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First Records of Two Benthic Chaetognaths, *Spadella japonica* and *Paraspadella gotoi* (Chaetognatha), in the Coast of South Korea, with Taxonomic Reassessment Using Morphological and Molecular Data

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ABSTRACT

The phylum Chaetognatha includes the Spadellidae, characterized by exclusive benthic species. Over the recent decades, 10 species belonging to Spadellidae have been discovered, each with unique habitats worldwide, including sediments, marine caves, and hydrothermal vent sites, underscoring the fragmented and isolated spatial distribution of Spadellidae species. Here, we report the first presence of *Spadella japonica* and *Paraspadella gotoi* in South Korean waters, two species previously described from specimens collected in South Japan. Comparative morphological analysis identified several variations from the former descriptions of *S. japonica*, mainly a larger adult size, a higher number of grasping spines, and the presence of posterior teeth. We also provide new scanning electron microscopy observations of *P. gotoi* which especialy focuses on the morphology of the adhesive organs. Molecular analyses using new sequences of small subunit ribosomal RNA genes enable to reassess the relationships of Spadellidae within Phragmophora with the broadest taxonomic sampling to date. We also generated cytochrome c oxidase subunit I mitochondrial (mtCOI) barcodes and all species always formed monophyletic groups in the phylogenetic analyses. Additionally, we observed highly divergent mitochondrial lineages in an interbreeding population of *S. japonica*. The intra- and interspecific genetic distances of mtCOI sequences within the genus *Spadella* reveals that the divergence between distinct lineages within a species is nearly as significant as that observed between different species. While considering the limitations of the mtCOI gene in establishing an accurate genetic threshold for species delimitation due to the absence of marked barcode gap, it remains an effective molecular marker for identifying congeneric chaetognath species with a tree-based approach.

Keywords: Chaetognatha, benthic species, morphology, biogeography, phylogeny, DNA barcoding

INTRODUCTION

The phylum Chaetognatha, commonly known as arrow worms, encompasses a unique group of small marine metazoans characterized by their slender, translucent body and a distinct head adorned with two sets of grasping spines (hooks) encircling the mouth (Bone et al., 1991; Marlétaz et al., 2006) Within the phylum, the Spadellidae belonging to the Phragmophora consists exclusively of benthic species. Most Spadellidae species exhibit restricted or fragmented geographical ranges often linked to endemism (Müller et al., 2019), which can be attributed to their limited dispersal capabilities and distinct reproductive strategy. This limited dispersal capacity, coupled with a direct development strategy in which eggs are attached to substrates and hatchlings immediately adopt a benthic lifestyle, further reinforces their restricted geographical ranges (Harzsch et al., 2015).

Over the past three decades, numerous new species within

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the Spadellidae have been described, each reflecting distinct ecological and geographical patterns (Müller et al., 2019). In extreme environments, *Spadella antarctica* Casanova, 1991, was discovered in the Antarctic Ocean, and *Calispadella alata* Casanova and Moreau, 2005, was found near hydrothermal vents on the Mid-Atlantic Ridge. In temperate regions, *S. interstitialis* Kapp and Giere, 2005, was reported from *Amphioxus* sediments in the Ligurian Sea.

Meanwhile, the Canary Islands host multiple endemic species, including *S. nunezi* Casanova and Moreau, 2004, *S. duverti* Hernández, De Vera and Casanova, 2009, and *S. lainezi* Casanova, Hernández and Jiménez, 2006. In tropical and subtropical waters, *S. xcalakensis* Tovar and Suárez-Morales, 2007, and *Paraspadella nana* (Owre, 1963) were identified in the Caribbean Sea. Additionally, *S. valsalinae* Winkelmann, Gasmi, Gretschel, Müller and Perez, 2013, in the Northern Adriatic Sea and *S. kappae* Schmidt-Rhaesa and Vieler, 2018, off the French Atlantic coast illustrate the family's adaptation to shallow and benthic habitats. These findings emphasize the Spadellidae's strong association with localized environments and their limited dispersal capacity (Winkelmann et al., 2013).

Various Phragmophora species, such as *S. angulata* Tokioka, 1951, *S. japonica* Casanova, 1993, and *Paraspadella gotoi* Casanova, 1990, have been identified along the southern coast of Japan, but no members of Phragmophora species have been reported from South Korea until now. In this study, we present the first records of *S. japonica* and *P. gotoi* in South Korean neritic waters, contributing to the understanding of chaetognath diversity in the region. To support these findings, we provide a taxonomical reassessment, detailed morphological descriptions, and new genetic data, including small subunit ribosomal RNA (SSU rRNA) and cytochrome c oxidase subunit I mitochondrial (mtCOI) sequences.

The inclusion of genetic data, such as SSU rRNA and mtCOI sequences, is crucial for complementing morphological studies and improving the phylogenetic framework of Phragmophora species. Recent studies have raised questions regarding the monophyly of Phragmophora, emphasizing the need for more comprehensive molecular datasets (Gasmi et al., 2014). By including sequences of *S. japonica*, this study contributes to expanding the genetic data available for this group, supporting future research into the evolutionary relationships of Chaetognatha.

MATERIALS AND METHODS

Sampling and morphological examination

Samples were collected using a conical net (diameter: 0.45 m, mesh size: 200 μm), manually towed from the bottom layer to the surface layer (0−5 m depth), or horizontally through macroalgae on the seabed (1−2 m depth) (Fig. 1). After microscopic observation of living samples, chaetognath specimens were fixed with 5% neutralized formaldehyde for morphological description and 80% ethanol for molecular analysis. In the laboratory, specimens were examined under a stereomicroscope (Stemi 305; Carl Zeiss, Jena, Germany) and a microscope (Axiolab 5; Carl Zeiss) for gross morphology description. Micrographs were obtained using a camera (Axiocam 208 color; Carl Zeiss), and line drawings were created using a digital LCD tablet (SM P580; Samsung, Seoul, Korea).

A total of 24 specimens of *S. japonica* were analyzed for morphological features, with eight specimens further processed for molecular analysis and five specimens used for scanning electron microscopy (SEM). Among these, three specimens (MABIK IV00173451−MABIK IV00173452) were deposited in the Marine Biodiversity Institute of Korea. Morphological variation was assessed in a total of 281 individuals. For *P. gotoi*, five specimens were observed morphologically, with two used for molecular analysis and three subjected to SEM observations.

Scanning electron microscopy (SEM)

Specimens underwent a transition from 5% neutralized formaldehyde to a graded series of ethanol for dehydration. Following critical-point drying and gold/palladium sputtering, analysis was performed using a scanning electron microscope, specifically the Tescan VEGA3 SBU Low-Vacuum.

DNA extraction and gene amplification

To avoid contamination by prey still present in the gut, the specimens were used for DNA extraction after removing the intestine. Five microliters of proteinase K, 250 μL of 10% Chelex, and the tissue were transferred to a 1.5 mL centrifuge tube and incubated in a thermal block at 60°C for 1 h to completely dissolve the tissue. The extracted genomic DNA was used as a template for amplifying the target region. PCR amplification of the SSU rRNA and mtCOI barcode region employed primers 18SCI5 (5′-TTGATGAAACTCTGGATA ACTC-3′) and 18SCI3 (5′-GGACCTCTCTACATCGT TCG-3′) according to Gasmi et al. (2014) and LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) according to Folmer et al. (1994). The PCR mixtures contained 1 μL of each primer (10 μM), 1 μL of DNA template, 17 μL of deionized water, and PCR premix. The PCR protocol was as follows: initial denaturation at 94°C for 5 min, followed by 45 cycles of (94°C for 5 min, 50°C for 45 s, 72°C for 30 s), and a final extension at 72°C for 5 min. PCR products were sequenced at Macrogen Inc. (Seoul, Korea).

Molecular analysis

All sequences obtained (forward and reverse) were first checked using NCBI BLAST, then assembled and aligned with orthologous sequences available in GenBank (Table 1) using CLUSTALW 2.1 implemented in MEGA version v11.0 (Tamura et al., 2021). Molecular analyses drew upon two distinct datasets: the first encompassed two paralogous classes of SSU rRNA sequences, while the second consisted of mtCOI barcode sequences. The SSU rRNA primers used in this study are the same sequences utilized by Gasmi et al. (2014) and are designed to target the small subunit ribosomal RNA 18S, specifically tailored for chaetognaths. These primers are widely used in phylogenetic studies of chaetognaths due to their specificity and the inclusion of a substantial number of chaetognath species in the established datasets, which is why we adopted them for this study. The MODELTEST v3.0b4 program was used to identify the best evolutionary model based on the Bayesian Information Criterion. Phylogenetic reconstructions were conducted through Maximum Likelihood (ML) method. Topological robustness was determined using non-parametric bootstrap method (500 replicates). The sequences generated for this study have been deposited in GenBank and given accession numbers listed in Table 1. Alignments are available upon request.

SYSTEMATIC ACCOUNTS

Class Sagittoidae Tokioka, 1965
Order Phragmophora Tokioka, 1965
[1]*Family Spadellidae Tokioka, 1965

Diagnosis. Caudal segment nearly half of body length. Teeth anterior only or both anterior and posterior. Single lateral fin; modified appendages on posterior lateral fin present or absent; begins near caudal segment. Corona ciliata present on neck or absent.

[2]***Genus *Spadella* Langerhans, 1880***

Diagnosis. Caudal segment nearly half of body length. Both anterior and posterior teeth present or only anterior teeth. Collarette covering entire body or restricted to trunk. Single fin beginning near caudal septum and ending at caudal segment. Corona ciliata present on neck.

[3]****Spadella japonica* Casanova, 1993 (Figs. 2, 3)**

Spadella japonica Casanova, 1993: 359–365, figs. 1–3.

Material examined. Korea: Busan-si: Gangseo-gu, Daehang Port, 35°00′44.09″N, 128°49′32.53″E, 28 Apr 2022 and 3 May 2023, Choo S, Jeong YS Coll.; Suyeong-gu, Namcheon Port, 35°08′17.15″N, 129°06′49.91″E, 10 Jan and 15 Mar 2024, Choo S Coll.; Jeju-do: Jeju-si, Udo-myeon, Cheonjin Port, 33°29′30.38″N, 126°56′59.55″E, 28 May 2024, Choo S Coll..

Re-description. Body type: rigid, trasparent. Body length: 4.6–5.2 mm, with caudal segment comprising 45.9–53.0% of total length. Ventral nerve center: 24.0–42.3%, terminating at 33.7–35.7% of body length, measuring 485.5–555.9 μm. Grasping spines: 8–10 on each side of the cephalic region. Collarette: thick, covering neck and caudal segment, thinning at trunk level. Anterior teeth: elongated, slightly curved, 3–4; posterior teeth: shorter, straight, 0–2. Eyes: square-shaped with rounded edges, composed of one pigmented cell surrounded by sensory cells. Corona ciliata: oval, 49.1–85.3 μm wide, with a short forward projection near the mediodorsal line. Intestinal diverticula: present. Lateral fins: fully rayed, extending from caudal septum to posterior seminal vesicles. Caudal fin: fully rayed and rounded posteriorly. Adhesive papillae: ventral side of body, seminal vesicles, and lateral fins; dorsal sides of lateral fin edges.

Seminal vesicles: touch lateral and caudal fins, filled with reniform-shaped sperm mass in mature specimens. U-shaped posterior space forms a tube-like structure leading to a mediolateral protuberance for sperm release. Ovaries: bilateral, located dorsally along the intestine, extending to anterior trunk when mature. Oocytes: arranged in 2–3 dorsoventral columns, 208.2–289.4 μm in diameter (mean: 239.9 μm). Female genital orifices: elongated cupel, 258.5–282.2 μm long (mean: 270.6 μm), located at the caudal septum.

Distribution. Korea (new record), Japan (such as Misaki, Kominato and Tomioka) (Casanova, 1993).

Ecology. *S. japonica* was sampled from environments with a temperature range of 14–16°C and salinity levels between 30.70 and 32.46. The seabed consisted of a detritic assemblage of sand and fragmented bivalve shells, extending from rocky shores. Additional specimens were collected from a macroalgal bed attached to artificial substrates in Namcheon Port. This habitat included *Padina arborescens*, *Colpomenia sinuosa*, *Ulva australis*, *Undaria pinnatifida*, *Sargassaceae* spp., and *Pterocladiella tenuis*. The appearance of *S. japonica* in Korean waters coincided with the spring season (April and May), during which both adults and immature individuals were observed.

Remarks. Descriptions of *Spadella* species have traditionally relied on characteristics such as the shape, number, and posi-

Korean name: [1]*칼날화살벌레과 (신칭), [2]*칼날화살벌레속 (신칭), [3]*동양칼날화살벌레 (신칭)

Table 1. Details of the sequences used in dataset 1 (SSU rRNA class I and II) and dataset 2 (mtCOI)

Order	Family	Species	Accession No.		
			Dataset 1		Dataset 2
			SSU rRNA class I	SSU rRNA class II	mtCOI
Phragmophora	Spadellidae	*Spadella Japonica*	**OR149863**	–	**OR186515**
			OR149864	–	**OR186516**
			–	–	**OR186517**
			–	–	**PQ569054**
			–	–	**PQ569055**
			–	–	**PQ569056**
					PQ569057
					PQ569058
		Spadella valsalinae	KM519846	–	**OR206478**
		Spadella ledoyeri	DQ351883	–	–
		Spadella cephaloptera	KM519845	–	GU257478
			–	–	GU257470
			–	–	GU257473
			–	–	GU257479
			–	–	GU257480
			–	–	GU257481
			–	–	GU257469
			–	–	GU257476
			–	–	GU257477
			–	–	GU257475
			–	–	GU257471
			–	–	GU257472
			–	–	GU257483
			–	–	GU257482
			–	–	GU257487
			–	–	GU257486
			–	–	GU257485
			–	–	GU257484
		Paraspadella gotoi	D14362	–	NC006083
			PQ573781	–	**PQ613980**
			PQ573782	–	**PQ613981**
	Heterokrohniidae	*Heterokrohnia davidi*	AB617781	–	
		Heterokrohnia sp.	–	–	FJ602475
		Heterokrohnia mirabilis	–	–	GQ368394
		Heterokrohnia sp.	–	–	FJ602474
		Xenokrohnia sorbei	DQ351888	–	
	Eukrohniidae	*Eukrohnia bathypelagica*	KM519851	–	GQ368377
			–	–	GQ368380
		Eukrohnia fowleri	–	DQ351889	GQ368387
			–	–	GQ368386
		Eukrohnia hamata	DQ519852	–	KC633127
			–	–	GQ368390
		Eukrohnia macroneura	–	–	GQ368392
					GQ368393
Aphragmophora	Sagittidae	*Pseudosagitta lyra*	KM519836	DQ351892	GQ368411
		Sagitta bipunctata	KM519822	DQ351890	GQ368396
		Flaccisagitta enflata	KM519795	–	HQ700936
		Serratosagitta serratodentata	KM519828	–	–
		Caecosagitta macrocephala	KM519834	–	–
	Krohnittidae	*Krohnitta subtilis*	KM519840	–	–

Accession numbers for sequences generated in the study highlighted in bold.

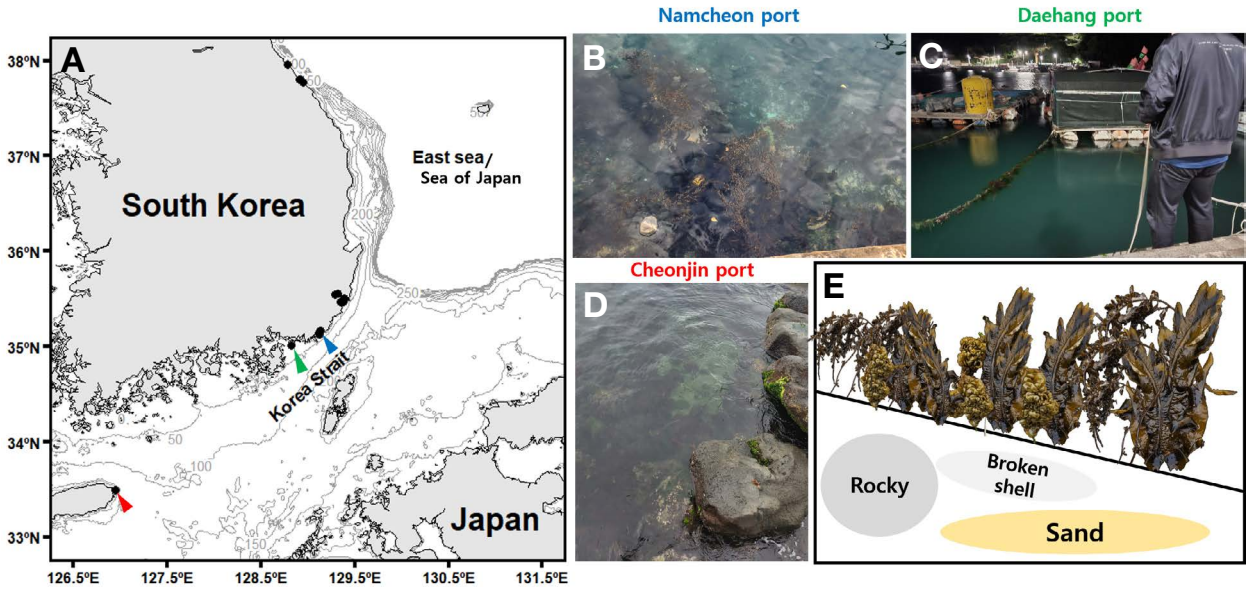

Fig. 1. Map of the sampling area and photographs showing the environment where the samples were collected. A, Map of South Korea with markers indicating the collection sites, B–D, Sampling locations at Namcheon Port, Daehang Port, and Cheonjin Port, respectively; E, Schematic of the sampling environment. Black dots indicate sampling stations where attempts were made to secure specimens. Colored arrowheads indicate stations where Spadellidae specimens were successfully secured.

tion of grasping spines and teeth, the presence/absence of posterior teeth rows, the shape and position of seminal vesicles, lateral fins, corona ciliata, and the appearance of female genital openings (Casanova, 1993; Casanova and Perez, 2000; Casanova and Moreau, 2004; Tovar and Suárez-Morales, 2007). This species was hitherto described from Southern Japanese waters at three different locations (such as Misaki, Kominato and Tomioka) (Casanova, 1993) and it is now also first recorded in southern coast of Korean waters. The morphology of the specimens was consistent with the original description of *S. japonica*, including key features such as the overall body outline, the trunk/tail ratio, distribution of the collarette, presence of female opening field, shape and position of the corona ciliata, lateral fins and seminal vesicles (Table 2). This set of highly valuable characteristics collectively supports that the Korean specimens belong to *Spadella japonica* (Table 2).

However, a difference was observed in body length, with larger specimens observed in Korean waters. Phenotypic variations in body size and the shape or position of lateral fins, has been well documented in several planktonic species (Müller et al., 2019) and is primarily attributed to changes in seawater temperature (Choo et al., 2024). Specifically, lower temperatures are associated with larger specimens. This trend appears to hold true for *S. japonica*, as the waters off Busan, Korea, are significantly colder than those in southern Japan (Han et al., 2023). The holotype and other paratypes were collected in August and September, whereas specimens from Korea were collected in March and May, during which the water temperatures ranged from 14 to 16°C. Although the original description does not specify the exact water temperature, it can be inferred that the Korean specimens were collected in relatively colder conditions, considering the latitude and seasonal factors. Additionally, a distinct morphological variation was noted in the distribution of adhesive papillae and number of posterior teeth. Specimens from Japan lacked posterior teeth (Casanova, 1993), while those from Korea exhibited 0 to 2 posterior teeth, depending on their developmental stage. Casanova's SEM micrograph does not align with his own description (fig. 2, p. 361), pointing out the presence of only 3–4 slightly curved anterior teeth. However, a careful examination revealed the presence of a straight, short tooth below the three anterior ones, similar in shape, position, and orientation to the posterior teeth identified in this study (Fig. 3D). Nevertheless, the number of posterior teeth must be approached with caution because the plasticity of this character has been illustrated in several species, notably in *S. valsalinae*, which has 1 to 3 posterior teeth (Winkelmann et al., 2013). *S. cephaloptera* usually exhibits from 1 to 3 posterior teeth, but their absence is not uncommon (Casanova, 1993). Finally, a critical morphological feature emphasized in Casanova's (1993) original description—the "papillate organ" on the head and caudal septum—was not observed on the body surface of the Korean specimens.

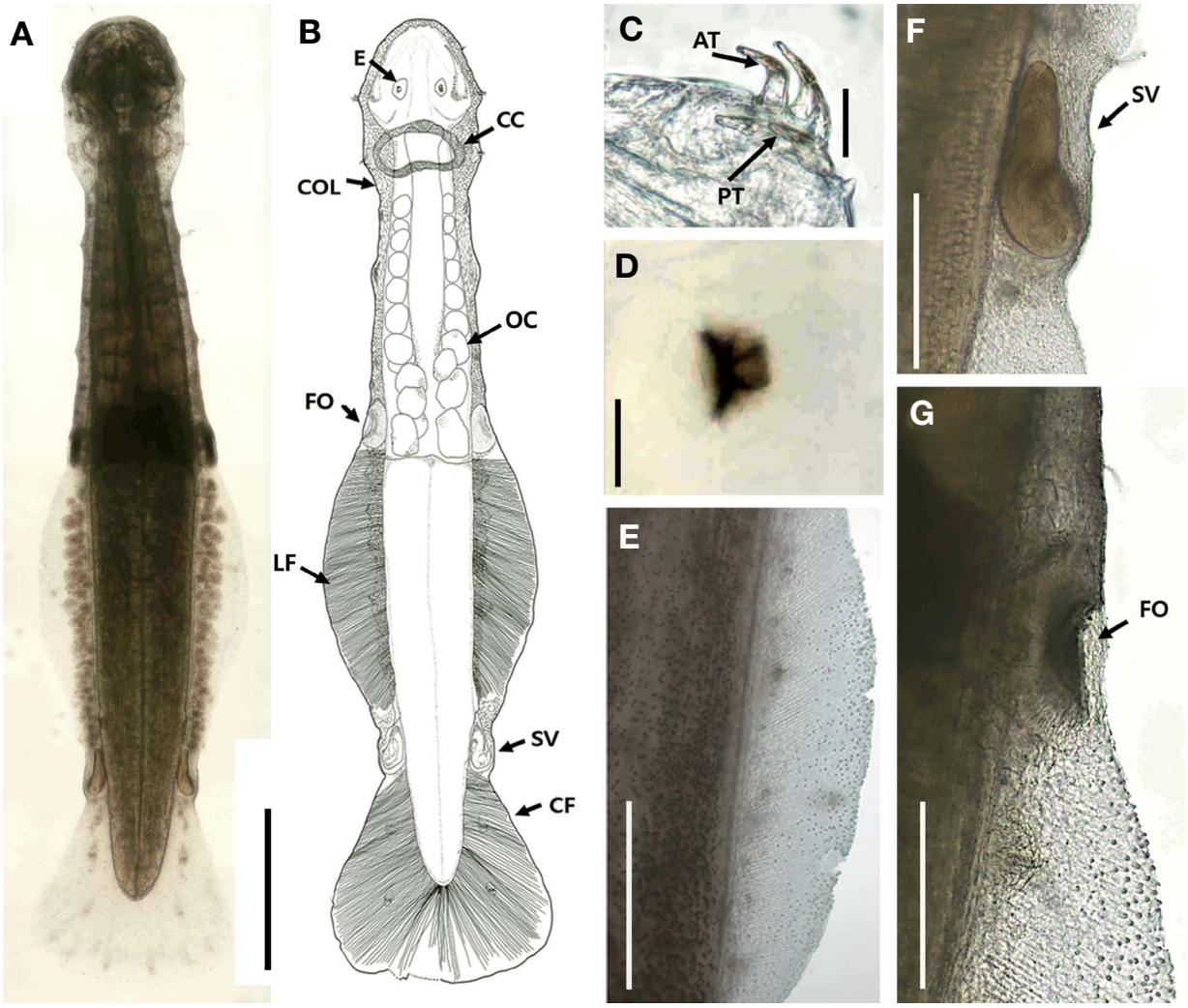

Fig. 2. Habitus of *Spadella japonica* in Korea waters. A, Light micrograph of a specimen fixed with formalin 5%, dorsal view; B, Semi-schematic drawing showing the habitus of *Spadella japonica*; C, Detail of the anterior (AT) and posterior teeth (PT); D, Detail of the eye and the pigmented cell; E, Ventral side of the caudal region with numerous adhesive papillae; F, Detail of posterior caudal region showing the seminal vesicle (SV); G, Detail of posterior trunk region. Note around the female genital opening (FO) the development of the genital field forming an elongated cupel. CC, corona ciliata; CF, caudal fin; COL, collarette; E, eye; LF, lateral fin; OC, oocyte. Scale bars: A, B=1,000 μm, C=5 μm, D=10 μm, E=500 μm, F, G=200 μm.

One of the morphological characters of primary importance for species diagnosis in chaetognaths is the structure of the reproductive system. The seminal vesicles of *S. japonica* are in contact with both lateral and caudal fins and contain a reniform-shaped sperm mass with a U-shaped bent tube visible at the posterior part of the vesicles. This tube ends anteriorly and forms a small mediolateral protuberance at the place where the vesicle disrupts upon the release of sperm. A similar hook-shaped organization has been described in four other known species of *Spadella*, which emphasizes the *japonica* species group (Table 2), consisting of *S. angulata* (Tokioka and Hamatani, 1977), *S. boucheri* (Casanova and Perez, 2000), a species endemic to Japanese waters, as well as the Caribbean species *S. xcalakensis* (Tovar and Suárez-Morales, 2007) and the Californian species *S. bradshawi* (Bieri, 1974). Furthermore, the morphology of female genital openings of *S. japonica* is similar to the description provided for *S. xcalakensis* (Tovar and Suárez-Morales, 2007) (Fig. 3E). According to Casanova (1993), the unusual structure surrounding the female genital openings, named 'genital fields' by Tovar and Suárez-Morales (2007), should correspond to the cement gland initially described in the globally distributed species *S. cephaloptera*. Such a structure is absent in *S. angulata*, *S. boucheri*, and *S. bradshawi*, all of which exhibit a simple orifice,

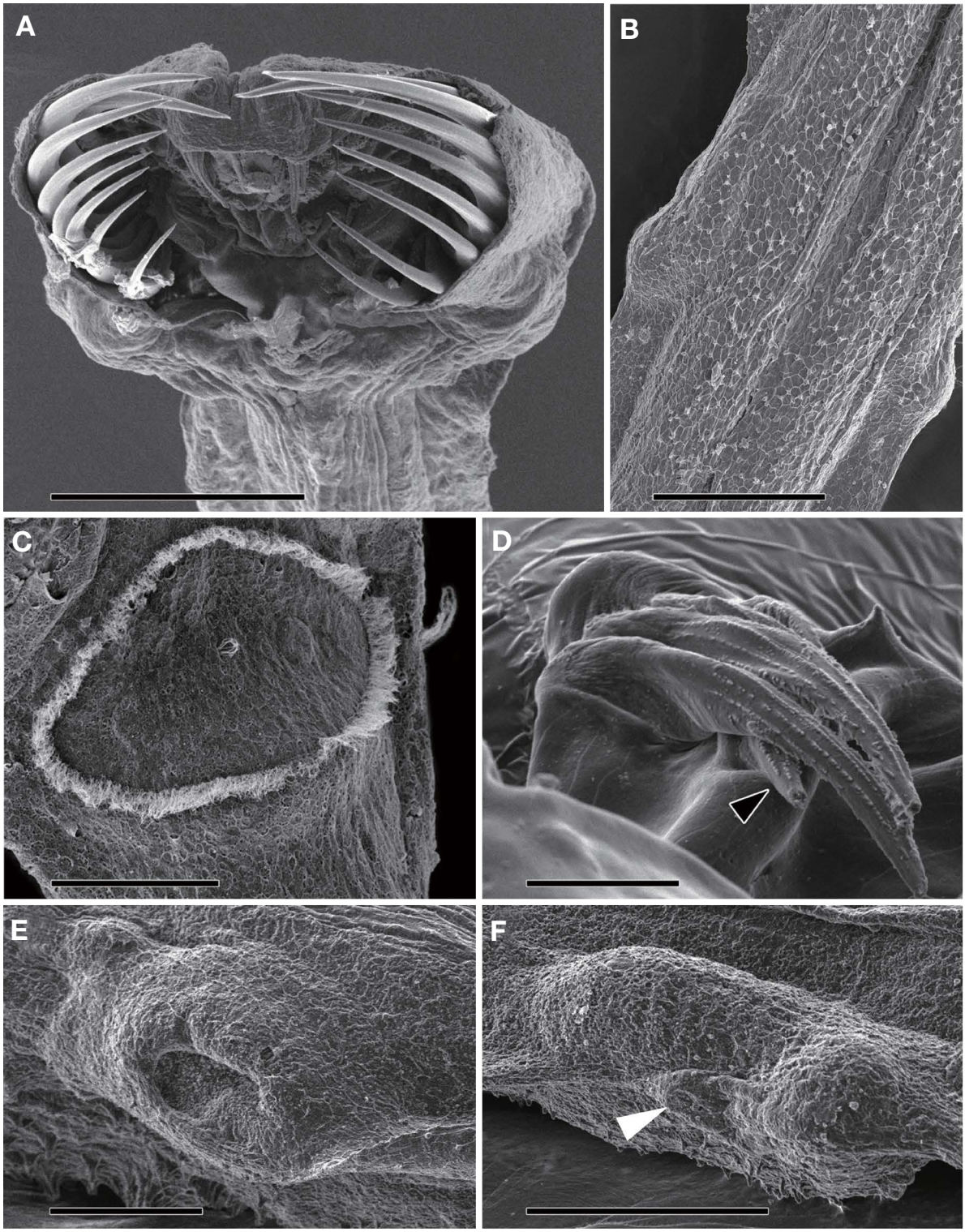

Fig. 3. Scanning electron micrographs of *Spadella japonica*. A, Ventral view of the head; B, Ventral view of the caudal segment. Note the presence of adhesive papillae on the ventral side of the body as well as the seminal vesicles and lateral fins; C, Detail of the corona ciliata; D, Detail of anterior and posterior teeth (black arrowhead); E, Detail of the left female genital opening. Note the elongated cupel surrounding the genital orifice; F, Detail of the left 'hook-shaped' seminal vesicle (lateral view). Note the mediolateral opening (white arrowhead) at the anterior tip of a U-shaped tube located posterior to the reniform sperm mass. Scale bars: A, B = 200 µm, C, E = 100 µm, D = 20 µm, F = 80 µm.

Table 2. Main diagnosis features of *Spadella* species belonging to the *japonica* species group

	S. boucheri (Casanova and Perez, 2000)	*S. angulata* (Tokioka and Pathansali, 1964)	*S. bradshawi* (Bieri, 1974)	*S. xcalakensis* (Tovar and Suárez-Morales, 2007)	*S. japonica* (Casanova, 1993)	*S. japonica* (this study, n=205)
Body length (mm)	1.1–1.3	2.5–5.8	5.5–6.5	3.7	3.25–3.75	4.6–5.2
Trunk/tail ratio (%)	50.0	48.6–57.9	53.0–54.0	49.7–55.0	48.6–51.8	45.9–53.0
No. of grasping spines	7	6–7	7–12	9–10	6–8	8–10
No. of anterior teeth	3	2–5	3–6	4	3–4	3–4
No. of posterior teeth	0	0–1	0	0	0	0–2
Adhesive papillae	On the ventral side, from tail to mid-trunk	Along the ventral and lateral side of the body in the posterior half of the trunk and in the anterior half of the caudal segment	Not described	Absent	On ventral part of body from head to tail, and on both ventral and dorsal sides of fins	On ventral part of the fins and body from the beginning of the ventral nerve centre till the end of the caudal segment and on dorsal sides of fin edges
Collarette	Not very developed. Largest in the neck region and dorsally on the head	Well developed in the neck region	Well developed in the neck region and along the trunk to the tail	Well developed in the neck region, stretches to caudal segment	Well developed in the neck region, then narrower on trunk	Well developed in the neck region, narrower on trunk then thicker on the tail
Intestinal diverticula	Absent	Present	Absent	Not described	Present	Present
Corona ciliata	Oval and small, its largest axis narrower than the width of the neck	Oval, transversely elongated with a forward projection near the middle	Massive and rounded	Oval, thick, with slight thickening near head	Oval and transversely elongate	Oval, transversely elongated with a forward projection near the middle
Lateral fin	Begging at posterior part of trunk and in contact posteriorly with the seminal vesicles	Beginning at the posterior part of the trunk and reaches posteriorly the seminal vesicles	Extend anteriorly beyond the transverse septum but do not reach the seminal vesicles	Beginning at posterior part of trunk ending before anterior part of seminal vesicles	Beginning at posterior part of trunk and reaching posteriorly to seminal vesicles	Beginning at the caudal septum and reaching posteriorly to seminal vesicles
Seminal vesicle	Hook-shaped when small, oval when mature. In contact with lateral and caudal fins	The thickening might be corresponding to the prominent protuberance. In contact with lateral fin and caudal fins	Small U-shaped tube projecting anteriorly. in contact with caudal fins but not with lateral fins	Slightly reniform when full, elongated when empty. in contact with caudal fins but not with lateral fin	Small, hook-shaped when empty. In contact with lateral fin and caudal fins	Reniform shape when full, small posterior U-shaped tube projecting anteriorly. In contact with lateral fin and caudal fins
Female genital opening	Simple orifice	Simple orifice	Simple orifice	Orifice at bottom of an elongated donut	Orifice at bottom of an elongated cupel	Orifice at bottom of an elongated cupel
Distribution	Japanese waters (Miyako island)	Malaysian and Japanese waters	Californian waters (San Diego, US)	Caribbean Sea (Mexico)	Japanese waters (Misaki, Kominato and Tomioka bays)	South Korean waters (Gadeok island)
Ecology	3–5 m depth in a coral reef lagoon (sandy floor)	50 m depth, sandy floor and Zostera belt	25–150 m depth coarse sand and high percentage of silt	1 m depth in a coral reef lagoon (coral and calcareous algae fragments)	Zostera belt, tide pool	3–5 m depth, sand and molluscan shell fragments

further linking the morphology of *S. japonica* to *S. xcalakensis*. Based on the similarity in reproductive system morphology, we considered the species found in the Pacific region, including *S. japonica*, as part of the japonica complex group.

[1]*Genus *Paraspadella* Salvini-Plawen, 1986

Diagnosis. Caudal segment less than half of body length. Only anterior teeth present. Collarette covering entire body or restricted to trunk. Single fin beginning near caudal septum and ending at caudal segment. Adhesive organ present at posterior lateral fins.

[2]****Paraspadella gotoi* Casanova, 1990 (Figs. 4, 5)**

Paraspadella gotoi: Casanova, 1990: 907–912 , figs. 1–2.

Material examined. Korea: Busan-si, Suyeong-gu, Namcheon Port, 35°08′17.15″N, 129°06′49.91″E, 10 Jan and 15 Mar 2024, Choo S Coll.

Re-description. Body type: thick, stout, reddish-orange color. Body length: 4.0–5.1 mm, with the caudal segment comprising 43.7–51.1% of the total length (Fig. 4A, B). Ventral nerve center: extends from 20.6–25.5% and terminates at 35.7–44.2% of the body length, ~640 μm in length. Grasping spines: 8–10 on each side of the cephalic region (Fig. 4D). Collarette: thick and uniform from head to trunk, slightly thicker at the caudal segment (Fig. 4A, B). Anterior teeth: elongated, 4–6, slightly curved; second teeth longest (Fig. 4C). No posterior teeth observed. Eyes: square-shaped, one pigmented cell surrounded by sensory cells (Fig. 2F). Corona ciliata: flattened pear-shaped, with three extended, regularly shaped processes over the neck (Figs. 4B). Intestinal diverticula: present.

Ovaries: two, dorsal along the intestine, extending to the neck in mature specimens, each with one column of conspicuous oocytes (Fig. 4A, B). Lateral fins: extend slightly anterior to the caudal septum; shape features successive symmetrical indentations, divided into small anterior and longer posterior regions near the female genital opening (Fig. 4A, B). Fully mature specimens display a prominent seminal receptacle causing this fin division. Caudal fin: spatulated; all fins fully rayed (Fig. 4A, E).

Seminal vesicles: touch lateral and caudal fins, containing reniform-shaped sperm mass when mature (Fig. 4A, B, E). Adhesive organs: two ventral pairs at the end of lateral fins with 8–10 finger-like processes; anterior pair contacts lateral fins, posterior pair connects seminal vesicles and caudal fin (Figs. 4E, 5A–C). Papillae: specialized epidermal cells on ventral epidermis at fin tips and adhesive organ processes, inflated and granular in appearance (Fig. 5D, E). The caudal fin edge has smaller, less-developed finger-like processes (Fig. 5E).

Distribution. Korea (new record), Japan (such as Reihoku) (Casanova, 1990).

Ecology. Specimens were collected from a macroalgal bed on artificial substrates comprising *Padina arborescens*, *Colpomenia sinuosa*, *Ulva australis*, *Undaria pinnatifida*, *Sargassaceae* spp., and *Pterocladiella tenuis*, in an environment with a temperature range of 14–15°C and salinity levels between 30.70 and 31.00. The specimens were found attached to algal leaves during collection.

Remarks. Like others Spadellidae, *Paraspadella* species are distinguished thanks to traditional morphological characters such as the type and position of seminal vesicles, the shape and number of teeth and grasping spines, the position and shape of the corona ciliata as well as the distribution of the colarette on the body surface. However, a more reliable criterion for diagnosing species within this genus is the characterization of adhesive organs (Casanova et al., 2003). According to Goto and Yoshida (1985), the adhesive organs function like ‘limbs’ and assist in positioning the body vertically during mating. The large anterior and posterior adhesive organs typically observed in *P. gotoi* allow the unambiguous identification of the Korean specimens as belonging to this species.

The Korean specimens closely resemble those described in the original description from Casanova (1990), including aspects such as the overall body outline, the adult size, the number and shape of the teeth and grasping spines, the morphology and position of the seminal vesicles, collarette, and adhesive organs. A difference lies in the size and number of oocytes, with Korean specimens having larger but fewer oocytes compared to their Japanese populations (Table 3).

On the basis of the number and morphology of adhesive organs, Casanova (1990) suggested that the most closely related species to *P. gotoi* is *Paraspadella sheardi* (Mawson, 1944). The characterization of two pairs of lateral fins, as described by Mawson (1944) for *P. sheardi* and by Casanova for Japanese specimens of *P. gotoi*, warrants reconsideration. During the ontogeny of *P. gotoi*, the lateral fins are divided at the caudal septum into a short anterior region and a longer posterior region as the female genital openings and seminal receptacles develop. Notably, immature specimens of *P. gotoi* from Korean waters exhibit undivided lateral fins, suggesting they should be regarded as single at this stage. Similar conclusions have been drawn in earlier studies in *Paraspadella anops* (Bowman and Bieri, 1989) and *Paraspadella schizoptera* (Feigenbaum, 1976). In contrast, members of the Sagittidae possess two distinct pairs of lateral fins, with the anterior fins remaining entirely separate from the posterior ones—a

Korean name: [1]*유사칼날화살벌레속 (신칭), [2]*유사칼날손가락화살벌레 (신칭)

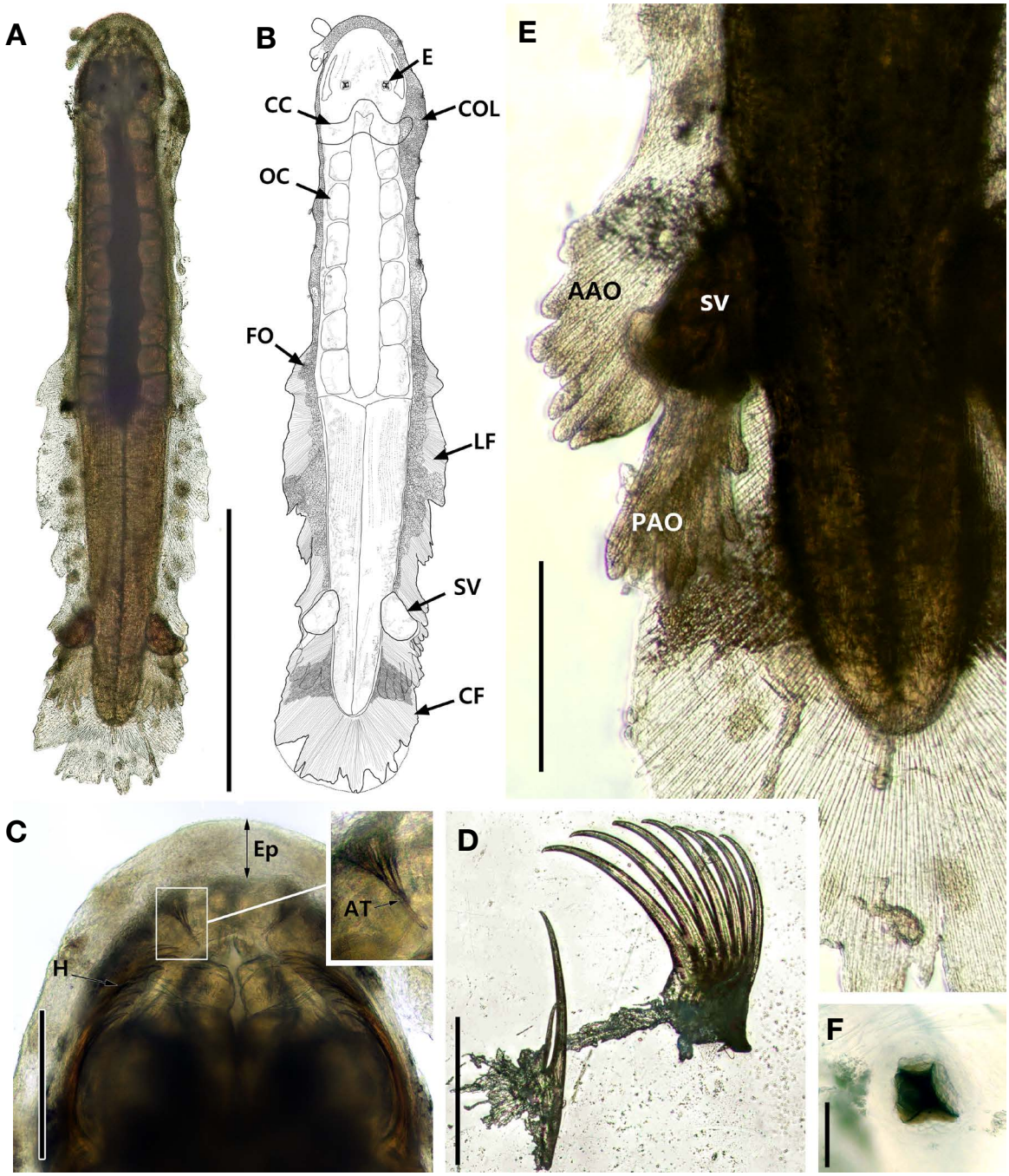

Fig. 4. Habitus of *Paraspadella gotoi* in Korea waters. A, Light micrograph of a specimen fixed with formalin 5%, dorsal view; B, Semi-schematic drawing showing the habitus of *Paraspadella gotoi*; C, Detail of the ventral side of the head with a clos-up of the anterior teeth; D, Grasping spines from the left side after dissection; E, Ventral side of the posterior caudal region at the level of the anterior (AAO) and posterior adhesive organs (PAO) and seminal vesicle (SV); F, Detail of the eye and the pigmented cell. AT, anterior teeth; CC, corona ciliata; CF, caudal fin; COL, collarette; E, eye; Ep, epidermis; FO, female genital opening; H, head; LF, lateral fin; OC, oocyte. Scale bars: A, B=2,000 μm, C, D=300 μm, E=200 μm, F=10 μm.

condition not observed in *P. gotoi*. Thus, attributing the presence of two pairs of lateral fins to the genus *Paraspadella* is misleading. Such a mischaracterization is particularly problematic because the presence of two distinct pairs of lateral fins is phylogenetically significant and is a synapomorphy of Sagittidae (Gasmi et al., 2014).

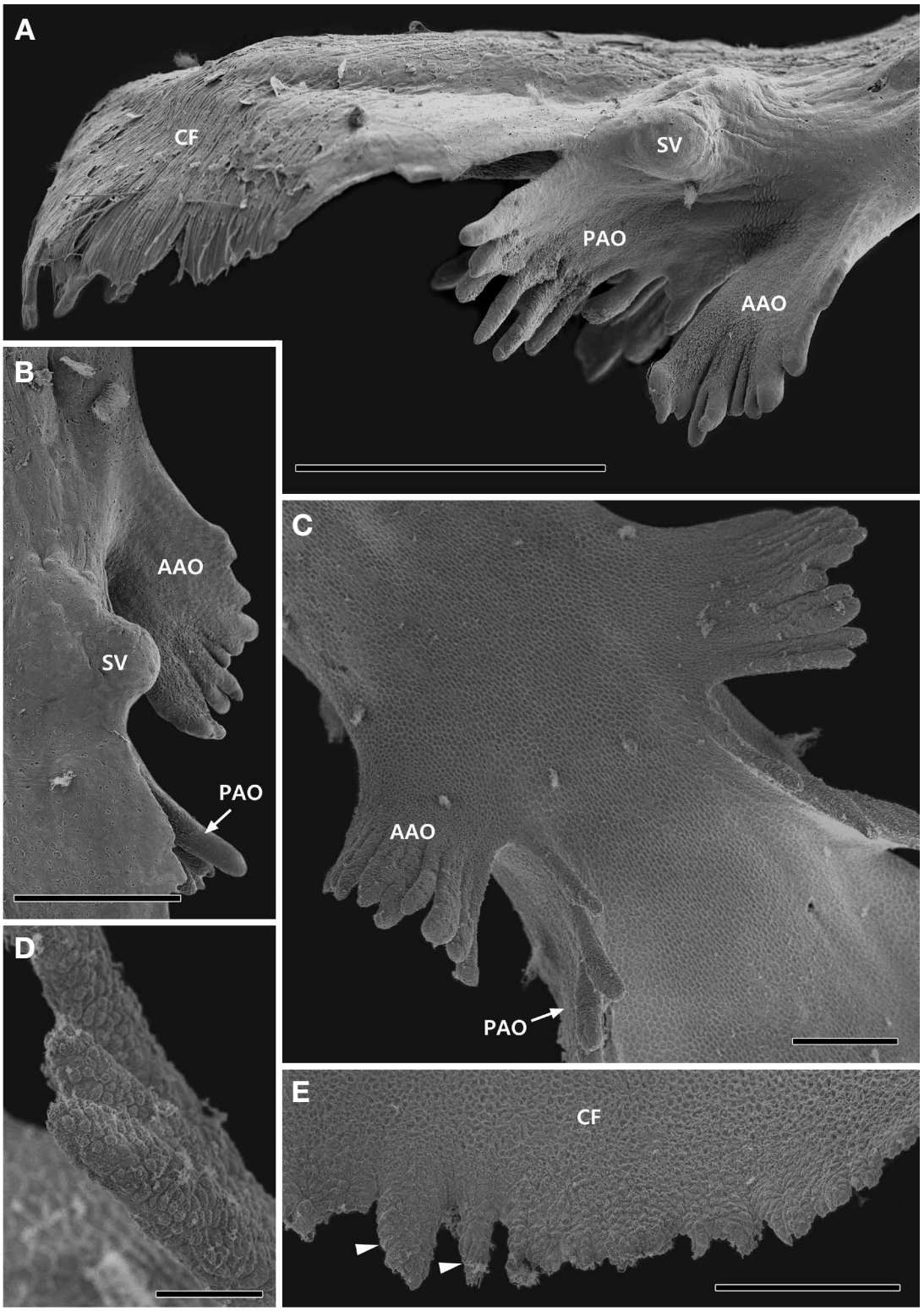

Fig. 5. Scanning electron micrographs of *Paraspadella gotoi*. A–C, Lateral, dorsal and ventral views showing the anterior (AAO) and posterior adhesive organs (PAO) and the right seminal vesicle (SV); D, Close-up showing the finger-like processes of the adhesive appendage, with specialized epidermal cells forming tiny papillae on their surface; E, Ventral view of the tip of the caudal fin (CF), showing the presence of specialized epidermal cells at the site of expansion, resembling short, finger-like processes (white arrowheads). Scale bars: A = 400 µm, B = 200 µm, C = 100 µm, D, E = 50 µm.

Table 3. Main diagnosis features of *Paraspadella* species similar to *P. gotoi*

	P. sheardi (Mawson, 1944)	*P. gotoi* (Casanova, 1990)	*P. gotoi* (this study, n = 5)
Body length (mm)	4.7–6.5	5.9	4.0–5.1
Trunk/tail ratio (%)	44–45	46.5–51	43.7–51.1
No. of grasping spines	11	8–10	8–10
No. of anterior teeth	3	4–6	4–6
No. of posterior teeth	0	0	0
Adhesive organ	Two pairs, anterior and posterior, separated, each with 10–11 processes	Two pairs, anterior and posterior more or less united, cocks-comb shaped, rayed as fins	Two pairs, anterior and posterior, united, cocks-comb shaped, rayed as fins, each with 8–10 finger-like processes
Adhesive papillae	Not described	Tiny, on posterior edges of lateral and caudal fins as well as extremity of adhesive organ processes	Tiny, on posterior edges of lateral and caudal fins as well as extremity of adhesive organ processes
Collarette	Not described	Thick, stretching from the neck to the end of the caudal segment	Thick, stretching from the neck to the end of the caudal segment. Uniform thickness from the head to the trunk, but becomes thicker at the caudal segment
Intestinal diverticula	Absent	Well developed	Well developed
Corona ciliata	Three cornered shape with regular outline	Three lenghtened, irregularly shaped processes	Flattened pear-shaped with three lenghtened processes with regular outline
Lateral fin	Fully rayed and divided in two parts. Anterior and posterior fins almost restangular, well separated.	Fully rayed and divided in two parts. Anterior fins triangular and posterior ones roundish, in close contact.	Fully rayed, divided in two parts by constriction at the level of female genital opening, extending from a region slightly anterior to the caudal septum. Irregurlarly outlined with several successive indentations
Seminal vesicle	Small, crescent shaped	Very large, crooked, touching both the lateral and caudal fins	Large, filled with a reniform-shaped sperm mass (crooked), touching both the lateral and caudal fins
Female genital opening	Lateral, between anterior and posterior fins	Ventral, below the junction of anterior and posterior fins	Simple orifice, lateral, slighlty below the beginning of the lateral fin
Distribution	Australian waters, Off Port Hacking to Ulladulla	Japanese waters, Amakusa island, Reihoku	Korean waters, Namcheon Port, Busan
Ecology	70–100 meters depth on fine sand and detritus without algae	Tide pools	1–3 meters depth, rocky floor with macro algae

Phylogenetic reconstruction and DNA barcode

Small subunit ribosomal RNA. The alignment comprised 36 SSU rRNA sequences, with a total length of 1,393 nucleotides, including 33 sequences from class I and 3 from class II (dataset 1). All positions with less than 75% site coverage were eliminated (partial deletion option). This dataset offers the opportunity to reappraise the relationships within Phragmophora with the broadest taxonomic sampling to date (Fig. 6A). The three sequences from the second paralogy class of SSU rRNA (class II) were used to root one paralogy class over the other. The best model of evolution, estimated using MODEL TEST, was the TN93 + Γ + I model (lnL = −6,200.655). Phylogenetic trees constructed using ML revealed the following branching order from the last common ancestor to the most derived family: Eukrohniidae + Heterokrohniidae + Spadellidae, Krohnittidae, and Sagittidae. The Aphragmophora order (Sagittidae + Krohnittidae) received a high bootstrap value (bv = 100), whereas the Phragmophora order (Heterokrohniidae + Eukrohniidae + Spadellidae) did not. Specifically, the monophyletic group consisting of Heterokrohniidae and Eu-

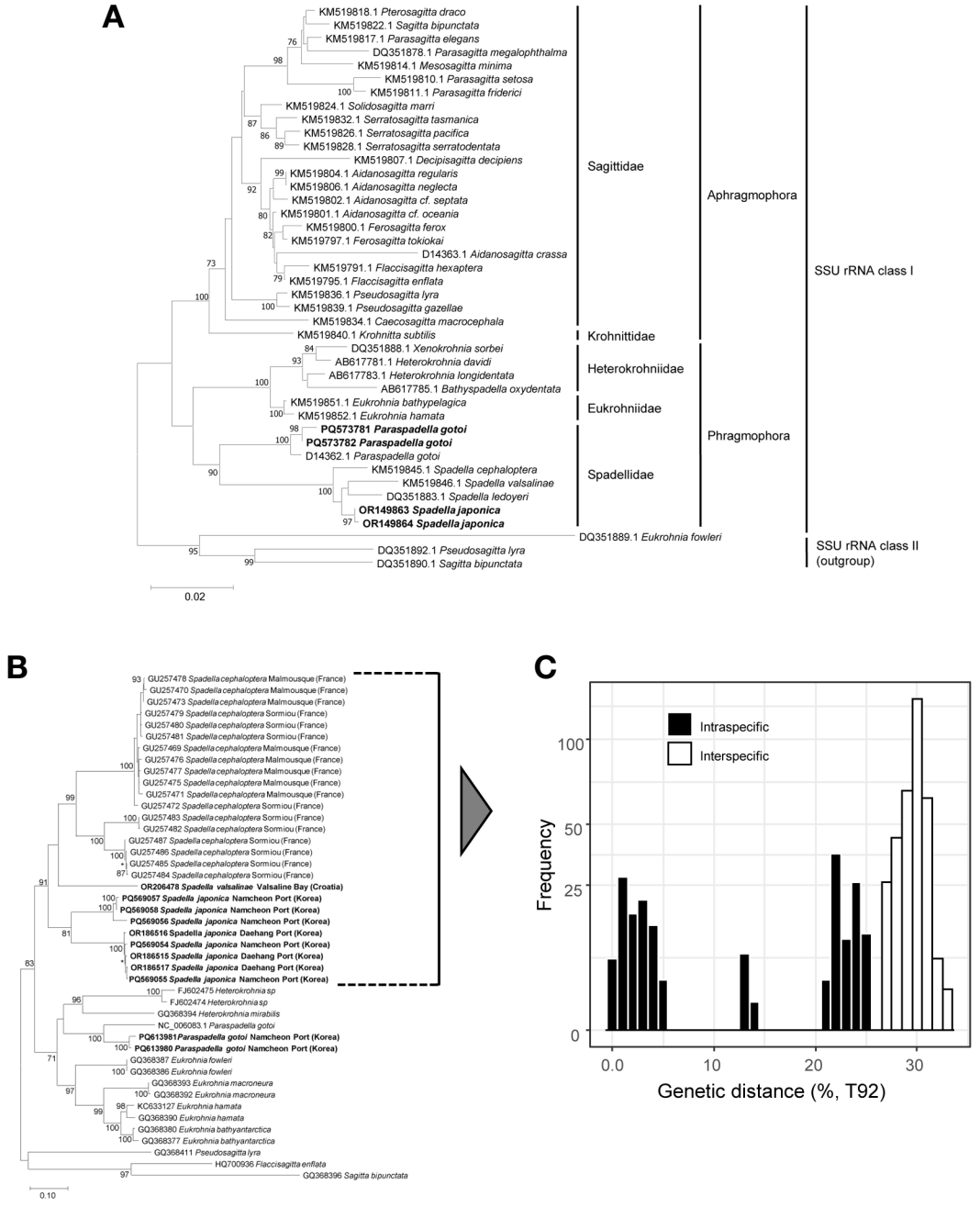

Fig. 6. A, Phylogenetic analysis based on class I small subunit ribosomal RNA sequences from 36 chaetognath species belonging to Phragmophora and Aphragmophora with Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods. The topology presented is the NJ reconstruction. The three class II sequences are used as outgroup. Nodes with bootstrap support values (bv) lower than 70 not shown or indicated by an asterisk; B, Phylogenetic analysis based on 41 cytochrome c oxidase subunit I mitochondrial sequences from 11 chaetognath species belonging to Phragmophora with NJ and ML methods. The topology presented is the NJ reconstruction. Three sequences obtained from Aphragmophora species are use as outgroup. Nodes with bv lower than 70 not shown or indicated by an asterisk; C, Frequency histogram of interspecific (white bars) and intraspecific (black bars) T92 genetic distances within *Spadella* genus, based on COI barcodes.

krohniidae received unambiguous support (bv = 100), but the sister-group relationship of this clade with Spadellidae was not supported (bv = 42). Within Spadellidae (bv = 100), the two sequences from *S. japonica* clustered with sequences

from other *Spadella* species and formed the sister-group to *S. ledoyeri* and *S. valsalinae*, albeit with low support (bv = 61). Within Sagittidae, most genera defined by Bieri (1991) received good support values in both methods, but *Parasagitta* and *Aidanosagitta* appeared polyphyletic.

Although the current dataset is limited, it permits a preliminary evaluation of the phylogenetic relationships within the *Spadella* genus. Phylogenetic reconstructions based on the SSU rRNA revealed genetic divergence in *S. cephaloptera* and *S. japonica*, with three and two distinct lineages, respectively. These findings indicate high levels of polymorphism within both species, which did not hinder the accurate identification of either species, as supported by high bootstrap values.

Cytochrome c oxidase subunit I. The alignment of 41 sequences of mtCOI was 640 nucleotides in length (dataset2). The best model of evolution estimated with MODELTEST was the T92 + Γ + I model (lnL = −7,433.063). ML phylogenetic reconstructions revealed the Eukrohniidae (bv = 97) and the Heterokrohniidae (bv = 96) to be monophyletic. Nevertheless, mtCOI analysis did not recover the monophyly of Spadellidae because of a sister-group relationship between *P. gotoi* and Heterokrohniidae. The resolution of the relationships at the generic level was well supported, and the sequences from *S. japonica* clustered within the *Spadella* genus, with high support in ML analysis (bv = 91). Overall, all the defined species represented by two or more individuals were recovered as distinct clades on highly-supported branches. ML bootstrap values were reliable measures for species identification, as nearly all identifiable species had all individuals grouped by bv > 95, the only exception being within Spadellidae with *S. japonica* (bv = 81).

The mtCOI-based DNA barcoding was employed to assess the effectiveness of DNA barcoding within the *Spadella* genus. For each species, intraspecific genetic distances within each lineage reached a maximum of 4.9%, with an average of 2.2%. High intraspecific genetic divergence between distinct lineages was observed in both species, ranging from 21.6% to 25.4%, with an average of 23.0%. To assess nucleotide variation within and among species, frequency distribution histograms of intraspecific and interspecific pairwise T92 distances were generated, along with tree-based analyses as recommended by the BOLD system.

Although interspecific divergence did not overlap with intraspecific divergence, the minimal difference between the lowest interspecific distance (26.5%) and the highest intraspecific distance (25.4%) resulted in the absence of a marked DNA barcode gap. Consequently, the COI sequences used in this study do not allow for the establishment of a molecular threshold for species delimitation within *Spadella*.

DISCUSSION

Towards effective phylogeny and species identification through molecular data within Spadellidae

The systematics of chaetognaths has been a subject of long-standing debate (Bieri, 1991; Perez et al., 2022). Recent advancements in molecular phylogeny have clarified some aspects of the relationships within this phylum (Miyamoto and Nishida, 2011; Gasmi et al., 2014; Perez et al., 2022). Our molecular analyses of the SSU rRNA gene provide an opportunity to re-evaluate relationships with a comprehensive taxonomic sampling within Phragmophora. By combining the SSU rRNA sequences used in Gasmi et al. (2014) and Miyamoto and Nishida (2011) with the sequences obtained in this study, molecular phylogeny once again did not support the Phragmophora and refutes the Monophragmophora/Biphragmophora hypothesis (Casanova, 1986). Regarding *S. japonica*, the SSU rRNA analysis supports its inclusion within the genus *Spadella* but does not allow for the inference of deeper relationships for two main reasons. First, the phylogenetic resolution of SSU rRNA sequences is limited in elucidating relationships at the infrageneric level. Furthermore, the taxonomic sampling is insufficient and lacks sequences from species morphologically close to our specimens, such as *S. xcalakensis*, *S. angulata*, *S. japonica*, and *S. bradshawi*.

Compared to other marine invertebrates, studies using DNA barcoding in Chaetognatha remain limited. According to previous results (Bucklin et al., 2010; Jennings et al., 2010; Nair et al., 2015; Peter et al., 2020), the mtCOI barcode region of chaetognaths exhibits a favorable variation pattern for distinguishing closely related species. Jennings et al. (2010) noted minimal geographical structure among planktonic chaetognaths, suggesting significant genetic mixing over large distances. In contrast, several other studies have indicated genetic variation in the nuclear and mitochondrial DNAs of various planktonic species, sometimes associated with cryptic speciation, including *Parasagitta elegans* and *Parasagitta setosa* (Peijnenburg et al., 2005, 2006; Marlétaz et al., 2017), *Caecosagitta macrocephala* (Miyamoto et al., 2010), *Eukrohnia hamata* (Kulagin, 2010; Miyamoto et al., 2012; Kulagin et al., 2014), *Flaccisagitta enflata* (Ju, 2014; Lee et al., 2016), and *Pseudosagitta maxima* (Kulagin and Neretina, 2017). Only two studies have focused on a benthic species (Marlétaz et al., 2008, 2017). They highlight an extreme phenomenon of genetic polymorphism within the nuclear and mitochondrial genomes in interbreeding populations of *Spadella cephaloptera*. Cytochrome b transcripts, as well as COI and COIII, are split into distinct lineages separated by large molecular distances. Comparison with nuclear markers indicates that this

genetic diversity is not the result of cryptic speciation or past hybridization events (Marlétaz et al., 2017). Our analysis of COI sequences obtained from interbreeding populations of *S. japonica* reveals similar characteristics to those observed in *S. cephaloptera*, confirming the presence of distinct mitochondrial lineages in at least two geographically distant *Spadella* species. Additionally, we did not observe consistent double peaks in the Sanger chromatograms for *S. japonica*, ruling out explanations such as heteroplasmy. This aligns with a similar conclusion previously proposed for *S. cephaloptera* (Marlétaz et al., 2017). Finally, for both *Spadella* species studied, we observed well-supported monophyletic lineages gathering sequences isolated from specimens collected at different locations, an observation that rules out the effect of geographical structure. Thus, the notable intraspecific variation can be attributed to rapid molecular rates associated with extensive gene flow.

Such high molecular polymorphism could pose challenges, as the presence of highly divergent lineages for mitochondrial markers may significantly reduce their reliability for species identification and population genetics (Marlétaz et al., 2017). However, we showed that a tree-based approach still allows for accurate identification of closely related *Spadella* species. The barcode analysis in *Spadella* contrasts with previous analyses which found a marked gap for chaetognaths represented in a planktonic assemblage from Atlanctic Ocean (Bucklin et al., 2010; Jennings et al., 2010). This incongruence may be attributed to the limited taxonomic sampling in previous studies, which included a maximum of five sequences per species. The effectiveness of DNA barcoding relies on the extent of sampling, as a sufficiently large sample size is required to accurately estimate genetic divergence within a given species. Several studies indicate that the existence of a gap may be an artifact of insufficient sampling, both geographically and taxonomically (Wiemers and Fiedler, 2007; Bergsten et al., 2012; Čandek and Kuntner, 2015; Phillips et al., 2022). Thus, taxon distance thresholds should be computed as more specimens are collected (Phillips et al., 2022).

To establish a broad DNA barcode library, additional sampling efforts are needed to generate new sequences. One objective is to obtain additional specimens from the Japanese and Korean populations of *S. japonica*, and from the other members of the *japonica* group, e.g. *S. angulata*, *S. bradshawi*, and *S. xcalakensis*, which have fragmented geographical distributions across the entire Indo-Pacific basin and Caribbean Sea. Specifically, *S. angulata* is found in Malaysia (Tokioka and Pathansali, 1964), the Laccadive Islands (Nair and Rao, 1973), and Japan (Casanova, 1993), *S. bradshawi* off the Californian coast (Bieri, 1974), and *S. xcalakensis* in the Caribbean Sea (Tovar and Suárez-Morales, 2007). Additionally, we aim to investigate whether *S. xcalakensis* and *S. japonica*, which are morphologically highly similar and previously assigned to different names depending on their geographic locations (Caribbean-Atlantic or Indo-Pacific basins, respectively), represent sibling species that arose through allopatric speciation following the emergence of the Isthmus of Panama, as observed in numerous other marine phyla (Rocha and Bowen, 2008; O'Dea et al., 2016; Sawelew et al., 2022). Such a broad taxonomic sampling will enable us to better account for genetic variation and accurate species delimitation within Spadellidae and will offer a deeper understanding of their true diversity and evolution.

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CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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