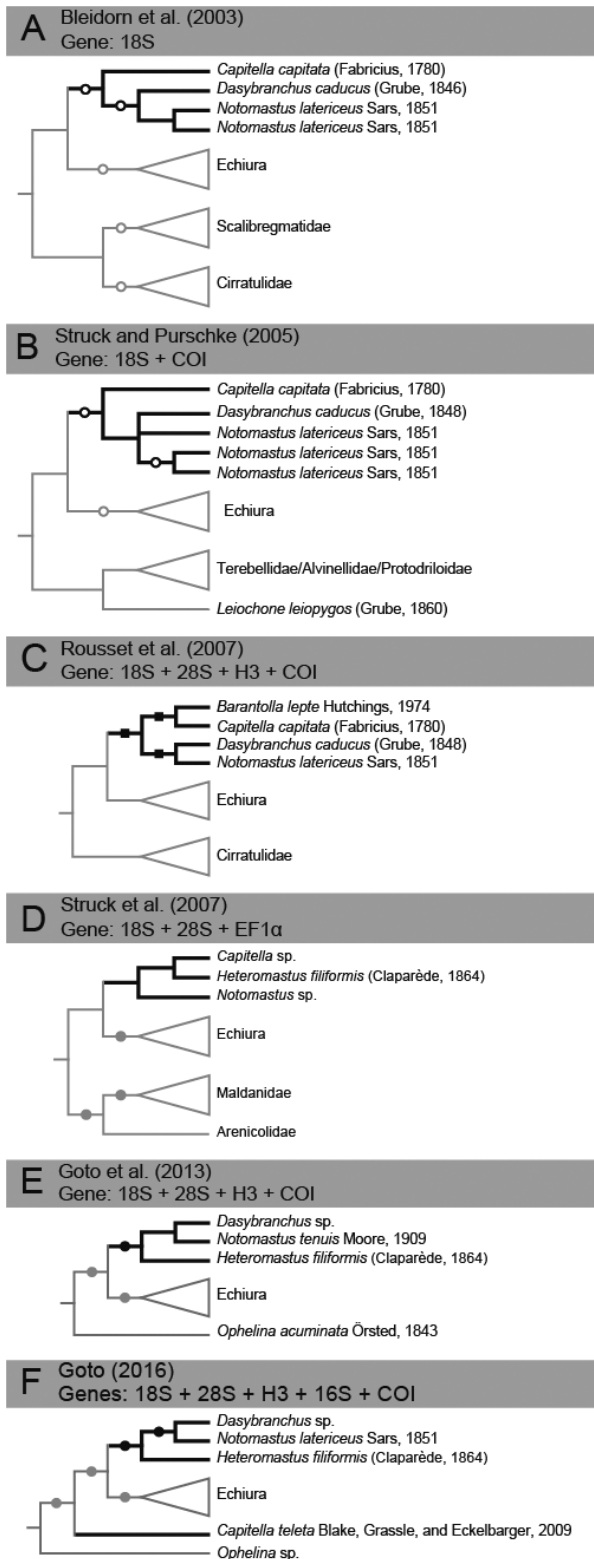
The family Capitellidae Grube, 1862 consists of free-living, benthic, earthworm-like annelids and contains about 190 species (Rouse and Pleijel, 2001; Read, 2010). It includes *Capitella teleta* Blake, Grassle & Eckelbarger, 2009, formerly known as “*Capitella* sp. I” (Grassle and Grassle, 1976), which is now used as a model annelid in the field of evolutionary developmental biology, and for which the complete genome has been sequenced (e.g., Ferrier, 2012; Seaver et al., 2012; Lauri et al., 2014; Seaver, 2016). Furthermore, some other species in the genus *Capitella* have been used as bioremediators of organically enriched sediments (e.g., Tsutsumi et al., 2005; Kinoshita et al., 2008) or environmental bioindicators of disturbed habitats (e.g., Reish, 1955; Grassle and Grassle, 1976; reviewed in Dean, 2008). While all capitellids are similar in external morphology, they have successfully colonized diverse environments. Although most species inhabit bottom substrata such as polluted sediments (e.g., Tsutsumi, 1987; Ahn et al., 1995; Méndez et al., 2001; Dean, 2008; Croquer et al., 2016), sandy beaches (e.g., Delgado et al., 2003; Incera et al., 2006; Papageorgiou et al., 2006; García-Garza and De León-González, 2011; Tomioka et al., 2012), and seagrass beds (e.g., Nakaoka et al., 2002; Omena and Creed, 2004; Eklöf et al., 2005; Tanner, 2005; Tomioka et al., 2013), some dwell among squid egg masses (e.g., Hartman, 1947), or burrow into molluscan shells (Blake, 1969) or whale bones

(e.g., Fujiwara et al., 2007; Amon et al., 2013; Silva et al., 2016; Sumida et al., 2016). Capitellidae is one of the few metazoan groups where some species exhibit poecilogony (e.g., Chia et al., 1996; Tsutsumi, 2005), or polymorphism in larval development, which has been reported only in some other polychaete groups (e.g., cirratulids, Petersen, 1999; spionids, David et al., 2014) and gastropod molluscs (e.g., Vedetti et al., 2012; McDonald et al., 2014). While capitellids are thus evolutionarily interesting, their phylogenetic relationships have scarcely been studied.

Since Eisig's (1887) extensive monograph on Capitellidae, this family has remained a taxonomically well-defined group (e.g., Hartman, 1947; Fauchald, 1977; Hutchings, 2000). In both morphology-based cladistic analyses (Rouse and Fauchald, 1997) and subsequent molecular analyses of annelid phylogeny (Bleidorn et al., 2003; Struck and Purschke, 2005; Rousset et al., 2007; Struck et al., 2007; Goto et al., 2013), Capitellidae has nearly always emerged as a monophyletic group (Fig. 1A–E). On the other hand, some 19th century naturalists (Carus, 1863; Malmgren, 1865; Haeckel, 1866; Quatrefages, 1866) did not regard Capitellidae as a group, and in Goto's (2016) multi-locus analysis of Echiura, the putative sister taxon to Capitellidae (Weigert et al., 2014), the four capitellid species included as outgroup taxa did not comprise a monophyletic group (Fig. 1F), although the author himself suggested that this might have been the result of poor taxon sampling of capitellids. The monophyly of capitellids thus requires further testing with increased taxon sampling.

Capitellidae presently includes 44 genera (Fauchald, 1977; Piltz, 1977; McCammon and Stull, 1978; Rullier and

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**Fig. 1.** (A–F) Partial tree topologies from selected, previous molecular studies that included more than two OTUs from Capitellidae. Nodes with open circles are supported with a bootstrap value (BS) greater than 90% in maximum-parsimony analyses and a posterior probability (PP) of 1.00 from Bayesian inference (BI). Nodes with solid circles are supported with a BS greater than 90% in maximum-likelihood analyses and 1.00 PP from BI. Nodes with solid squares are supported by a jackknife value greater than 90%.

Amoureux, 1979; Amaral, 1980; Ewing, 1982; Amoureux, 1983; Kirkegaard, 1983; Brown, 1987; Garwood and Bamber, 1988; Warren, 1991; Capaccioni-Azzati and Martín, 1992; Warren and Parker, 1994; Blake, 2000; Buzhinskaja and Smirnov, 2000; Green, 2002; Magalhães and Bailey-Brock, 2012), most of which are likely not monophyletic, and among which phylogenetic relationships remain uninvestigated. Rouse and Pleijel (2001: p. 44) remarked, “the taxonomy of Capitellidae is in serious need of revision”, because genera were, and still are, defined in a way that does not reflect phylogeny. Genera have been diagnosed based on unique combinations of morphological characters, such as the shape of the head, number of thoracic segments, arrangement/type of chaetae, presence or absence of branchiae, and shape of the pygidium (Fauchald, 1977; Blake et al., 2009). This ‘unique combination’ approach itself is unlikely to lead to monophyletic taxa (Sundberg, 1993; Sundberg and Pleijel, 1994), and one diagnostic character, the arrangement of chaetae, is known to change ontogenetically within individuals (Ewing, 1982; Fredette, 1982; George, 1984).

In this study, we conducted a molecular phylogenetic analysis of capitellid worms from Japanese waters to test the monophyly of Capitellidae and some of its constituent genera. This allowed us to also examine whether some of the generic diagnostic characters mentioned above are concordant with phylogenetic relationships.

## MATERIALS AND METHODS

### Sampling and identification

About 400 specimens of capitellid polychaetes were collected at 29 localities around Japan from 2012 to 2016. The anterior portions of specimens were fixed in 10% formalin–seawater and later transferred into 70% ethanol for morphological observation; the posterior or middle portions were preserved in 99% ethanol for DNA extraction. DNA was extracted from a few individuals from each locality, and then molecularly barcoded using the genes H3 (nuclear histone H3), 18S (nuclear 18S ribosomal RNA), and COI (mitochondrial cytochrome c oxidase subunit I). From 42 specimens from which a sequence was obtained for at least one of these markers, 31 were selected to represent putative species (operational taxonomic units, or OTUs) to be included in analyses (Table 1). As outgroup taxa, one individual each of the maldanid *Nicomache personata* Johnson, 1901 (partially sequenced by Kajihara et al., 2014) and the nauid oligochaete *Tubifex tubifex* (Müller, 1774) (cultured by Professor Takashi Shimizu, Hokkaido University) were sequenced (Table 1). All specimens not destroyed for DNA extraction have been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo (Table 1).

Capitellid specimens were identified to the genus level based on combinations of the following five characters: *i*) width of the head relative to the first segment (Type A, head half the width of the first segment; Type B, head same width as first segment) (Fig. 2A, B); *ii*) number of thoracic segments; *iii*) arrangement/type of chaetae; *iv*) presence or absence of branchiae; and *v*) shape of the pygidium. The 31 specimens selected for analysis, each representing a single, putative species, were identified as representing eight genera (Table 2). Because most specimens were missing the posterior portion of the body upon collection, characters *iv*) and *v*) were not included in the character-state analysis. The first character (head type) was consistent irrespective of the state of the proboscis (*i.e.*, whether it was extruded or retracted) or the degree of body contraction (ST pers. obs.).

**Table 1.** List of specimens used in this study, with species name, sampling locality or source, habitat, DDBJ/ENA/GenBank accession numbers for 18S, 28S, H3, and COI sequences, the museum catalogue number (ICHUM), and references.

| Species  | Sampling locality or source   | Habitat   | Accession number |          |          |          | Catalogue number | Reference                         |
|--|---|---|------------------|----------|----------|----------|------------------|-----------------------------------|
|  |   |   | 18S              | 28S      | H3       | COI      |                  |                                   |
| Ingroup  |   |   |                  |          |          |          |                  |                                   |
| <i>Barantolla</i> sp. 1  | Muroran, Hokkaido<br>(42.3049°N, 140.9885°E)                          | seagrass bed  | LC208025         | LC208055 | LC208087 | LC208116 | 5397             | this study                        |
| <i>Barantolla</i> sp. 2  | Oura Bay, Okinawa<br>(26.5540°N, 128.0416°E)                          | tidal mud flat  | LC208026         | LC208056 | LC208088 | LC208117 | 5398             | this study                        |
| <i>Capitella teleta</i> Blake, Grassle & Eckelbarger, 2009 (1) | Ainan, Ehime<br>(32.9206°N, 132.5189°E)                               | muddy sediments<br>beneath aquaculture<br>rafts   | LC208027         | LC208057 | LC208089 | LC120636 | 5167             | Tomioka et al. (2016), this study |
| <i>Capitella teleta</i> 2                                      | GenBank   | –   | JF509728         | –        | JF509713 | –        | –                | Andrade et al. (2011)             |
| <i>Capitella</i> aff. <i>teleta</i>                            | Gamo, Miyagi<br>(38.2578°N, 141.0144°E)                               | tidal mud flat  | LC208028         | LC208058 | LC208090 | LC120642 | 5130             | Tomioka et al. (2016), this study |
| <i>Capitella</i> sp. 1   | Monbetsu, Hokkaido<br>(44.3645°N, 143.3546°E)                         | seagrass bed  | LC208029         | LC208059 | LC208091 | LC120651 | 5399             | Tomioka et al. (2016), this study |
| <i>Capitella</i> sp. 2   | Akkeshi, Hokkaido<br>(43.0185°N, 144.8346°E)                          | seagrass bed  | –                | LC208060 | –        | LC208118 | 5400             | this study                        |
| <i>Capitella</i> sp. 3   | Onosato River, Osaka<br>(34.3765°N, 135.2509°E)                       | tidal mud flat  | LC208030         | LC208061 | LC208092 | LC012038 | 5401             | this study                        |
| <i>Capitella</i> sp. 4   | Manko-Higata, Okinawa<br>(26.1941°N, 127.6836°E)                      | tidal mud flat  | LC208031         | LC208062 | LC208093 | –        | 5402             | this study                        |
| <i>Capitella</i> sp. 5   | Hatsukaichi, Hiroshima<br>(34.2756°N, 132.2669°E)                     | tidal mud flat  | LC208032         | LC208063 | LC208094 | –        | 5403             | this study                        |
| <i>Decamastus</i> sp. 1  | Hachi-Higata, Hiroshima<br>(34.3260°N, 132.8979°E)                    | tidal mud flat  | LC208033         | LC208064 | LC208095 | –        | 5404             | this study                        |
| <i>Decamastus</i> sp. 2  | off Manabe Island<br>(34.3772°N, 133.6162°E)                          | unknown [a biological<br>dredge from the TR/V<br>Toyoshio-maru<br>(Hiroshima University)] | LC208034         | LC208065 | LC208096 | LC208119 | 5405             | this study                        |
| <i>Heteromastus</i> sp. 1                                      | Akkeshi Lake, Hokkaido<br>(43.0515°N, 144.8568°E)                     | tidal mud flat  | LC208035         | LC208066 | LC208097 | LC208120 | 5406             | this study                        |
| <i>Heteromastus</i> sp. 2                                      | Hachi-Higata, Hiroshima<br>(34.3260°N, 132.8979°E)                    | tidal mud flat  | LC208036         | LC208067 | LC208098 | LC208121 | 5407             | this study                        |
| <i>Heteromastus</i> sp. 3                                      | Nagura-Amparu,<br>Ishigaki Island, Okinawa<br>(24.4007°N, 124.1407°E) | tidal mud flat  | LC208037         | LC208068 | LC208099 | LC208122 | 5408             | this study                        |
| <i>Heteromastus</i> sp. 4                                      | Sone-Higata, Fukuoka<br>(33.8243°N, 130.9663°E)                       | tidal mud flat  | LC208038         | LC208069 | LC208100 | LC208123 | 5409             | this study                        |
| <i>Heteromastus</i> sp. 5                                      | Fushino River, Yamaguchi<br>(34.0215°N, 131.4154°E)                   | tidal mud flat  | LC208039         | LC208070 | LC208101 | LC208124 | 5410             | this study                        |
| <i>Heteromastus</i> sp. 6                                      | Edo River, Tokyo<br>(35.7007°N, 139.9252°E)                           | tidal mud flat  | –                | LC208071 | LC208102 | LC208125 | 5411             | this study                        |
| <i>Heteromastus</i> sp. 7                                      | Manose River, Kagoshima<br>(31.4487°N, 130.2949°E)                    | tidal mud flat  | LC208040         | LC208072 | LC208103 | LC208126 | 5412             | this study                        |
| <i>Heteromastus</i> sp. 8                                      | Koajiro-Higata, Kanagawa<br>(35.1635°N, 139.6296°E)                   | tidal mud flat  | LC208041         | LC208073 | –        | LC208127 | 5413             | this study                        |
| <i>Heteromastus filiformis</i><br>(Claparède, 1864)            | GenBank   | –   | DQ790081         | DQ790038 | –        | –        | –                | Struck et al. (2007)              |
| <i>Leiohrides</i> sp. 1  | Hachi-Higata, Hiroshima<br>(34.3260°N, 132.8979°E)                    | tidal mud flat  | LC208042         | LC208074 | LC208104 | LC208128 | 5414             | this study                        |
| <i>Leiohrides</i> sp. 2  | Koajiro-Higata, Kanagawa<br>(35.1635°N, 139.6296°E)                   | tidal mud flat  | LC208043         | LC208075 | LC208105 | –        | 5415             | this study                        |

Table 1. Continued.

| Species  | Sampling locality or source                               | Habitat  | Accession number |          |          |          | Catalogue number | Reference                                     |
|--|---|--|------------------|----------|----------|----------|------------------|---|
|  |   |  | 18S              | 28S      | H3       | COI      |                  |   |
| <i>Leiohrides</i> sp. 3  | Komi, Iriomote Island, Okinawa<br>(24.3511°N, 123.9381°E) | dead-coral debris sediment on tidal flat   | LC208044         | LC208076 | –        | –        | 5416             | this study                                    |
| <i>Mastobranchus</i> sp. 1                                       | Komi, Iriomote Island, Okinawa<br>(24.3511°N, 123.9381°E) | dead-coral debris sediment on tidal flat   | LC208045         | –        | LC208106 | LC208020 | 5417             | this study                                    |
| <i>Mediomastus opertaculeus</i> Tomioka, Hiruta & Kajihara, 2013 | Abashiri, Hokkaido<br>(44.0493°N, 144.2596°E)             | seagrass bed   | LC208046         | LC208077 | LC208107 | AB794985 | 4372             | Tomioka et al. (2013), this study             |
| <i>Notomastus</i> sp. 1  | Tama River, Tokyo<br>(35.5411°N, 139.4401°E)              | tidal mud flat   | LC208047         | LC208078 | LC208108 | LC208129 | 5424             | this study                                    |
| <i>Notomastus</i> sp. 2  | Yagachi Island, Okinawa<br>(26.6507°N, 128.0091°E)        | tidal mud flat   | LC208048         | LC208079 | LC208109 | LC208130 | 5425             | this study                                    |
| <i>Notomastus</i> sp. 3  | off Owase<br>(34.1611°N, 136.8436°E)                      | unknown [a biological dredge from the TR/V Seisui-maru (Mie University)]         | LC208049         | LC208080 | LC208110 | –        | 5426             | this study                                    |
| <i>Notomastus</i> sp. 4  | Suou-Nada<br>(33.7475°N, 131.5810°E)                      | unknown [a biological dredge from the TR/V Toyoshio-maru (Hiroshima University)] | LC208050         | LC208081 | LC208111 | –        | 5427             | this study                                    |
| <i>Notomastus</i> sp. 5  | Torinoe-Higata, Okayama<br>(34.4734°N, 133.5296°E)        | tidal mud flat   | LC208051         | LC208082 | LC208112 | LC208131 | 5428             | this study                                    |
| <i>Notomastus</i> sp. 6  | Takahashi River, Okayama<br>(34.5427°N, 133.7025°E)       | tidal mud flat   | LC208052         | LC208083 | LC208113 | LC208132 | 5429             | this study                                    |
| <i>Notomastus</i> sp. 7  | Fukiage Beach, Kagoshima<br>(31.4416°N, 130.2832°E)       | open sandy beach   | LC208053         | LC208084 | LC208114 | –        | 5430             | this study                                    |
| <i>Notomastus hemipodus</i> Hartman, 1945                        | GenBank<br>(Bamfield, Canada)                             | –  | HM746728         | –        | HM746759 | –        | –                | Paul et al. (2010)                            |
| <i>Notomastus latericeus</i> Sars, 1851                          | GenBank<br>(Bohuslän, Sweden)                             | –  | AY040697         | –        | DQ779747 | –        | –                | Siddall et al. (2001), Roussett et al. (2007) |
| <i>Notomastus tenuis</i> Moore, 1909                             | GenBank   | –  | DQ790084         | DQ790044 | –        | –        | –                | Struck et al. (2007)                          |
| Outgroup   |   |  |                  |          |          |          |                  |   |
| <i>Arhynchite hayaoi</i> Tanaka & Nishikawa, 2013                | GenBank   | –  | AB771462         | AB771474 | AB771487 | AB771495 | –                | Goto et al. (2013)                            |
| <i>Bonellia</i> sp.  | GenBank   | –  | AB771463         | AB771475 | AB771488 | AB771496 | –                | Goto (2016)                                   |
| <i>Ikedosome gogoshimense</i> Ikeda, 1904                        | GenBank   | –  | AB967989         | AB968002 | AB968013 | AB968026 | –                | Tanaka et al. (2014)                          |
| <i>Listriolobus sorbillans</i> (Lampert, 1883)                   | GenBank   | –  | AB967995         | AB968006 | AB968019 | AB968032 | –                | Tanaka et al. (2014)                          |
| <i>Lumbricus polyphemus</i> (Fitzinger, 1833)                    | GenBank   | –  | HQ728904         | HQ728938 | –        | –        | –                | James and Davidson (2012)                     |
| <i>Nicomache personata</i> Johnson, 1901                         | Oshoro Bay, Hokkaido<br>(43.3639°N, 141.4317°E)           | seagrass bed   | LC006051         | LC208085 | LC005496 | LC006052 | –                | Kajihara et al. (2014), this study            |
| <i>Ophelina acuminata</i> Örsted, 1843                           | GenBank   | –  | DQ790085         | DQ790045 | HM746761 | –        | –                | Struck et al. (2007)                          |
| <i>Tubifex tubifex</i> (Müller, 1774)                            | Cultured by Professor Takashi Shimizu                     | soft mud in a vat  | LC208054         | LC208086 | LC208115 | LC208133 | 5431             | this study                                    |

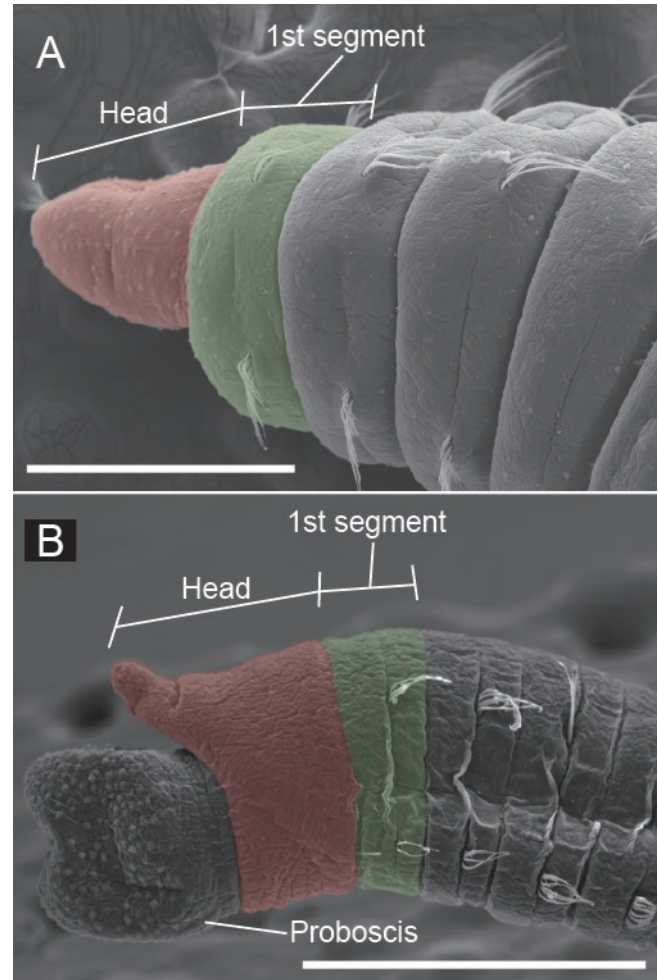
### DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from the middle or posterior portion of worms with a DNeasy Blood & Tissue Kit (Qiagen, Germany). Four regions [18S, 28S (nuclear 28S rRNA), H3, and COI] were sequenced for the 31 capitellid and two outgroup species. Primers used for PCR amplification and sequencing are listed in Table 3. Based on the sequence of *Mediomastus opertaculeus* Tomioka, Hiruta & Kajihara, 2013, a specific primer (Mastus\_COI\_F) was designed by using Primer3Plus (Rozen and Skaletsky, 2000). PCRs were performed with an iCycler thermal cycler (BioRad, USA) in 10- $\mu$ l reaction volumes each containing 1  $\mu$ l of total DNA template, 1  $\mu$ l of 10 $\times$  Ex Taq buffer (TaKaRa Bio, Japan), 2 mM each dNTP, 1  $\mu$ M each primer, and 0.25 U of TaKaRa Ex Taq DNA polymerase (5 U/ $\mu$ l; TaKaRa Bio) in deionized water. PCR conditions were 95°C for 90 s; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min (COI, H3) or 2 min (18S, 28S); and 72°C for 7 min. PCR products were purified following the method of Boom et al. (1990) with some modifications (Kobayashi et al., 2009). Terminator reactions were done with a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Life Technologies, USA) following the manufacturer's protocol. Sequencing was performed with a 3730 DNA Analyzer (Applied Biosystems, USA). Sequences were checked and assembled by using MEGA ver. 5.2.2 (Tamura et al., 2011). They have been deposited in the DDBJ/ENA/GenBank databases under accession numbers LC012038 and LC208025–LC208133. In addition, our dataset contained 37 sequences downloaded from public databases (Table 1).

### Phylogenetic tree construction

To reconstruct phylogeny, we analyzed a combined dataset (18S, 28S, H3, and COI) using maximum likelihood (ML) and Bayesian inference (BI). The outgroup, augmented with additional taxa chosen by reference to recent annelid phylogenomic studies (Struck et al., 2011, 2015; Weigert et al., 2014), contained eight species representing Sedentaria. Sequences were aligned by using MAFFT ver. 7 (Katoh and Standley, 2013) under the options *E-INS-i* for 18S and 28S, and *Auto* for H3 and COI. trimAl ver. 1.2 (Capella-Gutiérrez et al., 2009) was used to remove poorly aligned regions (18S, 693 bases including indels; 28S, 441 bases including indels; COI, six bases including indels; H3, five bases) with the *strict* option. The aligned dataset of 44 species (36 ingroup capitellids and 8 outgroup taxa) comprised 18S (1250 bases including indels), 28S (576 bases including indels), H3 (317 bases without indels), and COI (529 bases including indels).

For the ML analysis, the partition method implemented in RAxML ver. 8.0 (Stamatakis, 2014) was used with the GTR + G substitution model; nodal support values were obtained through ML analyses of 1000 bootstrap pseudoreplicates (Felsenstein, 1985; Tavaré, 1986). For the BI analysis, MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) was used with the following substitution models selected by PartitionFinder ver. 1.1.1 (Lanfear et al., 2012) for the partitions: K80 + I + G for 18S and H3 (first and third codon positions); GTR + G for 28S; HKY + I + G for H3 (second codon position) and COI (first and second codon positions); and GTR + I + G for COI (third codon position). A Markov chain Monte Carlo analysis (MCMC) was simulated for 10 million generations and sampled every 100 generations. The first 25% of the trees were discarded as burn-in. The trace files generated by the MCMC runs were inspected in Tracer ver. 1.6.0 (Rambaut



**Fig. 2.** SEM images showing the two types of head in Capitellidae, with the boundary between the head and first segment labeled. **(A)** *Capitella teleta* (Type A). **(B)** *Mediomastus opertaculeus* (Type B); the width ratio between the head and first segment is constant even when the proboscis is retracted. Scale bars: 1 mm.

**Table 2.** List of selected morphological character states of capitellid genera in this study. For shape of the head: A, head half the width of first segment; B, head same width as first segment. Asterisk (\*) indicates abdominal segments with some pairs of fascicles including both capillary chaetae and hooded hooks on dorsal side.

| Genus                | Number of thoracic segments | Number of segments with capillary chaetae | Presence or absence of branchiae | Shape of the pygidium         | Shape of the head |
|----------------------|-----------------------------|---|----------------------------------|-------------------------------|-------------------|
| <i>Barantolla</i>    | 11                          | 6   | unknown                          | unknown                       | B                 |
| <i>Capitella</i>     | 9                           | 7 or 9                                    | absent or unknown                | without appendages or unknown | A                 |
| <i>Decamastus</i>    | 10                          | 10  | unknown                          | unknown                       | B                 |
| <i>Heteromastus</i>  | 11                          | 5   | unknown                          | unknown                       | B                 |
| <i>Leiochrides</i>   | 12                          | 12  | present                          | unknown                       | B                 |
| <i>Mastobranchus</i> | 11                          | 11*                                       | unknown                          | unknown                       | B                 |
| <i>Mediomastus</i>   | 10                          | 4   | absent                           | with a caudal cirrus          | B                 |
| <i>Notomastus</i>    | 11                          | 11  | unknown                          | unknown                       | B                 |

**Table 3.** List of PCR and cycle sequencing primers used in this study with genes, primer names, sequences, and references.

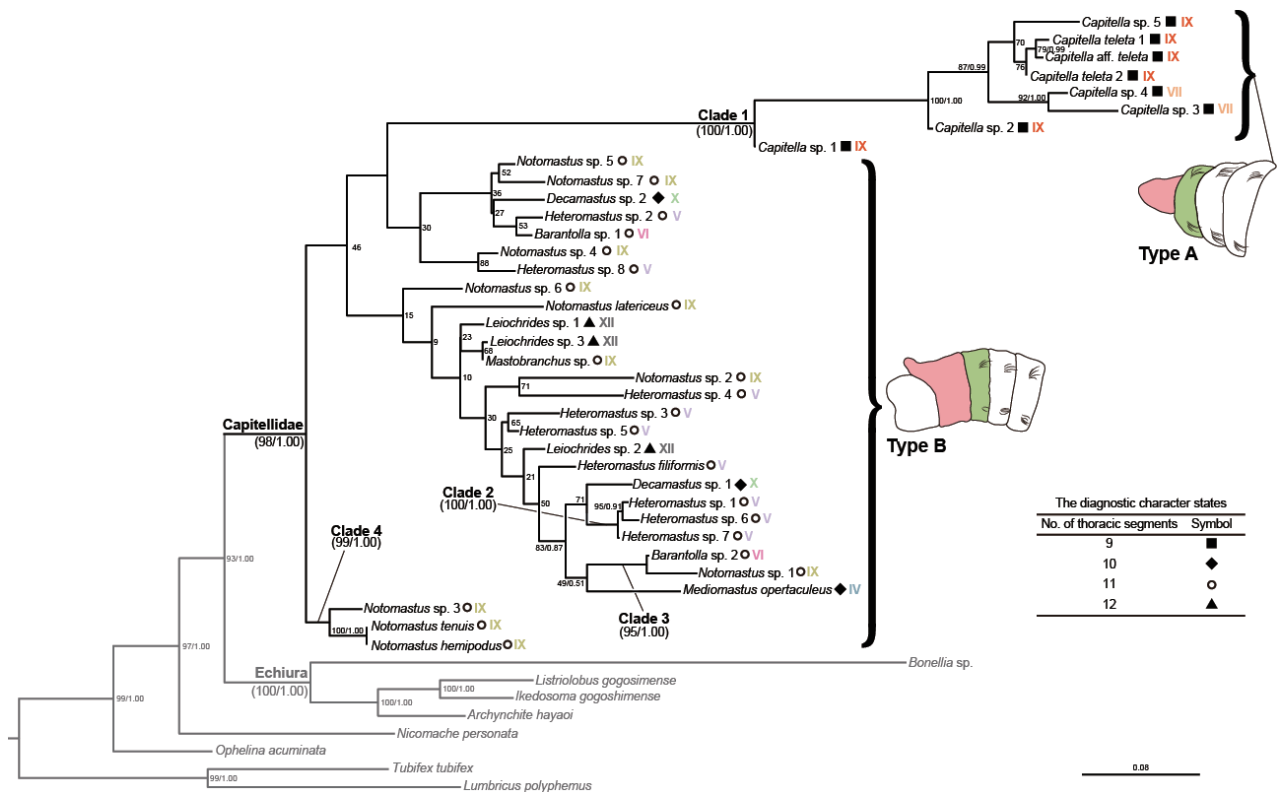
| Gene | Primer name  | Sequence (5' to 3')             | Reference                 |
|------|--------------|---------------------------------|---------------------------|
| 18S  | F1           | TACCTGGTTGATCCTGCCAG            | Yamaguchi and Endo (2003) |
|      | F2           | CCTGAGAAACGGCTRCCACAT           | Yamaguchi and Endo (2003) |
|      | F4           | GGTCTGTGATGCCCTYAGATGT          | Yamaguchi and Endo (2003) |
|      | R6           | TYTCTCRKGCTBCCTCTCC             | Yamaguchi and Endo (2003) |
|      | R8           | ACATCTRAGGGCATCACAGACC          | Yamaguchi and Endo (2003) |
|      | R9           | GATCCTTCCGCAGGTTACCTAC          | Yamaguchi and Endo (2003) |
| 28S  | LSU5         | ACCCGCTGAAYTTAAGCA              | Littlewood (1994)         |
|      | LSU3         | TCCTGAGGGAAACTTCGG              | Littlewood (1994)         |
|      | D2f          | CTTTGAAGAGAGAGTTC               | Littlewood (1994)         |
|      | 28Z          | CTTGGTCCGTGTTTCAAGAC            | Hillis and Dixon (1991)   |
| H3   | aF           | ATGGCTCGTACCAAGCAGAC            | Colgan et al. (1998)      |
|      | aR           | ATATCCTTRGGCATRATRGTGAC         | Colgan et al. (1998)      |
| COI  | LCO 1490     | GGTCAACAAATCATAAAGATATTGG       | Folmer et al. (1994)      |
|      | HCO 2198     | TAAACTTCAGGGTGACCAAAAAATCA      | Folmer et al. (1994)      |
|      | polyLCO      | GAYTATWTTCAACAAATCATAAAGATATTGG | Carr et al. (2011)        |
|      | polyHCO      | TAMACTTCWGGGTGACCAARAATCA       | Carr et al. (2011)        |
|      | Cap_COI_F    | GGAATTTGAGGTGGGCTTGT            | Tomioka et al. (2016)     |
|      | Cap_COI_R    | CACCACCACAGTATTATCA             | Tomioka et al. (2016)     |
|      | Mastus_COI_R | AAGTACGGGGTCTCCWCCWC            | this study                |

et al., 2014) to check whether the number of sampling generations and effective sample sizes (ESS) were large enough for reliable parameter estimates.

**RESULTS**

**Phylogenetic analyses**

The ML and BI trees were identical, with some differences at poorly supported terminal nodes; only the ML tree is shown here (Fig. 3). In the trees, Capitellidae is monophyletic, with nodal support of 98/1.00 (BS/PP), and forms the sister group to echiurans (93/1.00) (Fig. 3). Within Capitellidae, four clades appeared in both trees and had relatively high nodal support (greater than 95% BS and 1.00 PP). Clade 1 (100/1.00) includes all OTUs identified as *Capitella* and is separated from the sister clade by a long



**Fig. 3.** Maximum-likelihood tree based on a combined dataset of 18S, 28S, H3, and COI gene sequences. Numbers near nodes indicate the maximum-likelihood bootstrap support value in percent and posterior probability from Bayesian inference. Clades with both values shown at the basal node were congruent between the ML and BI trees. Clades with only the bootstrap value were not congruent between the two analyses. The number of thoracic segments is indicated by symbols (see the key at right), and the number of segments with capillary chaetae is indicated by roman numerals.

branch; Clade 2 (100/1.00) contains *Heteromastus* spp. 1, 6, and 7; Clade 3 (95/1.00) consists of *Barantolla* sp. 2 and *Notomastus* sp. 1; and Clade 4 (99/1.00) contains *Notomastus tenuis* Moore, 1909, *Notomastus hemipodus* Hartman, 1945, and *Notomastus* sp. 3. Other nodes in Capitellidae were poorly supported, with values lower than 90% BP and 0.99 PP (Fig. 3).

### Distribution of diagnostic characters

Figure 3 shows the distribution on the ML tree of the states of three key characters that have been used to diagnose capitellid genera: *i*) head shape, *ii*) number of thoracic segments; and *iii*) arrangement/type of chaetae. Clade 1 (exclusively comprising *Capitella*) is clearly distinct in that all the constituent members uniquely have the Type-A head and nine thoracic segments; the number of segments with capillary chaetae is either seven or nine, states not found in other capitellid OTUs included in the analysis.

No character states were found to be unique to or specific for the rest of the clades, all of which, however, have the Type-B head. The OTUs in Clades 2 and 4 share the same states for the three characters, but, again, these are not clade-specific. *Barantolla* sp. 2 and *Notomastus* sp. 1 in Clade 3 both have 11 thoracic segments, but this character state also occurs in many other OTUs included in the analysis.

### DISCUSSION

The expanded taxon sampling in our study supports the monophyly of Capitellidae, in agreement with several previous phylogenetic studies (e.g., Bleidorn et al., 2003; Struck and Purschke, 2005). The non-monophyly of Capitellidae in Goto (2016) may be attributable to inadequate taxon sampling (e.g., Hillis, 1998; Zwickl and Hillis, 2002) of Capitellidae or by different gene markers having been used in the analyses.

Most nodes except Clade 1 in our trees were weakly supported, and a better-supported tree might show a higher degree of OTUs forming clades by genus. However, a well-supported clade (Clade 3, Fig. 3) contained OTUs representing different genera, indicating that at least the genera *Barantolla* and *Notomastus* (and possibly also *Heteromastus*) are not monophyletic as currently diagnosed. Except for *Capitella*, three of the morphological characters traditionally used in defining capitellid genera (head type; number of thoracic segments; number of segments with capillary chaetae) are not informative for the purpose of delineating genera as natural groups.

Our results suggest that *Capitella* is monophyletic (Clade 1, Fig. 3). The implication of the monophyly of *Capitella* is that it should form the basis for research to illustrate evolutionary histories of habitat expansion [e.g., sand interstices (Green, 2002), whale bones (Silva et al., 2016), and squid mass egg (Hartman, 1947)], diversification of larval developmental forms [lecithotrophy, planktotrophy (Blake et al., 2009), and poecilogony (e.g., Chia et al., 1996; Tsutsumi, 2005)], and other interesting features found in this genus.

To better resolve relationships within Capitellidae, broader taxon sampling and additional genetic markers will both be necessary. If there is to be any hope of identifying

capitellid genera as natural groups based on morphology, the morphological characters traditionally used in defining genera will require a fine-scale reexamination, and new characters must be sought. In addition, future analyses including species showing specialized features as mentioned above will illustrate character evolution in *Capitella*.

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### COMPETING INTERESTS

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### AUTHOR CONTRIBUTIONS

ST designed the study, did morphological observation, performed experiments and analyzed the data. All authors wrote the manuscript. All authors read and approved the final manuscript.

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