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ORIGINAL PAPER

Genetic and morphological variation in *Pennella* sp. (Copepoda: Siphonostomatoida) collected from Pacific saury, *Cololabis saira*

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Abstract

In this paper, we describe three morphotypes of large parasitic copepods identified as *Pennella* Oken, 1815 (Siphonostomatoida, Pennellidae), which are found on the commercial fish Pacific saury *Cololabis saira* (Brevoort, 1856) (Beloniformes, Scomberesocidae). Of three morphotypes (*Pennella* sp. (PSP), parasite A (PA), and indeterminate individuals (IDI)), PSP had feather-like abdominal brushes and egg sacs which were characters typical of *Pennella*, PA lacked abdominal brushes and egg sacs, and IDI had few abdominal brushes but no egg sacs. Nucleotide sequence differences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear ribosomal internal transcribed spacer 1 (ITS1) regions between types were small and comparable with differences within a type, indicating that the three types were conspecific. Mean body length of PSP was significantly larger than those of PA and IDI, suggesting types PA and IDI were immature morphs of PSP or individuals that had ceased development prior to maturation.

Keywords Pacific saury · Parasitic copepod · Pennellidae · COI · ITS1 · Nucleotide sequence analysis · *Pennella*

Introduction

Parasitic copepods of the genus *Pennella* Oken, 1815 within the family Pennellidae Burmeister, 1835 are conspicuous because of its size (Kabata 1979). They are distinguished from other genera of Pennellidae by having a straight and elongate body, feather-like abdominal brush, and straight egg sac (Kabata 1979).

Pennella sp. or spp. also infests Pacific saury, *Cololabis saira* (Brevoort, 1856). Pacific saury is an epipelagic fish that is widely distributed in subarctic and subtropical North Pacific waters (Hubbs and Wisner 1980) and is commercially an important fish in Japan, China, Russia, Korea, and Taiwan, with landings fluctuating between 180,973 (1998) and 629,052 (2015) metric tons over the last 30 years (Food and Agriculture Organization of the United Nations 2017). The prevalence of *Pennella* sp. or spp. in Pacific saury varies between years, with parasites at times almost disappearing after large-scale infections that had persisted for several years (Hughes 1973; Nagasawa et al. 1988; Yamaguchi and Honma 1992). Maximum infection rate exceeded 30% in the western North Pacific in 1983 but declined to less than 1% after 1985 (Nagasawa et al. 1988; Yamaguchi and Honma 1992). Almost three decades later, a *Pennella* epidemic occurred in 2012, and relatively high infection rates continue until 2017. Sudden appearance of *Pennella* sp. on the Pacific saury had caused panic in fishermen, distributors, or consumers because of its large and eerie form (Nagasawa et al. 1984).

Pennella drills deeply into the host's flesh, sometimes reaching the heart, major blood vessels, or other internal organs, affecting host growth (Hughes 1973; Pascual et al. 1997). Despite the potential of this parasite to affect the host and to lower the commercial value, species of *Pennella*

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parasitizing Pacific saury have not yet been described. Hughes (1973) investigated parasitic composition of the eastern Pacific saury and described two types of *Pennella*, with individuals lacking abdominal brushes and egg sacs recognized as juveniles. We also found morphologically different *Pennella*-like parasites that had no or possessed only a small number of feather-like abdominal brushes. Accordingly, a re-evaluation of morphological characteristics used for differentiating species of *Pennella* infecting Pacific saury is necessary.

Systematics and taxonomy of the genus *Pennella* have been confusing. Although in excess of 30 species have been described (Kabata 1979), Hogans (1988a) reviewed and examined 31 of these and concluded only 7 were valid: *P. balaenoptera* Koren and Danielssen, 1877, *P. diodontis* Oken, 1815, *P. exocoeti* (Holten, 1802), *P. filosa* (Linnaeus, 1758), *P. instructa* (Wilson, 1917), *P. makaira* (Hogans, 1988), and *P. sagitta* (Oken, 1815). Given the state of taxonomic confusion, due in part to poor or incomplete descriptions, and morphological variation within and similarity between species, molecular genetic analysis might provide better insights into the taxonomic status of these taxa. Here we report results of genetic and morphological analyses of '*Pennella*-like parasites' collected from Pacific saury, based on which we discuss their temporal occurrence.

Materials and methods

Sampling

Pacific saury were caught from February to December, 2012 to 2015 in the North Pacific (Fig. 1; Table 1). Pre-fishing season (May to July) samples were collected by research vessel using surface trawl nets, frame nets, drift gill nets, or stick-held dip nets; fishing season (August to December) samples were collected from commercial fishing boats using stick-held dip nets or by research vessel using sea surface trawl nets. February samples were collected off Wakayama Prefecture from commercial fishing boats using stick-held dip nets. Infected fish collected by research vessels were frozen onboard and then transferred to the laboratory; fish collected by commercial fishing boats were packed on ice and brought to the laboratory. A total of 32,908 saury from 463 stations were examined, and fish infected by *Pennella* or *Pennella*-like parasites were detected at 221 stations (33°50–49°58'N, 136°00′E–168°59'W).

A total 1345 parasites were extracted from fish, but as they usually burrowed deep into the fish body, their collection intact was rendered difficult. A total of 1170 intact parasites were collected from 185 sampling stations from 33°50–49°58'N, 136°00′E–170°00'W.

Morphological analysis

The occurrence of egg sacs and abdominal brushes were examined, and total length (TL; from the anterior tip of the cephalothorax to the posterior end of the abdomen) was measured. Three morphotypes were noted based on abdominal brushes (see results). A subset of 20 intact individuals was extracted for detailed morphological and genetic analyses. These individuals have been deposited in the collections of the National Museum of Nature and Science, Tsukuba, Ibaraki Prefecture (NSMT-Cr 25618-25637).

The ratios of thee morphotypes to total numbers of parasite in each month were calculated. We also calculated ratios of the

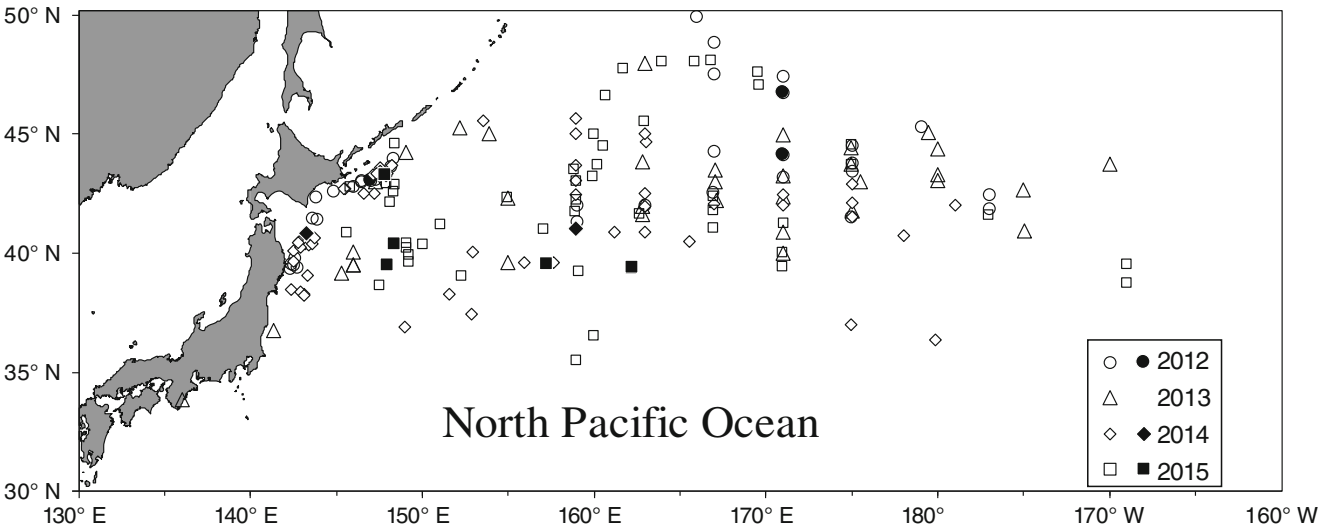

Fig. 1 Pacific saury (*Cololabis saira*) infected by *Pennella* sp. and/or *Pennella*-like parasite sampling stations. Symbols (circles, triangles, diamonds, and squares) indicate stations where infected saury were collected from 2012 to 2015, respectively; closed symbols indicate sampling stations where parasites used for detail morphological and genetic analyses were collected

Table 1 Pacific saury (*Cololabis saira*), *Pennella* sp., and *Pennella*-like parasitic copepod collection data

Year	Month	Pacific saury					No. parasite[c]
		Station No.[a]	No. fish[b]	Prevalence (%)	Latitude (N)	Longitude	
2012	Jun	5 (3)	1011	12.3	41° 19′–43° 01′	158° 59′ E–169° 00′ W	152
	Jul	30 (15)	5237	5.5	39° 57′–48° 51′	159° 00′ E–176° 59′ W	98
	Aug	2 (0)	248	20.6	43° 20′–43° 42′	149° 48′–150° 00′ E	0
	Sep	8 (8)	1358	7.9	42° 50′–49° 58′	145° 59′–165° 57′ E	88
	Oct	7 (6)	1269	3.6	39° 21′–42° 42′	142° 18′–145° 11′ E	52
	Nov	3 (3)	951	2.0	39° 24′–39° 35′	142° 23′–142° 43′ E	7
Subtotal			10,074	6.3	39° 21′–49° 58′	142° 23′ E–169° 00′ W	397
2013	Feb	1 (1)	30	3.3	33° 50′	136° 00′ E	1
	Jun	23 (20)	4444	1.2	39° 33′–45° 03′	154° 57′ E–179° 59′ W	42
	Jul	5 (4)	1387	0.4	40° 57′–43° 45′	175° 31′ E–170° 00′ W	5
	Aug	1 (1)	136	0.7	45° 02′	153° 53′ E	1
	Sep	3 (3)	380	0.8	42° 59′–45° 17′	147° 16′–152° 13′ E	3
	Oct	5 (5)	514	1.2	39° 09′–47° 59′	145° 19′–162° 58′ E	6
	Nov	1 (1)	100	1.0	36° 45′	141° 20′ E	1
	Dec	1 (0)	100	0.7	33° 30′	135° 00′ E	0
Subtotal			7143	1.0	33° 50′–47° 59′	136° 00′ E–170° 00′ W	58
2014	May	7 (6)	558	5.7	36° 22′–40° 31′	149° 00′–179° 53′E	40
	Jun	25 (23)	3163	3.4	37° 01′–46° 24′	150° 59′–175° 00′ E	106
	Jul	4 (2)	893	0.6	40° 44′–43° 35′	154° 59′ E–179° 00′ W	2
	Aug	2 (2)	185	4.3	43° 22′–43° 41′	147° 57′–148° 15′ E	9
	Sep	11 (11)	1256	2.3	40° 05′–45° 33′	142° 32′–153° 47′ E	31
	Oct	8 (8)	981	1.1	39° 40′–42° 42′	142° 31′–145° 31′ E	13
	Nov	6 (6)	724	1.0	38° 14′–40° 39′	142° 53′–143° 46′ E	9
	Dec	1 (1)	171	0.6	38° 29′	142° 21′ E	1
Subtotal			7911	2.5	36° 22′–46° 24′	142° 21′–179° 53′ E	211
2015	Mar	1 (1)	36	2.8	34° 34′–35° 28′	158° 55′–159° 02′ E	1
	Apr	4 (4)	456	9.4	39° 12′–39° 34′	158° 55′–162° 14′ E	26
	May	2 (2)	167	12.0	36° 31′–40° 22′	148° 58′–160° 01′ E	16
	Jun	21 (16)	2573	7.5	38° 39′–44° 32′	147° 32′ E–173° 00′ W	170
	Jul	11 (11)	1695	7.4	38° 43′–44° 59′	157° 06′ E–168° 59′ W	149
	Aug	1 (1)	167	8.4	44° 34′	148° 25′ E	15
	Sep	13 (12)	1301	5.2	42° 34′–48° 05′	147° 48′–169° 37′ E	68
	Oct	5 (5)	750	5.5	40° 23′–42° 46′	145° 40′–149° 08′ E	40
	Nov	4 (4)	635	3.0	39° 30′–41° 10′	147° 59′–151° 04′ E	18
Subtotal			7780	6.8	34° 34′–48° 05′	145° 40′–168° 59′ W	507
Total		221 (185)	32,908	4.3	33° 50′–49° 58′	136° 00′ E–168° 59′ W	1170

[a] Number of stations where infected Pacific saury were observed; numbers in parentheses are the number of stations from which parasites used for morphological analysis were collected

[b] Number of Pacific saury examined for parasites

[c] Number of parasites used for morphological analysis

numbers of *Pennella* sp. (PSP) that had egg sac to total numbers of PSP in each month.

After observation and measurement, parasites were preserved in 80% ethanol. We define prevalence as the ratio of infected to total number of saury examined from the 232 stations at which infected fish were collected.

Genetic analysis

DNA was extracted from egg sacs, trunk skin, abdominal brush, or one of the horns of aforementioned 20 intact individuals using Quick Gene-810 (Kurabo, Japan), since some individuals lacked egg sacs and/or abdominal brushes. A partial mitochondrial cytochrome oxidase subunit I (COI) region was amplified using invertebrate universal primers (LCO-1490 and HCO-2198) (Folmer et al. 1994). The entire stretch of nuclear ribosomal internal transcribed spacer 1 (ITS1) was amplified using primers kp2 and 5.8s (Presa et al. 2002). PCR reaction mixtures were preheated at 94 °C for 4 min, followed by 35 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 50 s), with a final extension at 72°C for 7 min. Our preliminary attempt using direct nucleotide sequencing for COI and ITS1 amplicons (*ca* 700 and 450 bp, respectively) frequently failed to obtain good electropherograms, which we suspected was caused by heteroplasmy or nuclear mitochondrial pseudogenes (NUMTs) for COI and intraspecific or intragenomic variation for ITS1. Therefore, we cloned PCR products using a pGEM-T Easy Vector System I (Promega),

and eight transformed colonies per individual were subjected to colony direct PCR using M13-20 forward and M13 reverse primers. The aforementioned amplification protocol but with an annealing temperature of 55°C was used for colony direct PCR. All PCR products of expected size (*ca* 950 bp for COI and 700 bp for ITS1, including the vector insert) were subjected to direct nucleotide sequence analysis using M13 primers. Nucleotide sequences determined are available in DDBJ/EMBL/GenBank for COI (accession no. LC198844–198871) and for ITS1 (accession no. LC198872–198895). Nucleotide sequence alignment and calculation of Kimura 2-parameter distance (K2P) between sequences were performed using MEGA6 (Tamura et al. 2013). Using MEGA6, phylogenetic relationships for COI sequences were assessed using a maximum likelihood (ML) analysis under HKY + G selected as the best fit model and a neighbor-joining (NJ) analysis based on K2P distance. Nucleotide sequences were subjected to a BLAST search in NCBI to find similar sequences in the GenBank database.

Results

Morphological analysis

Three morphotypes were noted; (1) individuals having typical *Pennella*-like morphology with feather-like abdominal brushes, which we designated as PSP (Fig. 2a, b); (2) those lacking abdominal brushes, designated parasite A (PA) (Fig. 2c, d); and (3) those with a few abdominal brushes, designated "indeterminate individuals" (IDI) (Fig. 2e, f).

Results of morphological and genetic analyses for 20 intact individuals, comprising eleven PSP, seven PA, and two IDI, are presented in Table 2. PSP had 20–24 brushes on one side row of the abdomen (Fig. 2b; Table 2); of these, two were short and unbranched, while nine had primary, secondary or sometimes tertiary branches. One IDI individual had a row of 16 small projections that probably represented a bud of abdominal brushes (Fig. 2f); a second individual had three

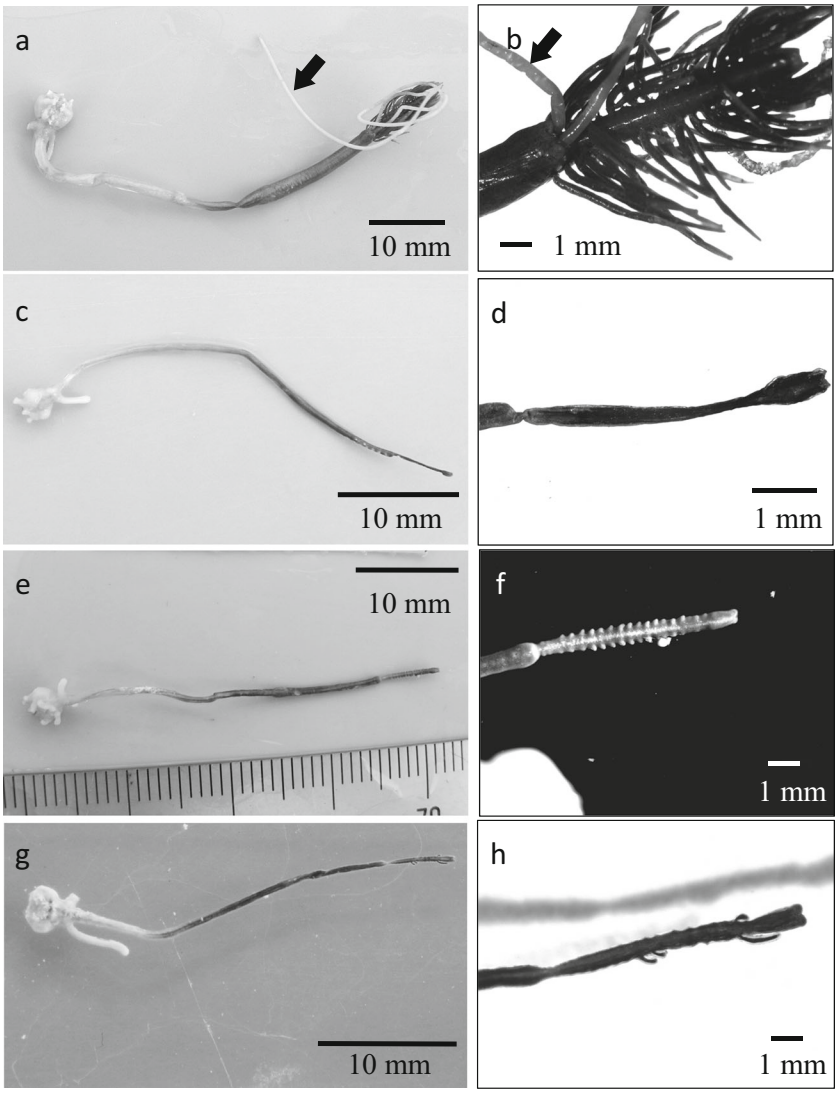

Fig. 2 Entire view (**a**, **c**, **e**, **g**) and enlarged abdominal portion (**b**, **d**, **f**, **h**) of three types of parasitic copepod collected from Pacific saury (*Cololabis saira*). **a, b** Representative *Pennella* sp. (type PSP, PS27-002) with feather-like abdominal brushes and egg sac (arrows). **c, d** Parasite A (type PA, PS27-620) lacking feather-like abdominal brushes and egg sacs. **e**–**h** Indeterminate individuals (**e** (type IDI: **e**, **f**, PS24-338; **g**, **h**, PS24-308) with small abdominal projections (**f**) or few abdominal brushes (**h**) and no egg sac. See Table 2 for specimen numbers

Table 2 Results of morphological and genetic analyses for *Pennella* sp. (PSP), parasite A (PA), and indeterminate individuals (IDI)

Individual	Date	Latitude (N)	Longitude (E)	SST	Type	TL (mm)	NAB	BA	ES	COI	ITS1
PS24-262	20 Jul 2012	42° 33′	166° 56′	13.0	PA	28	0		–		+
PS24-277	17 Jul 2012	48° 51′	167° 00′	10.7	PSP	52	22	2	+		+
PS24-307	16 Jul 2012	47° 25′	170° 58′	9.8	PSP	49	20	0	–	+	+
PS24-308	16 Jul 2012	47° 25′	170° 58′	9.8	IDI	33	3 (1)[a]	0	–		
PS24-319	15 Jul 2012	44° 08′	171° 00′	11.5	PSP	58	21	2	+		+
PS24-338	16 Jul 2012	46° 46′	171° 00′	10.4	PA	39	0		–	+	+
PS24-339	16 Jul 2012	46° 46′	171° 00′	10.4	PSP	54	23	2	+		+
PS24-344	16 Jul 2012	46° 46′	171° 00′	10.4	PA	52	0		–	+	+
PS24-561	22 Sep 2012	43° 04′	147° 02′	17.3	PSP	58	24	2	–	+	
PS24-580	5 Oct 2012	41° 25′	143° 51′	18.6	PSP	56	19	3	+		+
PS24-630	28 Oct 2012	39° 48′	142° 36′	16.9	PA	77	0		–		+
PS26-017	28 Jun 2014	41° 02′	158° 57′	14.5	PSP	58	22	2	+	+	
PS26-097	14 Jun 2016	42° 03′	166° 59′	9.4	PA	32	0		–		
PS26-101	6 May 2016	38° 45′	166° 16′	13.1	PA	29	0		–		
PS26-334	22 Sep 2014	40° 49′	143° 17′	18.0	PSP	62	22	1	+	+	
PS27-002	26 Apr 2015	39° 34′	157° 12′	13.6	PSP	62	22	1	+	+	
PS27-022	29 Apr 2015	39° 24′	162° 13′	14.0	PSP	39	22	0	–	+	
PS27-620	8 Sep 2015	43° 17′	147° 48′	14.8	IDI	44	16	0[b]	–	+	
PS27-708	30 Oct 2015	40° 25′	148° 24′	12.3	PA	44	0		–	+	
PS27-723	12 Nov 2015	39° 03′	147° 59′	12.0	PSP	69	23	3	+	+	

TL, total body length; NAB, number of abdominal brushes on one side; BA, branching number of abdominal processes; ES, egg sac present (+) or absent (−); COI, the individuals for COI nucleotide sequence analysis (+); ITS1, the individuals for ITS1 nucleotide sequence analysis (+); SST, sea surface temperature

[a] Number of abdominal brushes on left and right sides (in parentheses)

[b] Only small projections were observed instead of abdominal brushes

processes on one and one on the other side of the abdomen (Fig. 2h). Eight of 11 PSP had a pair of egg sacs, but three did not (Table 2); no egg sac was observed in any PA or IDI individual.

The three morphotypes usually had three pairs of well-branched larger antennary processes on the outer edge of the cephalothorax. These antennary processes surrounded many smaller inner processes, sometimes becoming lobe- or cup-like in larger individuals (Kabata 1979). PSP individuals had three pairs of branched antennary processes (Fig. 3a–d), of which the shape of the outer processes was variable sometimes anteriorly projecting toward the outside (Fig. 3a, d) or curved inwards or outwards (Fig. 3b, c). Both PA and IDI individuals usually had three pairs of outer-branched antennary processes and a small inner process (Fig. 3e, g), though exceptional individuals had only two pairs of outer-branched antennary processes (Fig. 3f). The extent of branching of the outer processes and the number of inner processes in PA and IDI were lower than in PSP. The nine PAs lacking antennary processes had segmented abdomens with no or very small lateral horns (Fig. 3f). Some small PA that appeared to have recently metamorphosed had three pairs of small, unbranched outer antennary processes (Fig. 3i).

Temporal variation in parasite abundance

The prevalence of parasitism was relatively high from May to August, but never exceeded 10% after September (Table 1). Of 1170 parasites, 256 were attributed to PSP (21.8%), 886 to PA (75.7%), and 28 to IDI (2.5%) (Table 3). Of 256 PSP, 156 (60.9%) had egg sacs. No egg sac was observed in all PA and IDI.

Ratios of the numbers of PSP (with and without egg sacs), PA and IDI to total numbers of individuals in each month are shown in Table 3 and Figs. 4 and 5. Considerable monthly (February to December) and yearly (2012 to 2015) differences are apparent. Excluding months for which less than ten individuals were collected, PSP was least abundant in August of 2015 (0.0% of all individuals encountered) and most abundant in June of 2012 (36.8%) to total numbers of individuals encountered (Table 3; Fig. 5a). There were significant differences in the ratio of PSP to total numbers of individuals between June and October, June and November, and July and

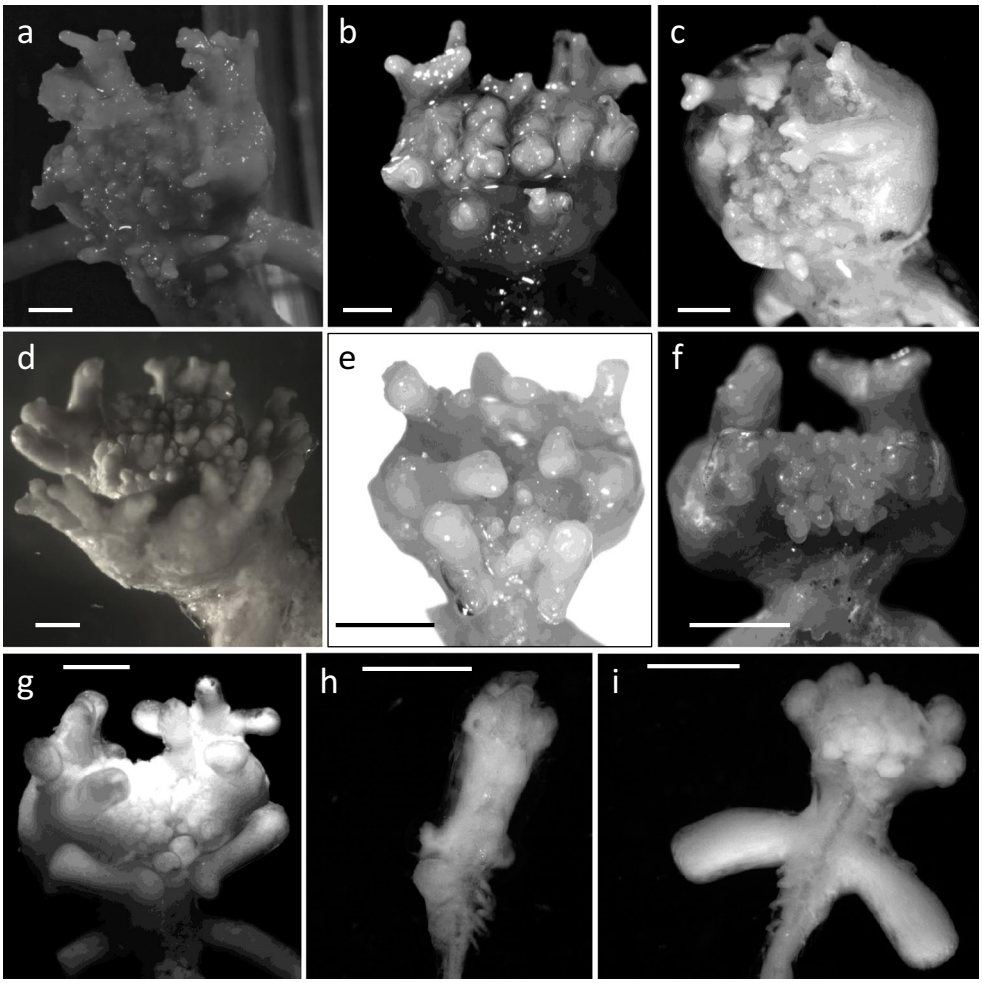

Fig. 3 Variation in antennary processes on the cephalothorax of representative *Pennella* sp. from Pacific saury (*Cololabis saira*) (**a**–**d** PSP; **e**, **f**, **h**, **i** PA; **g** IDI): **a** PS27-723, **b** PS26-017, **c** PS27-002, **d** PS24-277, **e** PS24-338, **f** PS24-344, **g** PS27-620, **h** PS26-101 (PA lacking cephalothorax antennary processes, no lateral horns, with segments), and **i** PS26-097 (small PA, possibly just after final metamorphosis, with three pairs of unbranched antennary process on the outer edge of cephalothorax). See Table 2 for specimen numbers. Scale bars, 1 mm

October in 2012. No significant difference in the numbers of PSP to total numbers of individuals was apparent between 2014 and 2015 (Tukey's multiple comparison test, $P > 0.05$).

We also investigated monthly changes in the ratio of numbers of PSP with egg sacs to total numbers of PSP. The numbers of PSP with an egg sac were usually greater than those without one, but differences in the ratios of PSP with and without egg sacs between months within a year and between years were not significant except for June and July of 2015 (Fig. 5b).

Parasite total length

Size (TL) distributions of PSP with and without egg sacs, and PA and IDI are shown in Fig. 6. PSP TL ranged 33–90 mm, with mean 55.8 ± 9.1 mm SD; mean PSP TL with egg sacs (57.3 ± 8.7 mm) was significantly greater than PSP without (53.4 ± 9.2 mm) (*t* test $P < 0.05$). PA and IDI TL ranged 17–80 and 29–64 mm, with means 39.4 ± 7.3 and 41.6 ± 7.4 mm, respectively, both significantly smaller than corresponding PSP.

Genetic analysis

Among a subset of 20 intact individuals aforementioned, PCR amplification followed by nucleotide sequence determination was successful in 11 (seven PSP, three PA and one IDI) for COI and nine (six PSP and three PA) for ITS1 (Table 2). Of 88 clones sampled from 11 individuals (seven PSP, three PA, and one IDI), 52 (one to eight clones per individual) had the expected size of a COI insert, from which nucleotide sequences (656–659 bp) were successfully determined. One to six haplotypes were observed per individual, with one haplotype being identical to the host fish. Five haplotypes having stop codons based on invertebrate mitochondrial code. Excluding the host fish haplotype and COI-like pseudocopies, 28 haplotypes (658 bp) were available for K2P calculation (Table 4). Mean K2P between haplotypes within individuals ranged 0.15–0.46%. Mean K2P between individuals within PSP and PA was 0.52 ± 0.14 and 0.44 ± 0.16%, respectively, and those between PSP, PA, and IDI ranged from 0.55 ± 0.14 to 0.65 ± 0.20%. At the time of writing, no *Pennella* COI data has been reported, but data for five other pennellid species in two genera

Table 3 Monthly occurrence of *Pennella* sp. (PSP), parasite A (PA), and indeterminate individuals (IDI) collected from Pacific saury, *Cololabis saira*, off the coast of Japan from 2012 to 2015

Year	Month	PSP No. (%)	PSP (egg sac) No. (%)	PSP (no egg sac) No. (%)	PA No. (%)	IDI No. (%)	Total No.
2012	Jun	56 (36.8)	41 (27.0)	15 (9.9)	96 (63.2)	0 (0.0)	152
	Jul	27 (27.6)	18 (18.4)	9 (9.2)	69 (70.4)	2 (2.0)	98
	Sep	15 (17.0)	13 (14.8)	2 (2.3)	71 (80.7)	2 (2.3)	88
	Oct	5 (9.6)	2 (3.8)	3 (5.8)	43 (82.7)	4 (7.7)	52
	Nov	2 (28.6)	2 (28.6)	0 (0.0)	5 (71.4)	0 (0.0)	7
	Total	105 (26.4)	76 (19.1)	29 (7.3)	284 (71.5)	8 (2.0)	397
2013	Feb	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1
	Jun	10 (23.8)	5 (11.9)	5 (11.9)	30 (71.4)	2 (4.8)	42
	Jul	2 (40.0)	1 (20.0)	1 (20.0)	3 (60.0)	0 (0.0)	5
	Aug	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1
	Sep	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	3
	Oct	2 (33.3)	1 (16.7)	1 (16.7)	4 (66.7)	0 (0.0)	6
	Nov	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1
	Total	15 (25.4)	8 (13.6)	7 (11.9)	42 (71.2)	2 (3.4)	59
2014	May	6 (15.0)	4 (10.0)	2 (5.0)	34 (85.0)	0 (0.0)	40
	Jun	16 (15.1)	11 (10.4)	5 (4.7)	87 (82.1)	3 (2.8)	106
	Jul	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	2
	Aug	2 (22.2)	2 (22.2)	0 (0.0)	7 (77.8)	0 (0.0)	9
	Sep	7 (22.6)	4 (12.9)	3 (9.7)	24 (77.4)	0 (0.0)	31
	Oct	2 (15.4)	2 (15.4)	0 (0.0)	11 (84.6)	0 (0.0)	13
	Nov	3 (33.3)	2 (22.2)	1 (11.1)	6 (66.7)	0 (0.0)	9
	Dec	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1
	Total	36 (17.1)	25 (11.8)	11 (5.2)	172 (81.5)	3 (1.4)	211
2015	Mar	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1
	Apr	3 (11.5)	2 (7.7)	1 (3.8)	23 (88.5)	0 (0.0)	26
	May	1 (6.3)	1 (6.3)	0 (0.0)	15 (93.8)	0 (0.0)	16
	Jun	32 (18.1)	20 (11.8)	12 (7.1)	135 (79.4)	3 (1.8)	170
	Jul	22 (14.8)	11 (7.4)	11 (7.4)	122 (81.9)	5 (3.4)	149
	Aug	0 (0.0)	0 (0.0)	0 (0.0)	14 (93.3)	1 (6.7)	15
	Sep	23 (33.8)	5 (7.4)	18 (26.5)	44 (64.7)	1 (1.5)	68
	Oct	13 (32.5)	7 (17.5)	6 (15.0)	24 (60.0)	3 (7.5)	40
	Nov	6 (33.3)	2 (11.1)	4 (22.2)	10 (55.6)	2 (11.1)	18
	Total	100 (19.9)	48 (9.5)	52 (10.3)	388 (77.1)	15 (3.0)	503
Total		256	156	100	886	28	1170

(*Haemobaphes* Steenstrup & Lütken, 1861, and *Lernaeenicus* Le Sueur, 1824) were available (accession numbers are presented in Fig. 7). K2P distances between species in different genera and our *Pennella* were large (24.9 to 43.8%). The ML phylogenetic tree constructed using COI nucleotide sequences of 28 haplotypes and five pennellid species is shown in Fig. 7a. Essentially, the same topology was obtained in the NJ tree. All *Pennella* and *Pennella*-like parasites formed a cohesive clade, diverging substantially from other species, without notable morphotype clustering (PSP, PA, and IDI) (Fig. 7a). The NJ-unrooted radiation tree based on K2P distance between 28 haplotypes (Fig. 7b) revealed that different haplotypes observed within individuals tended to be similar one another.

ITS1 nucleotide sequences were analyzed in five PSP and four PA, and a number of sequences determined, number of paralogues detected, and mean K2P distances are presented in Table 5. Nucleotide sequences of one to seven clones per individual (42 sequences in total) were determined. All sequences were of the same length (425 bp), comprising the 3′ end of 18S rDNA (12 bp), the entire ITS1 (379 bp), and the 5′ end of 5.8S rDNA (34 bp). No similar sequence for the ITS1 region was found in GenBank. No indel was observed among sequences but within-individual nucleotide, sequence variation was usually observed. One to five different sequences (= paralogues) were observed within individuals; 24 paralogues in total were obtained. K2P distance between paralogues within individuals ranged 0.26–1.47%. Mean K2P distances within each of PSP and PA were 1.07 ± 0.25 and 0.75 ± 0.22%, respectively, and that between PSP and PA was 0.94 ± 0.19%. The NJ-unrooted radiation tree of 24 paralogues (based on K2P distance) revealed little similarity among paralogues within individuals (Fig. 8).

Discussion

The results of genetic analysis performed in the present study indicate PA and IDI to be *Pennella*, and all examined individuals to be conspecific. Mean K2P distances in COI sequences

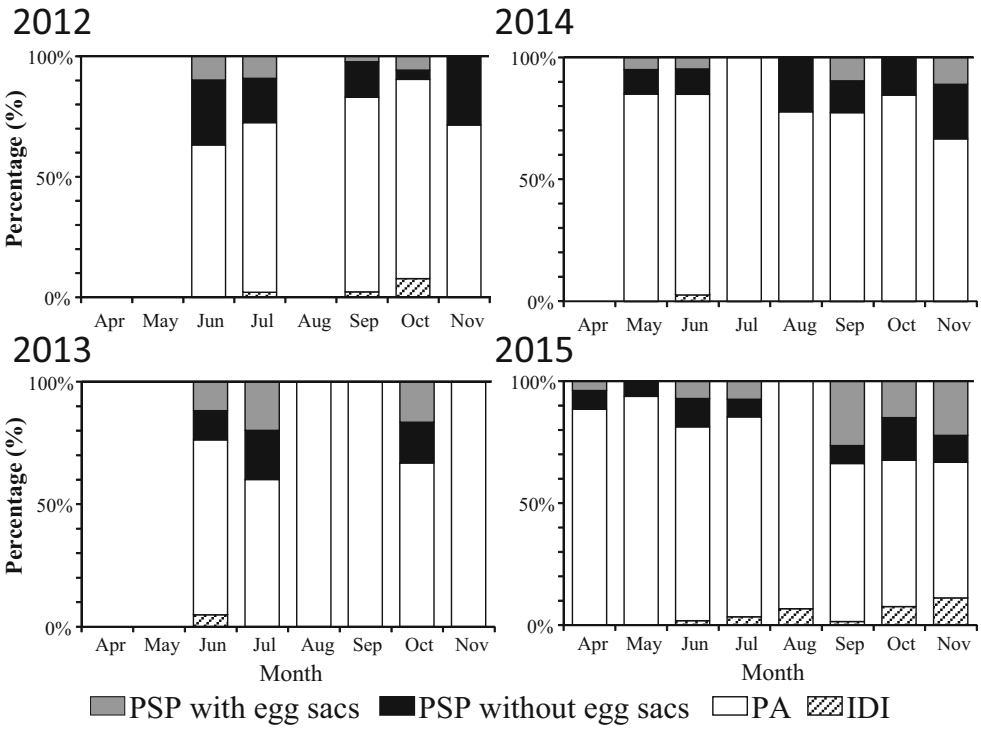

Fig. 4 Monthly percentage compositions for *Pennella* sp. (PSP) with egg sacs (gray), PSP without egg sacs (black), parasite A (PA, white), and indeterminate individual (IDI, mesh). Data from December to March not included due to small sample size

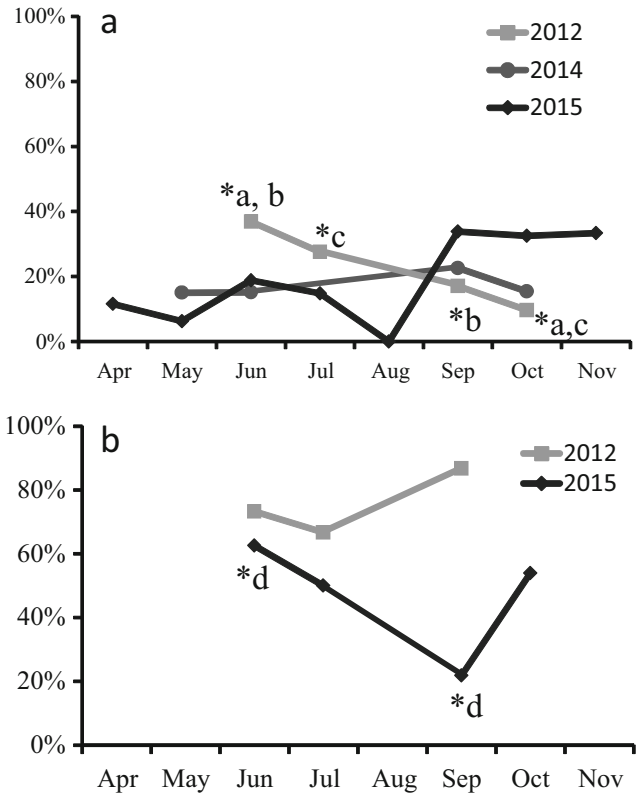

Fig. 5 Ratios of **a** numbers of PSP to total numbers of individuals and **b** numbers of PSP (with egg sacs) to total numbers of PSP (with and without egg sacs) for months in which more than ten individuals were caught. **a*, **b*, **c*, and **d* show combinations in which significant difference is detected (Tukey's multiple comparison test, $P > 0.05$)

among three morphotypes were similar or less than intra-specific divergence in the parasitic copepods *Haemobaphes pannosus* Kabata, 1979 (0.52 ± 0.29%) and *Lernaeenicus sprattae* (Sowerby, 1806) (1.13 ± 0.26%) (Raupach et al. 2015). COI nucleotide sequence diversity between congeneric copepod species is usually greater than 10% (Bucklin et al. 1999, 2003; Øines and Heuch 2005; Machida et al. 2006; Machida and Tsuda 2010; Yasuike et al. 2012). ITS1 is thought to evolve rapidly, and intra- and inter-specific nucleotide sequence variations in ITS1 are known to vary considerably among taxa (Chow et al. 2009). Intra-specific ITS1 sequence variation in Japanese spiny lobster (*Panulirus japonicus* (von Siebold, 1824)) ranged 0.6–0.9%, while that in the copepod *Paracalanus parvus* (Claus, 1863) ranged 0.0–7.9% (Chow et al. 2009). Intra-genomic and intra-specific nucleotide indels and/or a variable number of repetitive elements in ITS1 are observed in wide range of animal taxa (Chow et al. 2006, 2009; Bower et al. 2008). Nucleotide sequence substitution in the ITS1 region within and between PSP and PA was common, but no indel between sequences was observed.

An important finding in the present study is that heteroplasmy may be common in *Pennella* sp. Since K2P distance between different COI haplotypes within an individual was often less than between individuals (Fig. 7b), differentiation in the maternal lineage, rather than paternal mtDNA transmission, may be major source for heteroplasmy. In contrast, no such affinity was apparent among different ITS1

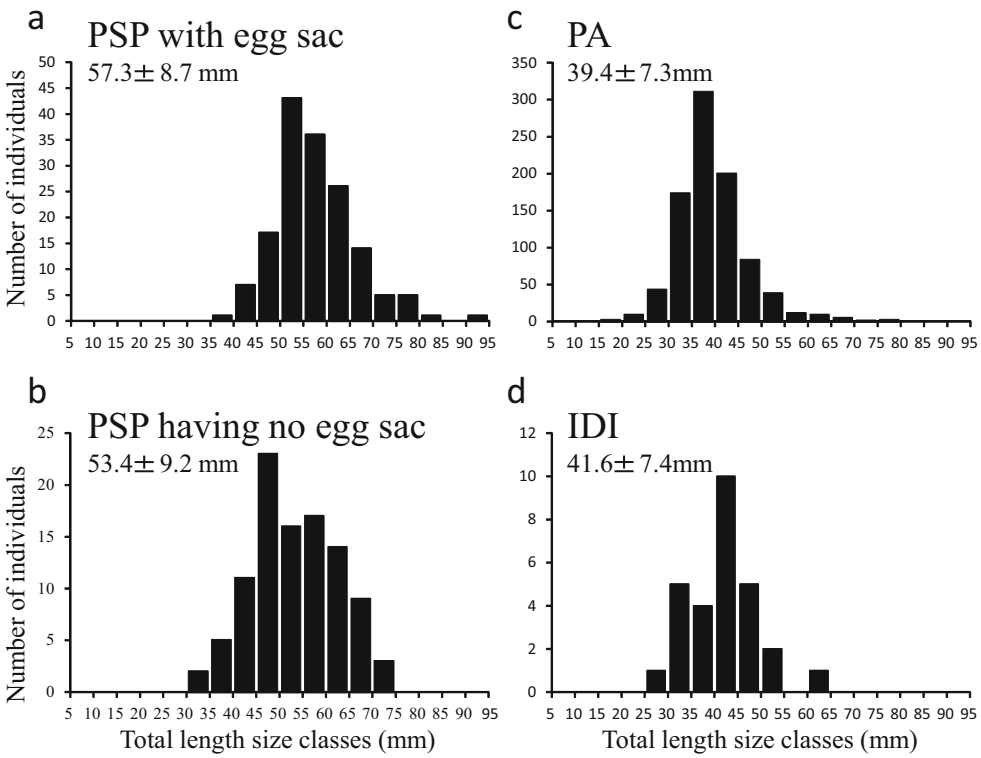

Fig. 6 Total length (TL) distributions **a** Typical *Pennella* sp. (PSP) with feather-like abdominal brushes and egg sacs **b** PSP lacking an egg sac **c** Parasite A (PA) lacking abdominal brushes and egg sacs.**d** Indeterminate individuals (IDI) with few abdominal brushes but no egg sac

paralogues within an individual, suggesting inter-genomic differentiation was the source of variation.

Our analyses corroborate Hughes (1973) inference that *Pennella*-like parasites lacking an abdominal brush were juvenile stages of the same species. The fact that mean TL of PA (Figs. 2c and 3e, f) was significantly smaller than that of PSP (Figs. 2a and 3a–d), and all PA lacked egg sacs, strongly suggested that PAs were either immature or had ceased developing even after their final molt. *Pennella* lacking abdominal brushes have not previously been reported in saury from the central and western North Pacific (Kosaka et al. 1985; Watanabe et al. 1985; Nagasawa 1984; Nagasawa et al. 1984, 1985, 1988; Yamaguchi and Honma 1992). We examined one Pacific saury specimen caught in 1988 (No. 19693) accessioned into the Meguro Parasitological Museum, Japan, on which we found two types of *Pennella* (PSP and PA). Individuals of another species, *P. oxyporhamphi* Sebastian, 1966, lacking an abdominal brush have also been reported

Table 4 Summary of COI nucleotide sequence analyses

Individual	Type	Number of sequence			Mean K2P distance (% ± SE)		
		Determined	Haplotype	With stop codon	Within individual	Among individuals	Between types
PS24-307	PSP	6	3	0	0.20 ± 0.15	0.52 ± 0.14	0.55 ± 0.14 to 0.65 ± 0.20
PS24-561	PSP	6	2	0	0.15 ± 0.15		
PS26-017	PSP	8	6	0	0.36 ± 0.13		
PS26-334	PSP	1	1	0	–		
PS27-002	PSP	2	2	0	0.46 ± 0.25		
PS27-022	PSP	2	2[a]	0	–		
PS27-723	PSP	5	3	1	0.31 ± 0.20		
PS24-338	PA	7	5	1	0.38 ± 0.17	0.44 ± 0.16	
PS24-344	PA	4	2	0	0.15 ± 0.15		
PS27-708	PA	6	3	1	0.15 ± 0.15		
PS27-620	IDI	5	5	2	0.41 ± 0.20	–	

[a] One host fish haplotype excluded from K2P calculation

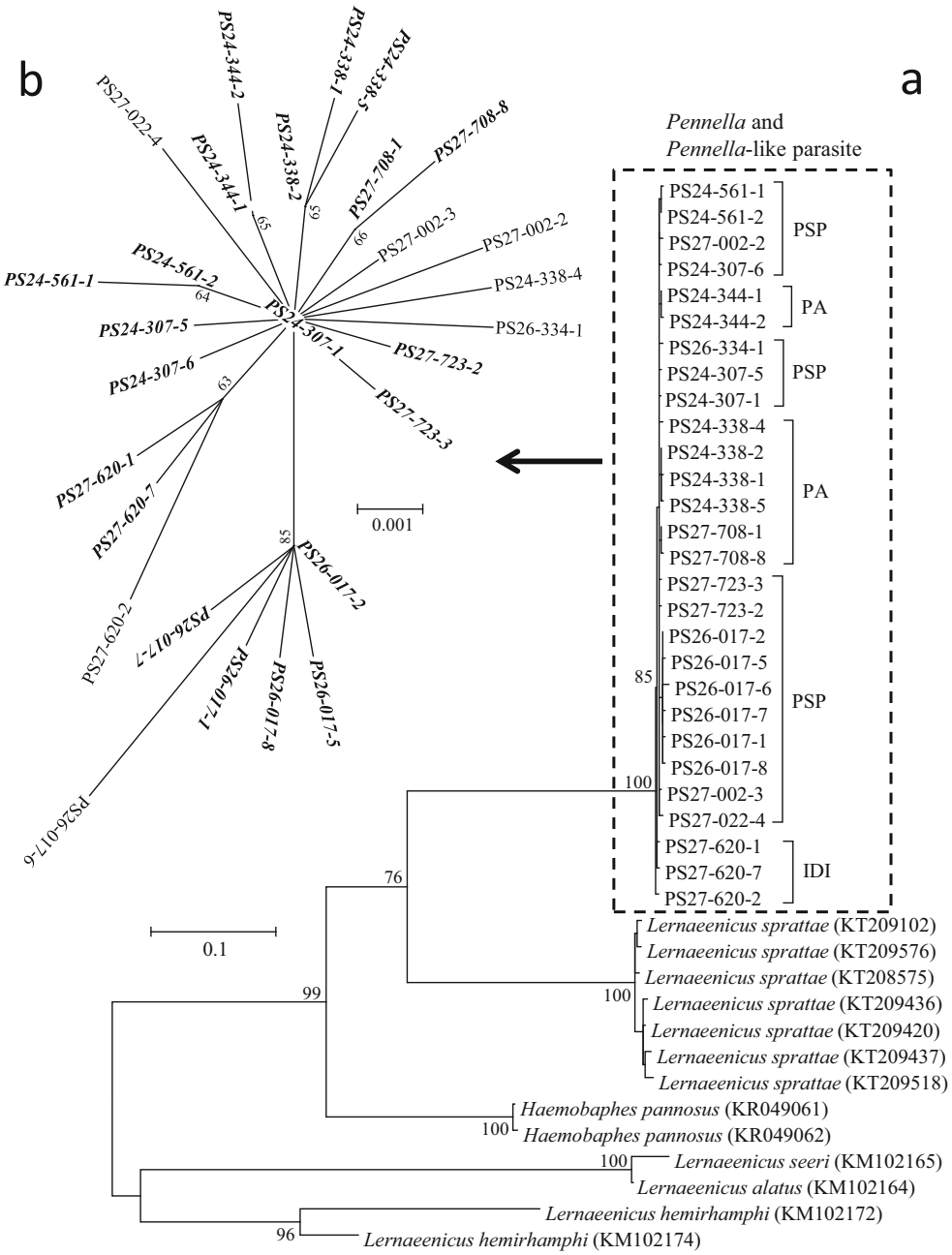

Fig. 7 **a** Maximum likelihood phylogenetic tree (based on the HKY + G model) using COI sequences of 28 haplotypes in seven *Pennella* sp. (PSP), four *Pennella*-like (PA and IDI) individuals, and five pennelid species belonging to two different genera (*Haemobaphes* and *Lernaeenicus*). The bootstrap values (1000 resampling) less than 50% are not shown. **b** Neighbor-joining radiation tree of 28 COI haplotypes based on K2P distance. Note that different haplotypes observed within individuals tend to gather more closely together (bold italic)

from subadult flying fish (*Oxyporhamphus micropterus* (Valenciennes, 1847)) off India (Sebastian 1966). Additionally, *P. diodontis* from the Moorish idol (*Zanclus cornutus* (Linnaeus, 1758)) had neither abdominal brushes nor egg sacs (Lazarus and Sreenivasan 1977).

Whether all PA transform to PSP with brushes after their final molt is unknown. It is possible that IDI with few abdominal brushes, or with small projections that probably represent a bud of abdominal brushes, may represent transitional individuals between PA and PSP stages. However, IDI was much less abundant than either PA and PSP, and no significant difference was observed in ratio of PSP to all individuals between May and October except 2012, and the ratio was rather decreasing in 2012. These facts suggest that most PA remain in a developmental stage before reaching PSP. Pacific saury migrate northward after spring, where they stay in habitat with temperatures usually below ~ 10 °C; after autumn, they start returning to southern regions with higher temperatures, where spawning occurs during winter (Fukushima 1979). It is possible that low temperatures inhibit development of PA to PSP. Since the life span of Pacific saury is less than 2 years, PA, especially those parasitized to relatively older

Table 5 Summary of ITS1 nucleotide sequence analyses

Individual	Type	Number of sequences		Mean K2P distance (% ± SE)		
		Determined	Paralogue	Within individual[a]	Among individuals	Between types
PS24-277	PSP	1	1	–	1.07 ± 0.25	0.94 ± 0.19
PS24-307	PSP	6	3	0.89 ± 0.38		
PS24-319	PSP	7	4	0.53 ± 0.26		
PS24-339	PSP	5	4	1.47 ± 0.41		
PS24-580	PSP	7	3	0.53 ± 0.29		
PS24-262	PA	5	5	0.58 ± 0.25	0.75 ± 0.22	
PS24-338	PA	2	2	0.26 ± 0.27		
PS24-344	PA	4	4	1.11 ± 0.38		
PS24-630	PA	5	5	0.69 ± 0.27		

[a] Distance between different paralogues

fish, must metamorphose rapidly to reproduce. As we saw no marked increase in PSP, many likely die during PA stages.

Wilson (1917) used TL to differentiate *Pennella* taxa into two groups: those larger than 100 mm and those smaller than 50 mm. Of seven species recognized by Hogans (1988a), three (*P. balaenopterae*, *P. filosa* and *P. instructa*) and four (*P. diodontis, P. exocoeti, P. makaira* and *P. sagitta*) might belong to large- and small-sized groups, respectively (Hogans 1988b). The size and shape of antennary processes have been also diagnostic features of these size groups, in which the antennary processes in the small-sized group are reported to be large and branched (Kirtisinghe 1935; Lazarus and Sreenivasan 1977; Kabata 1979; Hogans 1988a, b). The outer antennary processes of *P. filosa* are unbranched and do not modify into lobe-like antennary processes (Kabata 1979; Hogans 1987a, b), while those of *P. balaenopterae* and *P. instructa* are small, uniform, and unbranched (Hogans 1986, 1987a, b). Our *Pennella* sp. from Pacific saury had remarkably large antennary processes surrounding smaller inner antennary processes (Fig. 3a–g). In large individuals, these larger processes frequently had a lobe-like form (Fig. 3d). Few of our *Pennella* sp. exceeded 50 mm TL; none exceeded 100 mm TL (Fig. 6). Both size and antennary characteristics suggest our *Pennella* sp. belongs to the small-sized group. Since the variation in shape and arrangement of antennary processes in our *Pennella* sp. was greater than that previously described for *Pennella* species in the small-sized group (Kabata 1979; Hogans 1988a, b), we could not distinguish our *Pennella* species (from Pacific saury) from those in former studies (Eberhardt 1954; Hughes 1973; Nagasawa 1984; Nagasawa et al. 1984, 1985, 1988; Kosaka et al. 1985; Watanabe et al. 1985; Yamaguchi and Honma 1992). None of the four other small-sized species (*P. diodontis*, *P. exocoeti*, *P. makaira* and *P. sagitta*) has been reported from saury, but species identification based on morphological characters alone may be difficult. Further sampling of *Pennella* spp. from different hosts, and both morphological and genetic analyses are necessary to resolve taxonomic problems in this group.

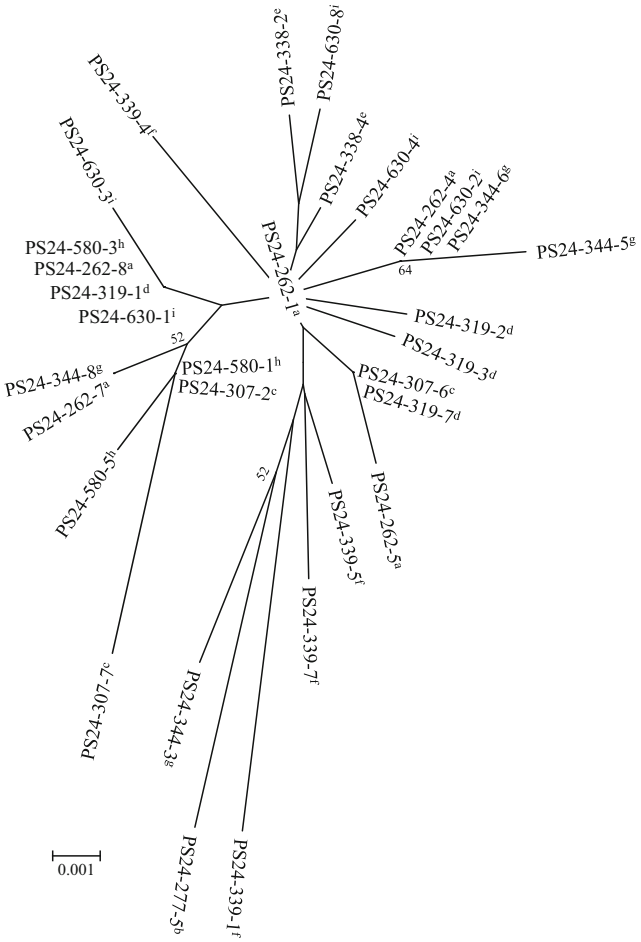

Fig. 8 Neighbor-joining unrooted radiation tree of 31 ITS1 clones (24 paralogues) based on K2P distance. Clones carrying the same alphabet are from the same individual, showing little affinity among different paralogues within individuals

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Sampling and field studies All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities and are mentioned in the acknowledgements, if applicable.

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