Note

Cryptic diversity of the tube-dwelling polychaete *Phyllochaetopterus* in the Shinkai Seep Field, Mariana Trench

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Abstract: *Phyllochaetopterus* (Annelida: Chaetopteridae) is a diverse genus of tube-dwelling polychaetes found in a wide range of marine environments from subtidal to abyssal depths, including chemosynthesis-based ecosystems. The Shinkai Seep Field (SSF) is a serpentinite-hosted system in the Mariana Trench, where the deepest-known *Phyllochaetopterus* polychaetes inhabit the surfaces of brucite/carbonate chimneys. Despite all specimens collected from SSF being morphologically consistent with *P. polus* originally described from a deep-sea hot vent on the Mid-Atlantic Ridge, molecular barcoding using the mitochondrial cytochrome *c* oxidase subunit I (COI) gene revealed at least three cryptic lineages, none of which corresponded to *P. polus*. Phylogenetic reconstruction recovered *P. polus* embedded among the three SSF lineages, confirming their close relationship. These results warrant careful examination of *Phyllochaetopterus* from other regions using integrative taxonomy in order to understand its true diversity and pinpoint further taxonomically informative morphological characters.

Key words: Chaetopteridae, deep sea, serpentinitization, chemosynthesis-based ecosystem

Phyllochaetopterus is a diverse polychaete genus with about 20 valid species living in a wide range of marine environments from shallow to deep waters, including chemosynthesis-based ecosystems such as hydrothermal vents and hydrocarbon seeps (Nishi & Rouse 2014). The Shinkai Seep Field (SSF) is the world's deepest known serpentinite-hosted seep field, located at a depth of ~5700 m deep in the southern Mariana forearc on the landward slope of the Mariana Trench (Ohara et al. 2012). The SSF is characterized by both sedimented area dominated by the vesicomyid clam, *Abyssogeton mariana* (Okutani et al. 2013), and rocky areas with brucite/carbonate chimneys where *Phyllochaetopterus* worms and the provannid snail, *Provanna cingulata*, are the most conspicuous animals (Fig. 1; Okumura et al. 2016; Chen et al. 2016). Though deeper than the carbonate compensation depth, these chimneys are sustained by geofluid input through slow seepage, enriched with hydrogen and methane (Okumura et al. 2016). The SSF represents the deepest record (5687 m) of the genus *Phyllochaetopterus* and is over 1.5 km deeper than

Phyllochaetopterus polus from the Ashadze-1 field, a serpentinite-hosted hydrothermal vent field on the Mid-Atlantic Ridge (4085 m; Morineaux et al. 2010). The taxonomic identity and systematic position of the SSF *Phyllochaetopterus*, however, have not been examined to date. Here, we present the first results of morphological and molecular examinations, using the barcoding region of the mitochondrial cytochrome oxidase *c* subunit I (COI) gene, for previously unidentified SSF *Phyllochaetopterus* specimens.

Phyllochaetopterus tubes were collected from two brucite/carbonate chimneys (type II chimneys 'Chim 4' and 'Chim 10' in Okumura et al. 2016) located in a single chimney site ('CH site 3' in Okumura et al. 2016) during dives #1402 (Chim 10, July 2014) and #1433 (Chim 4, July 2015) of the deep-submergence vehicle (DSV) *Shinkai 6500* in R/V *Yokosuka* cruises YK14-13 and YK15-11. A detailed *in situ* visual observation of the *Phyllochaetopterus* tubes was also performed during the remotely operated vehicle (ROV) *Kaiko* (with vehicle *Mk-IV*) dive #709 on-board R/V *Kairei* cruise KR16-04 (Fig. 1). The mineral compositions of both chimneys were similar, and they are thought to have formed from the same source of geofluids (Okumura et al. 2016). Some tubes among

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Fig. 1. *In situ* observations of *Phyllochaetopterus* in the Shinkai Seep Field. A: Brucite/carbonate chimneys in ‘CH site 3’ (Okumura et al., 2016) overgrown by tubes of *Phyllochaetopterus*; B: Close-up of *Phyllochaetopterus* worms, arrowheads indicating live worms with extended palps visible. Photos taken on ROV *Kaiko* dive #709 in R/V *Kairei* KR16-14 cruise.

the many collected were empty, yielding eight live worms in *Shinkai 6500* dive #1402 and 32 live worms in #1433, which were preserved in 99.5% ethanol. Genomic DNA was extracted, and COI barcoding was carried out following methods detailed in Chen et al. (2019), using the ‘universal’ primers designed by Folmer et al. (1994) with an annealing temperature of 50°C.

Sequence variability among the SSF specimens and other closely related species with available sequences on GenBank, were compared after alignment by Clustal X in MEGA 7.0.26 (Kumer et al. 2016) resulting in a final alignment of 525 bp (GenBank accession numbers MT491391–MT491430). A maximum likelihood (ML) phylogeny was reconstructed in MEGA 7.0.26 with 2,000 bootstrap replicates using the Hasegawa-Kishino-Yano substitution model with a Gamma distribution and invariant sites as selected by the Model Selection function in MEGA 7.0.26. Phylogenetic reconstruction by Bayesian inference was carried out by MrBayes (Ron-

quist et al. 2012) with 5,000,000 Markov chain Monte Carlo (MCMC) generations; the first 10,000 generations were removed as burn-in. A Bayesian implementation of the Poisson tree process (bPTP) model (Zhang et al. 2013) and the Automatic Barcoding Gap Delimitation (ABGD; Puillandre et al. 2012) methods were carried out on the respective web servers to identify barcoding gaps among sequences and estimate species delimitation. The parameters for bPTP were set to 100,000 generations, with the first 1,000 generations removed as burn-in. The ABGD $P_{\min}$ and $P_{\max}$ were set to 0.001 and 0.1, respectively.

All SSF specimens examined shared the morphological characteristics of *P. polus*, originally described from the Ashadze-1 field in the Mid-Atlantic Ridge (Morineaux et al. 2010). These include having minute second antennae, nine chaetigers in the anterior region (A-region), two chaetigers in the middle region (B-region), trilobed notopodia in the B-region, one or two A4 chaetae in each notopodium, and

Fig. 2. Maximum likelihood tree of *Phyllochaetopterus* based on 525 bp of the barcoding region of the mitochondrial COI gene (Bayesian and ML methods resulted in identical topologies). OTUs of newly obtained sequences are represented by GenBank accession numbers, with asterisks indicate the samples from Chim 4. Non-SSF sequences were retrieved from GenBank with their accession numbers shown in the parenthesis. Node values are ML bootstrap values/Bayesian posterior probabilities. Results of ABGD and bPTP species delimitation analyses are shown with colors or patterns indicate the resulting partitions. Please refer to specimen voucher information on GenBank for specific sample numbers including the U.S. Fish and Wildlife Service special use permit numbers.

more than 40 chaetigers in the posterior region (C-region). However, COI barcoding sequences revealed three distinct, cryptic lineages (Fig. 2), which we tentatively call SSF1 (five individuals), SSF2 (34 individuals), and SSF3 (one individual). A clear barcoding gap >7% (up to 41 bp out of

525 bp, average 39.206 ± 0.585) in the pairwise uncorrected distances was observed between SSF1 and SSF2. There was no sequence variation among the SSF1 individuals and only a maximum variation of 3 bp was seen in SSF2. The third SSF lineage, SSF3, exhibited >8% pairwise distances and a

43,000±0.000 bp difference to SSF1, and >6% pairwise distances and 32,265±0.567 bp difference to SSF2.

Phylogenetic reconstruction (Fig. 2) confirmed the close relationships between *P. polus* and the three SSF *Phyllochaetopterus* lineages, as inferred by their morphological similarity. *Phyllochaetopterus polus* was recovered sister to SSF2, the pair in turn being sister to SSF3, while SSF1 was the most basal of the four. The uncorrected sequence divergences between *P. polus* and the three SSF lineages were >8% and 46,000±0.000 bp for SSF1, >6% and 34,265±0.567 bp for SSF2, and >8% and 46 bp for SSF3. Although such divergences are suggestive of these lineages representing different species, results from the two species delimitation methods used were contradictory. The bPTP analysis indicated that all four lineages correspond to separate species, whereas the ABGD analysis suggested that all four lineages represent intraspecific variation of *P. polus* (Fig. 2).

Similar cases of cryptic diversity have been reported for other animals in deep-sea chemosynthesis-based communities. For example, the vesicomyid clams *Phreagena soyoae* and *Phreagena okutanii* are a pair of sister-species that overlap morphologically and co-occur in hydrocarbon seeps in Sagami Bay (Kojima & Ohta 1997). Morphometric analyses showed that although the two species differed in shell form statistically, some individuals from both species had overlapping features and were difficult to distinguish based on morphology alone. The microhabitats preferred by the two species in Sagami Bay differed in both temperature and salinity (Kojima & Ohta 1997, Watanabe et al. 2013), indicating ecological and physiological differences such as thermo- and/or osmoregulation capabilities, between the two clam species. Previous studies found no underlying geochemical differences between the two brucite/carbonate chimneys hosting *Phyllochaetopterus* in mineralogy and fluid chemistry, and the stable isotopic compositions of the animals did not differ between the two chimneys (Okumura et al. 2016, Onishi et al. 2018). As such, little evidence for differences in habitat preferences among SSF *Phyllochaetopterus* lineages exists now and we also cannot exclude the possibility of sampling bias regarding their preferred distributions. Nevertheless, it is possible that finer investigations of local conditions may reveal fine differences in their microhabitats.

Carbon and nitrogen stable isotopic compositions of *P. polus* from the Ashadze-1 vent field on the Mid-Atlantic Ridge were similar to the SSF lineages, indicating that they are detritus feeders mainly consuming chemoautotrophic bacteria (Fabri et al. 2011, Onishi et al. 2018). Although the sulfur ($\delta^{34}\text{S}$) stable isotopic composition differed between *P. polus* (+6.87‰) and the SSF lineages (+21.6‰, Fabri et al. 2011; Onishi et al. 2018), this likely reflect geochemical differences between hydrothermal vents, where *P. polus* occurs, and the SSF which is a serpentinite-hosted seep. The former, Ashadze-1 field is a high-temperature, black-smoker type serpentinite-hosted hydrothermal vent field with minerals in chimneys showing sulfur isotopic compositions ($\delta^{34}\text{S}$) ranging from +3.7‰ to +14.1‰ (Fouquet et al. 2008, Firstova et

al. 2013), whereas the SSF is a low-temperature serpentinite-hosted seep field with the fluid $\delta^{34}\text{S}$ isotopic composition of +20.3‰ to +20.4‰ (Onishi et al. 2018). Taken together, these suggest that the *Phyllochaetopterus* lineages in the SSF have similar feeding ecology with the closely related *P. polus*, from which they appear to be morphologically indistinguishable based on characters currently used to differentiate *Phyllochaetopterus* species. Nevertheless, they indeed rely on local food sources available at the different chemosynthesis-based ecosystems they live in.

Cryptic speciation is common in annelids and has also been documented in other vent and seep polychaetes, such as the amphinomid genus *Archinome* (Borda et al. 2013) and the ampharetid genus *Amphisamytha* (Zhou et al. 2019). More in-depth examinations of the morphological characters may reveal reliable morphological characteristics that differentiate the lineages discovered by DNA barcoding, and in some cases life history characters may prove to be the key (Sigwart & Chen 2018). Our results warrant further studies of the global *Phyllochaetopterus* from multiple perspectives including not only morphology and molecular barcoding but also physiological and ecological aspects, in order to better understand its diversity and the differences among lineages.

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