

Subdividing the Common Intertidal Hermit Crab *Pagurus minutus* Hess, 1865 (Decapoda: Anomura: Paguridae) Based on Molecular, Morphological and Coloration Analyses

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(Received 15 April 2018; Accepted 23 November 2018; Published 30 December 2018; Communicated by Benny K.K. Chan)

Citation: Jung J, Jung J, Kim W. 2018. Subdividing the common intertidal hermit crab *Pagurus minutus* Hess, 1865 (Decapoda: Anomura: Paguridae) based on molecular, morphological and coloration analyses. Zool Stud 57:61. doi:10.6620/ZS.2018.57-61.

Jibom Jung, Jongwoo Jung, and Won Kim (2018) A phylogenetic study was conducted to investigate whether distinct genetic groups are present within the East Asian *Pagurus minutus*. In this study, 167 individuals of *P. minutus* were collected along the coasts of South Korea, east coast of Honshu, west coast of Kyushu, Okinawa Islands of Japan, and Taiwan. The collection of *P. minutus* was divided into three groups based on the differences in cytochrome *c* oxidase subunit I (COI) sequences and morphological and color characters: Major Group (MAG), Minor Group (MIG), and Taiwan-Okinawa Group (TOG). MAG commonly inhabits the entire coast of South Korea (except for the northeast coast), east coast of Honshu, and west coast of Kyushu in Japan. MIG predominantly inhabits the northeast coast of South Korea, while a small proportion inhabits the west coast of South Korea and west coast of Kyushu in Japan. TOG is restricted to Taiwan and the Okinawa Islands of Japan. The COI divergence among MAG, MIG, and TOG was larger than the minimum interspecific divergence of the other *Pagurus* species. Little ingroup COI divergences exist in the MAG and MIG, but distinct ingroup COI divergence is present between the two subgroups of TOG inhabiting Taiwan and Okinawa Islands. MAG, MIG, and TOG show minor differences among morphological characters. Each specimen of these three groups has distinguishing color patterns. These differences in molecular, morphological and color characters suggest that *P. minutus* are separated into three groups at the species level, and this subdivision of *P. minutus* shows that additional phylogenetic studies of other hermit crabs and common marine decapod species in East Asia are needed.

Key words: Common species, Phylogeny, COI, Color pattern, Biogeography.

BACKGROUND

Pagurus minutus Hess, 1865 is one of the most dominant intertidal hermit crab species in East Asia, distributed from the Primorye of East Russia, Japan (from the southern coast of Hokkaido to Okinawa Islands), Korea, the northeast coast of China, to Taiwan (Komai and Mishima 2003). *Pagurus minutus* mainly inhabits sandy

or muddy coasts, although a few individuals live along the rocky coasts (Komai and Mishima 2003). This species is abundant on the Korean coast and temperate Japanese coast (Kim 1973; Komai and Mishima 2003).

The coloration of *P. minutus* individuals varies between two previous studies (see Komai and Mishima 2003; McLaughlin et al. 2007). While the description on the base color of ambulatory legs

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was consistent across the two studies, neither study showed consistent descriptions of the color patterns of the lateral surfaces of ambulatory legs. Komai and Mishima (2003) provides a fuller description of the color pattern of living *P. minutus*, including the pattern of the lateral surfaces of ambulatory legs, which is described as a grayish brown or olive-drab base with a single, dark brown median longitudinal stripe. On the other hand, McLaughlin et al. (2007) presents a color pattern distinct from the description of Komai and Mishima (2003). This pattern had dark grayish brown or olive-drab legs, similar to the description of Komai and Mishima (2003); however, the colored figure of McLaughlin et al. (2007) showed that Taiwanese *P. minutus* lacked stripes on the lateral surfaces of the propodi of each ambulatory leg.

We investigated the color patterns and genetic lineage of *P. minutus* collected from South Korea in order to give a clear account of its morphology and genetics in the area. This study found two genetic groups of *P. minutus*, each with a fixed diagnostic color pattern (Fig. 1A and 1B) and geographical distribution. Based on this finding on South Korean *P. minutus*, the present study further analyzed *P. minutus* collected in Japan and Taiwan in order to strengthen the previous finding and to determine the separation between *P. minutus* genetic groups. Thus, the present study sought to determine both whether *P. minutus* has distinct groups by using COI sequences, morphological characters, and colorations, and the geographical distribution of the identified groups of the present *P. minutus* collection by extending our previous study and defining additional groups. In addition, we discuss the phylogenetic relationship between *P. minutus* groups and *Pagurus* species in East Asia in order to elucidate the unique characters of the *P. minutus* groups.

MATERIALS AND METHODS

We studied the molecular, morphological and color characters of total 167 individuals of *Pagurus minutus* collected from 14 sites in South Korea (113 individuals), 7 sites in Japan (46 individuals), and 1 site in Taiwan (10 individuals) (Table 1). To examine the molecular characters of these samples, tissues were excised from one of the pereopods of each of the specimens in order to extract the total DNA using QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). The universal primers LCO1490, HCO2198 and jgLCO, jgHCO

were used to amplify the COI sequence (Folmer et al. 1994; Geller et al. 2013). A PCR solution included 1 μ L of DNA template, 1 μ L of each primer (10 μ M), 0.3 μ L of Go Taq DNA polymerase (Promega, Madison City, WI, USA), 5 μ L of 5x color Go Taq reaction buffer, 1 μ L of dNTP mixture (10 mM) and 15.7 μ L of distilled H₂O (total 25 μ L). The amplification protocol entailed the following steps: 5-10 min denaturation at 94°C followed by 38-42 cycles of 1 min at 94°C, 1.5 min at 45-48°C and 2 min at 72°C and a final extension of 10 min at 72°C. PCR products were identified in 1% agarose gels and sequenced by Cosmogenetech Co. (Seoul, South Korea) and Macrogen Inc. (Seoul, South Korea).

The COI sequences obtained by DNA extraction were analyzed and edited with SeqMan 5.0 (DNASTAR, Madison City, WI, USA). In order to investigate the nucleotide diversity of collection sites, the haplotypes of *P. minutus* were obtained by multiple sequence alignments and DNA Sequence Polymorphism (DnaSP) Version 6 software (Rozas et al. 2017). All the COI haplotypes of *P. minutus* and COI sequences of 17 *Pagurus* species (Table 1) were used in a molecular evolutionary genetics analysis of MEGA7 program (MEGA, PA, USA) (Kumar et al. 2016). All *P. minutus* sequence data in this study are available in Genbank (Table 1). Among the 17 sequences of the *Pagurus* species, 13 sequences were obtained from our samples from South Korea in MADBK and 4 sequences of *Pagurus* species were downloaded from Genbank. The maximum likelihood analysis of these sequences was based on the Kimura 2-parameter model (Kimura 1980) with 5 rate categories of gamma distribution (+ G) and invariable sites (+ I), which gained the lowest Bayesian Information Criterion (BIC) scores by Model Selection command of MEGA7. The consistency among topologies was assessed using bootstrap values with 1,000 replications. Minimum Spanning Networks (Bandelt et al. 1999) of the *P. minutus* haplotypes was constructed via PopART (Leigh and Bryant 2015) to investigate gene flow among them. Interspecific and intraspecific sequence divergences were obtained based on the 'Compute Pairwise Distances' command of MEGA7.

We examined the morphological characters of the *P. minutus* samples in this study by macroscopy, dissection microscope, and a digital caliper. Shield length (sl, mm), from the distal tip of the rostrum to the midpoint of the posterior margin of the shield, is given to indicate the size

of the specimens. Length of ocular peduncle was measured from the distal tip of the cornea to the midpoint of the posterior margin of the ocular peduncle (Fig. 2). To examine the relative length

of ocular peduncle, the length of ocular peduncle divided by sl was calculated. To examine the relative length of dactyl of ambulatory legs, the length of dactyl divided by propodus was

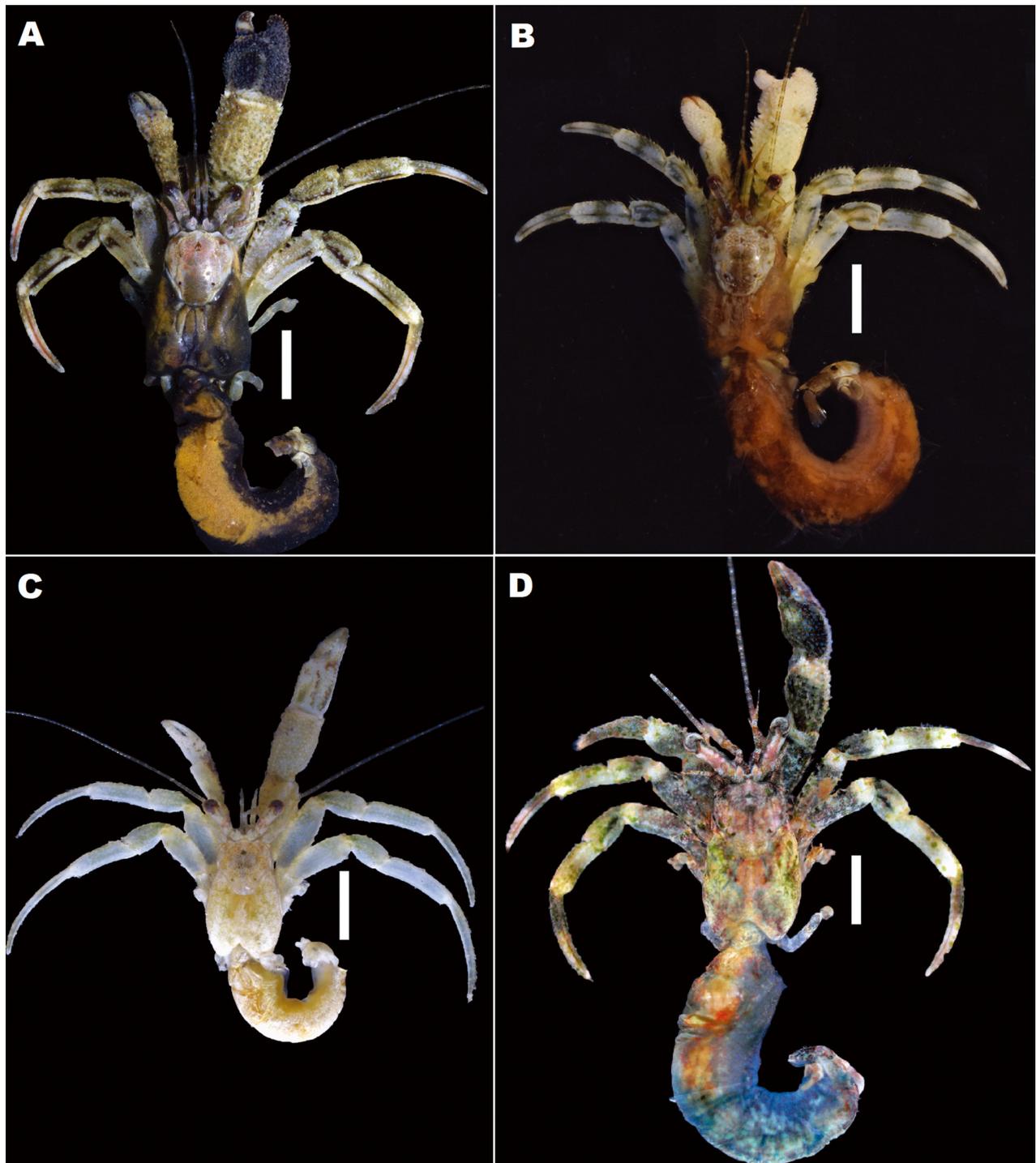


Fig. 1. Coloration of three *Pagurus minutus* groups. A, Major Group (MAG) (male, sl 5.5 mm, MADBK 160706_032); B, Minor Group (MIG) (male, sl 2.5 mm, MADBK 160749_002); C, D, Taiwan-Okinawa Group (TOG) (C, male, sl 2.5 mm, MADBK 160750_001; D, male, sl 3 mm, MADBK 160750_002). Scale bars: A = 5 mm; B-D = 3 mm. C, D, photographed by Ryuta Yoshida (Marine and Coastal Research Center, per. contact).

calculated. In addition, we examined dorsodistal spine on dactyl of ambulatory leg 2 and the lateral protuberance on dactyl and propodus of ambulatory leg 2 of females.

The coloration of these samples was also examined by macroscopy and dissection microscope. The color pattern of the lateral surface of ambulatory legs, especially background color, stripe pattern of propodus, and stripes pattern of propodus, was examined in detail.

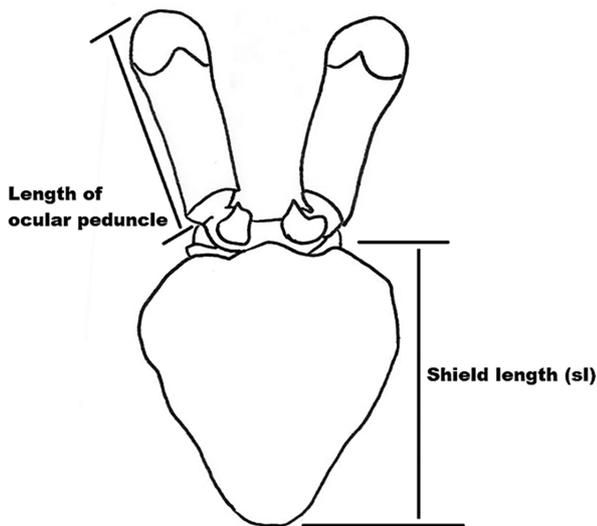


Fig. 2. Diagrammatic figures of shield length (sl) and length of ocular peduncle.

These specimens were deposited in the Marine Arthropod Depository Bank of Korea, Seoul National University, Seoul, South Korea (MADBK) and Natural History Museum and Institute, Chiba, Japan (CBM).

RESULTS

Based on the differences in molecular, morphological and color characters of all of the samples, we classified our 167 *P. minutus* individuals into three groups: 129 individuals of Major Group (MAG), 15 individuals of Minor Group (MIG), and 23 individuals of Taiwan-Okinawa Group (TOG). MAG was predominantly found in three areas: the entire South Korean coastline except for the northeast coast, the east coast of Honshu, and the west coast of Kyushu in Japan. On the other hand, MIG was dominant in northeastern South Korea, while a few individuals were also found along the west coast of South Korea and the west coast of Kyushu, which overlapped with the distribution of MAG. TOG was only found in Taiwan and the Okinawa Islands of Japan (Fig. 3).

Molecular Analysis

Mitochondrial nucleotide COI sequences (658 bp) corresponding to 69 haplotypes were

Table 1. Geographical locations and sample information of the three groups of *Pagurus minutus* and other 17 *Pagurus* species in this study in order of reference, species name, region and north latitude of coordination

Species or groups	Region	Location	Specimen Number	Coordination	Individual Number	Accession Number	Nucleotide diversity (average over loci)
<i>P. minutus</i> : Major Group (MAG)	South Korea	Silmido (M)	MADBK 160706_057	37°24'09"N 126°23'32"E	13	KY321942, KY321943, KY321945, KY321948, KY321953- KY321956	0.004325 ± 0.002736
		Seonjaedo (E)	MADBK 160706_058	37°17'13"N 126°30'39"E	3	KY321943, KY321948	0.002026 ± 0.002082
		Seosan (S)	MADBK 160706_070	36°52'27"N 126°21'59"E	3	KY321943, KY321948, KY321954	0.005066 ± 0.004403
		Taeon (A)	MADBK 160706_055	36°24'58"N 126°21'43"E	8	KY321943, KY321948, KY321955, KY321976	0.003474 ± 0.002472
		Boryeong (B)	MADBK 160706_106	36°14'44"N 126°32'12"E	8	KY321942- KY321947, KY321973, KY321974	0.006079 ± 0.003955
		Pohang (P)	MADBK 160706_046	36°09'52"N 129°16'29"E	7	KY321942, KY321943, KY321955, KY321967	0.004053 ± 0.002804
		Seocheon (C)	MADBK 160706_095	36°07'52"N 126°35'18"E	10	KY321942, KY321943, KY321945, KY321948, KY321954, KY321957, KY321968	0.003968 ± 0.002652

Table 1. (continued)

Species or groups	Region	Location	Specimen Number	Coordination	Individual Number	Accession Number	Nucleotide diversity (average over loci)
		Eocheongdo (O)	MADBK 160706_059	36°07'12"N 125°58'49"E	2	KY321943, KY321957	0.006079 ± 0.006797
		Hampyeong (H)	MADBK 160706_032	35°09'36"N 126°22'23"E	11	KY321942, KY321948- KY321952, KY321975	0.003647 ± 0.002443
		Goseong (G)	MADBK 160706_003	35°00'46"N 128°29'52"E	4	KY321948, KY321950	0.002026 ± 0.001860
		Jindo (D)	MADBK 160706_074	34°31'37"N 126°12'56"E	10	KY321948, KY321958- KY321964	0.005100 ± 0.003227
		Jeju (J)	MADBK 160706_042	33°33'00"N 126°41'25"E	5	KY321948, KY321961, KY321965, KY321966	0.003647 ± 0.002766
		Seogwipo (W)	MADBK 160706_040	33°19'10"N 126°50'38"E	13	KY321948, KY321950, KY321969- KY321972	0.002455 ± 0.001743
	Japan	Miyako (V)	MADBK 160706_120	39°58'94"N 141°94'84"E	4	MG214611	
		Hatsutsu ura(R)	MADBK 160706_121	38°32'90"N 141°14'04"E	10	KY321942, KY321950, KY321953, MG214619, MG214620,	0.002470 ± 0.002150
		Ushibashi (U)	MADBK 160706_122	37°99'14"N 140°91'67"E	5	KY321942, KY321950, MG214617, MG214618	0.003950 ± 0.003650
		Unoo (Q)	MADBK 160706_123	37°79'98"N 140°97'02"E	9	KY321948, KY321950, KY321953, MG214611- MG214616	0.005700 ± 0.006710
		Kumamoto (F)	MADBK 160706_124	32°65'58"N 130°51'04"E	4	KY321942, KY321950, KY321953, MG214621	0.002530 ± 0.002490
<i>P. minutus</i> : Minor Group (MIG)	South Korea	Kujin (K)	CBM-ZC 14611	38°26'52"N 128°27'55"E	3	KY321937, KY321938	0.001013 ± 0.001264
		Sokcho (L)	MADBK 160749_003	38°12'51"N 128°36'03"E	2	KY321938	0.001520 ± 0.002149
		Gangneung (N)	MADBK 160749_001 MADBK 160749_002	37°54'25"N 128°49'32"E	2	KY321938	
		Taeon (A)	MADBK 160749_004	36°24'58"N 126°21'43"E	2	KY321934, KY321940	0.001520 ± 0.002149
		Boryeong (B)	MADBK 160749_005	36°14'44"N 126°32'12"E	3	KY321934- KY321936	0.002026 ± 0.002082
		Eocheongdo (O)	MADBK 160749_006	36°07'12"N 125°58'49"E	2	KY321936, KY321939	0.004559 ± 0.005265
	Japan	Kumamoto (F)	MADBK 160749_007	32°65'58"N 130°51'04"E	1	MG214622	
<i>P. minutus</i> : Taiwan-Okinawa Group (TOG)	Japan	Okinawa (Y)	CBM-ZC 14612 MADBK 160750_001	26°32'71"N 127°83'58"E	1 4	MG214623 MG214623- MG214625	0.002440 ± 0.002920
		Iriomote (I)	CBM-ZC 14613	24°27'68"N 123°88'05"E	8	MG214623, MG214626- MG214629	0.003600 ± 0.004100
	Taiwan	Magong, Penghu (T)	MADBK 160750_002	23°57'48"N 119°58'62"E	10	MG214630- MG214637	0.002870 ± 0.003760
<i>P. brachiomastus</i> *	East Asia				1	JN590063	
<i>P. ochotensis</i> *					1	JN590062	
<i>P. pectinatus</i> *					1	JN590060	
<i>P. proximus</i> *					1	KC347562	
<i>P. constans</i>	South Korea	Busan	MADBK 160705_001	35°08'17"N 129°09'37"E	1	MG214639	
<i>P. filholi</i>		Seogwipo	MADBK 160707_002	33°08'36"N 126°30'55"E	1	KY321979	
<i>P. japonicus</i>		Jeju	MADBK 160710_001	33°17'58"N 126°09'34"E	1	MG214647	
<i>P. lanuginosus</i>		Tongyeong	MADBK 160712_017	34°38'52"N 128°15'35"E	1	MG214646	
<i>P. maculosus</i>		Jeju	MADBK 160722_005	33°19'00"N 126°07'44"E	1	MG214645	
<i>P. middendorffii</i>		Kujin	MADBK 160713_010	38°30'44"N 128°27'06"E	1	MG214649	
<i>P. nigrivittatus</i>		Ulleung	MADBK 160725_002	37°28'12"N 130°49'07"E	1	MG214644	
<i>P. quinquelineatus</i>		Uljin	MADBK 160742_001	37°28'12"N 130°49'07"E	1	MG214648	
<i>P. rubrior</i>		Ulleung	MADBK 160717_023	37°28'12"N 130°49'07"E	1	MG214643	
<i>P. spina</i>		Ulleung	MADBK 160726_003	37°28'12"N 130°49'07"E	1	MG214642	
<i>P. rathbuni</i>		Kujin	MADBK 160731_001	37°56'53"N 128°48'39"E	1	MG214641	
<i>P. undosus</i>		Samcheok	MADBK 160745_001	37°04'51"N 129°26'12"E	1	MG214640	
<i>P. trigonocheirus</i>		Yangyang	MADBK 160720_009	37°56'53"N 128°48'39"E	1	MG214638	

*: Genbank sequences.

obtained from 167 individuals of *P. minutus*. The most common haplotypes were Hap15 (27 individuals, 11 sites) and Hap10 (17 individuals, 8 sites) of MAG. MAG includes 129 individuals and 46 haplotypes (Hap09-Hap54); MIG includes 15 individuals and 8 haplotypes (Hap01-Hap07 and Hap55), and; TOG includes 23 individuals and 15 haplotypes (Hap56-Hap70) (Fig. 4).

In the phylogenetic tree of this study, *P.*

minutus in East Asia was clearly separated into MAG, MIG, and TOG. (Fig. 5). Table 2 summarizes the COI pairwise distances of the three groups. The COI divergences among these three groups exhibited larger genetic gaps than the minimum interspecific divergence of the *P. lanuginosus* and *P. maculosus* (5.59%).

South Korean and Japanese MAG populations were weakly separated (Fig. 4). A

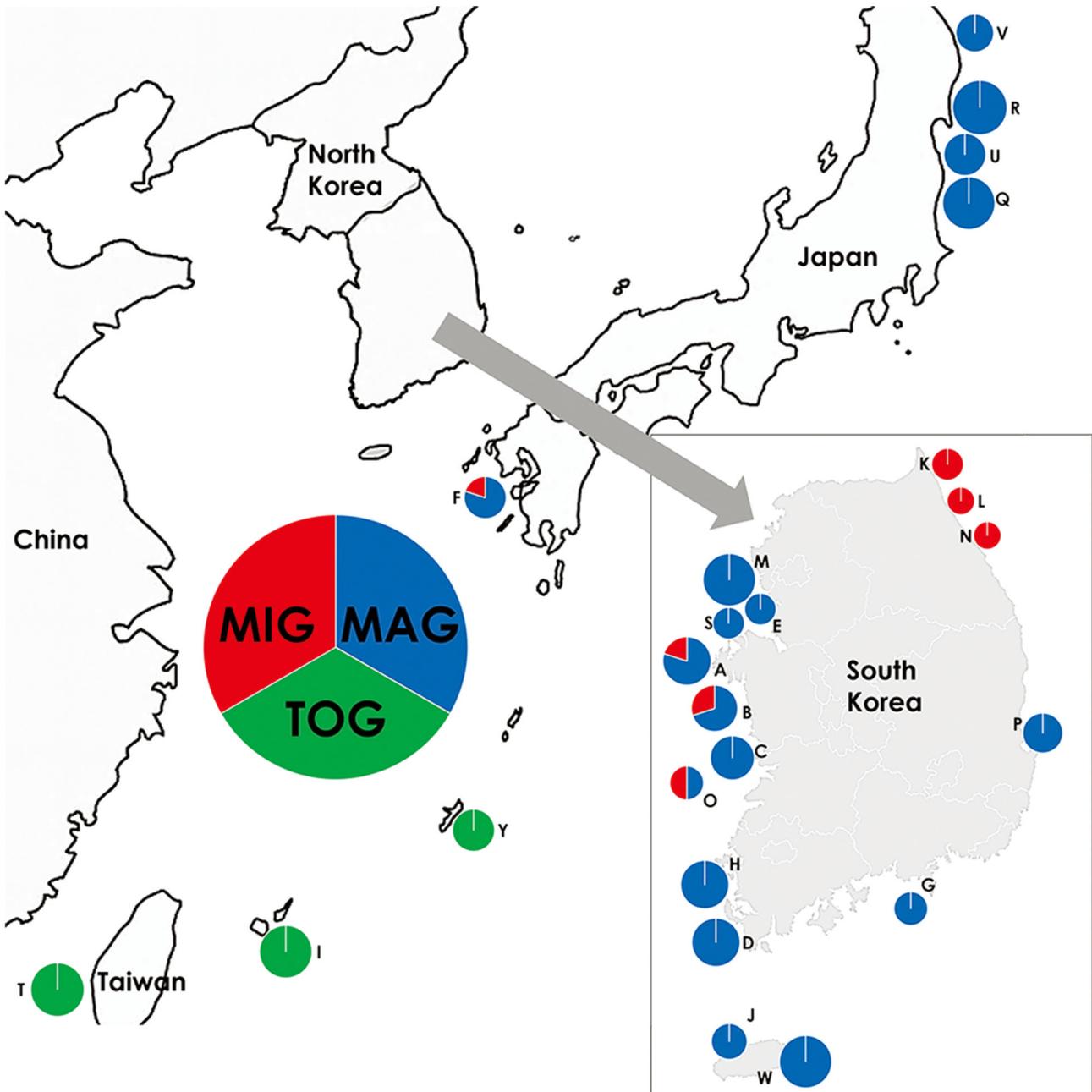


Fig. 3. Distribution map of three groups of *Pagurus minutus* in East Asia. The abbreviation locations refer to table 1. The pie-graph represent the size and proportions of individuals belonging to the Major Group (MAG, blue), Minor Group (MIG, red) and Taiwan-Okinawa Group (TOG, green).

little divergence was found within TOG, which was divided into the Taiwan subgroup and the Okinawa subgroup (Table 2). The COI divergence of them was smaller than the minimum interspecific divergence of the congeneric species in this study

(5.59%). Each of the three groups was separated into an independent group distinct from the other *Pagurus* species, which were also separated as a single clade (Fig. 5).

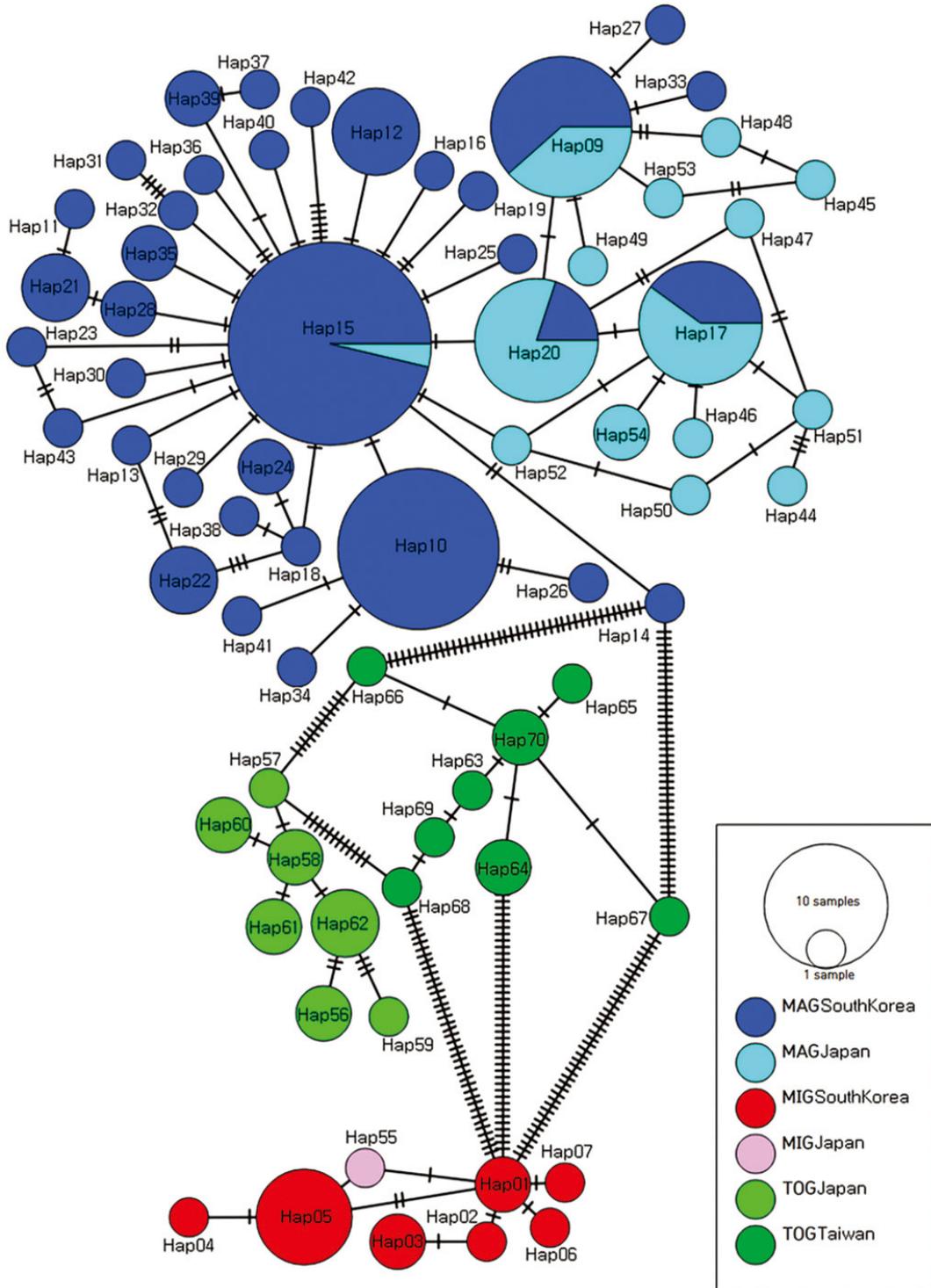


Fig. 4. The haplotypes network of three *Pagurus minutus* groups in East Asia. The abbreviation: Major Group (MAG); Minor Group (MIG); Taiwan-Okinawa Group (TOG). TOGJapan means Okinawa subgroup of TOG.

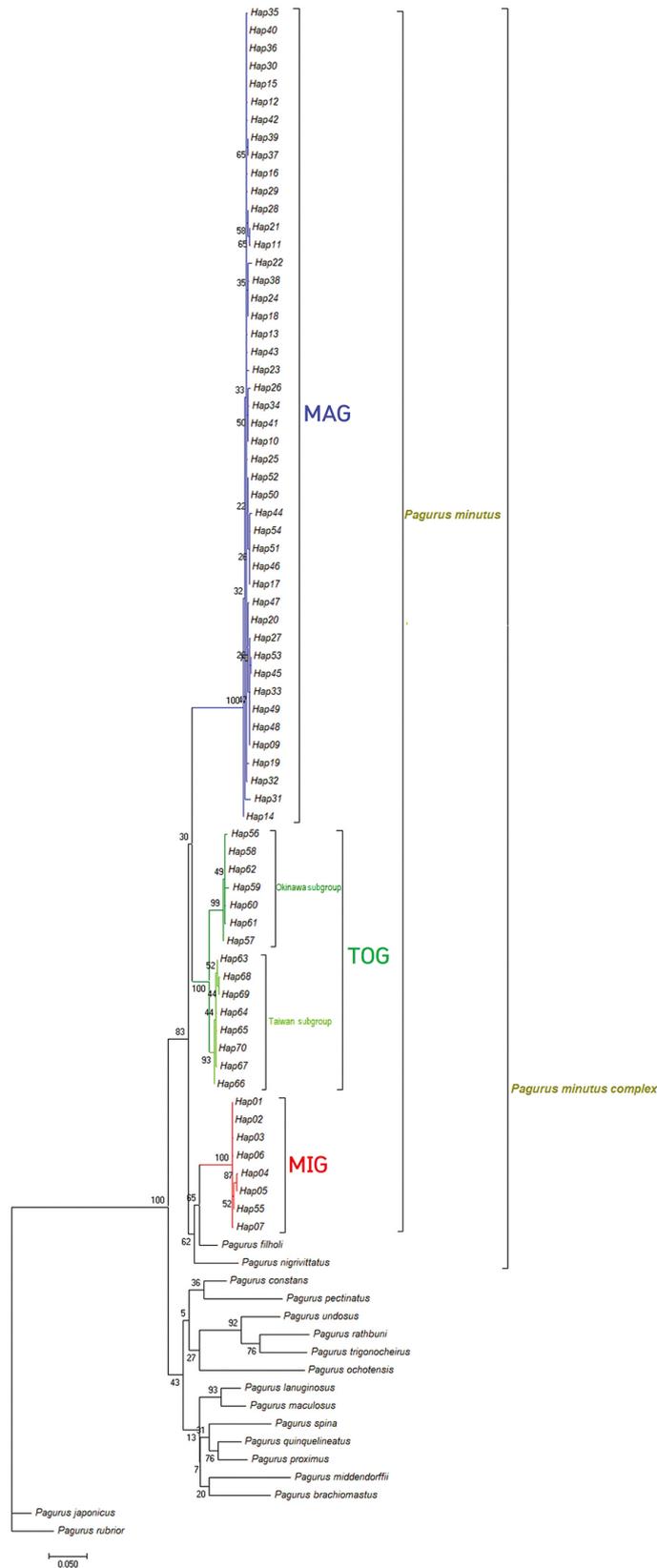


Fig. 5. Maximum likelihood rooted trees of haplotypes of three *Pagurus minutus* groups and congeners in East Asia. Each tree refers to COI tree of Major Group (MAG), Minor Group (MIG), and Taiwan-Okinawa Group (TOG) and the two subgroups (Okinawa and Taiwan subgroup). Bootstrap values were calculated from 1,000 replicates.

Morphological Analysis

MAG, MIG, and TOG were morphologically different although the difference is relatively subtle. Detailed morphological characters of MAG, MIG, and TOG are shown in from figures 6-11. The three groups showed differences in the length of the ocular peduncle and the dactyl of the ambulatory legs; MIG had the shortest length, whereas TOG had the longest length. The three groups were different in the number of dorsodistal spines on the dactyl of ambulatory legs in large individuals (Fig. 12). The female specimens of MAG had lateral protuberances on dactyl and propodus

of ambulatory leg 2, whereas not the female specimens of MIG and TOG (Fig. 12). Detailed results are shown in table 3.

Coloration Analysis

MAG, MIG, and TOG had different background colors and the stripe patterns of the ambulatory legs between them. The background color seen on the lateral surfaces of ambulatory legs of MAG and TOG was similar, whereas the color of MIG was different (Fig. 1). The stripe pattern of MAG and MIG is similar in the lateral surfaces on the propodi of the ambulatory legs,

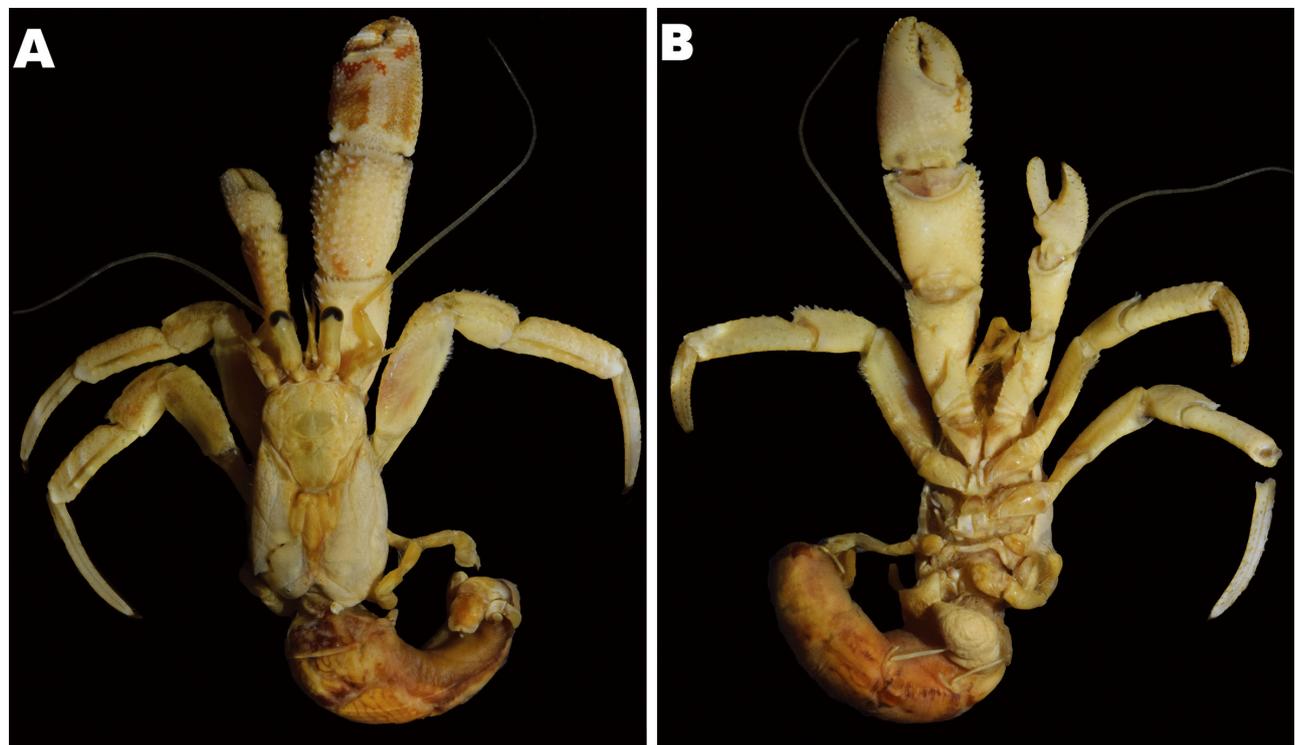


Fig. 6. Major Group (MAG) of *Pagurus minutus*, male, sl 7.0 mm, MADBK 160706_070, right ambulatory leg 2 and dactyl of left ambulatory leg 2 detached. A, dorsal view; B, ventral view. Scale bars = 7 mm.

Table 2. COI sequences pairwise distances for each group and subgroup of *Pagurus minutus* using pairwise distance (%) (the bracket in the value cell represents the mean value of each distance)

Groups	Distances (%)			
	Within group or subgroup	Between groups or subgroups		
		1	2	3
1. Major Group (MAG)	0-1.80 (0.62)			
2. Minor Group (MIG)	0-0.79 (0.40)	9.82-11.46 (10.66)		
3. Taiwan-Okinawa Group (TOG): Okinawa subgroup	0-0.99 (0.45)	8.90-11.03 (9.92)	9.12-10.74 (9.70)	
4. Taiwan-Okinawa Group (TOG): Taiwan subgroup	0.20-0.79 (0.44)	8.00-9.85 (9.06)	7.76-8.88 (8.23)	2.42-3.67 (3.06)

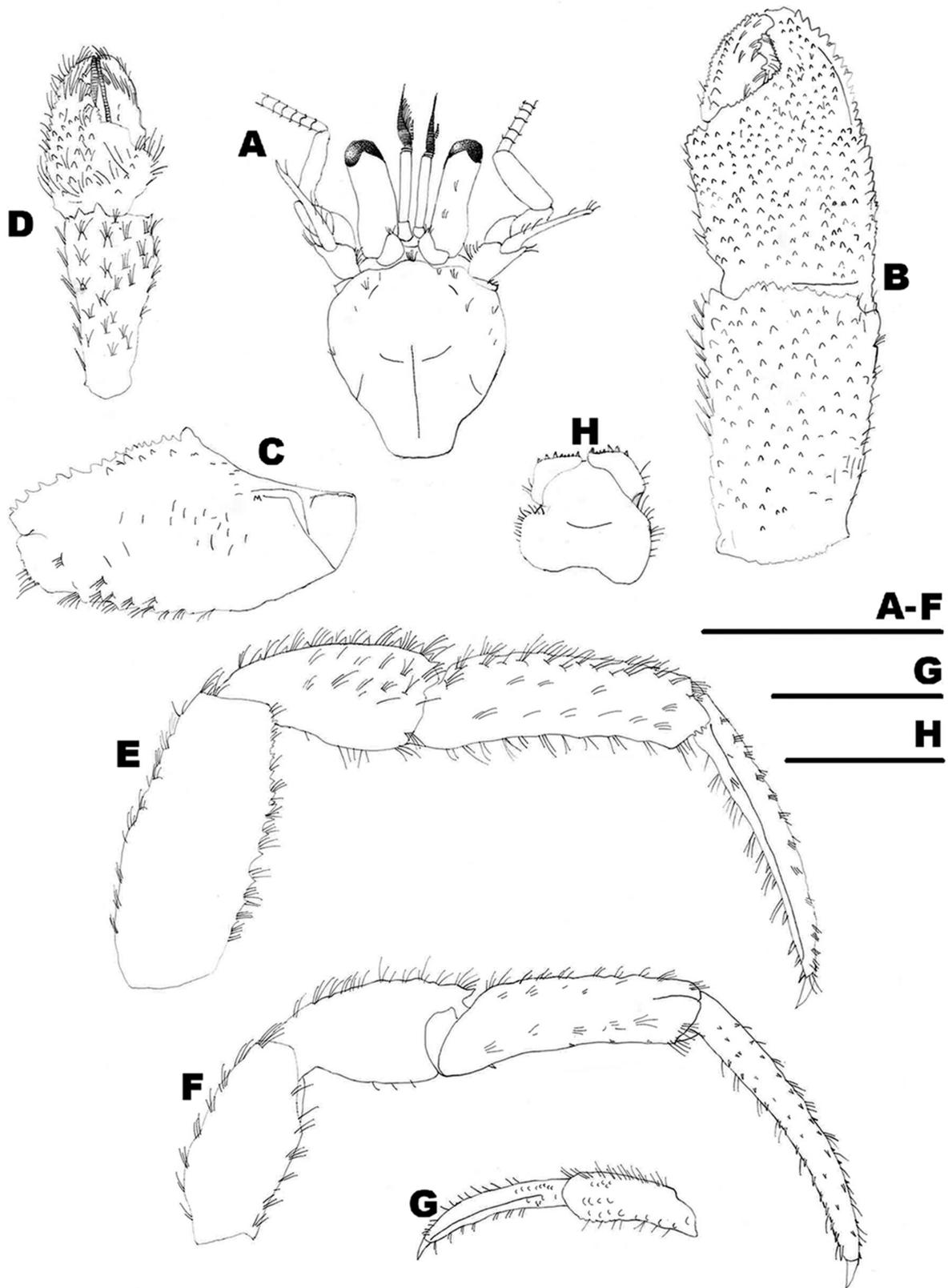


Fig. 7. Major Group (MAG) of *Pagurus minutus*. A, shield and cephalic appendages, dorsal view; B, right cheliped, dorsal view, dorsal setae of chela omitted; C, merus of right cheliped, lateral view; D, left cheliped, dorsal view; E, left ambulatory leg 1, lateral view; F, left ambulatory leg 2, mesial view; G, dactyl and propodus of left ambulatory leg 2, lateral view; H, telson, dorsal view. A-F, H: male, sl 7.0 mm, MADBK 160706_070; G: female, sl 4.7 mm, MADBK 160706_057. Scale bars: A-F = 7 mm; G = 5 mm; H = 3 mm.

whereas not the stripe pattern of TOG (Fig. 12). The three groups were also different in the stripe number of lateral surfaces on the carpi of ambulatory legs (Fig. 12). Detailed results are shown in table 4.

DISCUSSION

Taxonomic status of the three groups of *Pagurus minutus*

This study suggests that 167 individuals of *P. minutus*, a common hermit crab species in East

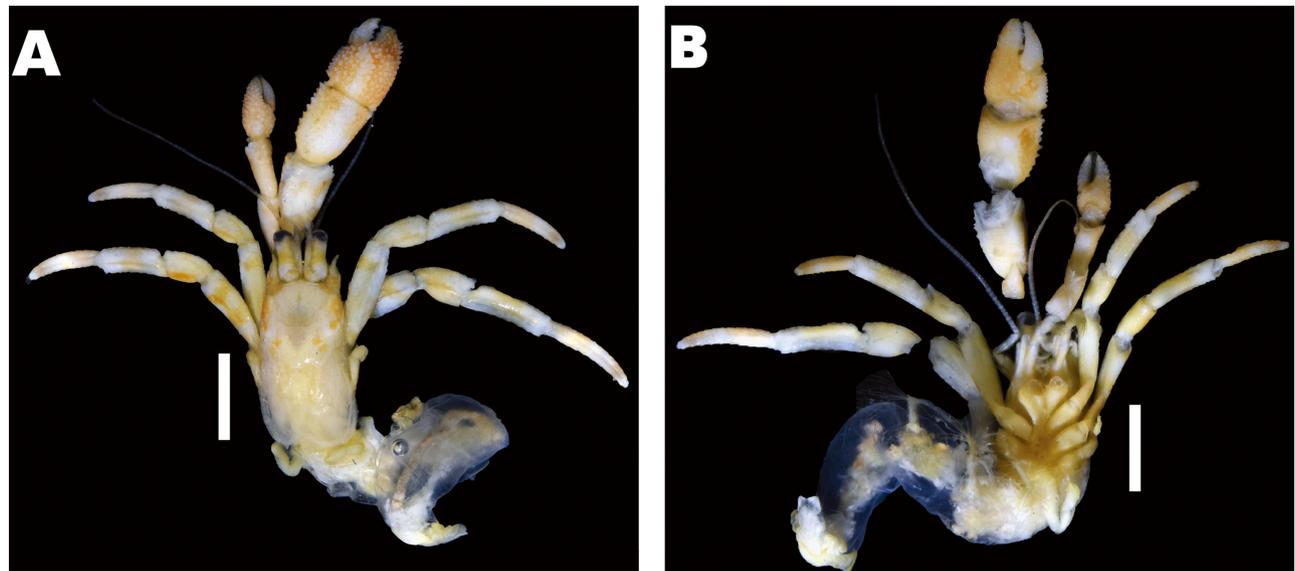


Fig. 8. Minor Group (MIG) of *Pagurus minutus*, female, sl 3.2 mm, MADBK 160749_001, right and left chelipeds and right ambulatory leg 2 detached. A, dorsal view; B, ventral view. Scale bars = 3 mm.

Table 3. Summary of morphological differences among the three groups of *Pagurus minutus*

Morphological characters	Groups		
	Major Group (MAG)	Minor Group (MIG)	Taiwan-Okinawa Group (TOG)
The relative length of ocular peduncle to shield	0.7-0.9 (Fig. 7A)	0.6-0.7 (Fig. 9A)	0.7-1.0 (Fig. 11A)
The relative length of dactyl of ambulatory legs to propodus	1.2-1.6 (Fig. 12A)	1.0-1.2 (Fig. 12B)	1.2-1.7 (Fig. 12C)
Dorsodistal spine on dactyl of ambulatory leg 2 in large male individuals (sl > 4 mm)	5-9 (Fig. 12A)	3-8 (Fig. 12B)	1-3 (Fig. 12C)
The lateral protuberance on dactyl and propodus of ambulatory leg 2 of female	Present (Fig. 12A)	Absent (Fig. 12B)	Absent (Fig. 12C)

Table 4. Summary of color pattern differences among the three groups of *Pagurus minutus*

Groups	The color pattern of the lateral surface of ambulatory legs		
	Base	Propodus	Carpus
Major Group (MAG) (Fig. 1A)	Light yellowish brown to olive-drab	With one median longitudinal stripe	With one median longitudinal stripe
Minor Group (MIG) (Fig. 1B)	Cream	With one median longitudinal stripe	With two longitudinal stripes
Taiwan-Okinawa Group (TOG) (Fig. 1C, 1D)	Light yellowish brown to olive-drab	Without stripe	With or without one median longitudinal stripe

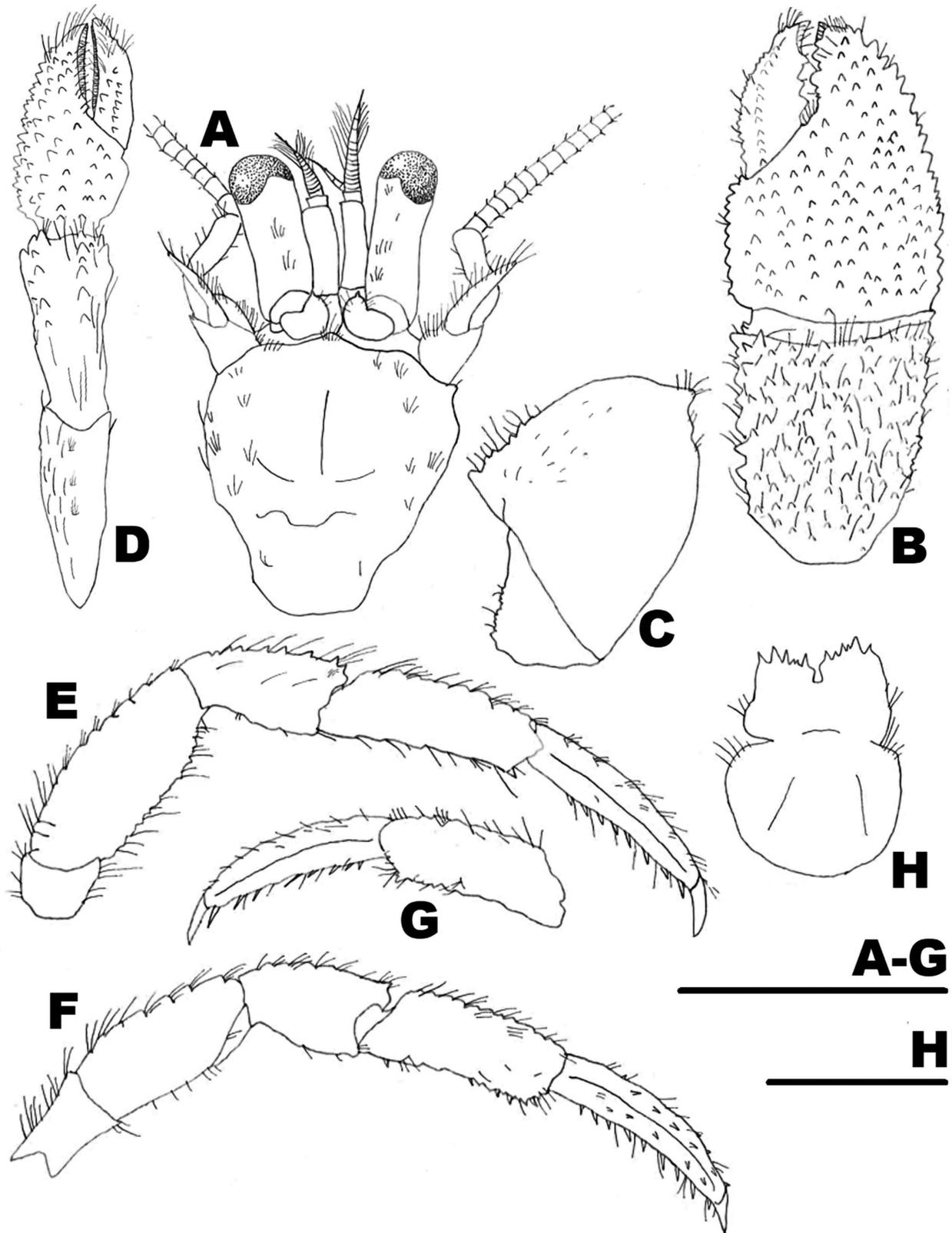


Fig. 9. Minor Group (MIG) of *Pagurus minutus*, female, sl 3.2 mm, MADBK 160749_001. A, shield and cephalic appendages, dorsal view; B, chela and carpus of right cheliped, dorsal view, setae of chela omitted; C, merus and ischium of right cheliped, lateral view, dorsal setae of chela omitted; D, left cheliped, dorsal view; E, left ambulatory leg 1, lateral view; F, left ambulatory leg 2, mesial view; G, dactyl and propodus of left ambulatory leg 2, lateral view; H, telson, dorsal view. Scale bars: A-G = 3 mm; H = 1 mm.

Asia, should be separated into three groups—MAG, MIG, and TOG—based on molecular and color characters. The taxonomic status of these three groups should be addressed at the species level for three reasons. First, these three groups were distinguished based on the molecular, morphological, and color characters, suggesting that each of the three groups is a distinct evolutionary unit that shares fixed diagnostic characters with the phylogenetic species concept of Cracraft (1983). Second, no mitochondrial gene exchange among the three groups implies that these three groups are sexually isolated and individually evolved, corresponding to the biological species concept of Mayr (1942). Third, their divergences in COI sequences are larger than the estimated minimum interspecific divergence between two closely related species of *Pagurus*: *P. lanuginosus* and *P. maculosus*. The taxonomic statuses of *P. lanuginosus* and *P. maculosus* were already confirmed by sexual isolation (Imafuku and Ikeda 1995) and morphological differences (Komai and Imafuku 1996). In addition, the minimum interspecific divergence of this study (5.59%) was similar to the minimum interspecific divergence of *Pagurus* (6.43%) (da Silva et al. 2011b) and Lithodidae (6.38%) (da Silva et al. 2011a). Therefore, these divergences of the three groups are considered large enough to confirm that they are taxonomically distinct at the species level.

Based on the morphological differences among the three *P. minutus* groups, the speciation of MAG, MIG, and TOG is considered as pseudocryptic speciation. The defining feature of a pseudocryptic species is that it can be identified by minor morphological characters, which is not the case in cryptic species (see Knowlton 1986 1993; Bickford et al. 2007; Lajus et al. 2015). A number of cryptic marine decapod species were uncovered based on differences in non-morphological characters such as DNA sequences and color patterns. These species include the fiddler crabs *Uca mordax* and *U. burgersi* (Von Hagen 1983, both are now considered as genus *Minuca* by Shih et al. 2016), the associated snapping shrimps *Alpheus* spp. (Knowlton and Keller 1985), the reef coral-associated crabs *Trapezia* spp. (Castro 1996), the thin-striped hermit crabs *Clibanarius vittatus* and *C. symmetricus* (Negri et al. 2014), and the kuruma shrimps *Penaeus japonicus* and *P. pulchricaudatus* (Tsoi et al. 2014). On the other hand, a few pseudocryptic marine decapod species were also discovered based on morphological differences, along with other characters. They include the common Japanese intertidal crabs *Hemigrapsus penicillatus* and *H. takanoi* (Asakura and Watanabe 2005), the red ghost crabs in the *Neosarmatium meinerti* species complex (Ragionieri et al. 2012), and the fiddler crabs *Tubuca urvillei* and *T. alcocki* (Shih et al.

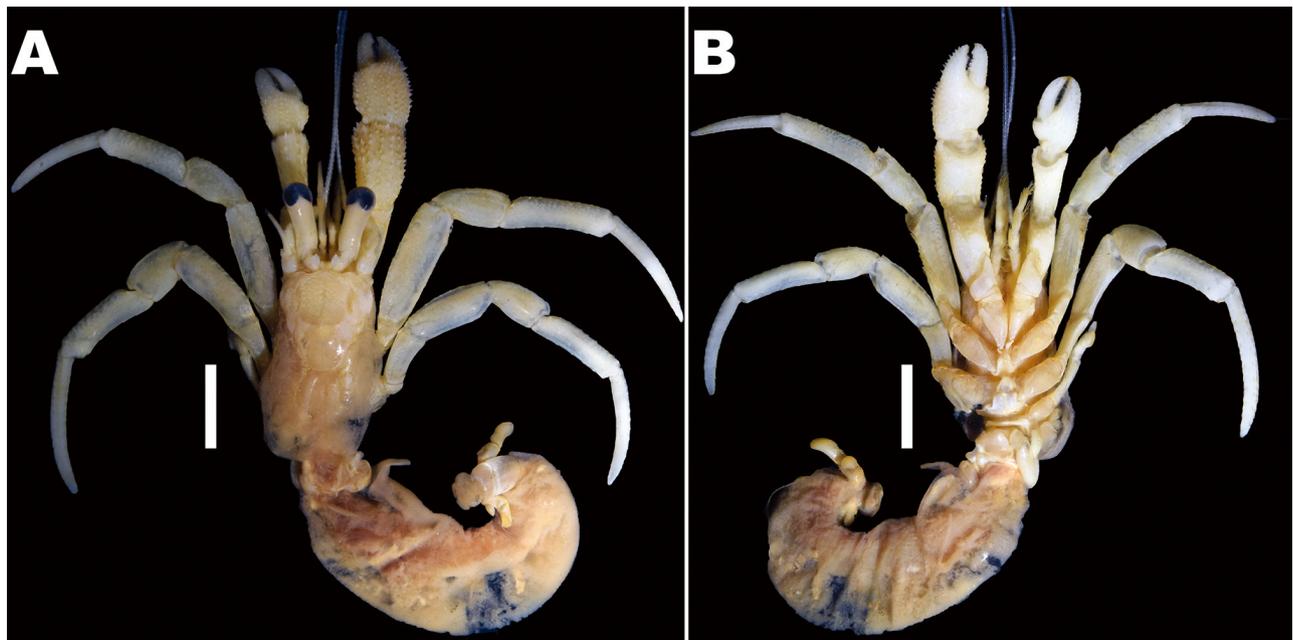


Fig. 10. Taiwan-Okinawa Group (TOG) of *Pagurus minutus*, male, sl 3.1 mm, CBM-ZC 14612. A, dorsal view; B, ventral view. Scale bars = 3 mm.

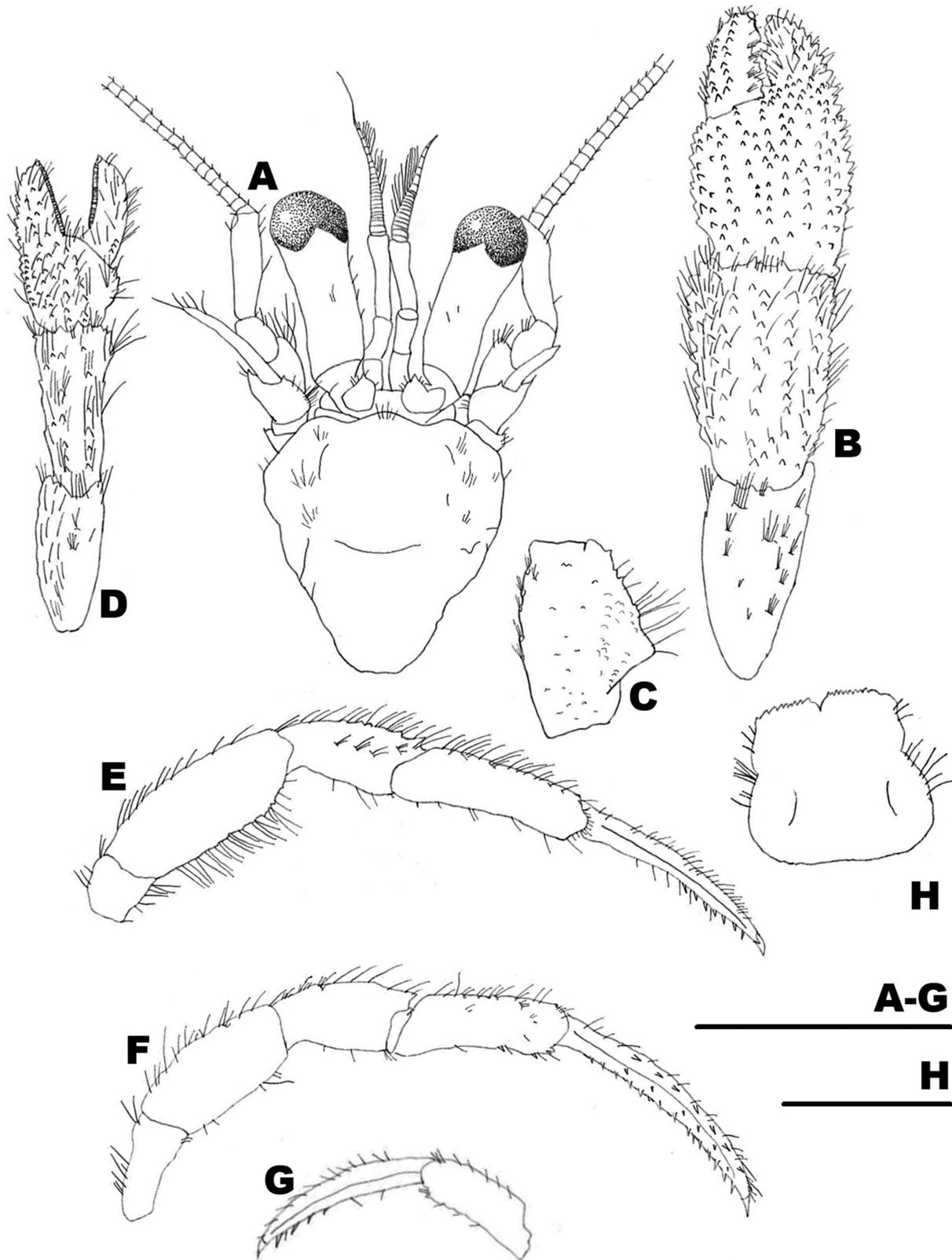


Fig. 11. Taiwan-Okinawa Group (TOG) of *Pagurus minutus*. A, shield and cephalic appendages, dorsal view; B, right cheliped, dorsal view, dorsal setae of chela omitted; C, merus of right cheliped, lateral view; D, left cheliped, dorsal view; E, left ambulatory leg 1, lateral view; F, left ambulatory leg 2, mesial view; G, dactyl and propodus of left ambulatory leg 2, lateral view; H, telson, dorsal view. A-F, H: male, sl 3.1 mm, CBM-ZC 14612; G: female, sl 2 mm, MADBK 160750_002. Scale bars: A-G = 3 mm; H = 1 mm.

2018).

Although this study provides strong evidence justifying the taxonomic status of MAG, MIG, and TOG at a species level, more research is needed in order to report each group as independent new species. This is because the only one remaining syntype *P. minutus* specimen is the holotype male; the holotype is too old to examine its molecular characters; the coloration of the holotype was briefly reported to be dirty gray; and the type locality is unknown (Hess 1865; Komai and Mishima 2003). Because holotype's characters are so uncertain, it is difficult to determine which group is true *P. minutus*. In addition, this study did not analyze any specimen from Russia or China, although *P. minutus* is distributed from southeast Russia, Japan, Korea, China to Taiwan. Further study is needed to denominate the scientific name of MAG, MIG, and TOG.

Ingroup COI divergence of TOG

Differences in the distribution pattern and COI sequences of the two TOG subgroups—the Taiwan and Okinawa subgroups—could be explained by the geographical and genetic barriers between these groups. Oceanographic variation and distribution of the *Tetraclita* species

in Taiwan and Okinawa (Chan et al. 2008) represent evidence that this barrier exists. In order to define the two TOG subgroups, we further investigated the differences in morphological and color characters between CBM-ZC 14613 and MADBK 160750_002, but only minor differences in color pattern on the lateral surfaces of carpi of ambulatory legs were found (Taiwan subgroup: with a median stripe; Okinawa subgroup: without any stripe). However, while no significant differences in morphological characters were found, Terry et al. (2000) suggested that *Girella nigricans*, a western North American coral reef fish, is undergoing incipient speciation based on geographical and genetic differences. On a related note, the geographical and genetic differences present in these two subgroups lead us to assume that TOG has undergone incipient speciation. However, since the molecular divergence and morphological difference are minor, the present study does not distinguish the two subgroups but considers them both to be TOG.

Previous records of *P. minutus* and comparisons to three groups of *P. minutus*

Pagurus minutus described by Komai and Mishima (2003) and MAG are regarded as conspecific. According to the redescription of *P. minutus* by Komai and Mishima (2003), there is one longitudinal stripe on each propodus and carpus of the lateral surface of the ambulatory legs, similar to MAG. In addition, they described the presence of lateral protuberance on the dactyl and propodus of ambulatory leg 2 of female *P. minutus*, which is also found in MAG. Furthermore, the other morphological characters of *P. minutus* described by Komai and Mishima (2003) and the characters of MAG are similar.

Makarov (1938) described *P. dubius* (Ortmann, 1892), a junior synonym of *P. minutus* (Komai and Mishima 2003), collected from eastern Russian waters. The relative length of the ocular peduncle to the shield of this specimen is 0.6, and the relative length of the dactyl of ambulatory legs is 1.2. We believe that *P. dubius* described by Makarov (1938) is MIG according to the two morphological characteristics similar to those of MIG. However, Makarov (1938) noted that *P. dubius* is mainly found in a slightly deep subtidal area with a depth of 50-120 m, but MIG is not. Additional studies on the specimens of Makarov (1938) and Russian samples are needed to identify this matter precisely.

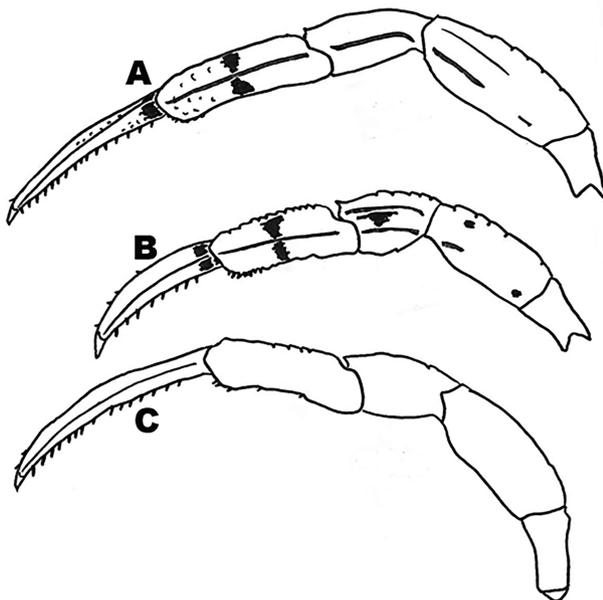


Fig. 12. Semi-diagrammatic figures of morphological characteristics and color patterns on the lateral surface of 2nd ambulatory legs of the female of three *Pagurus minutus* groups. A, Major Group (MAG); B, Minor Group (MIG); C, Taiwan-Okinawa Group (TOG), Okinawa subgroup.

McLaughlin et al. (2007) reported *P. minutus* in Taiwanese waters with unnumbered figures. This species is regarded as TOG because it shares some features with the specimens of TOG collected in Taiwan (MADBK 160750_002), including the collection site, color pattern, and morphological characters (*i.e.*, lateral and mesial armature on the dactyl and propodus of ambulatory leg 2 of female). However, the samples of Taiwanese *P. minutus* examined by Komai and Mishima (2003) are considered MAG. The results suggest that MAG and TOG are mixed in the Taiwanese population of *P. minutus*.

Morphological, coloration and biogeographical comparisons of *P. filholi*, *P. nigrivittatus*, and the three groups of *P. minutus*

The maximum likelihood phylogram (Fig. 5) branches of *P. filholi* and *P. nigrivittatus* were located in a single clade with MAG, MIG, and TOG, despite larger divergences than the minimum interspecific divergence (Table 5). Therefore, we compared specimens of *P. filholi* and *P. nigrivittatus* in MADBK and previous descriptions of these two species with those of MAG, MIG, and TOG in order to distinguish the three groups of *P. minutus* from *P. filholi* and *P. nigrivittatus* in terms of morphology, coloration, and biogeography.

The common morphological features of MAG, MIG, TOG, *P. filholi*, and *P. nigrivittatus* are characterized by the presence of a large tubercle on the ventral surface of the merus of the right cheliped, although the tubercle of *P. nigrivittatus* is absent in a few specimens (see Sandberg and McLaughlin 1993; Komai 2003; Komai and Mishima 2003). These organisms also contain a similar right palm covered by numerous small spinules (see Komai and Mishima 2003). In addition, the right chelae of five species show sexual dimorphism, with an elongated subovate in males and the subovate and distinctly delimited

dorsomesial margin by a row of small spines in females (see Komai 2003; Komai and Mishima 2003). Furthermore, the left palms of the five organisms have a conspicuously elevated midline on the dorsal surface (see Komai 2003; Komai and Mishima 2003). On the other hand, three groups of *P. minutus* share a few morphological features with *P. filholi*. Komai and Mishima (2003) described morphological affinity of *P. minutus* and *P. filholi* as a lack of setae on right palm, and we found that MIG and TOG also have similar characteristics. The common morphological features of MIG, *P. filholi*, and *P. nigrivittatus* are characterized as the relatively short length of the ocular peduncle and the dactyl of the ambulatory legs (see Sandberg and McLaughlin 1993; Komai and Mishima 2003). Table 6 shows the results.

The common color patterns of MAG, MIG, TOG, *P. filholi* and *P. nigrivittatus* are characterized by the presence of two transverse stripes on the corneas, a median transverse band on the ocular peduncles, and regularly placed numerous white articles on the antennal flagella (see Arima 2014). Three groups of *P. minutus* and *P. filholi* contain a median white part on the dactyli of the ambulatory legs as well (see Arima 2014). On the other hand, we found that MAG and *P. nigrivittatus* share the following characteristics: the chelae have a dark gray-brown base; the lateral surfaces of ambulatory legs have a median longitudinal stripe; and the propodi and carpi of the ambulatory legs display median gray-brown patches (see Komai 2003; Komai and Mishima 2003; Arima 2014). These common features of MAG were seen in MIG and TOG, but a different base color of chelae was found in MIG, and no longitudinal stripes on the lateral surfaces of the propodi (and carpi of Okinawa subgroup) of the ambulatory legs were found in TOG. Meanwhile, MIG and *P. nigrivittatus* share two stripes on the lateral surfaces of the carpi of ambulatory legs. In addition, both TOG and *P. filholi* have propodi (and carpi of Okinawa

Table 5. COI sequences pairwise distances between three groups of *Pagurus minutus*, *Pagurus filholi*, and *Pagurus nigrivittatus* using pairwise distance (%) (the bracket in the value cell represents the mean value of each distance)

<i>Pagurus minutus</i> groups	Distances from other species (%)	
	1. <i>P. filholi</i>	2. <i>P. nigrivittatus</i>
1. Major Group (MAG)	8.35-9.33 (8.90)	9.95-10.91 (10.45)
2. Minor Group (MIG)	5.79-6.38 (6.02)	7.76-8.35 (7.98)
3. Taiwan-Okinawa Group (TOG)	5.98-7.56 (6.78)	7.76-9.34 (8.41)

subgroup) of ambulatory legs without longitudinal stripes.

With respect to the common biogeographical features, the geographical distribution and habitats of MAG and *P. filholi* overlap in the Jeju Island of Korea to the northeast coast of Honshu of Japan (see McLaughlin 1976; Komai and Mishima 2003; Arima 2014) (*P. filholi* is described as *P. geminus*, junior synonym of *P. filholi*, in McLaughlin (1976)). On the other hand, the geographical distributions and habitat depths of *P. nigrivittatus* overlaps with that of MAG (see McLaughlin et al. 2007). The geographical distributions of MIG overlaps with that of *P. filholi* except for the Yellow Sea. The geographical distribution and habitats of TOG and *P. nigrivittatus* overlap in the Okinawa Islands of Japan to Taiwan.

In spite of these common features, MAG, MIG, and TOG show distinct morphological, color, and biogeographical characteristics from those of *P. filholi* and *P. nigrivittatus*. Morphologically, MAG and MIG are distinguishable from *P. filholi* and *P. nigrivittatus* by the presence of ambulatory legs with the dactyl, which is distinctly longer than propodus, as well as with the propodus having a tuberculate ventral margin in the second ambulatory leg (see Komai 2003; Komai and Mishima 2003). In addition, three groups of *P. minutus* differ from *P. nigrivittatus* by the presence of a row of dorsal spines on the dorsal margin of the carpus of the first ambulatory leg (see Komai 2003; Komai and Mishima 2003).

With respect to coloration, MAG and *P. filholi* are distinguished by the colors of the ambulatory legs (Miyake 1978), chelipeds, and shield (see

McLaughlin et al. 2007; Arima 2014). These differences in MAG are also found in MIG and TOG. On the other hand, we found that three groups of *P. minutus* are distinguishable from *P. nigrivittatus* by the presence of white median stripes of chelae and the number of stripes on the lateral surface of the ambulatory legs, which is one in MAG, 0-1 in TOG, 1-2 in MIG, and 2-3 in *P. nigrivittatus* (see Komai 2003; Komai and Mishima 2003).

The geographical distributions of MAG differ from those of *P. filholi*: MAG thrives in the Yellow Sea unlike *P. filholi*; *P. filholi* inhabits the region from northeastern South Korea to Kamchatka of East Russia unlike MAG (see McLaughlin 1976; Komai and Mishima 2003). Meanwhile, *P. nigrivittatus* inhabits from the Jeju Island of South Korea to the Boso Peninsula of Japan, but neither MIG nor TOG has been found yet.

This study suggests that MAG, MIG, TOG, *P. filholi*, and *P. nigrivittatus* should be treated as components of the *P. minutus* complex. A species complex is defined as a group of related independent species (Brown et al. 1995). According to this definition, these five *Pagurus* organisms are regarded as a species complex based on their molecular and morphological characteristics. Molecularly, these organisms are distinct from each other but grouped as a single clade from the other *Pagurus* species in the phylogram (Fig. 5). In addition, these five species are morphologically distinct from one other, but the species complex of these organisms is distinguishable from the congeneric by the characters of chelipeds.

Table 6. Common morphological features of three groups of *Pagurus minutus*, *Pagurus filholi*, and *Pagurus nigrivittatus*

Morphological characters	Groups or species				
	Major Group (MAG)	Minor Group (MIG)	Taiwan-Okinawa Group (TOG)	<i>P. filholi</i>	<i>P. nigrivittatus</i>
Ventral large tubercle on the merus of the right cheliped	Present (Fig. 7C)	Present (Fig. 9C)	Present (Fig. 11C)	Present	Mostly present
Numerous dorsal small spinules on the right palm	Numerous (Fig. 7B)	Numerous (Fig. 9B)	Numerous (Fig. 11B)	Numerous	Numerous
Midline on the dorsal surface of the left palm	Elevated (Fig. 7D)	Elevated (Fig. 9D)	Elevated (Fig. 11D)	Elevated	Elevated
Setae on the right palm	Lack (Fig. 7B)	Lack (Fig. 9B)	Lack (Fig. 11B)	Lack	Numerous
The relative length of ocular peduncle to shield	0.7-0.9 (Fig. 7A)	0.6-0.7 (Fig. 9A)	0.7-1.0 (Fig. 11A)	0.5-0.7	0.6-0.7
The relative length of dactyl of ambulatory legs to propodus	1.2-1.6 (Fig. 12A)	1.0-1.2 (Fig. 12B)	1.2-1.7 (Fig. 12C)	0.5-1.1	0.8-1.1

CONCLUSIONS

We suggest that *Pagurus minutus* in East Asia be separated into three groups based on differences in molecular, morphological and color characters. The taxonomic status of these three groups is suggested at species level based on (1) the differences in molecular, morphological and color characters, (2) the nonexistent shared COI haplotypes, and (3) the large divergence in the distances of COI sequences.

This study is the first phylogenetic study of *Pagurus* species in East Asia, which underscores the need for phylogenetic studies of other hermit crabs and common marine decapod species in East Asia aimed at identifying their taxonomical diversities and molecular and biogeographical characteristics.

List of abbreviations

sl, shield length.

COI, cytochrome c oxidase subunit I.

MADBK, Marine Arthropod Depository Bank of Korea, Seoul National University, Seoul, South Korea.

CBM, Natural History Museum and Institute, Chiba, Japan.

MAG, Major Group.

MIG, Minor Group.

TOG, Taiwan-Okinawa Group.

Acknowledgments: This work was supported, in part, by a grant from the Marine Biotechnology Program (20170431) funded by the Ministry of Oceans and Fisheries, Korea. Financial support was also provided to “Graduate Program for the Undiscovered Taxa of Korea (NIBR201524202)”. We thank Ji Yeong Shin, Hanna Kim and Juri Park (Department of Science Education, Ewha Womans University); Yunho Her, Dong-yun Han (Department of Biotechnology, Sangji University) Jin-Hyeop Jeong, Nayoung Yang, Beong-ju Che and Ji-hyeon Shin (School of Biological Sciences, Seoul National University) for the molecular experiments of COI. We also appreciate Dr. Tin-Yam Chan (Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan), Dr. Tomoyuki Komai (Natural History Museum and Institute, Chiba, Japan), Dr. Takeshi Yuhara (Graduate School of Life Science, Tohoku University, Miyagi, Japan), Dr. Masanori Taru (Faculty of Sciences, Tokyo Bay Ecosystem Research Center, Toho University, Chiba, Japan), and Dr. Ryuta Yoshida (Tateyama

Marine Laboratory, Marine and Coastal Research Center, Ochanomizu University, Chiba, Japan) for assistance with *P. minutus* samples and photos from Taiwan and Japan. We appreciate the editor and two reviewers for excellent comments on this manuscript. We thank Mrs. Hyunsoon Kim for proofreading this manuscript.

Authors’ contributions: Jibom Jung and Jongwoo Jung designed the study. Jibom Jung and Won Kim performed the field work and analyzed the specimen. Jibom Jung, Jongwoo Jung, and Won Kim contributed to the acquisition of the sequence data. Jibom Jung and Jongwoo Jung contributed to the analysis and interpretation of the sequence data. Jibom Jung and Won Kim drafted of the manuscript. Jongwoo Jung participated in the critical revision of the manuscript.

Competing interests: The authors declare that they have no conflict of interest.

Availability of data and materials: All sequence data in this study are available in GenBank. All examined and sampled specimens are available in the Marine Arthropod Depository Bank of Korea, Seoul National University, Seoul, South Korea (MADBK) and Natural History Museum and Institute, Chiba, Japan (CBM).

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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