Zoological Studies **57:** 16 (2018)
doi:10.6620/ZS.2018.57-16

Zoological Studies

Open Access

Validation of Male *Pontella spinipes* Giesbrecht, 1889 (Copepoda: Calanoida: Pontellidae) Based on Morphological and Mitochondrial *COI* Gene Sequence Analysis

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(Received 13 November 2017; Accepted 16 March 2018; Published 17 April 2018; Communicated by Ryuji Machida)

Citation: Francis SV, Nishida S, Nandan SB. 2018. Validation of male *Pontella spinipes* Giesbrecht, 1889 (Copepoda: Calanoida: Pontellidae) based on morphological and mitochondrial *COI* gene sequence analysis. Zool Stud **57:**16. doi:10.6620/ZS.2018.57-16.

Sanu Vengasseril Francis, Shuhei Nishida, and Sivasankaran Bijoy Nandan (2018) The neustonic copepods of the family Pontellidae - *Pontella spinipes* Giesbrecht, 1889 and *P. diagonalis* Wilson, 1950, both first described on the basis of female specimens exhibit very similar morphology and overlapping geographic ranges in the Indian Ocean. While several taxonomists have described males of each species, there has been no definitive evidence for female-male matching (link female and male of the same species) in the two species. In the present study, an analysis of mitochondrial cytochrome *c* oxidase subunit I (mtCOI) sequences in the specimens collected from the Arabian Sea revealed that female *P. spinipes* Giesbrecht, 1889, and male *P. diagonalis sensu* Silas and Pillai (1973) are genetically identical, providing evidence that the latter is actually *P. spinipes*. These findings emphasize that it is necessary to re-examine the female-male correspondence of other related species, formerly based on morphology alone, using molecular-genetic analysis as applied in the present study.

Key words: *Pontella spinipes*, *Pontella diagonalis*, Mitochondrial cytochrome *c* oxidase subunit I, Female-male correspondence, Indian Ocean.

BACKGROUND

The calanoid copepod *Pontella spinipes* Giesbrecht, 1889, was described from the Arabian Sea using female specimens. Wolfenden (1905) described a male from Maldive and Laccadive Islands as *P. spinipes* but Silas and Pillai (1973) assumed it to be *P. diagonalis*. Later, the specimens referred to as male *P. spinipes* were described by Sewell (1912), Silas and Pillai (1973), Pillai (1975), and Mulyadi (2000). Meanwhile, *P. diagonalis* Wilson, 1950 was also described on the basis of a female specimen from off Jolo Island, the Philippines, and the males referred to as *P. diagonalis* were described from Indian Ocean by Silas and Pillai (1973), Pillai (1975) and from Indonesian waters by Mulyadi (2000). Researchers believe that there is no distinction between male and females in either species, but there is no morphological evidence to support this claim. Moreover, their geographic ranges, which overlap in the Indian Ocean, and morphological similarity (Wolfenden 1905; Silas and Pillai 1973) arouse our suspicion over the proposed female-

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male matching. The female-male matching means linking female and male of the same species due to the previous descriptions based only on either the female or male specimens or also the morphological resemblance of co-occurring congeners (see also Francis and Nishida 2018). During a taxonomic study on copepods in the coastal waters of the Indian Ocean, we collected female *P. spinipes* and males assumed to be *P. diagonalis* by the above authors [hereafter referred to as "male *P. diagonalis sensu* Silas and Pillai (1973)"], often co-occurring in the same net samples, but did not find female *P. diagonalis* or males so far assumed to be *P. spinipes*. Under this circumstance, this study aimed at examining the female-male correspondence of *P. spinipes* using integrative morphological and molecular genetic analyses.

MATERIALS AND METHODS

The *Pontella* copepods were collected during FORV *Sagar Sampada* cruises in April 2015 from the Arabian Sea (Cruise No. 338, Stations 9, 45 and 48) and in November 2016 from the Bay of Bengal (Cruise No. 353, Stations 2 and 12) (Fig. 1, Table 1) using a plankton net (mesh size: 200 μm; mouth area: 0.28 m^2). The net was towed horizontally just below the water surface at a speed of 1 kn for 10 min. For morphological and molecular analysis, the samples were fixed in 4% buffered formalin and 95% ethyl alcohol, respectively. The preservative was changed after 24 h in the alcohol preserved samples. The *Pontella* specimens for morphological examination were sorted from the original samples; oral parts and swimming legs were dissected in 50:50 solution of glycerine and distilled water. Line drawings were made using a drawing tube attached to a Lynx - bright-field compound microscope (LM 52- 1704). The specimens were identified to species level based on Silas and Pillai (1973). The alcohol-preserved specimens were hydrated in 0.5-mL sterile distilled water for 10-12 hours at room temperature prior to DNA extraction (Sanu et al. 2016). Genomic DNA was extracted from adult individual copepods using the DNeasy Blood & Tissue Kit (Qiagen) following the spin column protocol. The polymerase chain reaction (PCR) mixture consisted of 25 μL Master Mix (Takara Clontech EmeraldAmp® GT PCR Master Mix), 1 μL forward primer, 1 μL reverse primer, 8 μL template DNA, and 15 μL distilled deionized H_2O. The amplification primers were LCO-1490 F (5'-GGTC AACAAATCATAAAGATATTGG-3') and HCO-2198 R (5'-TAAACTTC AGGGTGACCAAAAAATCA-3'), used for amplifying mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene sequences (Folmer et al. 1994). Amplification was carried out in Agilent technologies thermal cycler (Model no: Sure cycler 8800). The amplification protocol was denaturation at 94°C for 1 min., annealing at 37°C for 2 min. and extension at 72°C for 3 min; 40 cycles were performed. Amplified products exhibiting intense bands after agarose gel (1.2%) electrophoresis was purified and sent to SciGenom Labs (SciGenom Labs Pvt, Ltd. Ernakulam, India) for sequencing. Obtained sequences were assembled using BioEdit 7.0.9 (Hall 1999) and alignment was performed using ClustalX (Thompson et al. 1997). Intraspecific pairwise sequence distance and maximum likelihood tree (ML tree) were estimated using Kimura 2- parameter model in MEGA5 (Tamura et al. 2011). Bootstrap analysis was performed using 1000 pseudo replications. Intraspecific aligned sequences were submitted to the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov).

RESULTS

SYSTEMATICS

Order Calanoida Sars, 1903
Family Pontellidae Dana, 1852
Genus *Pontella* Dana, 1846
Pontella spinipes Giesbrecht, 1889

Synonymy: Female: *Pontella spinipes* Giesbrecht, 1889, P. 28; 1892, P. 462, 477, 774, Plate 24 (Fig. 30), Plate 40 (Figs. 2, 23, 24); Wolfenden, 1905, P. 1020-1021; Silas and Pillai, 1973, P. 826-827, Fig. 21a, b, Fig. 22a.

Not female *Pontella spinipes*, Mulyadi, 2011, P. 1523, Fig. 6.

Male: *Pontella spinipes*, Wolfenden, 1905, P. 1020-1021. *Pontella diagonalis*, Silas and Pillai, 1973, P. 824-826, Fig. 21g, h, I, Fig. 22e (mislabeled as *P. securifer*); Pillai, 1975, P. 131-132, Fig. 1h, i; Mulyadi, 2000, P. 185-186, Fig. 4a-f, Mulyadi, 2011, P. 1515-1519, Fig.3.

Not male *Pontella spinipes*, Sewell, 1912, P. 373-374, plate 24 (Figs. 1-4); Silas and Pillai, 1973, P. 826-827, Fig. 21c, d, k, Fig. 22b; Pillai, 1975, P. 133-134, Fig. 2a, b; Mulyadi, 2000, P. 193-194, Fig. 13a-d, 2011, P. 1523-1525, Fig. 7.

Material examined

A total of 150 females and 97 male *P. spinipes* specimens were collected (Table 1), of which 10 females and 10 males were used for genetic analysis. The other specimens were incorporated into the copepod collection at the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology (Catalog entry numbers MBM/DBT/13/16 for female MBM/DBT/14/16 for male specimens). Of these, 15 specimens, each of females and males, were examined for the following description.

Female: Total length 3.75-4.17 mm (mean: 3.97 mm, *n* = 15). Body robust (Fig. 2A). Antennule of 23 segments, reaching posterior margin of last pedigerous somite (Fig. 2B). Rostrum bifurcate; upper frontal lens absent; anterior and posterior lower frontal lenses present; diameter of posterior lens 1.3 times that of anterior lens (Figs. 2C, D). Three blue and rounded processes present mid-dorsally on first three pedigerous somites. Fourth and fifth pedigerous somites separate. Posterolateral corners of fifth pedigerous somite produced posteriorly into large pointed lobes, of which left one larger than right and reaching near posterior margin of genital somite. Crescent shaped lobular process present on either side between the pointed lobes of fifth pedigerous somite and insertion of urosome. Urosome two segmented; second segment invisible in dorsal view. Genital somite bulged on its right lateral margin, extending dorso-posteriorly and completely covering second urosomal somite, with small process on right dorsal surface appearing

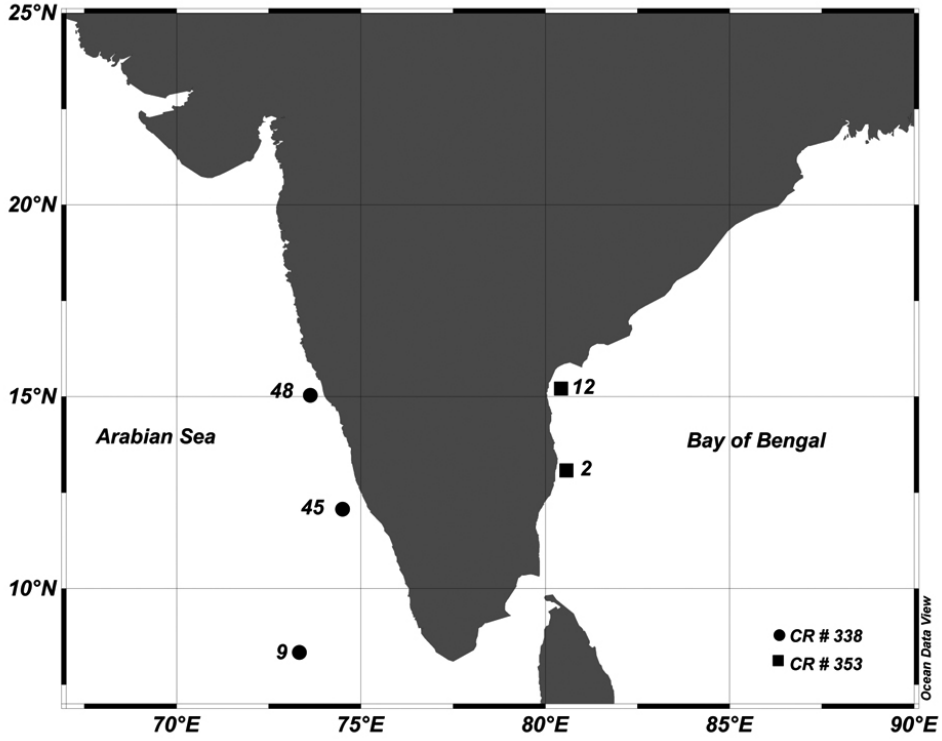

Fig. 1. Sampling locations in the Arabian Sea and Bay of Bengal.

Table 1. Summary of sampling data and number of female and male *Pontella spinipes* collected

Cruise	Station	Latitude (N)	Longitude (E)	Date (D.M.Y)	Number of specimens
338	9	08°15' 28.80"	73°0' 36.00"	14.04.15	67 ♀, 42 ♂
338	45	12°14' 24.00"	74°19' 12.00"	22.04.15	16 ♀, 5 ♂
338	48	14°5' 24.00"	73°40' 12.00"	24.04.15	24 ♀, 13 ♂
353	2	13°5' 14.68"	80°14' 48.12"	15.11.16	7 ♀, 4 ♂
353	12	15°1' 12.22"	80°12' 43.70"	17.11.16	44 ♀, 33 ♂

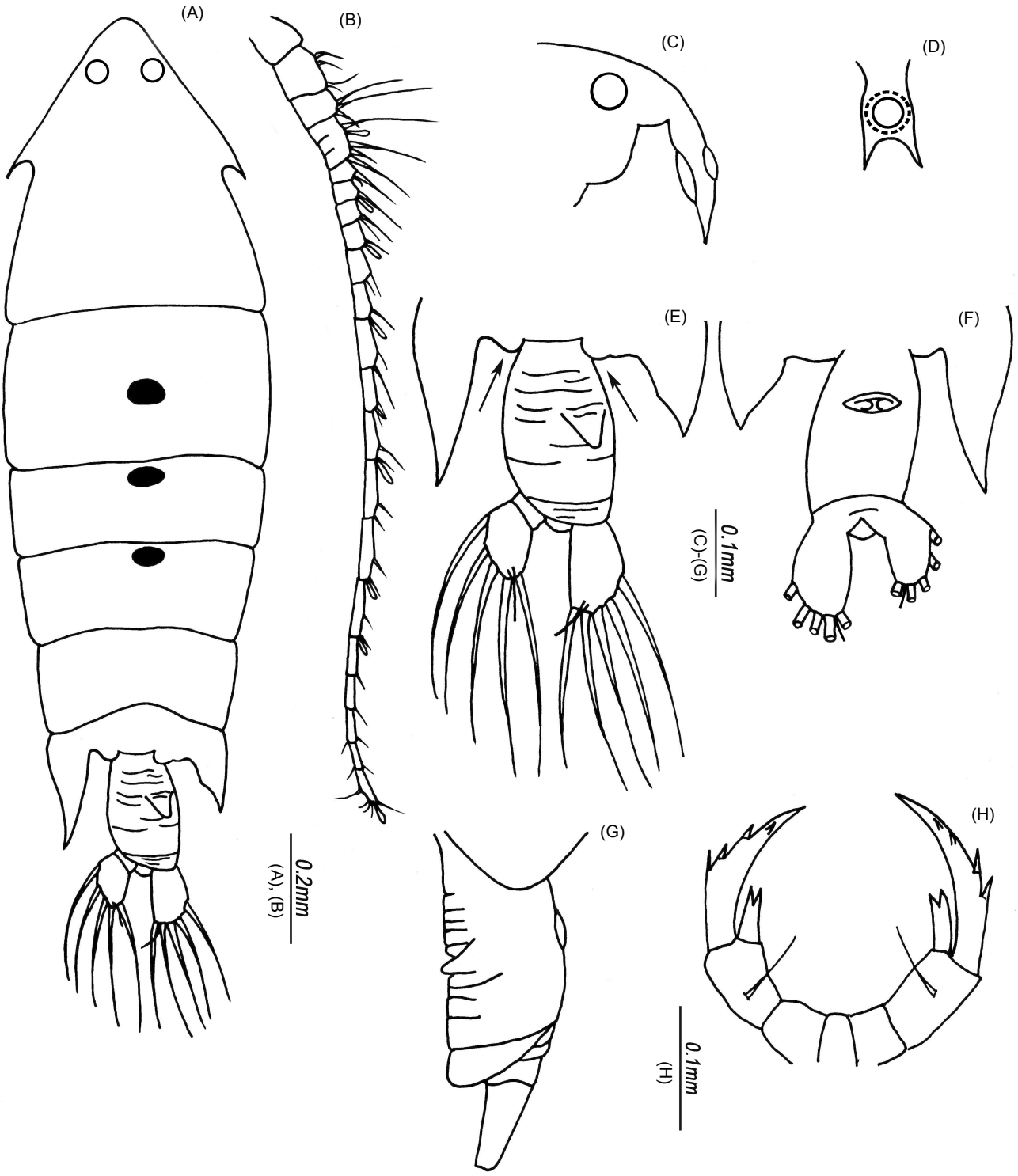

Fig. 2. *Pontella spinipes*, female. (A) Habitus, dorsal view; (B) right antennule; (C) head, right lateral view; (D) rostrum, anterior view, showing anterior and posterior frontal lenses in solid and dotted lines, respectively; (E) urosome, dorsal view; (F) urosome, ventral view; (G) urosome, right lateral view; (H) 5th leg, posterior view. Arrow indicates crescent shaped lobular process present on either side between the pointed lobes of fifth pedigerous somite and insertion of urosome.

as conical projection in right lateral view. Irregular ridges and raised areas present on dorsal surface of genital somite (Fig. 2G). Caudal rami asymmetrical, right ramus larger (Figs. 2E and F). Fifth leg asymmetrical, right leg slightly larger than left; exopod of each leg acuminate and curved with four lateral spinules; endopod about half length of exopod and bifid apically (Fig. 2H).

Male: Total length 3.31-3.73 mm (mean: 3.57 mm, *n* = 15). Fourth and fifth pedigerous somites separate. Posterolateral ends of fifth pedigerous somite produced into symmetrical, acuminate lobes (Fig. 3A). Rostrum well developed with upper frontal lens and anterior and posterior lower frontal lenses (Figs. 3B, C); diameter of anterior lower frontal lens 1.4 times those of upper- and posterior lower frontal lenses. Urosome composed of five somites; genital somite asymmetrical with lobe-like projection on left side. Caudal rami nearly symmetrical, each with five long plumose and one small setae. Right antennule (Fig. 3D) geniculate; ancestral segment XIV with stout, elongated spine terminating in bent tapering apex. Fused segments XIX-XX with proximally-oriented scalene-triangular ridge with anterior row of denticles. Fused segments XXI-XXIII with anterior process on proximal 1/3, with 3 stout and 2 minute teeth, of which middle one longest (Fig. 3E); distal 2/3 of anterior margin with denticulate plate; segment ends distally in falcate spur. Segments XXIV-XXVIII completely fused. Fifth leg with one seta on each basis. Chela on right leg well developed; outer margin of first exopodal segment with three unequal thumb-like processes basally, of which medial one longest and slightly curved and distal one with basal seta, and semicircular process at mid- to slightly distal part. Second exopodal segment slender, curved and pointed with two setae near base and seta at distal third. Left leg first exopodal segment with inner medial seta and outer distal spine; second exopodal segment with inner seta near distal third, outer seta near distal third, outer distal seta, two unequal distal spines, of which inner one 1.5 time longer than medial, and two rows of setules along inner margin (Fig. 3F).

Remarks: The present female specimens agree with *Pontella spinipes* Giesbrecht, 1889 as redescribed by Giesbrecht (1982), Wolfenden (1905), and Silas and Pillai (1973), and distinguished from the two closely similar species - *P. securifer* Brady, 1883 and *P. diagonalis* Wilson, 1950 - in the following characters: (1) the left pointed lobe of the fifth pedigerous somite is much larger than the right one (the lobes are subequal in *P. diagonalis*; left one slightly larger than the right one in *P. securifer*); (2) genital somite bulged on its right lateral margin, extending dorso-posteriorly and completely covering second urosomal somite with a small process on right dorsal surface (on the lateral side of genital somite a conspicuous digitiform process is present in *P. securifer*, while a sharply pointed, curved process is present in *P. diagonalis*; see also Tanaka, 1964; Silas and Pillai, 1973; Jeong et al. 2008 for re-descriptions). There have been slight differences between these authors in the number of lateral spinules on the exopod of leg 5: 4 in Giesbrecht (1982) and the present specimens, 3-4 in Silas and Pillai (1973), and 2 in Wolfenden (1905); this may be assumed as intraspecific variation. The female described as *P. spinipes* by Mulyadi (2011) differs from the present specimens and those described by Giesbrecht (1982) and Silas and Pillai (1973) in (1) the shape of genital somite (much more swollen laterally on the right side than the latters) and (2) the length of the endopod relative to the exopod of the fifth leg (less than 1/4 compared to about 1/2 in the latters. Note that the former length ratio is based on Fig. 6d of Mulyadi (2011) while his text (p. 1523) describes this ratio as about 1/2).

As shown in the list of synonymies, male *P. spinipes* had been described under the name *P. diagonalis* (Silas and Pillai 1973; Pillai 1975; Mulyadi 2000), except Wolfenden (1905) whose description of male *P. spinipes* accords with the present specimens, although morphological details of the specimen are unknown since the author did not present any illustrations [note that the figures of male *P. diagonalis* as described by Mulyadi (2011: Fig. 3) appear to have been copied from Mulyadi (2000: Fig. 4)]. Accordingly, the males described as *P. spinipes* by Sewell (1912), Silas and Pillai (1973), Pillai (1975), and Mulyadi (2000) are considered to belong to another species and distinguished from true male *P. spinipes* (= male *P. diagonalis sensu* Silas and Pillai, 1973) by (1) differences in the shape and size of the processes on the claw of right fifth leg and (2) the size and number of teeth on the elevated process of the fused segments XXI-XXIII of the right antennule (3 conspicuous teeth in *P. spinipes*) ; note that Silas and Pillai (1973) mislabeled the right antennule of their “male *P. diagonalis*” as *P. securifer* (Silas and Pillai 1973: Fig. 22e), as evidenced from their text sentence stating presence of 3 stout subequal teeth on the 19th segment (Silas and Pillai 1973: 825) which accords with their fig. 22e.

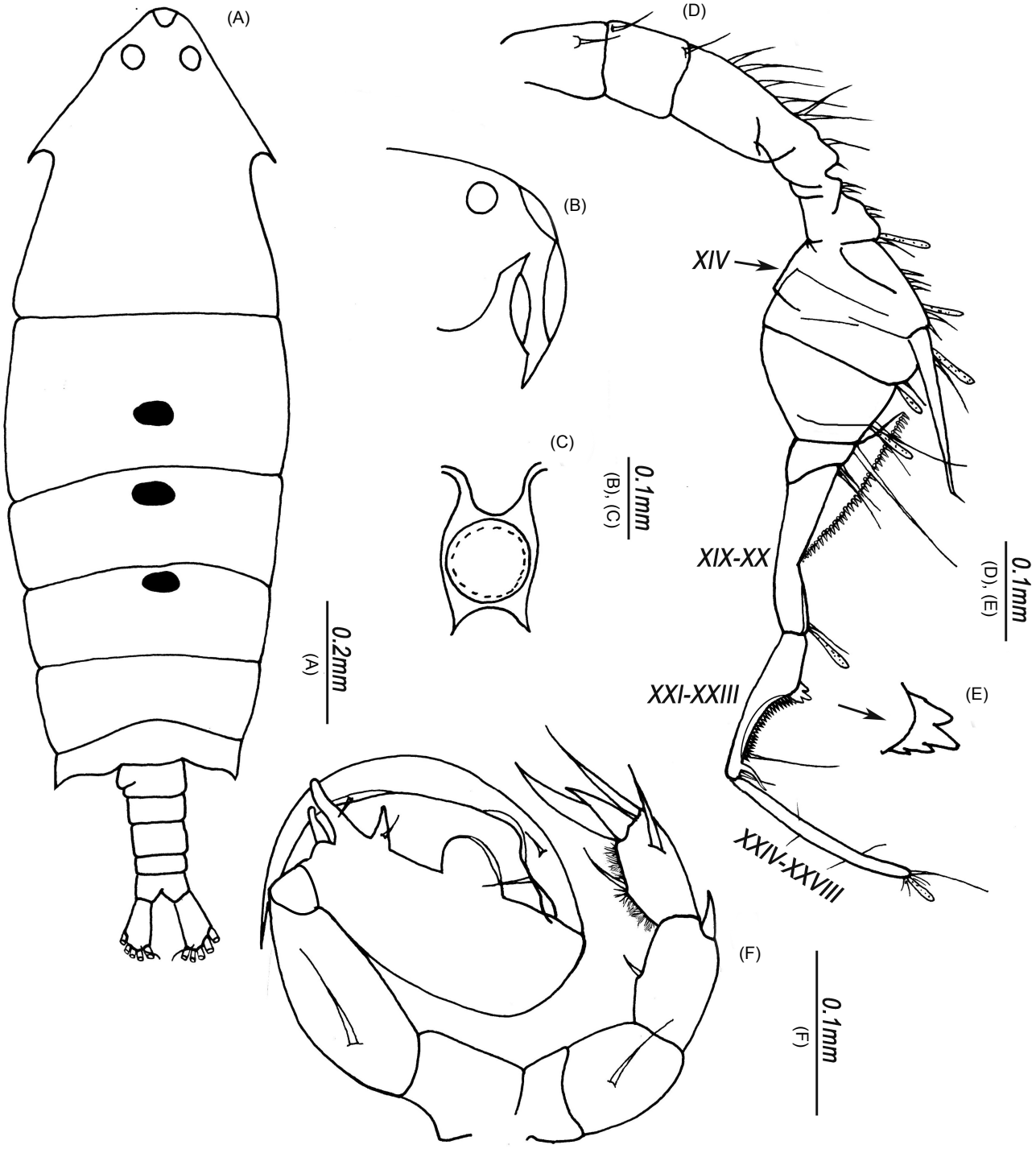

Fig. 3. *Pontella spinipes*, male. (A) habitus, dorsal view; (B) head, right lateral view; (C) rostrum, anterior view, showing anterior and posterior lower frontal lenses in solid and dotted lines, respectively; (D) right antennule, number of ancestral segments indicated with Roman numerals; (E) teeth on fused segments XXI-XXIII of right antennule; (F) 5th leg, posterior view.

Molecular analysis

The mtCOI sequences were successfully generated using the primer pair, reaction mix, and the thermal regime described above. The developed sequences of female *P. spinipes* and male *P. diagonalis sensu* Silas and Pillai (1973) were submitted to the NCBI database and assigned the following accession numbers: KT186887 to KT186891and KT267166 to KT267170 for female *P. spinipes*, KT282363 to KT282372 for male *P. diagonalis sensu* Silas and Pillai (1973). The base pair length for the developed sequences was 639 bp for the male *P. diagonalis sensu* Silas and Pillai (1973), and 660 bp for the female *P. spinipes*. In order to confirm the phylogenetic relationship of these specimens, an ML analysis was performed and pairwise sequence distances were generated and analyzed using the developed sequences as well as the mtCOI sequences of their congeneric species acquired from NCBI database (Table 2). *Acartia bispinosa* Carl, 1907 was selected as the out-group. The ML tree clearly exhibited the differential assemblage of congeneric species of the genus *Pontella* (Fig. 4). The female *P. spinipes* and male *P. diagonalis sensu* Silas and Pillai (1973) got arrayed within a single clade with the 100% bootstrap value which is distinct from the sequence of *P. rostraticauda* Ohtsuka, Fleminger and Onbe, 1987 (AB206446). In addition, *P. fera* Dana, 1849 (KT186882, KT186883) sequences got assembled next to the latter. The clade containing *P. sinica* Chen and Zhang, 1965 (KT336558, KT336559) and *P. chierchiae* Giesbrecht, 1889 (JQ714071) is sister to *P. fera*. As expected, the out-group *A. bispinosa* (KP068672) exhibited a diverged array. In order to justify the results of the phylogenetic tree, genetic distance persisting within the selected individuals was analyzed. The level of intra- and interspecific divergence persisting within the genus *Pontella* was evident from distance matrix data (Table 3). Specifically, female *P. spinipes* and male *P. diagonalis sensu* Silas and Pillai (1973) exhibited 0 - 0.2% intraspecific sequence divergence while all the other selected species showed considerable genetic divergence, justifying the findings of ML tree.

DISCUSSION

The present genetic analysis demonstrates that the male *Pontella* specimens described by Silas and Pillai (1973), Pillai (1975), and Mulyadi (2000) under the name *Pontella diagonalis* actually belong to *Pontella spinipes* Giesbrecht 1889; the latter was described in detail by Giesbrecht (1982) based on females. A possibility of sequence mismatch between conspecific female and male due to introgression may be ruled out since the sequences' specimens were collected from multiple distinct areas of the Arabian Sea. Another alternative explanation is that pseudogenes were amplified; this is suspected when two or more sequences are amplified from a single specimen. However, since only a single sequence was amplified from all the specimens examined, there is essentially no possibility of pseudogene amplification. Another possibility, that male *P. spinipes* and female *P. diagonalis* have exactly the same sequence, is also highly unlikely considering the generally accepted differences between congeneric copepod species, including those in *Pontella*, is much greater than ca.10% in *COI*. Accordingly, the males described as *P. spinipes* by Sewell (1912), Silas and Pillai (1973), Pillai (1975), and Mulyadi (2000) are considered to belong to another, unknown species. Proving this necessitates the molecular-genetic analysis that the present study applied, which should include

Table 2. Details of sequences incorporated and species abbreviations used in the molecular analysis, as applied in table 3

Species	Abbreviation	Accession numbers	Remarks
Pontella sinica Chen and Zhang, 1965	PSI	KT336559	Obtained
P. chierchiae Giesbrecht, 1889	PC	JQ714071	Obtained
P. fera Dana, 1849	PF	KT186882, KT186883	Obtained
P. diagonalis sensu Silas and Pillai, 1973	PD ♂	KT282363 to KT282372	Developed
P. spinipes Giesbrecht, 1889	PS ♀	KT186887 to KT186891 and KT267166 to KT267170	Developed
P. rostraticauda Ohtsuka, Fleminger and Onbe, 1987	PR	AB206446	Obtained
Acartia bispinosa Carl, 1907	AB	KP068672	Obtained

Table 3. Distance matrix showing inter and intraspecific percent divergence of *Pontella spinipes* and other species in the genus *Pontella*. See table 2 for specimen and species codes

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	|KT336559|PSI													
2	|KT336558|PS	0.0												
3	|JQ714071|*PC*	20.0	20.0											
4	|KT186882|*PF*	21.9	21.9	23.4										
5	|KT186883|*PF*	22.1	22.1	23.4	0.2									
6	|KT282367|PD♂	23.2	23.2	23.2	23.5	23.5								
7	|KT282368|PD♂	23.2	23.2	23.2	23.5	23.5	0.0							
8	|KT282366|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0						
9	|KT282369|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0					
10	|KT282370|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0				
11	|KT282365|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0			
12	|KT282371|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0		
13	|KT282364|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
14	|KT282372|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	|KT282363|PD♂	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	|KT267168|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	|KT267169|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	|KT267167|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	|KT267170|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20	|KT267166|PS♀	23.2	23.2	23.2	23.5	23.5	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
21	|KT186888|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	|KT186887|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	|KT186889|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	|KT186890|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	|KT186891|PS♀	23.2	23.2	23.2	23.5	23.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	|AB206446|PR	21.8	21.8	19.0	23.8	23.8	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4
27	|KP068672|*AB*	29.5	29.5	27.8	30.3	30.5	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0

		14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	|KT336559|PSI														
2	|KT336558|PS														
3	|JQ714071|*PC*														
4	|KT186882|*PF*														
5	|KT186883|*PF*														
6	|KT282367|PD♂														
7	|KT282368|PD♂														
8	|KT282366|PD♂														
9	|KT282369|PD♂														
10	|KT282370|PD♂														
11	|KT282365|PD♂														
12	|KT282371|PD♂														
13	|KT282364|PD♂														
14	|KT282372|PD♂														
15	|KT282363|PD♂	0.0													
16	|KT267168|PS♀	0.0	0.0												
17	|KT267169|PS♀	0.0	0.0	0.0											
18	|KT267167|PS♀	0.0	0.0	0.0	0.0										
19	|KT267170|PS♀	0.0	0.0	0.0	0.0	0.0									
20	|KT267166|PS♀	0.2	0.2	0.2	0.2	0.2	0.2								
21	|KT186888|PS♀	0.0	0.0	0.0	0.0	0.0	0.0	0.2							
22	|KT186887|PS♀	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0						
23	|KT186889|PS♀	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0					
24	|KT186890|PS♀	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0				
25	|KT186891|PS♀	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0			
26	|AB206446|PR	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.4		
27	|KP068672|*AB*	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.5	

P. diagonalis females and the females and males that have been identified as closely related to *P. securifer*.

Wolfenden (1905) noted the co-occurrence of a male (= male *P. diagonalis sensu* Silas and Pillai (1973)) and 3 *P. spinipes* females in a plankton sample. Silas and Pillai (1973) mentioned the co-occurrence of males (= male *P. diagonalis sensu* Silas and Pillai (1973)) and females of *P. spinipes* along with "*P. diagonalis*", the gender of which was not specified but may have included males that were really *P. spinipes*. In the present study we collected *Pontella fera* Dana, 1849, *P. sinica* Chen & Zhang, 1965, and *P. denticauda* A. Scott, 1909 at Stn 9 and *P. fera* at Stns 2, 12, 45, and 48, along with *P. spinipes*, many females and males of which were collected together at all stations (Table 1), but *P. diagonalis* did not occur at all. All these observations are consistent with the present results of female-male correspondence based on genetic information, in that female and male *P. spinipes* have been collected together in many occasions. It should also be noted that several species of *Pontella* have often been collected together in same stations and/or plankton-net tows (*e.g.* Brady 1883; Sewell 1912; Sherman 1964; Silas and Pillai 1973; Mulyadi 2000; this study). This would suggest the presence of mechanisms for co-existence of multiple congeneric species in relatively small areas and the two dimensional habitat in their neustonic life, either by differentiating their habitat water, as defined by various physico-chemical and/or biotic factors (Sherman 1964), and/or food resources (Ohtsuka 1985), inviting further research on their microhabitats and feeding ecology.

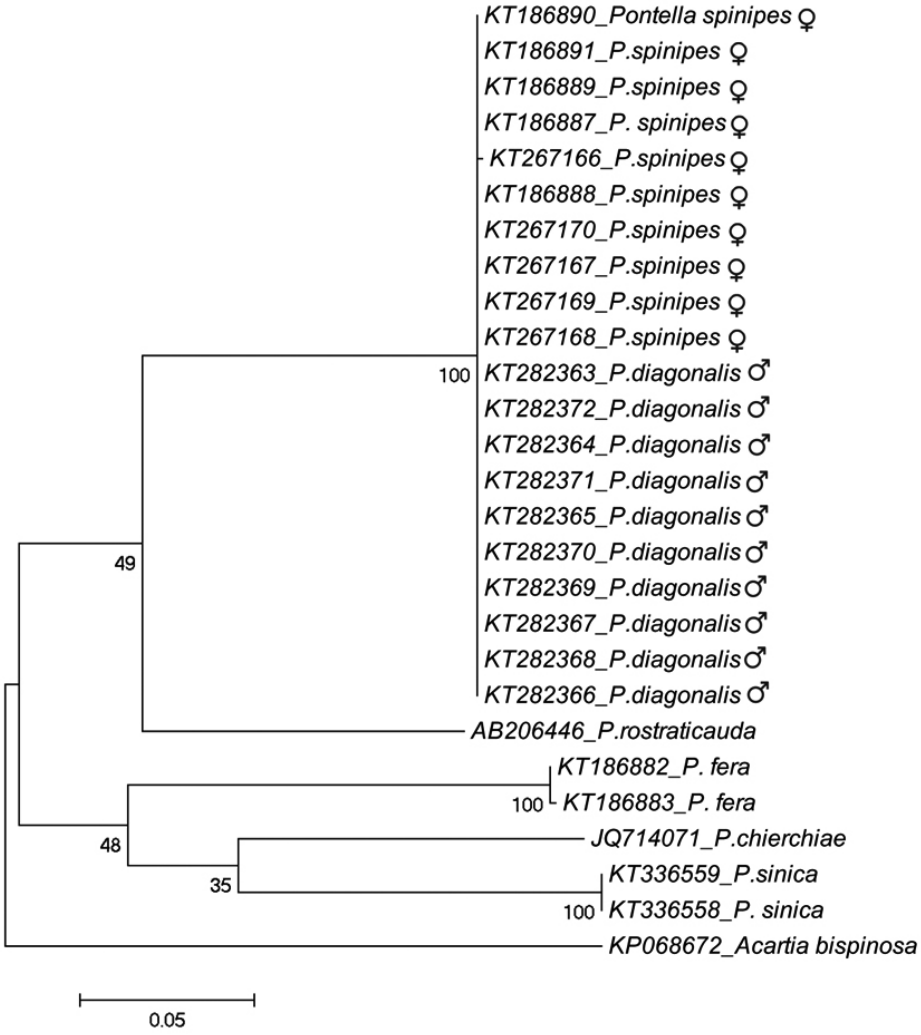

Fig. 4. Maximum likelihood tree for *Pontella spinipes* and other *Pontella* species (taken from GenBank with their accession numbers) based on 1000 bootstrap pseudoreplicas.

Acknowledgments: The study was funded by the Department of Biotechnology, Govt. of India. The authors are thankful to Dr. Rosamma Philip, Head of the Department of Marine Biology, Microbiology, and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology for letting them use facilities; they gratefully acknowledge the assistance rendered by Central Marine Living Resources and Ecology (CMLRE), Ministry of Earth Sciences, Govt. of India and the officers and scientific support staff onboard the FORV *Sagar Sampada* for their efforts during sampling. Thanks also to the research fellowship provided to SVF under the University Grants Commission Basic Science Research programme of Govt. of India and support from the Core-to-Core Program (B. Asia-Africa Science Platforms) of the Japan Society for the Promotion of Science to SN.

Authors' contributions: SVF and SN designed the study. SVF conducted the field work and collected samples. SVF prepared the figures and molecular work. SVF, SN and SBN analysed the data and wrote manuscript. All authors participated in revising the manuscript. All authors read and approved the final manuscript.

Competing interests: The authors have no competing interests to declare.

Availability of data and materials: Data are available from accession numbers (KT186887-KT186891 and KT267166 to KT267170 for female *P. spinipes*, KT282363 to KT282372 for male *P. diagonalis sensu* Silas and Pillai, 1973) and DNA sequences are available in GenBank (www.ncbi.nlm.nih.gov).

Consent for publication: Not applicable.

Ethics approval consent to participate: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. No experiments were done on living animals in this study.

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