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Ciliate species diversity and host–parasitoid codiversification in *Pseudocollinia* infecting krill, with description of *Pseudocollinia similis* sp. nov.

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ABSTRACT: All parasitoid apostome ciliates infecting krill in the northeastern Pacific are currently assigned to the genus *Pseudocollinia*. Each krill specimen is apparently infected by only 1 *Pseudocollinia* species. We describe *Pseudocollinia similis* sp. nov., discovered infecting the krill *Thysanoessa spinifera* off Oregon, USA. Its protomite-tomite stage resembles that of *P. beringensis*, which infects *T. inermis* (type host species), *T. longipes*, and *T. raschii* females in the Bering Sea. These ciliates have similar numbers of somatic kineties (18–21 vs. 16–20) and typically have 3 oral kineties. Furthermore, these 2 apostomes are sister species on gene trees based on sequences of small subunit rRNA (0.06% difference) and cytochrome *c* oxidase subunit 1 (*cox1*; 30% difference). *P. brintoni* and *P. oregonensis* are closely related as a separate group from *P. similis* and *P. beringensis*. The similar tree topologies based on the *cox1* sequences of 21 host krill individuals representing 6 krill species (*Euphausia pacifica*, *Nyctiphanes simplex*, *T. inermis*, *T. longipes*, *T. raschii*, and *T. spinifera*) and the apostomes isolated from these krill suggest host–parasitoid codiversification. However, this hypothesis was statistically rejected by an approximately unbiased test in which the host tree topology was used to model parasitoid evolution ($p \leq 0.05$).

KEY WORDS: Euphausiids · Apostomatida · Small subunit rRNA · SSUrRNA · Cytochrome *c* oxidase subunit 1 · *cox1* · Oregon

INTRODUCTION

Endoparasite apostome ciliates infecting the hemocoel of euphausiids were first reported by Capriulo & Small (1986), who described a new species, *Collinia beringensis*, infecting the krill *Thysanoessa inermis* in the Bering Sea. Gómez-Gutiérrez et al. (2003) demonstrated that these ciliates are actually endoparasitoids and could have significant impacts on euphausiid populations (see also Capriulo et al. 1991). Further investigations of these ciliates in the context of population ecology of euphausiids of the eastern North Pacific Ocean involved extensive sampling of 7 euphausiid species. This has resulted in the description of 2 additional species of ciliates and an analysis that placed these 3 ciliate species in the new apostome genus *Pseudocollinia* (*P. beringensis*, *P. oregonensis*, and *P. brintoni*; Gómez-Gutiérrez et al. 2006, 2012). Mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and small subunit rRNA (SSU rRNA) gene sequences of the most recently described species (*P. brintoni*) and the type species *P. beringensis*

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demonstrated that they are apostomate ciliates (Gómez-Gutiérrez et al. 2012). Here, we present an additional contribution in this series describing the new species *P. similis* sp. nov., identified based on genetic evidence, but with no apparent taxonomically distinctive morphological features, collected on the Oregon (USA) coast from its type host *T. spinifera*. We redescribe *P. oregonensis* morphologically, and we report, for the first time, its mitochondrial *cox1* and SSU rRNA gene sequences from its type host *Euphausia pacifica* collected from the Oregon coast. We also provide genetic sequence data for 11 additional *P. beringensis* isolates from *T. raschii* (9), *T. inermis* (1), and *T. longipes* (1) hosts and provide statistical and event-based analyses of the hypothesis that these 4 ciliate parasitoids have co-diversified with their 7 crustacean host species using *cox1* sequences. The success of our investigations demonstrates that this endoparasitoid apostome–krill species assemblage is very likely more broadly distributed, suggesting that exploration of krill populations in other parts of the world's oceans will lead to the discovery of new apostome parasitoid species or the discovery of zoogeographical range extensions in the distribution of extant *Pseudocollinia* species, which have currently been studied only for the northeastern Pacific Ocean region (Bering Sea to Gulf of California).

MATERIALS AND METHODS

Euphausiid collection

Pseudocollinia oregonensis infecting *Thysanoessa spinifera* (n = 29) and *Euphausia pacifica* (n = 43) specimens, identified by the opaque to orange appearance (Fig. 1), were collected during 10 oceanographic cruises (2000–2002) on the continental shelf off the coasts of Washington, Oregon, and California, USA (46° 21′ to 41° 54′ N, 124° 19′ to 125° 01′ W). *P. beringensis* infecting *T. raschii* (n = 51), *T. inermis* (n = 6), and *T. longipes* (n = 4) were collected during 4 oceanographic cruises in the Bering Sea (April to May and June to July 2009, May and June 2010; 55–62° N, 160–178° W). All specimens were collected using a 60 cm mouth diameter Bongo net or a 1 m^2 MOCNESS net. Body length (back of the eye to the base of the telson) of specimens collected in the Bering Sea was measured and converted to total length using species-specific equations (Harvey et al. 2012). Measurements were made using a calibrated micrometer in a dissecting stereomicroscope. Specimens were preserved either in 96% ethanol, Bouin's fixative, or 4% formalin buffered with sodium borate, following the methods described by Gómez-Gutiérrez et al. (2003, 2006, 2012). In the present study, only specimens preserved with 96% ethanol were used for correlative genetic and morphological analyses (both analyses done for ciliates derived from the same krill host specimen).

Fig. 1. Euphausiids *Thysanoessa spinifera*, host of *Pseudocollinia similis* sp. nov., and *Euphausia pacifica*, host of *P. oregonensis*, showing healthy specimens (upper example in each panel) and those in advanced infection stage with parasitoid ciliates of the genus *Pseudocollinia* (lower examples). Photograph of healthy *T. spinifera* taken by Phillip Colla; the other photographs were taken by Jaime Gómez-Gutiérrez

Ciliate collection, preservation, and staining

The cephalothorax of ethanol-preserved krill was opened and the masses of infecting ciliates were

transferred into 1.5 ml microcentrifuge tubes. The ciliates were rinsed with distilled water and stained by the quantitative protargol stain as described by Montagnes & Lynn (1993).

DNA sequence analysis of ciliate–krill mitochondrial *cox1* gene and SSU rDNA

Ciliates from 2 host specimens of *T. spinifera*, 3 host specimens of *E. pacifica*, 1 host specimen each of *T. longipes* and *T. inermis*, and 9 host specimens of *T. raschii* were also used for sequencing the SSU rDNA, ITS1-5.8S-ITS2-5′-LSU rDNA region, and *cox1* genes, following the procedures described in detail by Gómez-Gutiérrez et al. (2012). The forward primer F388dT was used for *P. oregonensis* and the forward primer F298dT for *P. beringensis* and *P. similis* (Strüder-Kypke & Lynn 2010). These *Pseudocollinia* sequences obtained from specimens collected in different regions (i.e. northeast Pacific including the Bering Sea, Washington, Oregon, and California coasts; Bahía Magdalena, located on the southwest coast of the Baja California peninsula, and Gulf of California, Mexico) were compared to each other and to previously published *Pseudocollinia* sequences (*P. brintoni* isolated from *Nyctiphanes simplex* and *P. beringensis* isolated from *T. inermis* and *T. raschii*; Gómez-Gutiérrez et al. 2012). In the present study, we added the sequence from a *T. longipes* specimen, a new host species, collected in the Bering Sea. We also report the first genetic sequences of *P. oregonensis* obtained from *E. pacifica* collected from the Oregon coast.

The mitochondrial *cox1* sequences of the host specimens were amplified, sequenced, and catalogued at the Biodiversity Institute of Ontario, Guelph, Canada. The gene sequences are available on BOLD and GenBank under accession numbers (HQ965947–HQ965965).

Sequence analyses followed procedures described in detail by Gómez-Gutiérrez et al. (2012). An alignment of *cox1* sequences was imported into MEGA ver. 5.2 (Tamura et al. 2011), while an alignment of SSU rDNA sequences was imported into the Dedicated Sequence Editor (De Rijk & De Wachter 1993). Four different phylogenetic analyses — maximum likelihood (ML, using RaxML; Stamatakis et al. 2008), Bayesian inference (BI, using MrBayes; Ronquist & Huelsenbeck 2003), maximum parsimony (MP, using PAUP 4.10; Swofford 2002), and neighbor joining (NJ, using PHYLIP ver. 3.6.9; Felsenstein 2009) — were performed on the alignments of SSU rDNA and *cox1* genes to construct trees as described by Gómez-Gutiérrez et al. (2012). The general time reversible model for nucleotide substitution, with gamma-distributed substitution rates and invariable sites, was identified as the best model for both genes using jModelTest ver. 2.1 (Guindon & Gascuel 2003, Darriba et al. 2012). These parameters were used in ML and BI analyses. The data were re-sampled 500 (ML) and 1000 (MP, NJ) times. The MP analysis was performed with a random addition (n = 5) of the species and the tree bisection-reconnection branch-swapping algorithm in effect.

Test of codiversification hypothesis

To test the hypothesis of codiversification (defined as the simultaneous diversification of 2 species lineages, especially of a parasite and its host), the host topology of the *cox1* gene sequences was used as the constrained tree for the ciliate *cox1* sequences using the approximately unbiased (AU) and Shimodaira-Hasegawa (SH) test as implemented in CONSEL ver. 01.j (Shimodaira & Hasegawa 2001, Shimodaira 2002). For these tests, the constrained tree is taken as the null hypothesis. If the test demonstrates a statistically significant difference (i.e. $p \leq 0.05$), then the null hypothesis is rejected and the constrained tree cannot be chosen as an explanation for the phylogenetic relationships.

We further analyzed the evolutionary associations among the euphausiid host species and the *Pseudocollinia* ciliate species with 2 reconciliation tools: CoRe-Pa ver. 0.5.1 (Merkle et al. 2010) and Jane ver. 4 (Conow et al. 2010). Phylogenetic trees inferred from the *cox1* sequences of (1) 1 representative of each krill host species and the corresponding endoparasitoid ciliates and (2) 1 representative each of the 6 krill host species and 1 representative each of the 4 endoparasitoid ciliate species were imported into CoRe-Pa and Jane, respectively, using the default options. One analysis was run with the default costs of each event as defined in the program (Jane: cospeciation = 0, duplication = 1, host switch = 2, loss of parasite = 1, failure to diverge = 1; CoRe-Pa: cospeciation = 0, lineage sorting = 1, duplication = 2, host switch = 3). The cost value of each event takes into account the likelihood of this event. Both programs employ the maximum parsimony principle to find a solution, and the resulting tree will show minimal total cost. In CoRe-Pa, we ran 100 000 random cycles with and without root-to-root mapping enforced. A statistical test was performed in Jane over

1000 cycles, with 500 randomizations in which random ciliate phylogenies were constructed (beta parameter = −1) in order to test whether the observed codiversifications are due to chance.

RESULTS

Distribution patterns of krill in the Bering Sea

We detected infections of krill by *Pseudocollinia*, presumably *P. beringensis*, in its type host species *Thysanoessa inermis*, and in 2 other abundant krill species, *T. raschii* and *T. longipes*. These krill hosts were distributed in either neritic (*T. raschii*) or oceanic (*T. inermis* and *T. longipes*) waters (Fig. 2).

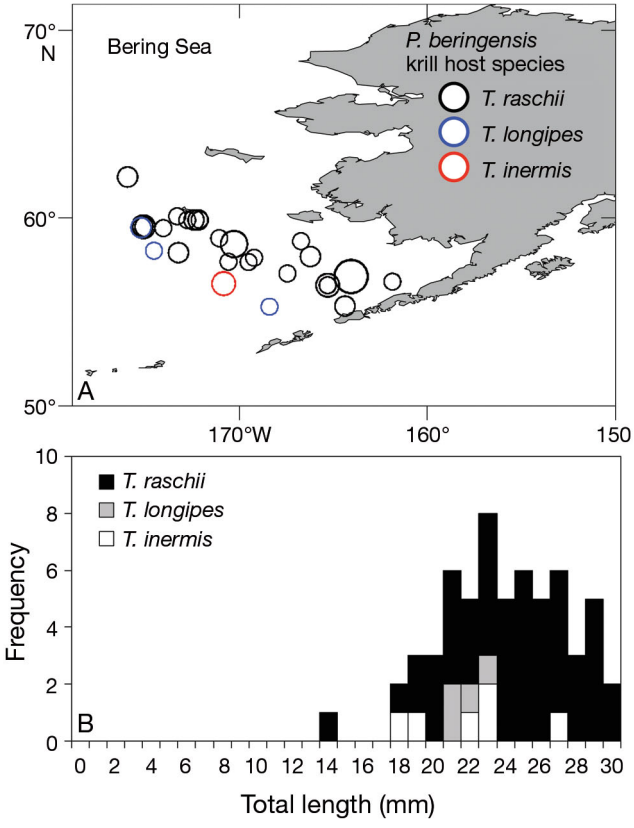

Fig. 2. *Thysanoessa* krill species in the Bering Sea infected with *Pseudocollinia* species. (A) Area of study and oceanographic stations where *Thysanoessa* species infected with *P. beringensis* were collected (2009–2010). The size of the circle is proportional to the number of infected specimens collected (range 1–6 krill). (B) Size-frequency distribution of infected individuals per krill species. Note that krill were not randomly sampled from the entire population

Prevalences of *Pseudocollinia* spp.

Prevalence of *Pseudocollinia* infection in the Oregon region has been estimated only for a few oceanographic stations. A total of 64 *Euphausia pacifica* specimens collected at 4 oceanographic stations yielded 10 *E. pacifica* infected with *P. oregonensis* (prevalence = 15.6%). A total of 95 *T. spinifera* specimens collected from 7 oceanographic stations yielded 8 *T. spinifera* infected with *Pseudocollinia* spp. (prevalence = 8.4%; see Table 2 in Gómez-Gutiérrez et al. 2006). It is difficult to estimate prevalence of infected euphausiids since it is not easy to identify infected animals from preserved samples, and generally large catches of euphausiids preclude examining all individuals prior to preservation.

Symptoms of infection by *Pseudocollinia* spp.

Gómez-Gutiérrez et al. (2006) described the symptoms of infection of *E. pacifica* by *P. oregonensis*. The *E. pacifica* collected in the present study showed a similar etiology of infection, where all stages in the life cycle of *P. oregonensis* were observed, although only protomite-tomite stages have been stained so far (see below).

Healthy *T. spinifera* are typically transparent, but infected individuals show beige to orange coloration and the cephalothorax becomes swollen as the infection progresses (Fig. 1). The swimming pattern of the infected specimens is usually normal, but just before death they may swim in small circles or sink to the bottom of the incubation bottle, suggesting nervous system damage. The apostome endoparasitoid observed in the hemocoel of *T. spinifera* showed all the ciliate life-cycle stages described for the other *Pseudocollinia* species (i.e. phoront, trophont, tomont, and tomite in the host, and protomite-tomite and phoront outside the host).

Protomite-tomite morphology of *Pseudocollinia similis* sp. nov.

Protargol staining of ethanol-fixed *P. similis* sp. nov. infecting *T. spinifera* revealed mostly ovoid protomite-tomite stages with a blunt anterior end (Figs. 3A–C & 4A–C, Table 1). The cells were 44–57 µm long and 26–38 µm wide (average 50.1 × 32.1 µm, n = 33). They possessed a long and narrow macronucleus that stretched to almost the

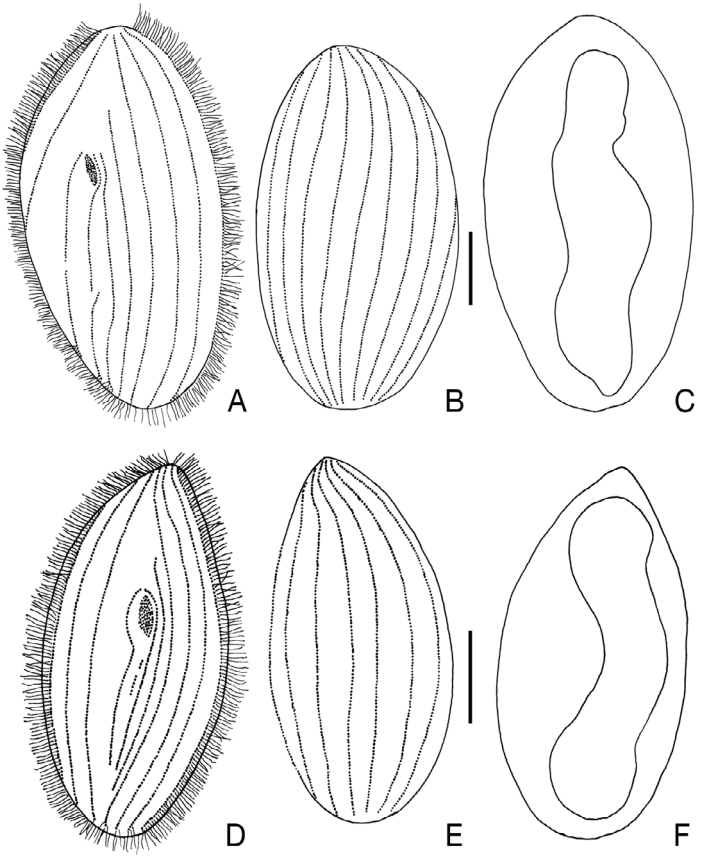

Fig. 3. Schematic drawings of (A–C) *Pseudocollinia similis* sp. nov. and (D–F) *P. oregonensis*. (A) Ventral view of the holotype of *P. similis* sp. nov., showing the dense ciliature and the dense field of oral kinetosomes on the left side of the oral cavity. There are 3 'oral' kineties: 1 on the right side of this oral field and 2 on the left side. (B) Dorsal view of the holotype. (C) Outline from another cell shows the size and shape of the macronucleus. (D) Ventral view of *P. oregonensis* showing the dense ciliature and the dense field of oral kinetosomes on the left side of the oral cavity. There are 2 'oral' kineties: 1 on the right side of this oral field and 1 on the left side. (E) Dorsal view of the cell in (D). (F) Outline of the cell in (D) showing the size and shape of the macronucleus. Scale bars = 10 µm

length of the cell (40–48 µm long, 5.6–11 µm wide, average 45 × 8.5 µm, n = 26; Figs. 3C & 4C); a micronucleus was not observed. There were on average 20 kineties (18–21, n = 33) that extended from pole to pole, except for 4 kineties. Three of these kineties, which might be called 'oral' kineties (or ciliary rows) and are numbered Kinety 1 (K1), Kinety n (Kn), and Kinety n-1 (Kn-1), curve around the oral cavity opening and terminate (Figs. 3A & 4A,B). The fourth kinety, Kinety n-2, curves anteriorly and right above the oral cavity before terminating in a non-ciliated region anterior to the oral cavity (Figs. 3A & 4A,B). The oral cavity, which is about 1/3 the cell's length from the anterior end

Table 1. Morphometrics of the tomite-protomite stage of *Pseudocollinia* species, parasitoids of northeastern Pacific euphausiids. Data are means ± SD (range); n = 33 for all measurements except where indicated. BL/W: body length/width (µm); AOA: anterior to oral apparatus (µm); MaL/W: macronuclear length/width (µm). Host euphausiids are from the genera *Nyctiphanes*, *Euphausia*, or *Thysanoessa*

Species	BL	BW	AOA	AOA/BL	MaL	MaW	Kineties		Host(s)		Reference(s)
							Total	Oral	Type	Other	
P. brintoni	31.1 ± 1.8 (28–35)	16.6 ± 2.3 (13–21)	9.9 ± 1.8 (5–14)	0.32 ± 0.05 (0.18–0.45)	22.8 ± 3.1[a] (18–28)	5.1 ± 3.1[a] (2–11)	16 ± 0.65 (14–18)	2	*N. simplex*		Gómez-Gutiérrez et al. (2012)
P. oregonensis	40.1 ± 1.9 (36–44)	21.3 ± 2.2 (18–26)	13.5 ± 1.4 (11–17)	0.34 ± 0.03 (0.28–0.41)	31.5 ± 7.6[b] (11–38)	7.1 ± 2.3[b] (2–19)	18 ± 0.53 (16–18)	2	*E. pacifica*		Gómez-Gutiérrez et al. (2006)
P. beringensis	37.1 ± 4.4 (30–46)	21.9 ± 3.1 (16–27)	12.2 ± 2.1[a] (7–16)	0.33 ± 0.04[a] 0.23–0.39)	27.4 ± 3.2[c] (22–36)	9.9 ± 2.2[d] (5–14)	19 ± 0.92 (16–20)	3	*T. inermis*	*T. raschii*, *T. longipes*	Capriulo & Small (1986), Gómez-Gutiérrez et al. (2006), this study
P. similis sp. nov.	50.1 ± 3.5 (44–57)	32.1 ± 3.5 (26–38)	14.9 ± 2.7 (8.3–21.1)	0.30 ± 0.05 (0.17–0.39)	45 ± 2.2[e] (40–48)	8.5 ± 1.5[e] (5.6–11)	20 ± 0.61 (18–21)	3 ± 0.25 (2–4)	*T. spinifera*	*T. gregaria*?[f]	This study

[a]n = 32; [b]n = 11; [c]n = 27; [d]n = 28; [e]n = 26; [f]No genetic data available for confirmation

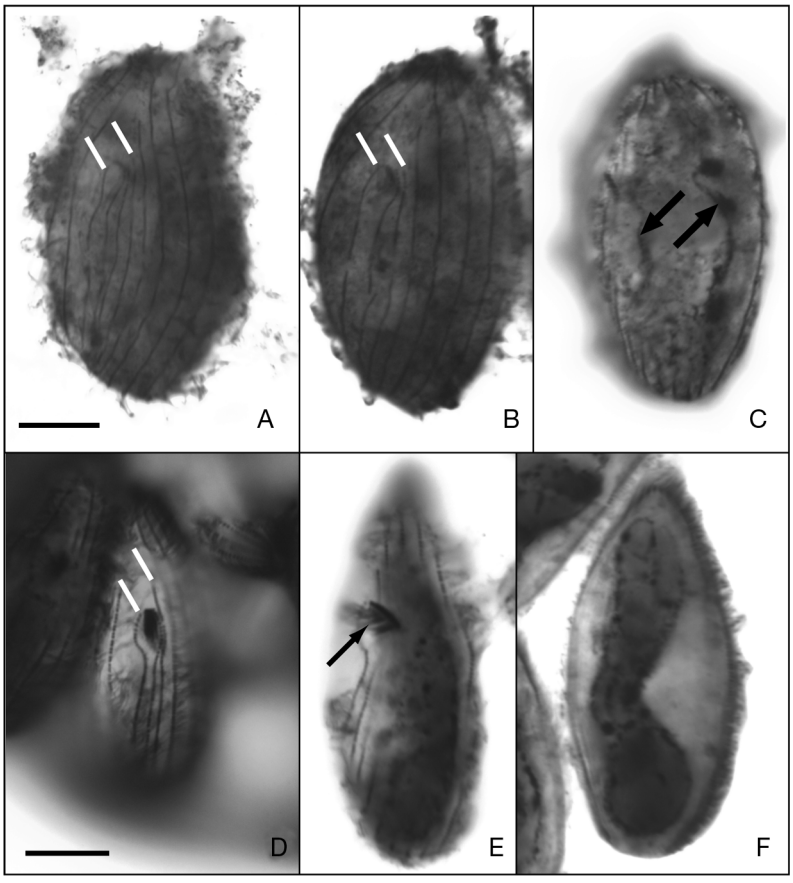

Fig. 4. Protargol-stained protomite-tomite stages of *Pseudocollinia* species. (A–C) *P. similis* sp. nov. that infects *Thysanoessa spinifera*. (A) Ventral view of specimen showing dense ciliature with Kinety 1, the rightmost 'oral' kinety (left white bar) and Kinety n-2, the leftmost 'oral' kinety (right white bar) indicated. Note that there are 4 'oral' kineties, Kinety 1, Kinety n, Kinety n-1, and Kinety n-2 in this specimen (cf. Fig. 3A). (B) Ventral view of holotype with the typical 3 'oral' kineties indicated (white bars). (C) Another specimen with the macronuclear envelope indicated (arrows). Scale bar for A–C = 10 µm. (D–F) *P. oregonensis* infecting *Euphausia pacifica*. (D) Ventral view with Kinety 1, the rightmost 'oral' kinety (left white bar) and Kinety n-2 (right white bar) indicated. This species has 2 'oral' kineties, Kinety 1 and Kinety n (see Fig. 4A). (E) Lateral view at sagittal plane showing cone-shaped oral cavity with cilia of the oral field extending outward (arrow). (F) Another specimen with the macronucleus shown. Scale bar for D–F = 10 µm

(Table 1), appears to be cone-shaped and lined on its left wall with a dense field of ciliated kinetosomes or basal bodies (Figs. 3A & 4A,B). Measurements were made on ciliates from a *T. spinifera* host (specimen no. 29), as the ciliates from the other host (specimen no. 2), which were confirmed genetically as *P. similis* sp. nov. (see below), were not well stained. Nevertheless, a few well-stained individuals from this other host showed identical morphological characteristics.

Protomite-tomite morphology of *P. oregonensis*

Protargol staining of ethanol-fixed *P. oregonensis* infecting *E. pacifica* revealed mostly ovoid protomite-tomite stages with a pointed anterior end (Figs. 3D–F & 4D–F, Table 1). The cells were 36–44 µm long and 18–26 µm wide (average 40.1 × 21.3 µm, n = 33). They possessed a long and narrow macronucleus that stretched to almost the length of the cell (11–38 µm long by 2–19 µm wide, average 31.5 × 7.1 µm, n = 11; Figs. 3F & 4F); a micronucleus was not observed. There were on average 18 kineties (16–18, n = 33) that extended from pole to pole, except for 3 kineties (Figs. 3D & 4D). Two of these kineties, which might be called 'oral' kineties (or ciliary rows) and are numbered Kinety 1 (K1) and Kinety n (Kn) curve around the oral cavity opening and terminate (Figs. 3D & 4D). The third kinety, Kinety n-1, curves anteriorly and right above the oral cavity before terminating in a non-ciliated region anterior to the oral cavity (Figs. 3D & 4D). The oral cavity, which is about 1/3 the cell's length from the anterior end (Table 1), appears to be cone-shaped and lined on its left wall with a dense field of ciliated kinetosomes or basal bodies (Figs. 3D & 4D,E). Morphological measurements were made on ciliates from 2 *E. pacifica* hosts (specimen nos. 28 and 30); the ciliates of a third *E. pacifica* host (no. 31), were confirmed genetically as *P. oregonensis* (see below).

Gene sequences of *Pseudocollinia* species

The complete SSU rDNA gene sequence of *P. similis* sp. nov. is 1746 nucleotides (nt) in length, has a GC content of 41%, and is identical for both isolates. The ITS1, 5.8S rRNA, ITS2, and partial large subunit (LSU) rRNA gene sequences were obtained as well, and like the SSU rRNA, they are identical for both *P. similis* isolates.

The complete SSU rDNA gene sequence of *P. oregonensis* is 1746 nt in length and has a GC content of 41%. It is almost identical among the 3 isolates (difference of 3 nt). The ITS1, 5.8S rRNA, ITS2, and partial LSU rRNA gene sequences of the isolates of *P. oregonensis* are identical.

The divergence between the SSU rDNA gene sequences of these 2 species is 0.6%, while they show 3.2% divergence in their ITS and 5.8S gene regions. All *Pseudocollinia* sequences have been submitted to GenBank under accession numbers HQ591468–HQ591488 (see also Gómez-Gutiérrez et al. 2012).

The mitochondrial *cox1* genes of *P. similis* sp. nov. and *P. oregonensis* include inserts that are 458 and 449 nt long, respectively. The inserts are typical for ciliates but they are considerably longer than those of other ciliate species analyzed so far (Strüder-Kypke & Lynn 2010, Gómez-Gutiérrez et al. 2012). The lengths of the partial *cox1* gene sequences are 852 to 1012 nt due to the different forward primers used. As in all ciliates, the GC content is low at 29–30%. The 2 isolates of *P. similis* show 0.1% divergence, while the 2 isolates of *P. oregonensis* show 16% divergence. Isolates of the 2 species were 50% divergent, mainly due to considerable differences in the extremely variable insert regions. All *Pseudocollinia cox1* sequences have been submitted to GenBank under accession numbers HQ591489–HQ591507.

The phylogenetic analyses of the SSU rDNA sequences place the 4 *Pseudocollinia* species in a well-supported monophyletic clade with other apostome ciliates. *Pseudocollinia* species are most closely related to *Fusiforma themisticola*, recently described as a parasitoid in the hemocoel of the zooplanktonic hyperiid amphipod *Themisto libelulla* collected in the Canadian Beaufort Sea (Arctic Ocean) (Chantangsi et al. 2013). *Fusiforma* along with *Pseudocollinia* species are currently assigned to the family Pseudocolliniidae, which is in turn closely related to other known histophagous parasitoid apostome ciliates that infect other crustaceans like decapods and copepods (Fig. 5). *P. similis* sp. nov. and *P. oregonensis* are quite well separated in the tree inferred from SSU rDNA sequences, with *P. oregonensis* basal in the radiation next to *P. brintoni* (Fig. 5). A distinct genetic distance of 0.5 separates them in the tree inferred from *cox1* gene sequences and robustly shows that *P. similis* sp. nov. (temperate) and *P. beringensis* (Arctic) are sister species, while *P. oregonensis* (temperate) is the sister species of *P. brintoni* (subtropical), suggesting a latitudinal zoogeographic cline of the krill hosts (Fig. 6).

Codiversification of *Pseudocollinia* species and their euphausiid hosts

The topology of the *cox1* gene tree of the euphausiid hosts, generated by NJ analysis, was used to determine whether it might explain the diversification of the 4 currently known *Pseudocollinia* species in the northeast Pacific Ocean (Figs. 6 & 7). The multiple specimens of the 3 genera of euphausiids (*Nyctiphanes*, *Euphausia*, and *Thysanoessa*) form monophyletic clades, while the monophyly of multiple species in the genus *Thysanoessa* is robustly confirmed in those species sampled more than once. When comparing the *Pseudocollinia* and host phylogenetic trees, the branch leading to the 2 isolates of *P. similis* sp. nov. is basal among *Pseudocollinia* isolated from *Thysanoessa* spp., while *T. spinifera*, the type host for *P. similis*, is not basal in the *Thysanoessa* radiation (Figs. 6 & 7). It is likely that this difference in topology leads to the statistical rejection of absolute codiversification of these endoparasitoid ciliates and their krill hosts (i.e. AU, $p = 0.015$; SH, $p = 0.022$).

The event-based codiversification analyses of host and ciliates in CoRe-Pa and Jane models, with default cost parameters, obtained 2 trees and 1 tree, respectively (Fig. 7a). Both programs differentiate between host-dependent events (co-speciation, lineage sorting) and host-independent events (duplication, duplication with host switch, failure to diverge). Co-speciation defines the event that host and parasite speciate simultaneously. Lineage sorting (referred to as 'Loss' in Jane) happens when, after a host speciation, the parasite species remains on only 1 of the new host species. Duplication means that only the parasite species speciates, and both child species of the parasite are associated with the same host as the parent species. Duplication with host switch is counted as 1 event in which the parasite speciates and 1 of the child species immediately switches to a new host species (Charleston 1998, Keller-Schmidt et al. 2011). Failure to diverge (only in Jane) is the case if the host speciates and the parasite remains associated with both new host species. Depending on the input tree (6 and 4 ciliate species, respectively), the computed trees show 2 different solutions. CoRe-Pa does not allow multiple host species per parasite species, so the second tree input was only calculated with Jane. The first tree (6 hosts, 6 ciliates) reconstructed with both programs shows 3 codiversification events, 1 loss, no duplications, and 2 host switches (3-1-0-2), with a total cost of 5. The statistical test with Jane showed that only 18% of the

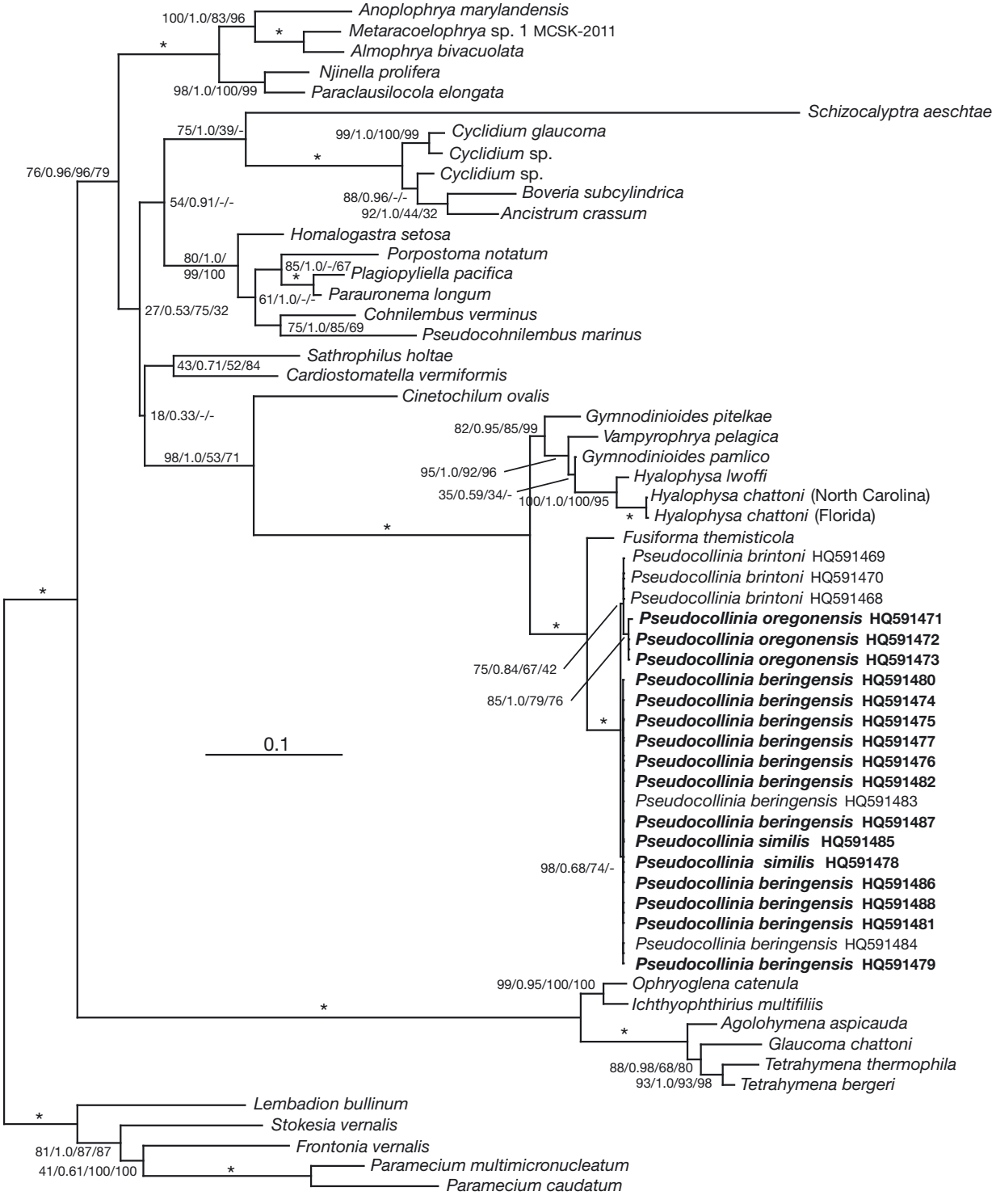

Fig. 5. Ciliate phylogenetic tree inferred from small subunit (SSU) rDNA gene sequences, computed with RAxML (Stamatakis et al. 2008), based on the general time reversible model with gamma distribution and an estimate of invariable sites. The first number at the nodes represents the bootstrap support for RAxML (maximum likelihood, ML); the second number represents posterior probability values of the Bayesian inference (BI) analysis; and the third and fourth numbers represent bootstrap values for maximum parsimony (MP) and neighbor joining (NJ), respectively. New sequences for *Pseudocollinia* spp. indicated in **bold** show unambiguously that they cluster with the other described species of the genus. Asterisks indicate full support in all analyses and dashes indicate support values <30%. Scale bar = 10 substitutions per 100 nucleotides

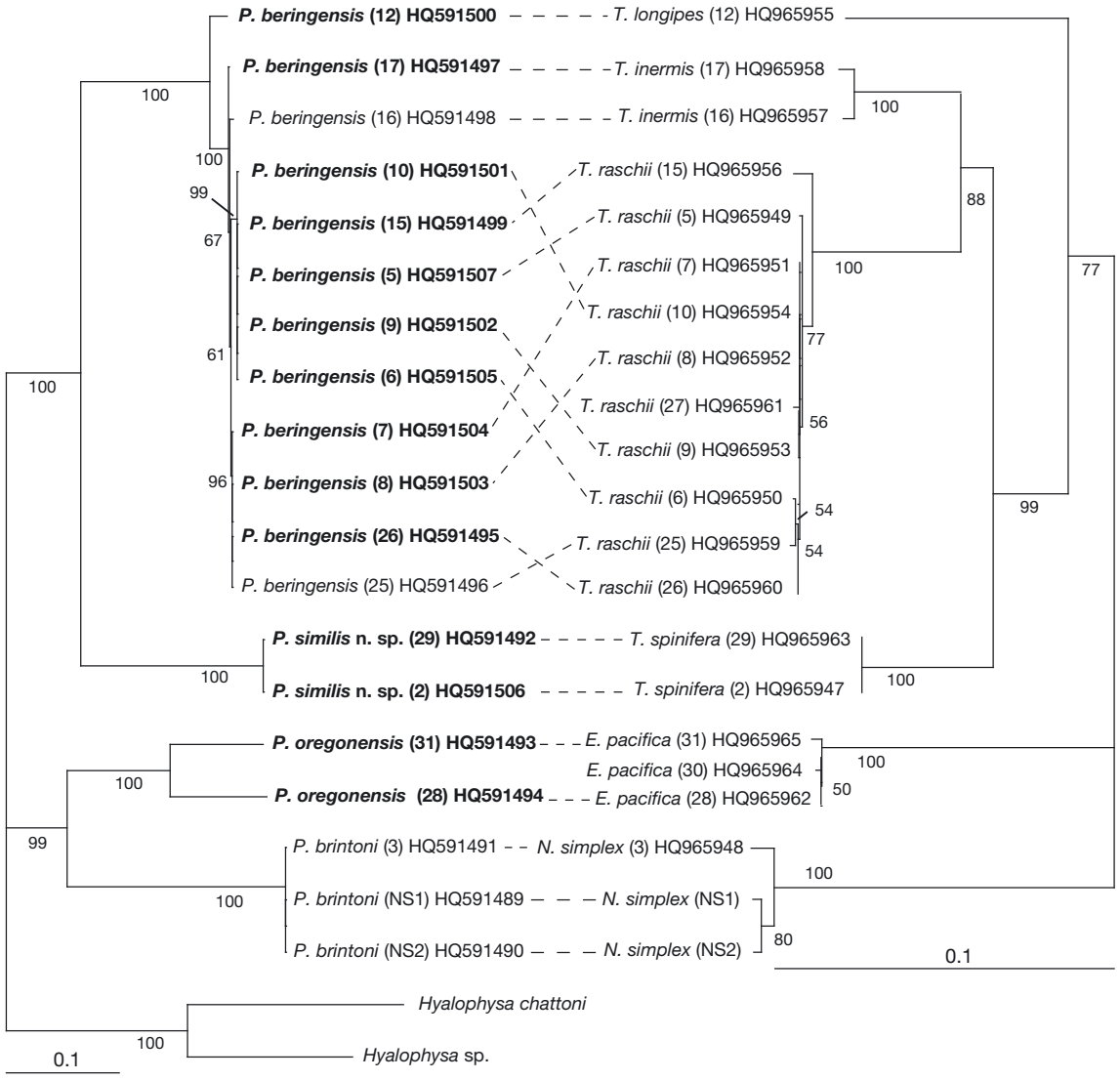

Fig. 6. Phylogenetic trees inferred from mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene sequences, computed with neighbor joining (NJ; Saitou & Nei 1987), based on the Kimura 2-parameter model (Kimura 1980). The left hand tree shows relationships among the apostome parasitoid ciliates of the genus *Pseudocollinia* while the right hand tree shows relationships among their krill hosts. The number at the nodes represents the bootstrap support for NJ. New sequences for *Pseudocollinia* spp. indicated in **bold** show unambiguously that these specimens can be assigned to this genus. *P. similis* sp. nov. and *P. oregonensis* are separated by 50% sequence divergence. Scale bars = 10 substitutions per 100 nucleotides

random trees showed a better (lower) total cost value than the minimum cost tree shown in Fig. 7a, suggesting that the retrieved codiversifications were not a random result. Enforced root-to-root mapping in CoRe-Pa resulted in a tree with 1 additional loss (3-2-0-2). The second tree (6 hosts, 4 ciliates) reconstructed with Jane shows 2 codiversification events, no loss, no duplication, 1 host switch, and 2 failure-to-diverge events (2-0-0-1-2), with a total cost of 4. The statistical test with Jane showed that only 4.6% of the random trees had a better (lower) total cost value than the minimum cost tree shown in Fig. 7b.

DISCUSSION

Comparing morphology and genetic diversity of *Pseudocollinia* endoparasitoid phases

With the discovery of *P. similis* sp. nov. there are now 4 species in this genus of parasitoids that infect euphausiids of the northeast Pacific Ocean (Capriulo & Small 1986, Capriulo et al. 1991, Gómez-Gutiérrez et al. 2003, 2006, 2012). Recently, the family Pseudocolliniidae was established to include what were then 3 known species in the type genus *Pseudocollinia* and

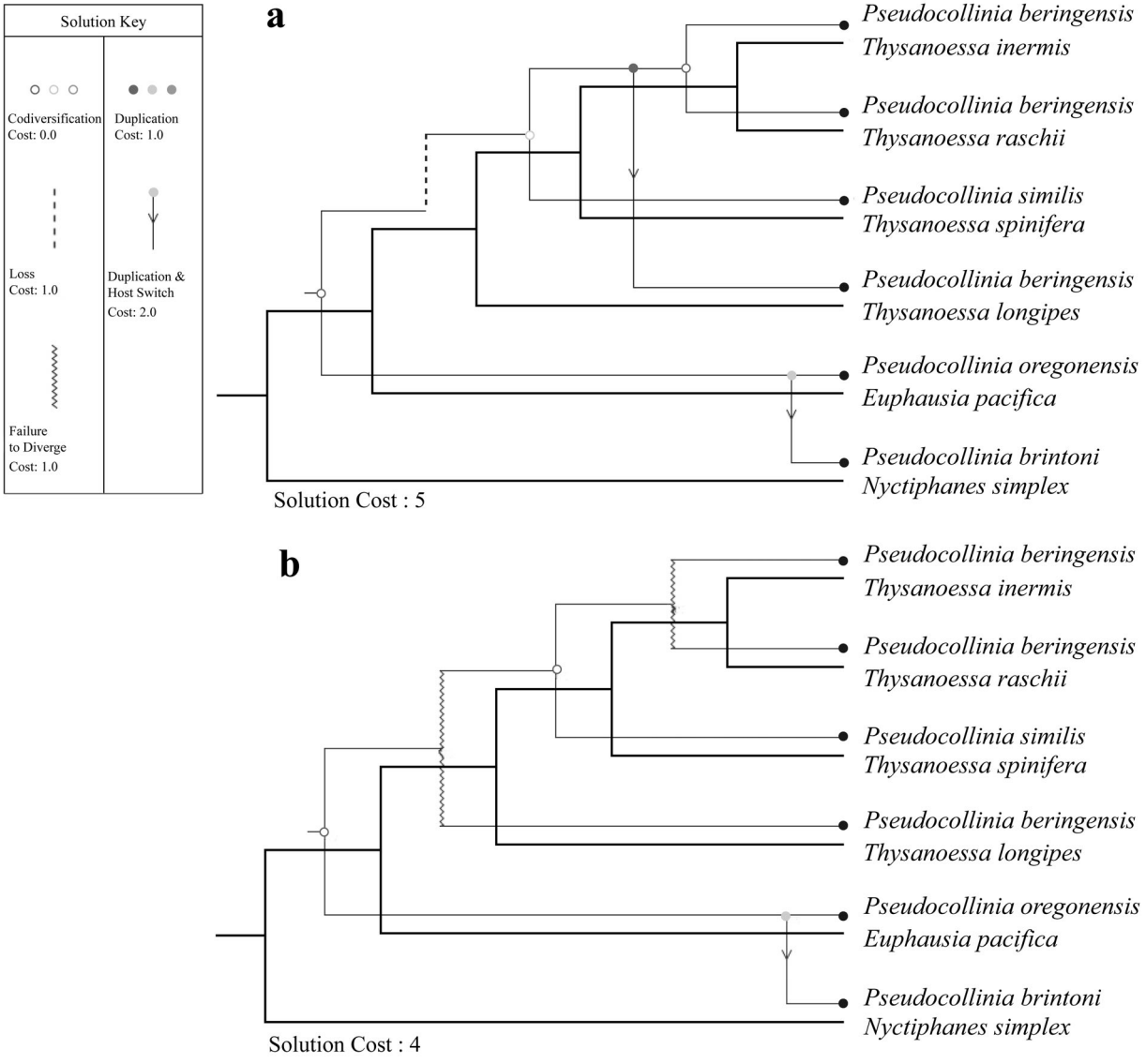

Fig. 7. The preferred reconstruction obtained by Jane analysis with default event costs. (a) Minimum cost tree with 6 ciliate species and 6 host species showing 3 codiversification events, 2 duplications with host switch, and 1 loss (3-1-0-2-0). (b) Minimum cost tree with 4 ciliate species and 6 host species showing 2 codiversification events, 1 duplication with host switch, and 2 failure to diverge events (2-0-0-1-2). The host tree topology is shown in black and the parasitoid tree topology in gray

Fusiforma themisticola, a newly discovered apostome that infects the hyperiid amphipod *Themisto libellula* (Chantangsi et al. 2013). Gómez-Gutiérrez et al. (2012) reexamined the type slide of *P. oregonensis* and determined that, while it could be placed in the genus *Pseudocollinia*, higher-quality protargol stains would be useful to characterize the protomite-tomite stage more fully. We have now provided this morphological and genetic characterization along with the characterization of *P. similis* sp. nov. (Table 1).

Gómez-Gutiérrez et al. (2006) provided characterization of the tomites of *P. oregonensis*, based on enumeration of features from scanning electron micrographs, staining with protargol, and cells in different stages of palintomy stained with hematoxylin and counterstained with Fast Green. The ranges that they provided for tomite stages obtained from the type host *Euphausia pacifica* overlap the ranges of our protargol-stained specimens from the same krill host species: 19–37 µm long × 15–30 µm wide (Gómez-Gutiérrez et al. 2006) vs. 36–44 µm long × 18–26 µm wide (Table 1); and total kineties of 16 (Gómez-Gutiérrez et al. 2006) vs. 16–18 (Table 1). Furthermore, like the original description of *P. oregonensis*,

we have also observed in our samples that the ovoid tomite with a pointed anterior end (cf. Fig. 5A in Gómez-Gutiérrez et al. 2006) and a cone-shaped oral cavity are features of this species (cf. Fig. 5I in Gómez-Gutiérrez et al. 2012). Thus, we assign this recent isolate from *E. pacifica*, the type host of *P. oregonensis*, to this species and provide a revised morphological characterization (see below) and genetically characterize the species using the SSU rDNA and *cox1* gene sequences.

Genetic characterization of ciliate species is becoming more common since extensive comparative molecular genetic research on the biological species of *Tetrahymena* (Kher et al. 2011) and *Paramecium* (Strüder-Kypke & Lynn 2010, Przyboś et al. 2013) using the mitochondrial *cox1* gene has demonstrated significant interspecific variation. For example, Kher et al. (2011) concluded that a >5% difference in *cox1* identifies different biological species of *Tetrahymena*, while Strüder-Kypke & Lynn (2010) concluded that this criterion rises to >20% for biological species of *Paramecium*. Biological species of *Tetrahymena* can have identical gene sequences for SSU rRNA, and so it is best to avoid this gene as a criterion for genetic species identification for taxonomic purposes (Strüder-Kypke & Lynn 2010). While we know nothing about the sexual reproductive biology of *Pseudocollinia* species, we believe that choosing a 20% criterion for differences in the *cox1* barcode should conservatively support identification of genetic species with taxonomic value.

We provide a comparative table to demonstrate the similarities and differences among the 4 *Pseudocollinia* species identified to date from 6 euphausiid species distributed in the northeast Pacific (Table 1). It is virtually impossible to unambiguously identify these apparently cryptic species based only on morphology, as almost all morphometric characteristics of the tomites overlap, although *P. beringensis* and *P. brintoni* do not overlap in number of kineties, geographic distribution, or host species. Nevertheless, genetic characterization, especially using the *cox1* gene sequences, clearly separated these 4 parasitoid species: *P. similis* sp. nov. from *Thysanoessa spinifera* differs from *P. oregonensis* from *E. pacifica* by 50% based on the *cox1* gene barcode (Fig. 6). Furthermore, our data indicate that there are 2 sets of sister species characterized by different numbers of oral kineties. *P. brintoni* and *P. oregonensis* invariably have 2 oral kineties as tomites and are genetically separated using the *cox1* gene barcode by 35% (Fig. 6). They also parasitize different genera of euphausiids: *P. brintoni* infects *Nyctiphanes simplex* and *P. oregonensis* infects *E. pacifica* (Fig. 6, Table 1).

P. beringensis and *P. similis* sp. nov. typically have 3 oral kineties as tomites and are genetically separated by 30% using the *cox1* gene barcode (Fig. 6). These 2 species parasitize at least 4 species of the genus *Thysanoessa*: *P. beringensis* infects *T. inermis*, *T. longipes*, and *T. raschii* while *P. similis* infects *T. spinifera* (Fig. 6, Table 1). Nevertheless, *P. similis* sp. nov. has the most variable numbers of oral kineties. This appears to be correlated with breaks in these kineties in the cell equatorial region (Fig. 3A), suggesting a mechanism whereby the anterior fragment extends posteriorly and the posterior fragment extends anteriorly to increase the number of oral kineties by 1.

Codiversification of *Pseudocollinia* species and their euphausiid hosts

Gómez-Gutiérrez et al. (2006) claimed that *P. oregonensis* also infected *Euphausia pacifica*, *T. spinifera,* and *T. gregaria* on the Oregon coast (Table 1, Gómez-Gutiérrez et al. 2006). We now conclude, based on genetic evidence, that the *Pseudocollinia* species infecting *T. spinifera* is a different species, viz. *P. similis* sp. nov., while the genetic species *P. oregonensis* is assigned to those ciliates isolated from the type host *E. pacifica*, as defined by Gómez-Gutiérrez et al. (2006). We have been unable to collect additional infected specimens of *T. gregaria* to obtain gene sequences of its ciliate parasitoid, and so cannot identify with certainty the *Pseudocollinia* species that infects this euphausiid species. Further genetic studies could determine whether *P. oregonensis* can indeed infect other krill hosts in the diverse northeast Pacific or even in other regions of the world.

Our inferred phylogenetic tree of the euphausiid species is very limited in the number of species and lacks an outgroup. While the relationships among species of the genus *Thysanoessa* seem well resolved and the specimens of *E. pacifica* and *N. simplex* can be unambiguously identified as conspecific, the relationships between the 3 genera are not clear. Jarman (2001) analyzed nuclear rDNA of several krill genera and D'Amato et al. (2008) inferred a phylogenetic tree from mitochondrial SSU rRNA and *cox1* genes. The nuclear genes grouped *Euphausia* and *Nyctiphanes* as sister genera and basal to *Thysanoessa*, while the mitochondrial SSU rDNA genes placed *Euphausia* in the basal position of the 3 genera.

Parasites often codiversify or coevolve with their hosts, and recently gene sequences and inferred tree topologies have been used to test these hypotheses of codiversification (Johnson et al. 2004, Kuo et al. 2008, Cruaud et al. 2012, Rosenblueth et al. 2012, Du Toit et al. 2013). While event-based reconciliation tools show some codiversification of *Pseudocollinia* species with their host euphausiids, the AU/SH tests rejected absolute codiversification. It would appear that *P. beringensis*, which apparently has broader host infection capabilities than the other species of *Pseudocollinia,* can infect *T. longipes*, which is a basal species in the *Thysanoessa* radiation, while the *P. beringensis* isolate from *T. longipes* clusters robustly with all other *P. beringensis* isolates, albeit basal in this clade (Fig. 6). *P. similis* and *P. beringensis*, which diverge significantly in their *cox1* gene sequences (30%), show little divergence in their SSU rDNA and ITS1-5.8S-ITS2-5′-LSU rDNA sequences (0.06% and 1.4%). This may indicate a very recent speciation, which is not yet reflected in the more highly conserved genes.

Kulka & Corey (1984) discovered protozoans, identified as sporozoans, infecting *T. inermis* (prevalence of 2.8%) in the Bay of Fundy, east coast of Canada (Atlantic Ocean). Our re-examination of the published micrographs in this report, in light of our morphological and genetic work on Pacific krill, leads us to believe that the external and internal features of the cells, their size, and the mode of palintomic cell division are similar to those features of *Pseudocollinia* species described from Pacific krill. Possibly Kulka & Corey (1984) actually first discovered *P. beringensis*, which was formally described 2 yr later from the same krill host species, *T. inermis*, in the Bering Sea (Capriulo & Small 1986). If true, this greatly extends the current known biogeographic distribution range of *Pseudocollinia* to the northwest Atlantic Ocean, and suggests the possibility that *Pseudocollinia* species may infect other krill species worldwide.

Taxonomic summary of *Pseudocollinia oregonensis* and *P. similis* sp. nov.

Subclass: Apostomatia Chatton & Lwoff, 1928
Order: Apostomatida Chatton & Lwoff, 1928
Family: Pseudocolliniidae Chantangsi, Lynn, Rueckert, Prokopowicz, Panha, & Leander, 2013
Genus: *Pseudocollinia* Gómez-Gutiérrez, Strüder-Kypke, Lynn, Shaw, Aguilar-Méndez, López-Cortés, Martínez-Gómez, & Robinson, 2012

Pseudocollinia oregonensis Gómez-Gutiérrez, Peterson, & Morado, 2006

Diagnosis: All life cycle stages possess 16–18 somatic kineties; tomite stage, ovoid with pointed anterior end; oral cavity bordered by 2 'oral' kineties; infecting a broadcast-spawning euphausiid with subarctic biogeographic distribution.
Type host: *Euphausia pacifica* Hansen, 1911
Type location: Northeast Pacific, along the Oregon coast, USA (43° 13′ N, 124° 59′ W).
Endoparasitoid stage: In the hemocoel and inside the abdomen and appendages of the host euphausiids.
Free-living encysted stage: Forming clusters of phoronts on filaments, not confirmed with scanning electron microscopy (SEM) observations, but likely associated with bacteria, as observed with *P. brintoni* (Gómez-Gutiérrez et al. 2012).
Type material: Two protargol-stained slides of *P. oregonensis* cells in the tomite stage (Holotype USNM 1084004 and Paratype USNM 1084005) and 1 *Euphausia pacifica* female in advanced stage of infection (23.7 mm total length, Non-type USNM 1084006) were deposited in the International Protozoan Type Slide Collection of the Department of Invertebrate Zoology of the National Museum of Natural History, Smithsonian Institution, by Gómez-Gutiérrez et al. (2006). We have now deposited a voucher slide (Fig. 3D,E) with cells of isolate 30 circled in black on the underside of the slide (USNM 1231430 [IZ]).
Gene sequences: Gene sequences of *P. oregonensis* were deposited in GenBank under accession numbers HQ591473 (SSU rRNA) and HQ591494 (*cox1*) for isolate 28; HQ591471 (SSU rRNA) and HQ591493 (*cox1*) for isolate 31, and HQ591472 (SSU) for isolate 30.

Pseudocollinia similis Lynn, Gómez-Gutiérrez, Strüder-Kypke, & Shaw, 2014

Diagnosis: All life cycle stages possess 18–21 somatic kineties; tomite stage, ovoid with blunt anterior end; oral cavity bordered by 3 'oral' kineties; infecting a broadcast-spawning euphausiid distributed along the northeastern Pacific Ocean.
Type host: *Thysanoessa spinifera* Holmes, 1900
Other possible host: *Thysanoessa gregaria* G. O. Sars, 1883, but must be confirmed using gene sequences.
Type location: Northeast Pacific, along the Oregon coast, USA (43° 13′ N, 124° 59′ W).
Endoparasitoid stage: In the hemocoel and inside the abdomen and appendages of the host euphausiids.

Free-living encysted stage: Forming clusters of phoronts on filaments, not confirmed with SEM observation but likely associated with bacteria, as observed with *P. brintoni* (Gómez-Gutiérrez et al. 2012).

Type material: A protargol-stained slide of *P. similis* sp. nov. cells in the tomite stage (Holotype USNM 1231431 [IZ]) was deposited in the International Protozoan Type Slide Collection of the Department of Invertebrate Zoology of the National Museum of Natural History, Smithsonian Institution. The holotype (Figs. 3A,B & 4B) as a cell from isolate 29 is circled in black on the underside of the slide.

Gene sequences: Gene sequences of *P. similis* sp. nov. were deposited in GenBank under accession numbers HQ591478 (SSU rRNA) and HQ591506 (*cox1*) for isolate 2 and HQ591485 (SSU rRNA) and HQ591492 for (*cox1*) isolate 29.

Etymology: The species name derives from *similis* (Latin meaning 'like, resembling') and is given because this species is the fourth described species of *Pseudocollinia*, which appears to be a complex of cryptic species of parasitoids infecting euphausiids of the northeastern Pacific Ocean.

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