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Validation of three sympatric *Thoracophelia* species (Annelida: Opheliidae) from Dillon Beach, California using mitochondrial and nuclear DNA sequence data

CHRIS J. LAW, KELLY M. DORGAN & GREG W. ROUSE
Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA, 92093–0202.
Correspondence: Greg Rouse grouse@ucsd.edu

Abstract

Thoracophelia (Annelida, Opheliidae) are burrowing deposit feeders generally found in the mid- to upper intertidal areas of sandy beaches. *Thoracophelia mucronata* (Treadwell, 1914) is found along the west coast of North America, including at Dillon Beach, CA. Two additional species, *Thoracophelia dillonensis* (Hartman, 1938) and *T. williamsi* (Hartman, 1938) were also described from this beach. These three sympatric species have been primarily distinguished by branchial morphology, and efforts to determine the validity of the species have been based on morphological, reproductive and ecological studies. Here we demonstrate using mitochondrial and nuclear DNA sequence data that these three species are valid. Mitochondrial Cytochrome c subunit 1 (COI) sequences show uncorrected interspecific distances of ~9–13%. We found no inter—specific differences in body color or in hemoglobin concentration, but found that reproductive males were pinkish-red in color and had lower hemoglobin concentrations than purplish—red reproductive females.

Key words: hemoglobin, COI, ITS1, sympatric, polychaete

Introduction

Thoracophelia is a genus of Opheliidae that was erected by Ehlers (1897) for *Thoracophelia furcifera* Ehlers, 1897 from the Magellan region of southern Chile. *Thoracophelia* was subsequently made a subgenus within *Euzonus* Grube, 1866 by Hartman (1956). This arrangement was either generally accepted, or *Thoracophelia* was not used as a subgenus at all, until it was shown by Brewer *et al.* (2011) that *Euzonus* was a junior homonym of *Euzonus* Menge, 1854, which was erected for a millipede species. Brewer *et al.* (2011) suggested that the 15 currently accepted *Euzonus* species be placed within *Pectinophelia* Hartman, 1938, but Blake (2011), however, reviewed the taxonomic history of these taxa and demonstrated that *Thoracophelia* Ehlers, 1897 was the valid genus and listed 17 known species. *Thoracophelia* species are characterized by having the body divided into three distinct body regions: (1) an anterior cephalic region consisting of the prostomium and first two chaetigers; (2) a swollen thoracic region; and (3) a long narrow posterior region characterized by a ventral groove and branchiae. A transverse groove/lateral notopodial ridge separates the thoracic and posterior region (Santos *et al.* 2004). *Thoracophelia* species have been described from the high latitudes of each hemisphere, including the US, Australia, Brazil, Japan and New Zealand and are generally intertidal sand-dwelling species, though deep-sea taxa have been described (Blake 2011; Santos *et al.* 2004).

Three species of *Thoracophelia* are accepted as valid from the west coast of North America (Blake 2011). *Thoracophelia mucronata* (Treadwell, 1914) was described (as *Ophelina mucronata*) by Treadwell (1914) from 'in sand' at La Jolla in southern California, where they can reach very high densities of over 40,000 worms m^{-2} (McConnaughey & Fox 1949). *Thoracophelia mucronata* has been recorded as far north as Vancouver Island in Canada (Berkeley & Berkeley 1932; Dafoe *et al.* 2008a, 2008b), with other records from Oregon (e.g., Kemp 1986, 1988). Hartman (1938) described two new species in a new opheliid genus *Pectinophelia* from Dillon Beach in central California. These are now referred to as *Thoracophelia dillonensis* (Hartman, 1938) and *T. williamsi* (Hartman, 1938). Hartman (1944) also recorded *T. mucronata* from the same beach.

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Variations in branchial structure are the only characters that consistently differ among the three species: *T. mucronata* has 18 pairs of bifurcated smooth branchiae; *T. williamsi* has 16–17 pairs of bifurcated branchiae with pinnules; and *T. dillonensis* has 15 pairs of pectinate branchiae (Hartman 1969; Parke 1973). Color had also been used to distinguish species; however, color descriptions for the three species by different authors are inconsistent (Hartman 1938; Parke 1973). Parke (1973) assessed the validity of the three *Thoracophelia* species at Dillon Beach in an analysis of morphology, ecological distribution, reproduction, and larval development. His crossbreeding experiments produced hybrids; however not all interspecific crosses were successful. When they were successful, the interspecific success rates were considerably lower than intraspecific rates and the larvae did not develop as well. However, larvae of all three species and their various hybrids showed no differences in development to settlement. Parke (1973) also found differences in distribution in the beach sands and seasonal timing of peak gamete viability for each species. He suggested these were sufficient to impede gene flow and prevent hybridization, despite some overlap in reproductive periods. Based on distribution and temporal reproductive isolation, Parke (1973) concluded that the three sympatric *Thoracophelia* spp. were distinct species.

The aims of this study were to assess the validity of the three *Thoracophelia* spp. at Dillon Beach, California using analyses of mitochondrial and nuclear DNA sequences and a reassessment of the morphological differences among taxa. We also assessed whether physiological differences consistent with observed variation in gill morphologies occur among the three taxa by measuring hemoglobin concentrations.

Collection and morphology

Thoracophelia specimens were collected from Dillon Beach, CA, USA, on April 12–13, 2012. *Thoracophelia dillonensis* and *T. williamsi* were found sympatrically along the beach whereas *T. mucronata* were found only at the northern end and at lower abundance. We observed that the branching pattern of branchiae was the only morphological character that consistently distinguished the three species (Fig. 1). Branchial lengths varied considerably both among individuals and among segments on individual worms, especially for *T. williamsi*. Variations in color were inconsistent with previous descriptions (Parke 1973) and did not distinguish the three species. Instead, we observed intraspecific color differences between males and females—females with visible gametes tended to be reddish purple whereas males with sperm tended to be pinker with opaque coelomic fluid. We also observed considerable intraspecific variability in size, in contrast to the narrow and distinct size ranges described by Parke (1973). We found only small *T. williamsi* in the lower intertidal, but did not conduct extensive sampling.

Upon collection, specimens were sorted based upon branchial morphology and relaxed in 7.5% magnesium chloride in freshwater and fixed in 96% ethanol for DNA extraction or in 10% seawater formalin for deposition at the Benthic Invertebrate Collection at Scripps Institution of Oceanography (catalogue numbers SIO-BIC A3385-A3403). Live specimens of *T. dillonensis* and *T. williamsi* were used for hemoglobin analysis. Additional *T. mucronata* were collected from La Jolla Shores Beach, CA for comparison with *T. mucronata* from Dillon Beach and were used for hemoglobin analysis owing to insufficient numbers of *T. mucronata* being collected from Dillon Beach. Gill morphology was indistinguishable between *T. mucronata* from the two locations, but worms from Dillon Beach were much larger, 5–10x the mass of adult *T. mucronata* from La Jolla. Similar variation in size of this species has been observed from Oregon beaches with a bimodal size distribution likely corresponding to two annual cohorts (Dangott & Terwilliger 1986; Kemp 1988).

Phylogenetic analyses

Genomic DNA was extracted from specimens using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Three specimens of each nominal species from Dillon Beach and three specimens of *T. mucronata* from La Jolla Shores Beach were extracted. Approximately 700 base pairs of the mitochondrial *Cytochrome c subunit 1* gene (COI) were amplified using the universal primers polyLCO (5' GAYTATWTTCAACAAATCATA AAGATATTGG 3') and polyHCO (5' TAMACTTCWGGGTGACCAAARAATCA 3') (Carr *et al.* 2011) with temperature profiles of 95 C for 3 minutes, followed by 40 cycles of 95° C for 40 seconds, 42° C for 40 seconds,

72° C for 50 seconds, and final extension at 72° C for 5 minutes. Approximately 700 base pairs of the nuclear marker, *Internal transcribed spacer 1* (ITS1), with flanking regions of 18S and 5.8 rDNA were amplified using ITS18SFPOLY (5' GAGGAAGTAAAAGTCGTAACA 3') and ITS5.8SRPOLY (5' GTTCAATGTGTCCT GCAATTC 3') (Pleijel *et al.* 2009) with temperature profiles of 95° C for 4 minutes, followed by 45 cycles of 94° C for 30 seconds, 48° C for 30 seconds, 72° C for 1 minute, and final extension at 72° C for 8 minutes. PCR reactions were conducted with 2 mL of DNA template, 1 mL of forward and reverse primers, 12.5 mL GoTaq Green Master Mix (Promega), and 8.5 mL H_2O, for a total of 25 µL. ExoSAP-IT (Affymetrix) was used to purify PCR products. All sequencing was carried out at either Retrogen Inc. or Eurofin. Sequences were edited using Geneious 5.5.6 (http://www.geneious.com/) and then aligned with MAFFT 3.8 (Katoh and Kuma 2002) under default settings with no manual alterations. Sequences are deposited at Genbank with the numbers KC164680-KC164692 for the COI sequences and KC168069-KC168087 for the ITS1 sequences.

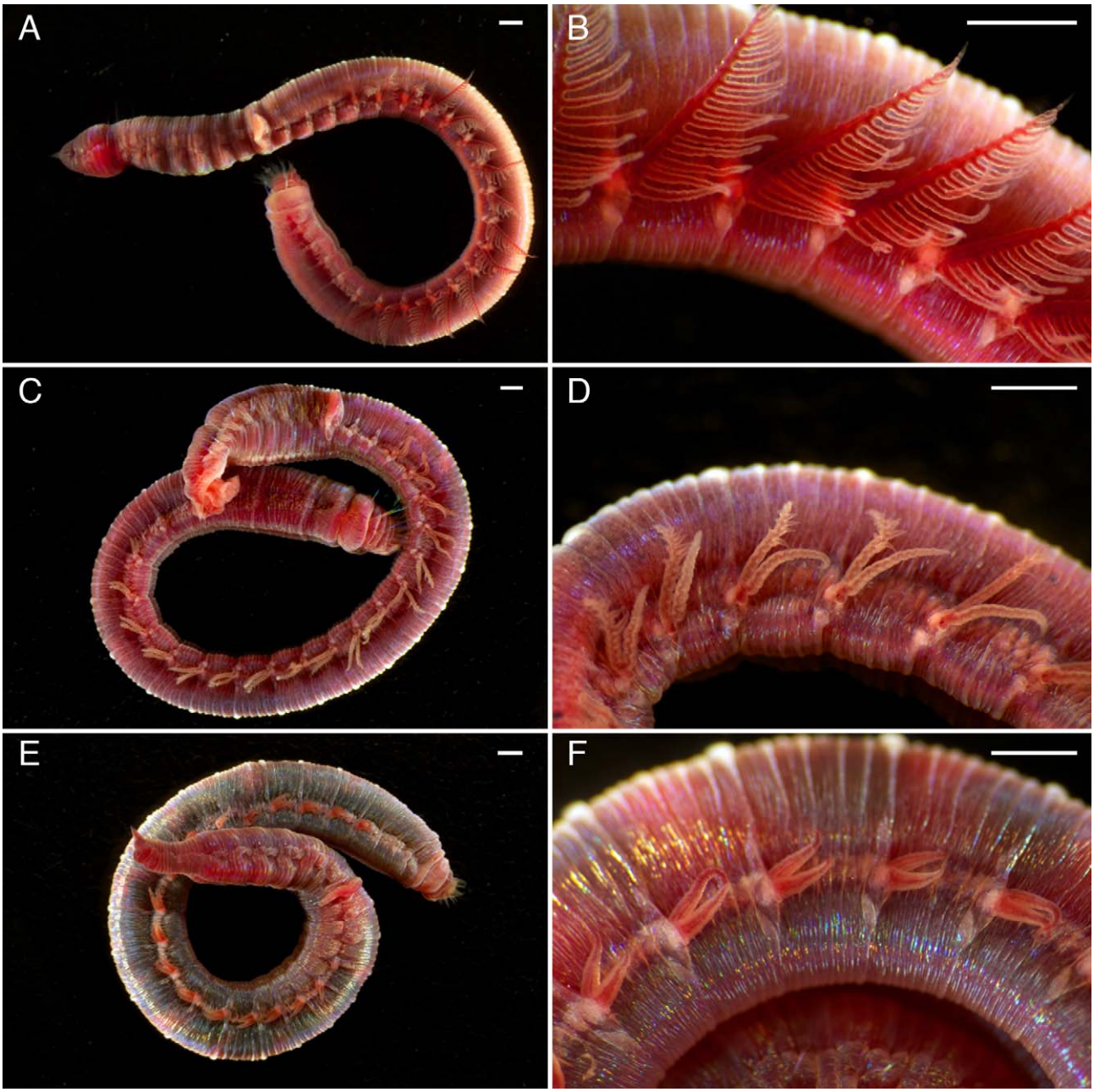

FIGURE 1. The three sympatric *Thoracophelia* spp. from Dillon Beach. **A,** *Thoracophelia dillonensis*. **B**, Pectinate branchiae of *T. dillonensis*. **C,** *Thoracophelia williamsi*. **D,** Bifurcated branchiae with pinnules of *T. williamsi*. E, *Thoracophelia mucronata*. **F,** Bifurcated branchiae of *T. mucronata.* Scale bars all 1 mm.

The mitochondrial and nuclear DNA sequences were analyzed separately. The opheliid *Ophelia limacina* (Rathke, 1843) (collected from Greenland) was used to root the tree based on the results shown in the mitochondrial analyses, but not in the ITS1 analysis due to problems with the alignment. Rooting of the ITS1 analysis was made according to the results obtained with the COI dataset. Parsimony analysis on each partition was conducted with PAUP* 4.0b10 (Swofford 2002) using heuristic searches with random stepwise addition of the terminals for 1,000 replicates, with the tree bisection re-connection (TBR) permutation algorithm and with maximum zero-length branches collapsed. The character matrix was equally weighted, and any gaps were treated as missing data. Clade support was assessed using jackknifing of sites on 1,000 replicates with 10 random additions per iteration. Maximum likelihood analyses was performed in RAxML 7.2.8 (Stamatakis 2006) under the General Time Reversible + Gamma (GTR + G) model, and bootstrap values were estimated using 1000 pseudoreplicates under the same model. Uncorrected and model-corrected distances were also using PAUP* 4.0b10. The appropriate model selected using jModelTest (Posada 2008) with the Akaike information criterion was General Time Reversible + Gamma (GTR + G) for both COI and ITS1, and the respective gamma shapes of 0.261 and 0.640.

The COI dataset consisted of 689 characters, of which 124 were parsimony-informative. Both parsimony and maximum likelihood analyses recovered a topology with all the *T. mucronata* terminals (Dillon and La Jolla Shores Beaches) forming a clade that was a sister group to *T. dillonensis* and *T. williamsi*, which were reciprocally monophyletic (Fig. 2A). The maximum GTR+G model-corrected distance between *T. mucronata* from Dillon Beach and La Jolla was 0.45% (uncorrected = 0.44%), suggesting that they represent a single species along the California coast, though the nuclear ITS1 data does suggest further investigation is warranted (see below). Sequencing of COI for numerous (>70) *Thoracophelia* individuals from La Jolla Shores Beach has revealed only a single species, *T. mucronata*, at this beach (Mandy Butler UCSD pers comm.).

Our COI data, however, clearly suggests that Dillon Beach has three sympatric species of *Thoracophelia*. The minimum GTR+G-corrected distance between *T. mucronata* and *T. dillonensis* was 20.7% (uncorrected = 11.9%), between *T. mucronata* and *T. williamsi* was 18.7% (uncorrected = 10.9%), and between *T. dillonensis* and *T. williamsi* was 13% (uncorrected = 8.8%). Maximum intraspecific distances for *T. williamsi* and *T. dillonensis* were 0.9% (uncorrected = 0.89%) and 0.3% (uncorrected = 0.29%), respectively.

The nuclear ITS1 dataset consisted of 713 characters of which 63 were parsimony-informative, and, consistent with the COI results, parsimony and maximum likelihood analyses revealed three clades of *Thoracophelia* corresponding to the current nominal species (Fig. 2B). The minimum GTR+G—corrected distance between *T. mucronata* and *T. dillonensis* was 3.4% (uncorrected = 3.2%), between *T. mucronata* and *T. williamsi* was 5.3% (uncorrected = 4.8%), and between *T. dillonensis* and *T. williamsi* was 2.7% (uncorrected = 2.6%). One of the three Dillon Beach *T. mucronata* ITS1 sequences was 2.8–4.1% (uncorrected = 2.6–3.8%) distant from the other *T. mucronata* sequences though nearly identical to one of the ITS sequences from La Jolla Shores (Fig. 2B). The low COI divergence within *T. mucronata* may therefore represent mitochondrial introgression <XREF>(Galtier *et al.* 2009), and further investigation into the status of *T. mucronata* as a single species may be warranted. The maximum intraspecific distances within *T. williamsi* and *T. dillonensis* were 0.73% (uncorrected = 0.71%) and 0.14% (uncorrected = 0.14%), respectively.

Hemoglobin analyses. Hemoglobin was measured spectrophotometrically using Drabkin's reagent (Sigma-Aldrich D5941) following standard procedures. Worms were held in seawater for > 24 hr to void gut contents, then homogenized with a glass-on-glass mortar and pestle in 3 mL (g wet mass)$^{-1}$ Drabkin's solution, in which all forms of hemoglobin are oxidized to methemoglobin, which reacts with potassium cyanide to form cyanmethemoglobin. A standard curve generated from known concentrations of bovine hemoglobin (Sigma-Aldrich H2500) was used to calculate hemoglobin concentration from absorbance measured at 540 nm of a 1:10 dilution of homogenate in Drabkin's solution. A total of 17 specimens of *T. dillonensis*, 8 of *T. mucronata*, and 18 specimens of *T. williamsi* were used. Before homogenization, worms were examined externally for presence of eggs or sperm. One worm of each species was sacrificed and gametes examined to confirm external observations. For *T. mucronata*, individuals were smaller and gametes were not apparent, but for *T. williamsi* and *T. dillonensis*, males appeared pinkish and coelomic fluid was cloudy, whereas females were red or even purple and eggs could be seen as spheres with a round nucleus (distinguishing them from less smooth cells without a clear nucleus found in coelomic fluid of both males and females).

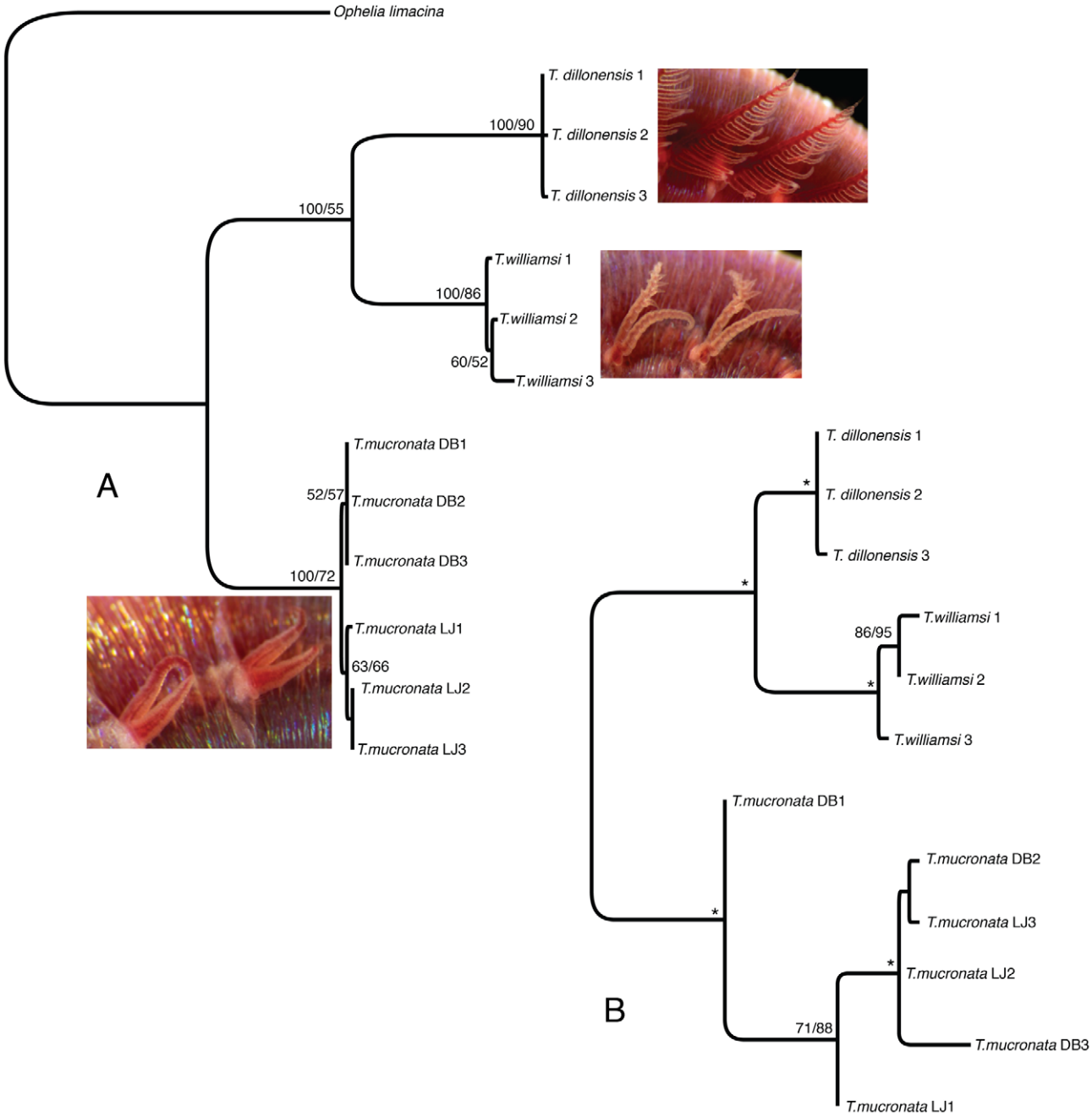

FIGURE 2. Phylogenetic results. **A,** Maximum likelihood tree from COI dataset rooted with *Ophelia limacina*. **B,** Maximum likelihood tree from ITS1 dataset rooted according to the result for the COI dataset. Support values are shown as jackknife from parsimony analysis and bootstrap from maximum likelihood respectively separated by /. * indicates 100% values for each support measure.

Hemoglobin concentration was independent of body weight for all three species (Fig. 3A), so results were combined (Fig. 3B). Mean hemoglobin concentration was higher for *T. dillonensis* than *T. williamsi* (ANOVA multiple comparison $p < 0.05$), although a significant gender effect and interaction term between species and gender ($p < 0.05$) suggests that this difference is driven by differences in female hemoglobin concentration between the two species. For *T. dillonensis*, females had significantly higher hemoglobin concentrations than males ($p < 0.05$), but for *T. williamsi*, the trend was not significant ($p = 0.13$).

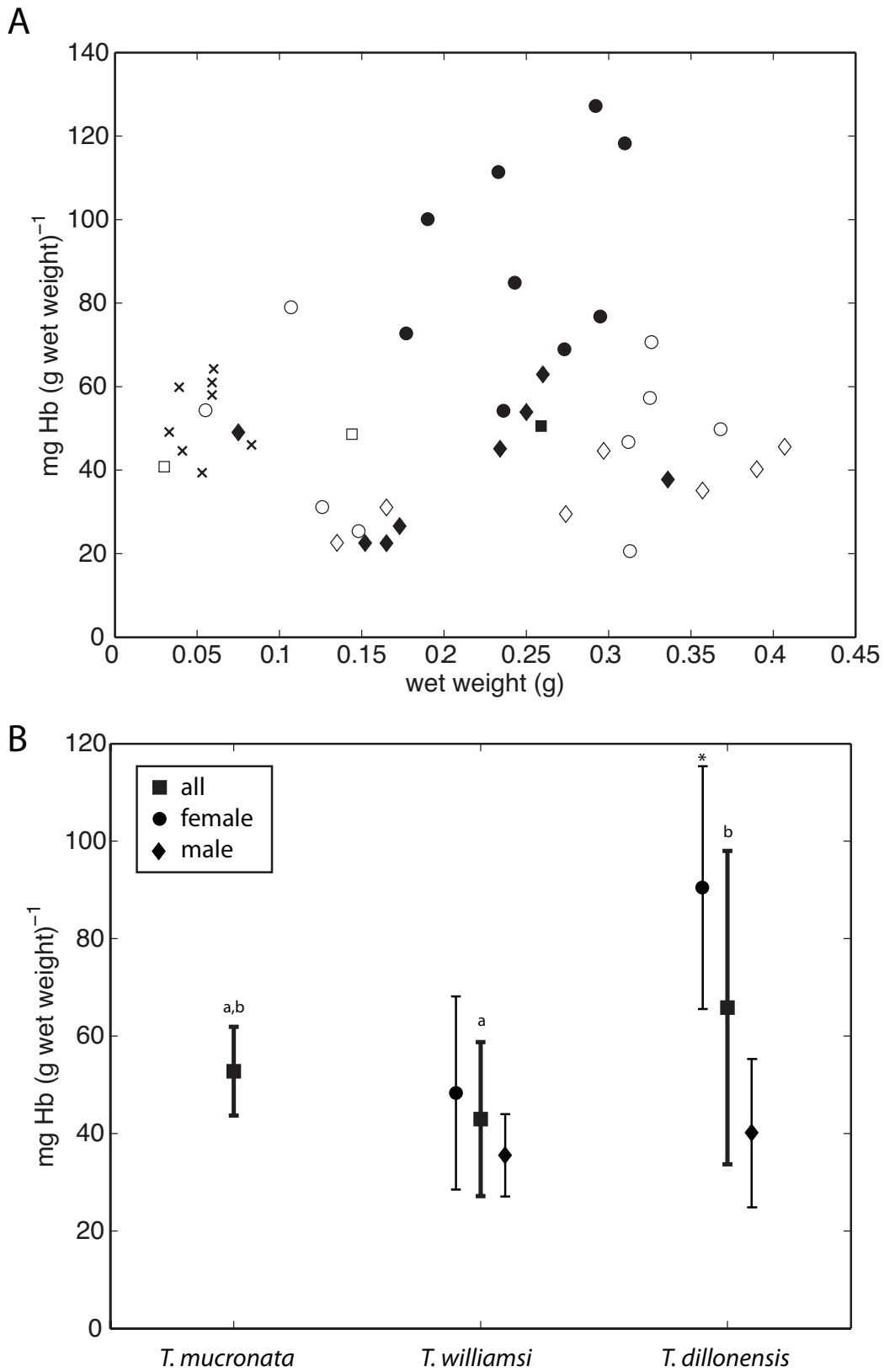

FIGURE 3. A, Hemoglobin concentration was independent of body weight ($p > 0.05$ for linear regression for all three species). For *T. mucronata* (x's), neither eggs nor sperm could be seen. For *T. williamsi* (open symbols) and *T. dillonensis* (closed symbols), circles indicate worms in which eggs could be clearly discerned, diamonds individuals for which sperm could be clearly discerned, and squares individuals for which sex was undetermined. **B,** Mean +/– 1 s.d. for each species (squares) and for females (circles) and males (diamonds) of *T. williamsi* and *T. dillonensis*. For combined gender data, *T. dillonensis* had significantly higher Hb concentration than *T. williamsi*, and for *T. dillonensis*, females had significantly higher Hb concentration than males (ANOVA $p < 0.05$).

Hemoglobin concentrations for *T. mucronata* were comparable to the mean concentration of 42 mg Hb (g wet weight)$^{-1}$ from specimens from Oregon that were bled into buffer rather than homogenized before hemoglobin was measured spectrophotometrically (Dangott & Terwilliger 1986). Higher hemoglobin concentrations in reproductive females are remarkable given the already high concentrations; Dangott & Terwilliger (1986) found that hemoglobin comprised 4.2% of wet weight and that worms were 78% water by weight resulting in hemoglobin comprising 19% of dry weight. For female *T. dillonensis*, hemoglobin comprised 9% of wet weight, and assuming comparable water content, 41% of dry weight. High hemoglobin concentrations may represent an additional cost of reproduction in this environment with variable oxic conditions. Parke (1973) found highest percentages of reproductive *T. williamsi* occurring in March and of *T. dillonensis* and *T. mucronata* in June and July, respectively, with substantial percentages of mature adults of both species in April. With only one sampling time, in April, we cannot distinguish whether the gender difference in hemoglobin concentrations that was stronger for *T. dillonensis* than for *T. williamsi* represents a species-specific difference or results from reproductive timing.

Conclusions

Our analyses of the COI and ITS1 datasets confirm that *Thoracophelia mucronata* ranges at least from southern to central California and validates the morphological (Hartman 1938, 1944) and other evidence (Parke 1973) for three sympatric species—*Thoracophelia mucronata*, *T. dillonensis*, and *T. williamsi* at Dillon Beach. It will be worth sampling sites where *Thoracophelia* has been recorded further north as *T. mucronata* (e.g., Berkeley & Berkeley 1932; Dafoe *et al*. 2008a, 2008b; Kemp 1986, 1988) along the North America west coast to assess if this species does have such as extensive range. It may be that the species that have hitherto only been recorded at Dillon Beach, *T. dillonensis* and *T. williamsi*, have more extensive ranges.

The finding that there are three *Thoracophelia* species at Dillon Beach raises questions about the evolution and maintenance of these morphologically similar species. Similar hemoglobin concentrations, as well as overlapping distributions suggest that habitat isolation is an unlikely explanation for the presence and maintenance of these sympatric species. Our results showing gender-related differences in hemoglobin concentration are intriguing and suggest that Parke's (1973) hypothesis of temporal reproductive isolation of species merits further study. The remarkably high hemoglobin concentration in female *T. dillonensis* raise questions about how oxygen limitation affects reproduction, and further research on reproductive timing may explain the sympatric distribution of *Thoracophelia* spp. and their survival in this extreme environment.

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