Culturing Putatively Obligate Epizoic Diatoms: Insights for the Evolution and Ecology of Diatoms and Their Host

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Abstract

Background: Our understanding of the importance of microbiomes on large aquatic animals—such as whales, sea turtles and manatees—has advanced considerably in recent years. Recent activity describing the epizoic diatoms growing on marine vertebrates suggests that these epibiotic diatom communities constitute diverse, polyphyletic, and compositionally stable assemblages that include both putatively obligate epizoic and generalist species. Here, we outline a successful attempt to culture putatively obligate epizoic diatoms without their hosts and propose further applications and research avenues in this growing area of study.

Results: We cultured cells of epizoic diatoms from multiple host species sampled in the wild and captivity. Analyzing the DNA sequences of these cultures, we found that several unique diatom taxa have independently evolved to occupy in epibiotic habitats. We created a library of reference sequence data for use in metabarcoding surveys of sea turtle and manatee microbiomes that will further facilitate the use of environmental DNA for studying host specificity in epizoic diatoms and the utility of diatoms as indicators of host ecology and health.

Conclusions: Our discovery that epizoic diatoms can be cultured independently from their hosts raises several questions about the nature of the interaction between these diatom species and their hosts. We encourage the interdisciplinary community working with marine megafauna to consider including diatom sampling and diatom analysis into their routine practices.

Background

Currently, the most common health indicators used to monitor cetaceans, sirenians and sea turtles include mortality rates, demographics, disease prevalence and frequency of stranding events. Since animal-associated microbiota may both affect and be affected by their host, both internal and external microbiome composition at any given time could also reflect mid- and longer-term effects of disturbances or stressors experienced by the animal [1]. New health and fitness indices based on compositional changes in the native microbiomes could be a valuable addition to comprehensive health assessment system in aquatic vertebrates [2].

Previous studies on the external microbiome of large aquatic vertebrates have focused on the bacterial and/or viral components. In contrast, epizoic microeukaryotes remain poorly explored despite the observation of diatoms on whales over a century ago [3, 4]. Diatoms (Bacillariophyta) are a diverse group of largely photosynthetic microalgae characterized by their uniquely shaped siliceous thecae (frustules) and are commonly found in the plankton and benthos of many different aquatic habitats. Recent studies have expanded the known diversity of epizoic diatoms through increased sampling of hosts to include sea turtles [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22], sea snakes [23] and manatees [24, 25].

Competition for limited resources among diatoms has led to niche partitioning and significant habitat specificity in some taxa. The epizoic diatom communities growing on aquatic vertebrates appear to be formed by a combination of opportunistic surface-associated taxa and putatively obligate epizoic (POE) taxa. While the opportunistic taxa are shared across the benthic habitats of the local environment, the POE taxa thus far have only been observed in the epizoic microbiome [7, 21, 26, 27]. This mixture of opportunistic and POE taxa is an intriguing assemblage, as it is potentially influenced by the host's biology (e.g. physiology, anatomy and host-specific prokaryotic microbiome) and behavior (e.g. long-distance migrations, diving, basking, and terrestrial nesting which expose epibionts to extremes in temperature, pressure, irradiance, nutrient concentration and desiccation) as well as the environment (e.g. mean temperature, salinity, nutrient load, local biocenoses). Moreover, the unique and highly specific diatom flora composition can be documented long past the death of the diatom cells by the weathering-resistant inorganic frustules. This has resulted in diatoms being utilized extensively for paleoecological reconstructions and bioindication in freshwater environments; for multiple reviews, see [28]. Similar diatom-based health indices may be developed for the marine animals and their habitats.

However, before this can happen, at least two issues must be addressed:

1) We must expand upon our knowledge of the specific molecular, genomic and ecological nature of the interactions between POE diatoms and their host and environment.

2) We need to simplify the identification of epizoic diatoms, which currently requires specialized equipment (such as electron microscopy) and literature that can be highly fragmented and incomplete, particularly in the case of marine diatoms.

In the future, both issues could be addressed by metagenomic and metabarcoding techniques, respectively. Currently, however, the dearth of reference data—both in annotated genome and transcriptomes as well as vouchered DNA barcodes for diatoms—would limit the effectiveness of either effort. For example, a metabarcoding attempt on sea turtle epiflora [29] failed to recover some of the diatom taxa identified in microscopical surveys, including the dominant POE taxon *Labellicula lecohuiana* Majewska, De Stefano & Van de Vijver. The authors acknowledged that this failure was likely due to the lack of any relevant reference sequences for the genus *Labellicula*. Further, the position of *Labellicula* in the molecular phylogeny of diatoms is unknown. This uncertainty significantly hinders any bioinformatic efforts to find sequence data even closely related to *Labellicula* among both the metabarcoding reads and the reference databases. Many other POE taxa have uncertain phylogenetic affinities within the raphid diatoms, including the "*Tripterion* complex" (*Tripterion* Holmes, Nagasawa & Takano, *Chelonicola* Majewska, De Stefano & Van de Vijver, *Poulinea* Majewska, De Stefano & Van de Vijver and *Medlinella* Frankovich, Ashworth & M.J.Sullivan), *Tursiocola* Holmes, Nagasawa & Takano and *Epiphalaina* Holmes, Nagasawa & Takano, which all have uncertain phylogenetic affinities within the raphid diatoms.

To address the aforementioned issues, the goal of the present study was to generate additional DNA sequence data from POE diatom taxa on sea turtles and sirenians. To reach this goal we have compiled

genetic data from diatoms isolated and cultured from sea turtles and manatees from the wild, rehabilitation and rescue centers as well as aquaria from the United States of America, The Bahamas, Croatia, Italy and South Africa.

Results

Culture Success

We successfully cultured >600 strains, both POE and opportunistic diatoms on the epizoic habitat. These were primarily from the southeastern US, the eastern coast of South Africa and the eastern Mediterranean. This manuscript focuses on 76 of these sequenced strains (Table 1) and the sequences from the single-cell DNA extractions of the non-photosynthetic *Tursiocola* spp. (Figures 1, 2). Sequence data from 21 additional diatoms are included (Figures S1, S2). While these additional sequenced diatom taxa were isolated from epizoic collections, they are known opportunistic taxa, occur in non-epizoic habitats, or their habitat preferences are unclear.

Table 1

POE diatoms cultured in this study, sorted by host species. POE diatoms are abbreviated and followed by the number of strains cultured from the indicated host: Ae = *Achnanthes elongata*, Ca = *Craspedostauros alatus*, Cd = *Craspedostauros danayanus*, Cm = *Craspedostauros macewanii*, Cca = *Chelonicola caribeana*, Cco = *Chelonicola costaricensis*, Csp = *Chelonicola sp.*, Ma = *Medlinella amphoroidea*, Pl = *Poulinea lepidochelicola*, Ps = *Proschkinia sulcata*, Pv = *Proschkinia vergostriata*, Td = *Tursiocola denysii*, Tg = *Tursiocola guyanensis*.

Host Species	Location	Host Status	POE Diatoms cultured (# of strains)	Total diatom strains cultured
Green Sea Turtle *Chelonia mydas*	Bahamas	wild animal, health assessment: “turtle1”	Cca (2), Td (2), Pv (1)	9
Green Sea Turtle *Chelonia mydas*	Durban, South Africa	aquarium resident: “Calypso”	Cco (1), Cm (3), Pl (2)	14
Green Sea Turtle *Chelonia mydas*	Durban, South Africa	aquarium resident: “Wasabi”	Ma (1), Pl (4)	12
Green Sea Turtle *Chelonia mydas*	Florida, USA	wild animal, nesting: “FL noname”	Ae (2), Tg (1)	6
Green Sea Turtle *Chelonia mydas*	Florida, USA	rehabilitation animal: “Fleming”	Ae (5), Pl (3), Pv (3)	22
Hawksbill Sea Turtle *Eretmochelys imbricata*	Texas, USA	aquarium resident: “Einstein”	Ca (2)	4
Hawksbill Sea Turtle *Eretmochelys imbricata*	Durban, South Africa	aquarium resident: “Tripod”	Ma (3)	11
Kemp’s Ridley Sea Turtle *Lepidochelys kempii*	Georgia, USA	wild animal, health assessment: “Z6”	Ae (3)	12

Host Species	Location	Host Status	POE Diatoms cultured (# of strains)	Total diatom strains cultured
Leatherback Sea Turtle *Dermochelys coriacea*	Kosi Bay, South Africa	wild animal, nesting: “ZA0019A/ZA1824E”	Cd (1)	7
Loggerhead Sea Turtle *Caretta caretta*	Durban, South Africa	aquarium resident: “Shiv”	Pl (3)	6
Loggerhead Sea Turtle *Caretta caretta*	Kosi Bay, South Africa	wild animal, nesting: “ZA00940/ZA10860”	Ma (1)	4
Loggerhead Sea Turtle *Caretta caretta*	Kosi Bay, South Africa	wild animal, nesting: “ZA1595E/ZA1826E”	Pl (1)	10
Loggerhead Sea Turtle *Caretta caretta*	Kosi Bay, South Africa	wild animal, nesting	Csp (2)	10
Loggerhead Sea Turtle *Caretta caretta*	Florida, USA	wild animal, health assessment: “A2”	Ae (7)	8
Loggerhead Sea Turtle *Caretta caretta*	Florida, USA	wild animal, health assessment: “CC032217a”	Cca (2), Pv (2)	19
Loggerhead Sea Turtle *Caretta caretta*	Florida, USA	wild animal, nesting: “FL Christine”	Cca (2)	6
Loggerhead Sea Turtle *Caretta caretta*	Brijuni Islands, Croatia	aquarium resident: “Lunga”	Ps (1)	3
Loggerhead Sea Turtle *Caretta caretta*	Bisceglie, Italy	rehabilitation animal: “Iracus”	Pl (3)	39

Host Species	Location	Host Status	POE Diatoms cultured (# of strains)	Total diatom strains cultured
Olive Ridley Sea Turtle *Lepidochelys olivacea*	Long Beach, California	Aquarium resident: “LoMain”	Pl (1)	14
Olive Ridley Sea Turtle *Lepidochelys olivacea*	Florida, USA	rehabilitation animal: “Harry”	Ae (2), Pl (3)	10
West Indian Manatee *Trichechus manatus latirostris*	Florida, USA	wild animal, health assessment: “FLMan40”	Ae (2)	11
West Indian Manatee *Trichechus manatus latirostris*	Georgia, USA	wild animal, health assessment: “CGA1605”	Ae (1)	23

Target POE taxa

POE taxa were identified based on the available literature and included diatom species that have only ever been observed in association with the epizoic habit being found on multiple animal specimens [6, 8, 10, 11, 14, 15, 16 24, 25, 30]. Among these were epizoic taxa typically reaching high relative abundances (>25%)—*Achnanthes elongata* Majewska & Van de Vijver, *Chelonicola costaricensis* Majewska, De Stefano & Van de Vijver, *C. caribeana* Riaux-Gobin, Witkowski, Ector & Chevallier, *Craspedostauros danayanus* Majewska & Ashworth, *Medlinella amphoroidea* Frankovich, Ashworth & M.J.Sullivan, *Poulinea lepidochelicola* Majewska, De Stefano & Van de Vijver, *Tursiocola* spp, as well as species often present on animals but never exceeding 10% of the diatom relative abundance—*Craspedostauros alatus* Majewska & Ashworth, *C. macewanii* Majewska & Ashworth, *Proschkinia sulcata* Majewska, Van de Vijver & Bosak and *P. vergostriata* Frankovich, Ashworth & M.J Sullivan. SEM images of some of these taxa sampled for DNA can be found in Figure 3. This list of POE taxa is not exhaustive as the full diversity of POE diatoms remains to be documented. Moreover, it does not include several probable POE species (e.g. *Achnanthes squaliformis* Majewska & Van de Vijver, *Navicula dermochelycola* Riaux-Gobin, Witkowski, Kociolek & Chevallier), which have not yet been isolated and cultured.

Molecular phylogenetic results

The currently recognized POE strains were predominantly located in two clades in the molecular phylogeny—*Achnanthes* sensu stricto+*Craspedostauros* (Fig. 1) and the clade containing the *Tripterion* complex, *Tursiocola* and *Proschkinia* (Fig. 2). With regards to *Achnanthes*, most of the sampled diversity comes from manatees and three species of sea turtles (green, Kemp's ridley and loggerhead) sampled in the southeastern US. These strains formed a well-supported clade (ML bootstrap support [bs] = 100%, BI posterior probability [pp] = 1.0) sister to the rest of the sequenced *Achnanthes* spp. The POE *Achnanthes* clade also sorted by host, with strains collected from manatee (100%/1.0 bs/pp) and sea turtle (100%/0.74 bs/pp) hosts in their own clades. The POE *Craspedostauros* taxa show a different pattern to the rest of the POE diatoms. Their clade included both POE and non-POE species, with POE taxon *C. danayanus* sister to *C. alyoubii* and *C. paradoxus* (96%/0.99 bs/pp) rather than to the POE *C. macewanii* and *C. alatus*.

The "*Tripterion* complex +" clade (Fig. 3) was resolved with strong support (100%/1.0 bs/pp). This clade contained *Stauroneis* Ehrenberg, *Craticula* Grunow, *Parlibellus* E.J.Cox, *Fistulifera* Lange-Bertalot, as well as some monoraphid genera such as *Schizostauron* Grunow and *Astartiella* Witkowski, Lange-Bertalot & Metzeltin. It also contained clades of the POE genus *Tursiocola* and those of the *Tripterion* complex genera, as well as *Proschkinia* Karayeva, which has both POE and non-POE species. The molecular data suggested no common origin for the POE clades; *Tursiocola* and the *Tripterion* complex are sister to non-POE taxa rather than each other, and the POE *Proschkinia* (*P. vergostriata* and *P. sulcata*) formed a clade sister (100%/1.0 bs/pp) to the rest of the *Proschkinia* spp.

Within *Tursiocola*, both nutritional types appear monophyletic, with the non-photosynthetic manatee-associated taxa (100%/1.0 bs/pp) and the photosynthetic sea turtle-associated taxa (100%/1.0 bs/pp) in their own clades. It should be noted, however, that there were only two photosynthetic *Tursiocola* taxa sampled. Tree topology in the *Tripterion* complex remained the same regardless of analysis, with *Chelonicola costaricensis* "Majewska21C" + *Poulinea lepidochelicola* (100%/1.0 bs/pp) sister to *Medlinella amphoroidea* + *Chelonicola* sp. "Majewska39A/40A" + *C. caribeana* (92%/0.74 bs/pp).

Only two clades in the *Tripterion* complex had any geographic variation: the *Poulinea* clade and *Chelonicola caribeana* clade. For *Poulinea*, strains collected in South Africa were not monophyletic, with "Majewska 17C" sister to the rest of the clade, which included strains isolated from the Adriatic, Florida, California and South Africa. It should be noted that the Florida clade represented strains collected from a single location—a rehabilitation facility—while the South African strains were isolated from collections of both wild and captive host animals. The *C. caribeana* clade, on the other hand, contained strains isolated exclusively from wild host animals in South Africa, Florida and the Bahamas, with the South African strains ("Majewska39A/40A") sister to the rest.

Discussion

This study helps to lay the groundwork for developing a database of DNA sequence data from POE diatoms. The use of DNA sequence data enables application of these epizoic diatom assemblages as

proxies for host and ecology without the need for an extensive knowledge of benthic diatom taxonomy. We generated DNA sequence data from 16 of the known POE diatom species for sea turtles and maintained 12 of these taxa in culture. This opens the door for future studies on the molecular, genomic and physiological nature of the epizoic diatoms. Moreover, our ability to culture POE diatoms without their hosts invites several questions about diatom-host interactions. Specifically, POE diatoms evidently do not require the host to survive and so we propose four non-exclusive hypotheses that could explain the specificity of POE to particular hosts in the wild:

H1: The epizoic environment creates a unique nutrient supply regime affecting bottom-up competition, with POE taxa being better competitors in the epizoic environment and/or worse competitors in a non-epizoic environment.

There are several ways that the epizoic host environment may increase nutrient availability relative to a more static environment. Firstly, there may be leakage of nutrient compounds through the skin of the host, directly raising N or P concentrations near the host. Although mammalian skin is generally highly impermeable, the barrier requirements of glabrous marine animals living in the slightly hypertonic seawater may be less rigorous than in terrestrial species [31]. There is some evidence to suggest mammals and reptiles have similar skin permeability [32]. Secondly, the movement of the host through the water will constantly reduce nutrient gradients along the host (and near the diatoms) much as stream flow or wave action does. As the animal-derived nutrients are unlikely to include silicon, the Si:P and/or Si:N supply ratios may be very low close to the animal surface, a condition that the POE diatoms may be better adapted to than opportunistic taxa. In the marine environment, rivers are one of the major silica sources [33] and higher Si supply rates are typical of shallow-water coastal habitats and non-mobile substrates.

Regardless of the particular mechanism(s) that might provide a different competitive environment, given that we have been able to culture so many of the epizoic diatoms we have encountered, the hypothesis that epizoic diatoms have different nutrient requirements and uptake capability than closely related benthic relatives should be testable.

H2: The POE flora requires prokaryotic microbes derived from the epizoic environment.

There is ample evidence that benthic microalgae, including diatoms, exist in a complex network of interactions (the “phycosphere”) with other microorganisms either adhering to their membranes and theca or attached via extracellular matrices [34, 35, 36]. Therefore, the biochemical signals and links to the epizoic habit may be derived from bacteria specific to the host rather than the host itself. There is some evidence already that large aquatic vertebrates can harbor unique bacterial assemblages [37, 38, 39, 40], but the interaction between epizoic bacteria and algae has barely been investigated. The epizoic diatoms isolated for this study were not grown axenically, so phycosphere-associated bacteria were transferred into culture with the diatoms. This association might have allowed the POE diatoms to grow in the absence of the epizoic habit.

H3: The POE flora has special adaptations to epizoic lifestyle.

Most of the POE species we have studied do not require the epizoic host to survive and divide. Thus, it is possible that these taxa are found on vertebrate hosts not because they are favored by such an environment, but because they are simply tolerant of it. While there is no evidence the epizoic diatoms are directly harming the host, unchecked growth of diatoms (and other algae) could increase drag and negatively affect rheological properties of the actively moving animals. Raphid diatoms are often biofilm stabilizers, depositing secreted polysaccharides which allow for further attachment by bacteria, fungi, macrophytes and invertebrates, including potential pathogens and organisms that can damage animal tissue. In addition, settlement of macroepibionts may significantly increase the host's weight, thus increasing the energetic cost of swimming and diving. Therefore, marine vertebrates have developed various mechanisms to prevent the settlement of pioneering surface-conditioning organisms and thereby limiting epizoic diatom growth [41, 42].

While biochemical deterrents to biofouling have evolved in many organisms, there is little evidence for such specialized secretions in sirenians and sea turtles. Production of bioactive antifouling compounds is especially unlikely in the case of the sea turtle carapace scutes, which are built from physiologically inactive anucleate cells [43]. Cetacean skin, which also harbors POE diatoms, is believed to lack any glands and its biochemical adaptation depends largely on keratinocytes present in both the basal layer of the skin and the most external stratum corneum. The nucleated cell layers are responsible for production of a gel-like matrix containing hydrolytic enzymes, such as glycosidases and peptidases [44]. Although it has been suggested that the enzymes might degrade adhesive polymers produced by some of the biofoulers [44], their main role seems to be the initiation of the desquamation process [45]. The latter could be one of the most efficient defense strategies against biofouling developed by many long-lived marine macroorganisms [42, 46]. In both glabrous marine mammals and reptiles, skin scales show highly homogenous topology with few microniches. Moreover, micro- and nanoornamentations (dermatoglyphics [47]) of the skin scales may decrease the number of contact points and thus prevent the attachment of microscopic biofilm precursors [45].

Host behavior may also influence settlement. West Indian manatees, for example, move from marine to brackish to freshwater habitats in response to seasonality and water temperature, potentially at a rate and duration to which opportunistic diatoms may not be able to adapt [27]. Basking behavior of sea turtles as well as passive drifting close to the ocean surface typical of sea snakes [48], which can also carry epizoic diatoms [23], can expose the diatoms to damaging levels of heat, irradiance and desiccation. Deep diving, long-distance migrations and nesting behavior shown by many sea turtle species may induce intolerable rates of change in temperature, salinity, nutrient availability (particularly silica, which can be limiting in the open ocean) and hydrostatic pressure on non-adapted, opportunistic diatom species. The POE diatoms may also be better adapted to resist drag, friction, and high shear regime induced by the animal movement as well as frequent dermal abrasion caused by grooming practices employed by either the host animal itself [49] or specialized grazers feeding on the epizoic flora of the host [27, 50].

Resistance to these antifouling measures as well as high tolerance to rapidly and continuously changing environmental conditions would allow the POE taxa to successfully colonize and dominate the animal surfaces. However, they would provide no competitive advantage against opportunistic diatom species on other substrates. This could explain why the POE taxa thrive in both their native epizoic habitat and artificial monoculture.

H4: There is a non-epizoic reservoir of these taxa that we have yet to discover

Large areas of the world's marine shallow benthic environment are poorly studied for diatoms, and therefore we cannot exclude the possibility that the POE taxa do exist outside of epizoic habitats. Even in localities that are relatively well-studied for benthic diatoms, variation in the composition and relative abundance in an assemblage due to substrate specificity and seasonality make the assembly of an exhaustive diatom flora extremely difficult. While a published flora exists for one of our collection sites, Florida Bay [51], and many small-celled diatom species have been reported, the relatively small size of the *Tripterion* clade POE taxa (< 20 μm) and their gross morphological similarity to other non-epizoic gomphonemoid diatoms (e.g. *Gomphoseptatum* Medlin, *Cuneolus* Giffen) makes it possible that valves in low concentrations, such as those that might have sloughed off host animals and entered the tychoplankton, might have been overlooked.

Environmental DNA surveys, such as metabarcoding, have an advantage over microscope-based surveys with regards to relatively small-sized taxa. The results of this study, however, indicate that POE diatoms can also be undetected in eDNA surveys. Based on the molecular phylogeny of the *Tripterion* clade, it is easy to see how these taxa might have remained undetected in a BLAST-based bioinformatic summary of OTUs, as there is significant genetic difference between the *Tripterion* clade and the only other sequenced representatives of the Rhoicospheniaceae—the freshwater taxon *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot. In fact, there are no morphological characters exclusive to the taxa in the molecular clade containing *Tursiocola* and the *Tripterion* clade that would cause a diatomist to expect a close match in sequence identity to the POE taxa. With curated sequence data now available for the most common POE taxa, we may find evidence for their occurrence in non-epizoic habitats through eDNA studies.

However, it should be remembered that the presence of a POE specimen (or POE DNA) in benthic samples does not discount that these taxa may be epizoic specialists. In fact, it is highly unlikely that, given enough time, one would not encounter a *Tripterion* or *Tursiocola* specimen in the benthos or plankton of Florida Bay. If a cell of a POE diatom species had just been shed—living or dead—this would have little bearing on whether the POE taxa are obligate epizoics. Similarly, the low relative abundances of POE taxa valves that might have been overlooked in the surface sediment, seaweed, and seagrass samples would suggest that non-epizoic substrates do not provide optimal habitats for POE species. While we clearly value floristic studies, the presence-absence data cannot unequivocally determine if a species is a POE.

The hypotheses listed above are not necessarily exclusive. Different species may be affected by different conditions. Moreover, potential specific adaptations to epizoic lifestyle developed by POE diatoms suggested by (H3), do not directly explain why they have not been found in other benthic habitats. It is

possible that some trade-off in obtaining those adaptations makes the POE taxa less competitive in non-epizoic benthic environments (H1). We know little about how the phycosphere might affect the competitive ability of diatoms, and/or whether the phycosphere may itself manufacture some critical compound only in an epizoic community (H2). Nonetheless, we argue that these hypotheses can form the beginning of a conceptual framework to understand POE taxa functioning as well as their ecological role.

What do POE diatoms tell us about raphid diatom evolution?

Based on our molecular phylogeny, it appears that the epizoic habit has evolved several times and in several different raphid diatom morphotypes: elongate biraphid (*Proschkinia* and *Tursiocola*, Fig. 3f & 3g, respectively) and monoraphid frustules (*Achnanthes*, Fig. 1e), asymmetric, clavate biraphid frustules (*Tripterion* complex, Fig. 3a) and thin oval monoraphid frustules (*Bennettella, Epipellis* [52]). These independent gains of the epizoic habit could be driven by the host biology and evolution. The various epizoic diatom lineages, if eventually resolved to be closely linked to a specific type of host animal, might have diverged from non-epizoic taxa under different ecological and evolutionary constraints and at different times corresponding to the emergence of various groups of marine megafauna.

Among others, the eco-physiological constraints shaping epizoic diatom speciation through adaptive radiation would include the nature and character of the animal substrate. Variations of the dermal layer of sirenians and sea turtles including the ultrastructure, topology, physiology (e.g. shedding patterns), and biochemistry (e.g. enzymatic activity) would require different attachment and colonization (and re-colonization) strategies, thus encouraging the development of specific adaptations. Such a specific adaptation is evidenced by *Melanothamnus maniticola* Woodworth, Frankovich & Freshwater, an epizoic red alga on manatees that has unique skin penetrating rhizoids that anchor the thallus to the deeper epidermis and permit the alga to persist as the host surface skin cells are shed [53]. In sea turtles, the carapace scutes are often retained or shed periodically, while the skin scales are either shed continuously (sea turtles) or the epidermis is renewed completely in a process called ecdysis (sea snakes [54]). These patterns differ from those observed in marine mammals in which skin shedding may be regulated by external factors such as temperature [46]. Similarly, animals with different diving regimes may host diatoms with different physiological and metabolic adaptations as various stages of photosynthesis will be differently affected by changes in hydrostatic pressure related to the depth, duration, and frequency of dives [55].

Moreover, the diversification dynamics in POE diatoms may be linked to the host animal behavior and lifestyle. The high niche heterogeneity, biodiversity, productivity, and nutrient concentrations typical of shallow-water habitats occupied by sirenians and some sea turtles may increase colonization rates by new species and favor benthic diatom immigration to the epizoic community, thus spurring the observed diversity of diatom forms associated with manatees [24, 25] or sea turtles using neritic foraging habitats (e.g. loggerheads; [21]). The opposite phenomenon could explain low epizoic diatom diversity on leatherback sea turtles [5, 30], and pelagic sea snakes [23] that spend significant (though not exclusively

[56] time feeding in the pelagic zone rather than on benthic organisms [57]. This follows the general pattern of low macro-epibiotic diversity on leatherbacks [58]. Epizoic diatom diversity might also be driven by intrinsic biotic factors, such as gregariousness and range of the host species as both factors may affect the new species encounter and colonization rates. However, in these systems in which epizoic diatom species richness is driven mainly by speciation rates as opposed to benthic species immigration, the total epizoic diatom diversity may remain low. The higher number of diatom taxa observed on neritic megafauna species as compared to open-water animals seem to support this hypothesis [20].

Currently, taxon sampling is still scattered, and while strains were isolated from multiple geographic localities (focused in the southeastern USA and South Africa at this time), much of the strain diversity in species-level clades come from a single collection. The Florida *Poulinea lepidochelicola* clade, for example, represents strains isolated exclusively from the Turtle Hospital rehabilitation facility in Marathon, Florida. Among the South African *P. lepidochelicola* strains, six strains (Majewksa 14C, Majewska 20C, HK630, HK638, HK639 and HK640) came from collections from three turtles at the uShaka Sea World facility in Durban, and likely represent one population. It is curious to note, however, that a morphological difference does exist between the sequenced *Medlinella amphoroidea* strains from South Africa and the type population of Florida Bay. The valve areolae of the former appear to be occluded by hymenes (Fig. 3d) as opposed to the volae of the type population [14]. Whether this corresponds to a genetic, and perhaps species differentiation remains to be seen, once the Florida Bay population is sequenced.

What might POE diatoms tell us about their hosts?

While we do not yet have enough information to assign any sort of host specificity to certain POE diatom taxa, we have enough DNA sequence data to suggest that some genetic differentiation among POE diatoms is occurring. While we do not know if the genetic distance between the Florida, Mediterranean and South African *Poulinea* strains is driven by speciation or intraspecific biogeography, they are genetically distinct. Data collected from loggerheads suggests little mixing between sea turtle individuals across ocean basins [59], with the Mediterranean population being distinct from the northeast Atlantic one, which is then distinct from northwest Atlantic (including the Gulf of Mexico) population. Even within closer geographic boundaries, such as the western Atlantic, there is demonstrated genetic distance between POE strains (*C. caribeana* of Florida and the Bahamas; *Achnanthes elongata* of Florida and Georgia) in DNA sequence markers which are generally considered too conserved to show intraspecific variation in diatoms [60, 61].

Future studies and molecular information from a larger number of POE diatom strains may reveal whether genetic diversity in epizoic diatoms reflects biogeographic, ecological, and behavioral patterns observed in the host animal populations. For example, it was demonstrated that sea turtle phylogeography is shaped by the sea turtle species thermal regime and habitat preference [62]. Provided the close relationship between epizoic diatoms and sea turtles holds up under the scrutiny of increased data sampling, it may be expected that POE diatoms associated with the cold-tolerant leatherbacks, which are able to use the southwestern corridors to migrate across the oceans, will be characterized by

lower genetic diversity than diatom taxa growing on tropical species such as green turtles, hawksbills, and olive ridley sea turtles, whose Atlantic and Indo-Pacific populations appear to be genetically distinct [63]. This knowledge may significantly advance our understanding about evolutionary relationships between diatoms and their animal hosts as well as shed more light on the mechanistic processes of divergence and adaptive evolution of diatoms and other marine microbes.

Conclusions

The stated goal of this study was to generate additional DNA sequence data from POE diatom taxa on sea turtles and sirenians. This goal was greatly aided by our ability to culture many of these POE diatoms away from their hosts, which raises several questions about the ecological requirements and adaptations of epizoic diatoms. The isolated strains of POE diatoms, which can be maintained in artificial conditions and without the animal hosts, provide opportunities to further study the nature of the unique relationship between the diatoms and marine megafauna in a laboratory setting.

The DNA sequence data generated from the obtained cultures provides us a solid foundation for a photovouchered reference library that could be used for future metabarcoding surveys. These could be used to further probe the substrate specificity of POE diatoms by searching for the DNA barcodes in other epizoic and non-epizoic habitats. The sequence library could also be useful to investigate on the potential of epizoic diatoms as non-invasive bioindicators for sea turtle and manatee health. Phylogenetic analysis of the POE diatom DNA sequences showed that multiple diatom lineages have evolved to the epizoic habitat. Future studies may determine the number of evolutionary leaps to the epizoic habitat and the number of host switches, shedding more light on the co-evolution of diatom-animal relationships.

Methods And Materials

Cultures and Microscopy

Diatoms were collected from the skin of West Indian manatees and the skin and carapace of six species of sea turtles (see Table 1 for details). These collections were made following the protocol outlined by Pinou et al. [64]. Individual diatom cells were isolated by micropipette into sterile f/2 culture medium [65] with a salinity matching the collection source. In the case of non-photosynthetic taxa (like some *Tursiocola*), individual cells were photovouchered and isolated into WGA whole-genome amplification cocktail [25].

Cultures were harvested into separate pellets for microscopy preparation and DNA sequencing. Pellets for microscopy were cleaned with hydrogen peroxide and nitric acid, rinsed to neutral pH and dried onto 22 x 22 mm and 12 mm coverslips for light microscopy (LM) and scanning electron microscopy (SEM), respectively. Permanent mounts for the LM slides were made with Naphrax® mounting medium (Brunel Microscopes, www.brunelmicroscopessecure.co.uk) and micrographs were taken with a Zeiss Axioskop. Coverslips for SEM were coated with iridium by a Cressington 208 Bench Top Sputter Coater (Cressington

Scientific Instruments, Watford, UK) and micrographs taken with a Zeiss SUPRA 40 VP scanning electron microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

DNA isolation, amplification and sequencing

Pellets for DNA sequencing were extracted using the DNeasy Plant Minikit, with an extra 45 s incubation in a Beadbeater (Biospec Products, Bartlesville, OK, USA) with 1.0 mm glass pellets for colony and frustule disruption. The nuclear-encoded ribosomal SSU and chloroplast-encoded *rbc*L and *psb*C markers were amplified by PCR using the primers outlined in Theriot et al. [66] in 25 µL reactions with 1-3 µL of template DNA, 0.5 µL of each primer, 0.25 µL of Taq polymerase, 12.5 µL of pre-mixed FailSafe Buffer E (Lucigen Corporation) and 8.25-10.25 µL of sterile water. PCR conditions were identical for *rbc*L and *psb*C: 94^{o}C for 3.5 min., 35 cycles of (94^{o}C for 30 sec, 48^{o}C for 60 sec., 72^{o}C for 2 min.), and a final extension at 72^{o}C for 15 min. PCR conditions for SSU were: 94^{o}C for 3.5 min., 35 cycles of (94^{o}C for 30 sec., 51^{o}C for 60 sec., 72^{o}C for 3 min.), and a final extension at 72^{o}C for 15 min. The amplicons were purified using an EXO-SAP protocol: a 3 µL of an EXO-SAP solution containing 0.5 µL of shrimp alkaline phosphatase, 0.25 µL of exonuclease I and 2.25 µL of sterile water were added to the PCR products and incubated at 37^{o}C for 30 min. followed by 80^{o}C for 15 min. Purified products were then sequenced on an ABI 3730 DNA Analyzers using BigDye Terminator v3.1 chemistry.

Sequence data were added to a dataset of raphid and araphid pennate diatoms, with *Asterionellopsis glacialis* used as an outgroup (see Table S1 for GenBank accession numbers). SSU data were aligned by the SSUalign program, using the covariance model outlined in Lobban et al. [67]. Data were initially partitioned by gene, by paired and unpaired sites in SSU secondary structure and codon position in *rbc*L and *psb*C. Model testing and grouping of partitions were performed by PartitionFinder 2 [68] using all nucleotide substitution models, linked branches, and rcluster search [69] settings for trees inferred by RAxML 8 [70]. The best model was chosen using the corrected Akaike information criterion (AICc). Maximum Likelihood and Bayesian Inference based phylogenies were inferred using IQ-TREE version 1.6.12 for Linux [71] with partitioned models [72] and multi-threaded MPI hybrid variant of ExaBayes version 1.5 [73], respectively. Nodal support for the maximum likelihood phylogeny was assessed using 1,000 bootstrap replicates via IQ-TREE. ExaBayes analyses included four independent runs with two coupled chains where branch lengths were linked. Convergence parameters included an average deviation of split frequencies (ASDSF) of less than or equal to 5% with a minimum of 10,000,000 generations. Bayesian nodal support was assessed using posterior probabilities, with the first 25% of the trees removed as “burn-in”.

Abbreviations

POE = putatively obligate epizoic, SEM = scanning electron microscope, bs = bootstrap support, pp = posterior probability

Declarations

Ethics approval and consent to participate

Sampling activities performed in the iSimangaliso Wetland Park (South Africa) were carried out under research permits issued by the South African Department of Environmental Affairs (RES 2017/73, RES 2018/68, and RES 2019/05). Sampling activities performed at Loggerhead Marinelife Center (Juno Beach, Florida) were conducted under Florida Fish and Wildlife Conservation Commission Permit #086 and 205. Sampling performed at the Sea Turtle Clinic (Bari, Italy) was conducted with the permission of the Department of Veterinary Medicine Animal Ethic Committee (Authorization # 4/19), while sampling in Croatia was carried out in accordance with the authorization of the Marine Turtle Rescue Center at Aquarium Pula by the Ministry of Environment and Energy of the Republic of Croatia.

Consent for Publication

Not applicable

Availability of data and materials

DNA sequence data generated for this study are published on the NCBI GenBank online sequence depository under the accession numbers listed in Table S1. Additional micrographs and cleaned voucher material from the sequenced cultures are available from lead author MPA.

Competing Interests

Authors declare no conflict of interest

Authors Contributions

MPA and RM contributed equally to the study design, culture isolation and manuscript writing and editing. MPA also extracted and sequenced DNA and constructed the phylogenetic datasets, while RM collected samples in South Africa and the Bahamas and managed South African collections. TF contributed to managing collections in the US and study design and interpretation, as well as manuscript editing. MS contributed to data interpretation and manuscript editing. SB and KF contributed to collecting samples in Croatia, extracting and sequencing DNA as well as study design, interpretation and manuscript editing. BV contributed to study design, data interpretation and manuscript editing. MA, JS, NIS, JRP and CAM collected samples in the US and contributed to data interpretation and manuscript editing. RN obtained permits for sample collection in South Africa and contributed to manuscript editing. NJR and MG obtained permits and coordinated sample collection in the Bahamas and contributed to data interpretation and manuscript editing. ECT and DWL contributed to the phylogenetic analysis of DNA data, data interpretation and manuscript editing. SRM contributed to culture maintenance, DNA work in the US and manuscript editing.

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Figures

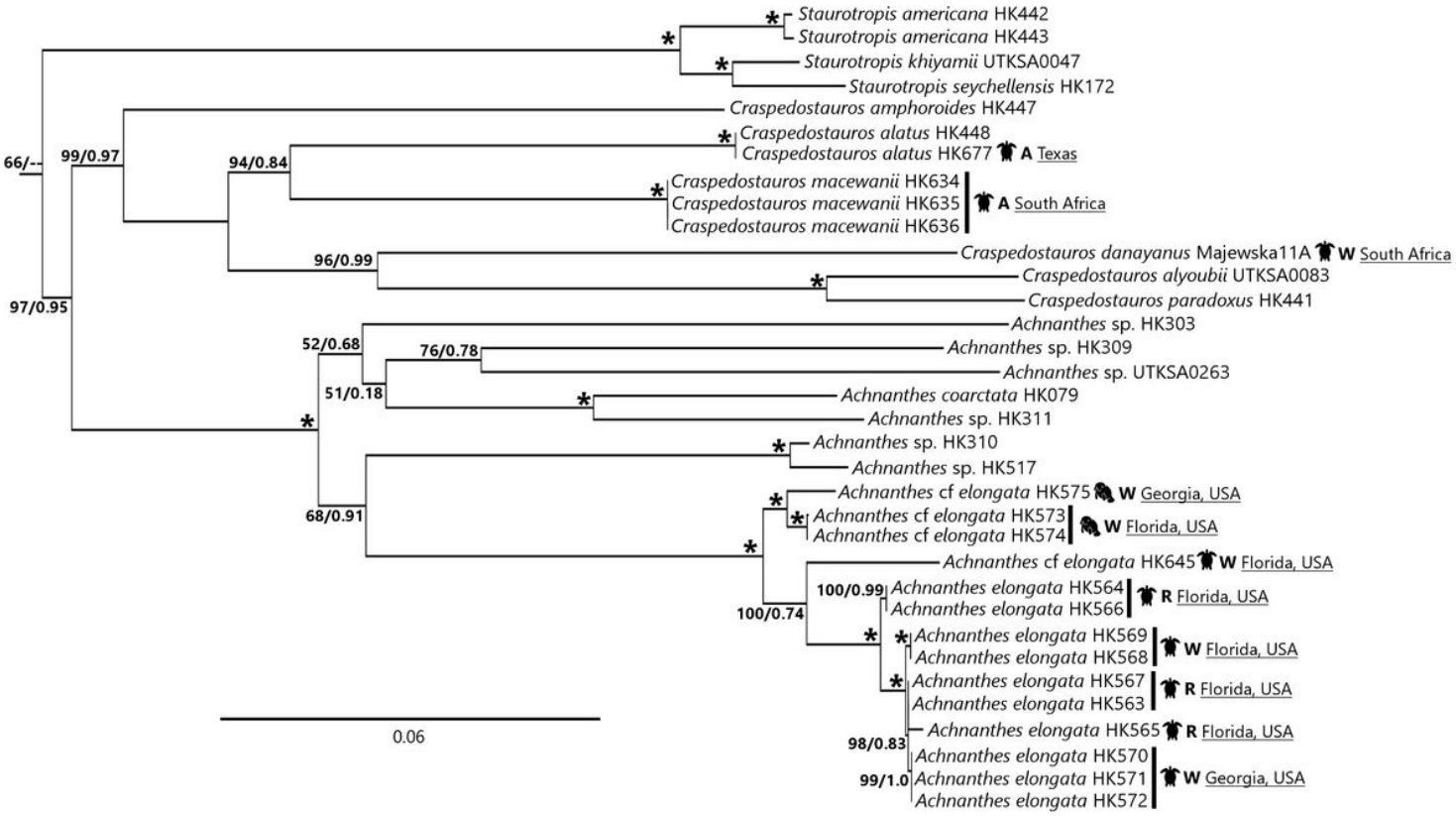

Figure 1

Maximum likelihood phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset, representing the Achnanthes, Craspedostauros and Staurotropis clades (complete tree shown in Figure S1). Support values (ML bootstrap support/BI posterior probability) shown above nodes; “*” = nodes with 100%/1.0 values. Taxon name followed by DNA extraction voucher number or strain ID. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild).

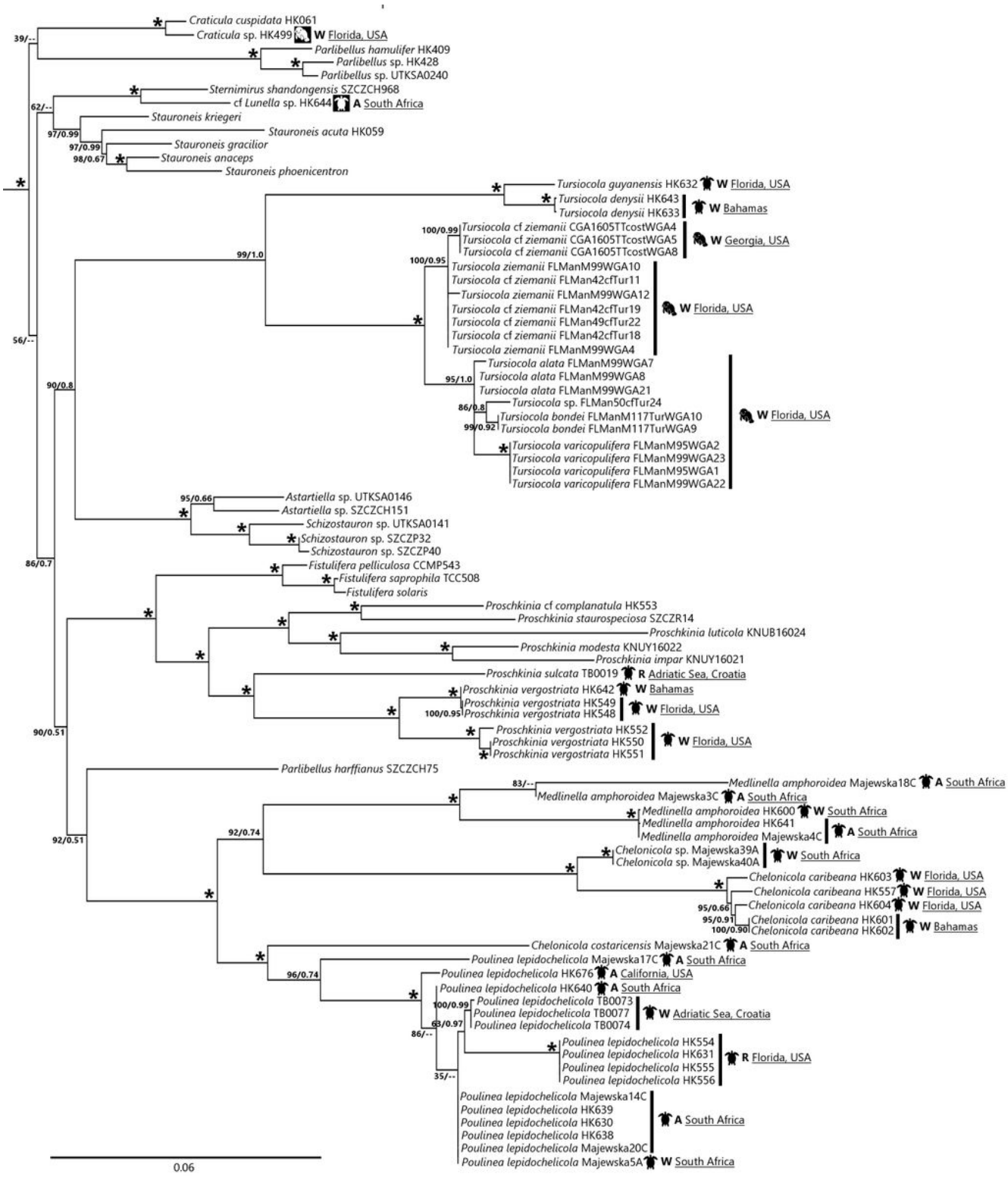

Figure 2

Maximum likelihood phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset, representing the clade containing the Tripterion complex, Tursiocola and Proschkinia clades (complete tree shown in Figure S1). Support values (ML bootstrap support/BI posterior probability) shown above nodes; “*” = nodes with 100%/1.0 values. Taxon name followed by DNA extraction voucher number or strain ID. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from

which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild). Black host icon = POE taxon; white host icon = unclear habitat preference.

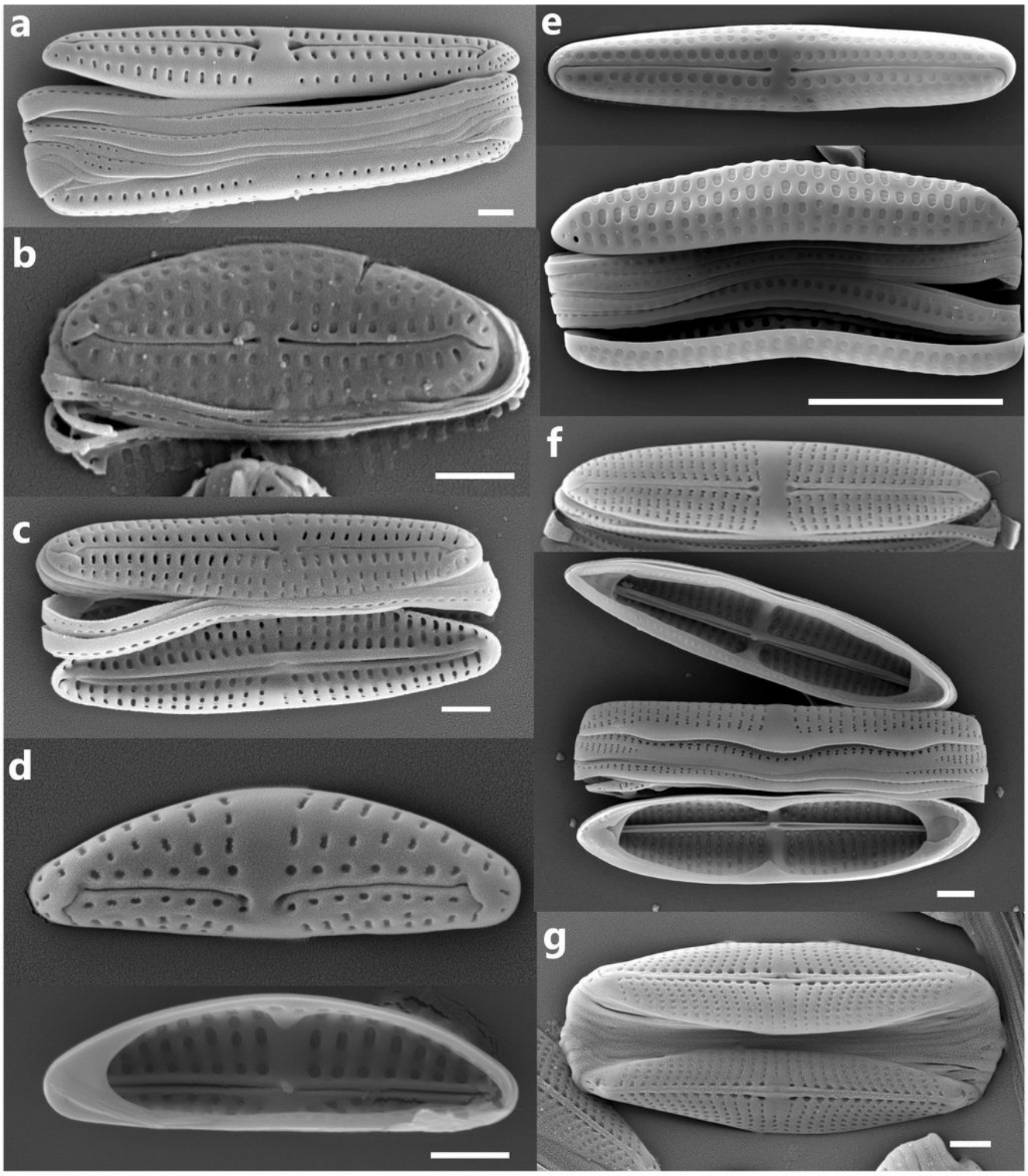

Figure 3

Scanning electron micrographs of some of the POE diatom taxa successfully cultured and sampled for DNA. a = Poulinea lepidochelicola HK630, complete frustule. b = Chelonicola cf costaricensis Majewska

21C, valve exterior. c = Chelonicola sp. Majewska 40A, complete frustule. d = Medlinella amphoroidea HK600 (valve exterior above, interior below). e = Achnanthes elongata HK563 (valve exterior above, complete frustule below). f = Tursiocola denysii HK633 (valve exterior above, complete frustule below). g = Proschkinia vergostriata HK552, complete frustule. All scale bars = 1 µm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.pdf
- FigureS2.pdf
- TableS1.doc