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Taxonomy and Phylogeography of the Freshwater Crab *Geothelphusa tawu* Species Complex (Crustacea: Decapoda: Potamidae) from Southern Taiwan and Offshore Islets

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The freshwater crabs *Geothelphusa tawu* Shy, Ng & Yu, 1994, *G. lutao* Shy, Ng & Yu, 1994 and *G. lanyu* Shy, Ng & Yu, 1994 from southern Taiwan and the offshore islets, Lyudao (Green I.) and Lanyu (Orchid I.) are closely related in morphology and genetics, and have been proposed to be the same species. Examination of a series of specimens collected from the distributional ranges of the three species indicated that key characters of the ambulatory legs and the male first gonopod (G1) are too variable to support the identity of three species. Based on the mitochondrial 16S rDNA and cytochrome oxidase subunit I (*COI*) sequences, the phylogenetic analysis did not recover three clades corresponding to the three species, but only a main clade without further clear grouping. The interspecific distances of nucleotides are also too small to support the species delimitation. We conclude that the three species should be treated as a single species. Additionally, unique haplotypes of *COI* have been found in Lyudao and Lanyu, which are hypothesized as two founder populations that colonized the islets from the Taiwan main island during glacial maxima.

Key words: Taxonomy, Phylogeography, 16S rDNA, COI, Founder populations, Morphology, Glaciations

BACKGROUND

Among the freshwater crabs in East Asia, the species diversity of *Geothelphusa* Stimpson, 1858 in the East Asian Arc is much higher than any other genera, except *Longpotamon* Shih, Huang & Ng, 2016 from continental China (Shih and Ng 2011; Shih et al. 2009 2016a). There are 38 species of *Geothelphusa* from Taiwan and adjacent islets (Shy et al. 1994 2020 2021), but some species complexes have been considered to have unsatisfactory taxonomy (Shih et al. 2004; Shy et al. 2020). The *Geothelphusa tawu* species complex

distributed in southern Taiwan and adjacent islets needs further study, as suggested by Shih et al. (2004). This complex includes *G. tawu* Shy, Ng & Yu, 1994 from the Hengchun Peninsula, and *G. lutao* Shy, Ng & Yu, 1994 and *G. lanyu* Shy, Ng & Yu, 1994 endemic to two offshore islets, Lyudao (= Green Island) and Lanyu (= Orchid Island), respectively (Fig. 1). Other freshwater crabs, including *G. ferruginea* Shy, Ng & Yu, 1994, *G. albogilva* Shy, Ng & Yu, 1994 and *Candidiopotamon rathbuni* (De Man, 1914) are also distributed in southern Taiwan. Populations of freshwater crabs found on offshore islets that are separated from the main island

Citation: Shih HT, Hsu JW, Chang K, Chen MW. 2023. Taxonomy and phylogeography of the freshwater crab *Geothelphusa tawu* species complex (Crustacea: Decapoda: Potamidae) from southern Taiwan and offshore islets. Zool Stud **62:**37. doi:10.6620/ZS.2023.62-37.

by more than 30 km might be expected to be different from populations on the main island. In Shih et al. (2004: fig. 2), however, the three recognized species have been suggested to be conspecific, based on an unresolved clade formed by the three species in a phylogenetic tree of mitochondrial 16S rDNA, together with small morphological differences. Additional genetic and morphology studies were referenced to conclusively establish that they are conspecific.

In our study, a morphological comparison of the key characters of ambulatory legs and male first gonopod (G1) used in Shy et al. (1994) was conducted on a series of specimens from the distributional ranges of *G. tawu*, *G. lutao* and *G. lanyu*. Our comparison



Fig. 1. Collection sites for species of the *Geothelphusa tawu* species complex from southern Taiwan, and the adjacent islets. Different lines indicate the possible biogeographic boundaries for each species found in southern Taiwan. For locality names, see table 1. Specimens of *G. tawu*, *G. lutao* and *G. lanyu* on the photographs are collected from Dawu R. (9 Jan. 2001), Lyudao (11 Sep. 1999) and Lanyu (8 Jan. 2008), respectively.

indicated that these characters were not discrete for each of these species, and some were found to be the same in one or more. In addition, the molecular evidence from 16S rDNA and cytochrome oxidase subunit I (*COI*) did not support the monophyly of the three species. Based on the available evidence in morphology and genetics, our study further proposes the three taxa belong to a single species.

MATERIALS AND METHODS

Specimens of the *G. tawu* species complex from around the distributional ranges (including Lyudao and Lanyu; Fig. 1) suggested in Shih et al. (2004 2007) were collected after several surveys. All specimens were preserved in 75–95% ethanol after collection and deposited into the Zoological Collections of the Department of Life Science, National Chung Hsing University (NCHUZOOL; see Table 1 for catalogue numbers). Additional specimens that had been deposited at the Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan (NTOU) were also used.

Measurements, all in millimeters (mm), are of the maximum carapace width (CW) and carapace length (CL). For the morphological comparison, only adult males (CW³ 12.0 mm) were selected. The abbreviations

P3, P4 and P5 are used for the third, fourth and fifth pereiopods (second, third and fourth ambulatory legs), and G1 for male first gonopods. As different authors may measure different positions from the photograph (Shih and Do 2014; PY Hsu and Shih 2018; Shih et al. 2019; Shy et al. 2020), the measurements of legs, including the dactyl length (DL) and propodus length (PL) of P3-5 and the merus length (ML) and merus width (MW) of P3 merus; as well as G1, including terminal segment length (TSL), total length of G1 (TLG1), synovial membrane length (SML) and synovial membrane width (SMW) are shown in figure 2. The two ratios of G1s were also measured on the photographs of holotypes of G. tawu, G. lutao and G. lanvu (Shy et al. 2020). Other characters of G1, including the terminal segment curvature and the existence of a tooth on the outer proximal margin of subterminal segment, were also examined, as they were sometimes used to distinguish different species of Geothelphusa (Shy et al. 1994 2020; Shy and Lee 2009). The morphological characters and terminology follow those in WJ Chen et al. (2007) and Shy et al. (2020).

Genomic DNA was isolated from muscle tissues using commercial kits, and the sequences of the 16S rDNA [~560 base pairs (bp)] and *COI* (616–658 bp) genes were obtained as described by Shih et al. (2016b) and verified with the complementary strand. The primers used were 1471 (5'-CCTGTTTANCAAAAACAT-3'),

Table 1.	Haplotypes of the 16	S rDNA and cytochrome	c oxidase subunit l	(COI) of the	Geothelphusa taw	<i>u</i> species
complex	and the related species	. Numbers within bracket	s correspond to the l	localities in fig	ure 1	

Species	Localities	Sample size	Catalogue no. of NCHUZOOL (unless indicated)	Haplotype of 16S	Access. no. of 16S	Haplotype of COI	Access. no. of COI
G. tawu	Taitung: Taimali R. [1]	1	13283	Gtw1	AB535446	Gtw-C1	AB535477
	Taitung: Dawu R. [2]	1	(uncatalogued)		AB127379		_
	Taitung: Dawu R. [2]	1	13058	Gtw3	AB127381	Gtw-C2	AB266300
	Taitung: Dawu R. [2]	1	13058	Gtw3	OQ822175		—
	Taitung: Dawu R. [2]	1	13155	Gtw2	OQ822176	Gtw-C3	OQ824907
	Taitung: Dawu R. [2]	1	13058		_	Gtw-C4	OQ824908
	Taitung: Dawu R. [2]	2	13058	Gtw4	OQ822177;		—
					OQ822178		
	Taitung: Dawu [3]	1	NTOU F10203	Gtw5	OQ822179	Gtw-C5	OQ824909
			(holotype)				
	Pingtung: Alangyi, Mudan [4]	1	17155	Gtw6	OQ822180	Gtw-C6	OQ824910
	Pingtung: Shuanliou, Danlu [5]	3	17152	Gtw2	OQ822181;	Gtw-C7	OQ824911;
					OQ822182;		OQ824912;
					OQ822183		OQ824913
	Pingtung: Shuanliou, Danlu [5]	1	17152	Gtw7	OQ822184	Gtw-C8	OQ824914
	Pingtung: (Damei, Mudan [6]	1	14320	Gtw9	AB127374	Gtw-C13	OQ824915
	Pingtung: Lilongshan, Shihzih [7]	1	17153	Gtw10	OQ822185	Gtw-C14	OQ824916
	Pingtung: Lilongshan, Shihzih [7]	1	17156	Gtw11	OQ822186	Gtw-C15	OQ824917
	Pingtung: Fenggang R., Danlu, Shihzih [8]	3	17163	Gtw2	OQ822187;	Gtw-C9	OQ824918;
					OQ822188;		OQ824919;
					OQ822189		OQ824920

Table 1. (Continued)

Species	Localities	Sample size	Catalogue no. of NCHUZOOL (unless indicated)	Haplotype of 16S	Access. no. of 16S	Haplotype of COI	Access. no. of COI
	Pingtung: Fenggang R., Danlu, Shihzih [8]	1	17163	Gtw8	OQ822190	Gtw-C10	OQ824921
	Pingtung: Fenggang R., Danlu, Shihzih [8]	1	13317	Gtw2	OQ822191	Gtw-C11	AB539523
	Pingtung: Fenggang R., Danlu, Shihzih [8]	1	13317	Gtw2	OQ822192	Gtw-C12*	OQ824922
	Pingtung: Shihwen, Chunrih [9]	1	13055	Gtw15	AB127375	Gtw-C20	AB266297
	Pingtung: Dahanshan, Chunrih [10]	4	12979	Gtw2	OQ822193; OQ822194; OQ822195; OQ822196		_
	Pingtung: Cijia, Chunrih [11]	3	13057	Gtw12	OQ822197; OQ822198; AB127376	Gtw-C16	OQ824923; OQ824924; OQ824925
	Pingtung: Cijia, Chunrih [11]	1	13056	Gtw13	AB127377	Gtw-C17*	AB266298
	Pingtung: Cijia, Chunrih [11]	1	13057	Gtw12	OQ822199	Gtw-C18*	AB266299
	Pingtung: Cijia, Chunrih [11]	1	13057	Gtw13	OQ822200	Gtw-C19	OQ824926
	Pingtung: Cijia, Chunrih [11]	1	13057	Gtw14	OQ822201		—
G. lutao	Taitung: Lyudao [12]	1	13060	Gtw16	AB127382	Glt-C1	AB266302
	Taitung: Lyudao [12]	1	13061	Gtw16	OQ822202	Glt-C2	AB266303
	Taitung: Haisenping, Lyudao [12]	1	14992	Gtw16	OQ822203	Glt-C3	OQ824927
	Taitung: Guanyin Cave, Lyudao [12]	2	17145	Gtw16	OQ822204; OQ822205	Glt-C1	OQ824928; OQ824929
	Taitung: Haishenping, Lyudao [12]	2	14993	Gtw16	OQ822206; OQ822207	Glt-C4	OQ824930; OQ824931
	Taitung: Haishenping, Lyudao [12]	1	14992	Gtw16	OQ822208	Glt-C1	OQ824932
	Taitung: Haishenping, Lyudao [12]	2	14992	Gtw16	OQ822209; OQ822210	Glt-C5	OQ824933; OQ824934
	Taitung: Lyudao [12]	1	NTOU F10200 (holotype)	Gtw16	OQ822211	Glt-C6*	OQ824935
G. lanyu	Taitung: Longmen R., Lanyu [13]	1	13059	Gtw2	AB127380	Gly-C1	AB266301
	Taitung: Longmen R., Lanyu [13]	1	13059	Gtw17	OQ822212	Gly-C1	OQ824936
	Taitung: Hongtou, Lanyu [13]	1	14326	Gtw17	OQ822213	Gly-C1	OQ824937
	Taitung: Hongtou, Lanyu [13]	1	14326	Gtw17	OQ822214	Gly-C1	OQ824938
	Taitung: Hongtou, Lanyu [13]	1	14326	Gtw17	OQ822215	Gly-C1	OQ824939
	Taitung: Hongtou, Lanyu [13]	1	14326	Gtw2	OQ822216	Gly-C1	OQ824940
	Taitung: Yeyou R., Lanyu [13]	1	17146	Gtw2	OQ822217	Gly-C3	OQ824941
	Taitung: Yeyou South R., Lanyu [13]	1	17147	Gtw2	OQ822218	Gly-C4	OQ824942
	Taitung: Yuren R., Lanyu [13]	1	17150	Gtw2	OQ822219	Gly-C5	OQ824943
	Taitung: Hongtou R., Lanyu [13]	1	17149	Gtw2	OQ822220	Gly-C6*	OQ824944
	Taitung: Longmen R., Lanyu [13]	1	17148	Gtw17	OQ822221	Gly-C6*	OQ824945
	Taitung: Langdao R., Lanyu [13]	1	17151	Gtw2	OQ822222	Gly-C7	OQ824946
	Taitung: Longmen R., Lanyu [13]	1	17142	G. 15	_	Gly-Cl	OQ824947
	Taitung: Longmen R., Lanyu [13]	1	17142	Gtw17	OQ822223	Gly-Cl	OQ824948
	Taitung: Yeyou R., Lanyu [13]	1	17154	Gtw2	OQ822224		
	Taitung: Yeyou R., Lanyu [13]	1	17154	Gtw18	OQ822225		
	laitung: Yuren K., Lanyu [13]	3	1/141	Gtw2	OQ8222226; OQ8222227; OQ8222228	Gly-Cl	OQ824949; OQ824950; OQ824951
	Taitung: Langdao R., Lanyu [13]	1	17143	Gtw2	OQ822229	Gly-C1	OQ824952
	Taitung: Langdao R., Lanyu [13]	1	17143	Gtw2	OQ822230	Gly-C2	OQ824953
	Taitung: Langdao R., Lanyu [13]	1	17143	Gtw19	OQ822231	Gly-C1	OQ824954
	Taitung: Lanyu [13] (holotype)	1	NTOU F10100 (holotype)	Gtw3	OQ822232	-	
total		69	· · · · · ·				
G. ferruginea	Pingtung: Maozaikengnei, Hengchun	1	13062	Gf-1	AB127383	Gf-C1	AB266304
	Pingtung: Nanrenshan, Manjhou	1	13282	Gf-2	OQ822233	Gf-C2	OQ824955
G. albogilva	Pingtung: Maozaikengnei, Hengchun	1	13053	Ga	AB127366	Ga-C	AB266295

*, sequence is shorter. See MATERIALS AND METHODS for abbreviations of universities.

1472 (5'-AGATAGAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996), 16L29 (5'-YGCCTGTTTATCAAAAACAT-3'), 16H10 (5'-AATCCTTTCGTACTAAA-3'), and 16H11 (5'-AGATAGAAACCRACCTGG-3') (Schubart 2009) for 16S; and LCO1490 (5'-GGTCAACAAATCATAA AGATATTGG-3'), HCO2198 (5'-TAAACTTCAGGGT GACCAAAAAATCA-3') (Folmer et al. 1994), COL14 (5'-GCTTGAGCTGGCATAGTAGG-3') (Roman and Palumbi 2004), jgLCO (5'-TITCIACIAAYCAYAA RGAYATTGG-3'), jgHCO (5'-TAIACYTCIGGRTG ICCRAARAAYCA-3') (Geller et al. 2013), LCOB (5'-CAAAYCATAAAGAYATYGG-3') and HCOex3 (5'-GCTCANACTACRAATCCTA-3') (Shih et al. 2022b), as well as the newly designed primers HCOex0 (5'-GAYTCTTTTTDCCDGAYTC-3') for COI. The sequences of the different haplotypes have been deposited in the NCBI GenBank database (accession numbers are summarized in Table 1).

As the sequences of *COI* are shorter than others by using the internal primer COL14, the missing data were designated as a '?' in the alignment of the Bayesian inference (BI) and maximum likelihood (ML) analyses, page 5 of 15

but the segments with missing data were excluded in network analysis and nucleotide pairwise comparison. Sequences of the related species, *G. ferruginea* and *G. albogilva* distributed in southern Taiwan and sometimes sympatric with *G. tawu*, were included in the phylogenetic analyses. For the combined 16S and *COI* dataset, the best-fitting models for sequence evolution of individual datasets were determined by PartitionFinder (ver. 2.1.1, Lanfear et al. 2017) and selected by the Bayesian information criterion (BIC). The obtained best models for the two individual datasets were HKY+I, and were subsequently used for the partitioned BI and ML analyses.

The BI was performed with the program BEAST (vers. 2.6.7, Bouckaert et al. 2019) and the divergence times among taxa were estimated by using a strict clock (Yule Model) with the substitution rates of 0.44% and 1.165% per million years for 16S and *COI*, respectively (Schubart et al. 1998). A Yule speciation process was conducted for the divergence within the *G. tawu* species complex. An HKY+I model with the parameters obtained from PartitionFinder was used for each gene. Two independent MCMC chains were run for 10



Fig. 2. Schematic drawings of the ambulatory leg (A) and the G1 (B) measurements used in this study. DL, dactyl length; PL, propodus length; ML, merus length; MW, merus width; TLG1, total length of G1, TSL, terminal segment length; SML, synovial membrane length; SMW, synovial membrane width.

million generations sampled every 1000 generations. The convergence of the two combined chains was determined by the ESS (> 200 as recommended) for each parameter in Tracer (vers. 1.7.2, Rambaut et al. 2018). Trees in the two chains were combined using LogCombiner (vers. 2.6.7, distributed as part of the BEAST package) and were assessed using TreeAnnotator (vers. 2.6.7, distributed as part of the BEAST package) with the default burnin cutoff (10% of sampled trees). A chronogram was constructed by FigTree (vers. 1.4.4, Rambaut 2018). A ML analysis was conducted in IQ-TREE (vers. 2.2.0, Minh et al. 2020) with the best models and 30,000 ultrafast bootstrap replicates (Hoang et al. 2017).

A TCS haplotype network of the 16S+COI haplotypes was generated using the program PopART (vers. 1.7, Leigh and Bryant 2015). Bp differences and the pairwise estimates of Kimura 2-parameter (K2P) distances (Kimura 1980) for genetic diversities between COI haplotypes were calculated with MEGA (vers. 11.0, Tamura et al. 2021).

Specimens examined: G. tawu: 1 \diamond (13.3 × 10.6 mm), NCHUZOOL 13283, Taimali R., Taitung, 30 Jan. 2007; 1 3 (14.5 × 11.5 mm), NCHUZOOL 13155, Dawu R., Taitung, coll. H.-T. Shih, 9 Jan. 2001; 1 $(17.3 \times 13.5 \text{ mm})$, 3 ? ? $(17.3 \times 13.4, 16.9 \times 13.4)$ 13.4, 13.4 × 10.3 mm), NCHUZOOL 13058, Dawu R., Taitung, coll. H.-T. Shih, 9 Jan. 2001; 1 ♀ (16.6 × 13.1 mm), NCHUZOOL 17155, Alangyi, Mudan, Pingtung, coll. Y.-J. Yang, 26 Mar. 2020; 2 & & (17.9) × 14.6, 17.8 × 14.7 mm), 2 $\stackrel{\circ}{\uparrow} \stackrel{\circ}{\downarrow}$ (18.6 × 14.3, 17.3 × 13.3 mm), NCHUZOOL 17152, Shuanliou, Danlu, Pingtung, 30 Mar. 2021; 1 ♀ (19.9 × 15.7 mm), NCHUZOOL 14320, Damei, Mudan, Pingtung, 9 Mar. 2000; 1 ♀ (18.5 × 14.3 mm), NCHUZOOL 17153, Lilongshan, Shihzih, Pingtung, 16 Jul. 2017; 1 3 $(16.5 \times 13.4 \text{ mm})$, NCHUZOOL 17156, Lilongshan, Shihzih, Pingtung, coll. S.-P. Wu, 7 Jul. 2020; 1 3 (19.5) × 15.8 mm), 2 $\stackrel{\circ}{\uparrow}$ $\stackrel{\circ}{\downarrow}$ (21.9 × 16.1, 16.3 × 13.2 mm), NCHUZOOL 13317, Fenggang R., Danlu, Shihzih, Pingtung, coll. H.-T. Shih, 11 Mar. 1999; 3 & & (19.3) × 15.6, 19.0 × 15.0, 17.5 × 13.6 mm), 3 $\stackrel{\circ}{\uparrow} \stackrel{\circ}{\downarrow}$ (17.9 × 14.1, 17.5 × 14.3, 13.8 × 11.2 mm), NCHUZOOL 17163, Fenggang R., Danlu, Shihzih, Pingtung, coll. H.-T. Shih, 11 Mar. 1999; 1 & (12.7 × 10.1 mm), NCHUZOOL 13055, Shihwen, Chunrih, Pingtung, 10 Sep. 2002; 1 \diamond (18.2 × 14.4 mm), 1 \updownarrow (17.8 × 14.4 mm), NCHUZOOL 17165, Shihwen, Chunrih, Pingtung, 10 Sep. 2002; 1 3 (18.1 × 13.5 mm), 2 broken $\Diamond \Diamond$, 1 \Leftrightarrow (19.2 × 14.7 mm), NCHUZOOL 12979, Dahanshan, Chunrih, Pingtung, 2 Mar. 1997; 1 & (16.4 × 13.3 mm), NCHUZOOL 13056, Cijia, Chunrih, Pingtung, 10 Sep. 2002; $4 \Leftrightarrow \Leftrightarrow (18.8 \times 14.7,$ $16.7 \times 13.2, 16.6 \times 13.1, 9.4 \times 7.3 \text{ mm}), 2 \neq \neq (22.1)$

× 16.8, 12.9 × 10.5 mm), 2 ovig. 2° (20.3 × 15.4, 18.8 × 14.7 mm), NCHUZOOL 13057, Cijia, Chunrih, Pingtung, 10 Sep. 2002; 1 & (15.6 × 12.1 mm), NCHUZOOL 17164, Cijia, Chunrih, Pingtung, 10 Sep. 2002; 1 $(17.5 \times 13.7 \text{ mm})$, 1 \neq (21.7 \times 16.6 mm), NCHUZOOL 17144, Cijia, Chunrih, Pingtung, 10 Sep. 2002. G. lutao: 1 & (18.5 × 14.2 mm), NCHUZOOL 13060, Lyudao, coll. H.-C. Liu, 11 Sep. 1999; 1 3 $(18.2 \times 14.3 \text{ mm})$, NCHUZOOL 13061, Lyudao, coll. H.-C. Liu, 11 Sep. 1999; 2 & & (12.6 × 9.8, 11.8 × 9.1 mm), NCHUZOOL 17145, Guanyin Cave, Lyudao, coll. Y.-H. Huang, 26 Sep. 2021; 7 Å Å (21.4 × 16.9, $19.2 \times 14.5, 18.9 \times 14.3, 16.9 \times 13.0, 15.7 \times 11.8,$ $14.8 \times 11.2, 14.5 \times 11.1 \text{ mm}$), $2 \ P \ P \ (21.2 \times 16.2, 14.5 \times 11.1 \text{ mm})$ 18.7×14.3 mm), NCHUZOOL 14992, Haishenping, Lyudao, 21 Apr. 2012; 4 (15.0 × 11.5, 13.8 × 11.0, $13.7 \times 10.8, 13.0 \times 9.9$ mm), $1 \stackrel{\circ}{+} (13.5 \times 10.5$ mm), NCHUZOOL 14993, Haishenping, Lyudao, 21 Apr. 2012. G. lanyu: 1 $(12.5 \times 9.7 \text{ mm})$, $3 \neq \uparrow (17.9 \times 10^{-5} \text{ cm})$ 14.1, 17.0×13.1 , 16.5×12.7 mm), 1 ovig. $\stackrel{\circ}{=}$ (17.9 × 14.0 mm), NCHUZOOL 17160, Lanyu, coll. H.-C. Liu & C.-H. Wang, 21–22 Mar. 1996; 1 Å (11.1 × 8.7 mm), NCHUZOOL 17151, Langdao R., Lanyu, 1 Apr. 2008; 1 \Leftrightarrow (12.8 \times 9.5 mm), 1 $\stackrel{\circ}{+}$ (20.6 \times 15.5 mm), NCHUZOOL 14326, Hongtou, Lanyu, 4 Apr. 2015; 1 & (19.7 × 15.1 mm), NCHUZOOL 17162; Hongtou, Lanyu, 4 Apr. 2015; 1 ♀ (9.3 × 7.0 mm), NCHUZOOL 17146, Yeyou R., Lanyu, 8 Jan. 2008; $2 \Leftrightarrow \Leftrightarrow (9.0 \times 7.4)$ 5.3 × 3.9 mm), NCHUZOOL 17154, Yeyou R., Lanyu, 8 Jan. 2008; 1 broken $\stackrel{\circ}{\rightarrow}$ (12.1 mm), NCHUZOOL 17147, Yeyou South R., Lanyu, 8 Jan. 2008; 1 ♀ (15.2 × 11.7 mm), NCHUZOOL 17150, Yuren R., Lanyu, 11 Jul. 2003; 2 \uparrow \uparrow (15.9 × 12.7, 13.0 × 10.3 mm), NCHUZOOL 17161, Yuren R., Lanyu, coll. T.-P. Tseng, 11 Jul. 2003; 2 & & (18.7 × 14.5, 12.0 × 9.1 mm), 1 $\stackrel{\circ}{=}$ (18.0 × 14.1 mm), NCHUZOOL 17141, Yuren R., Lanyu, coll. K. Chang et al., 28 Sep. 2022; 1 3 (16.3) × 12.7 mm), NCHUZOOL 17149, Hongtou R., Lanyu, 13 Jul. 2003; 1 ♀ (14.1 × 10.9 mm), NCHUZOOL 17148, Longmen R., Lanyu, 2 Apr. 2008; 2 👌 👌 (15.1 \times 12.1, 13.6 \times 10.9 mm), 1 ovig. $\stackrel{\circ}{\rightarrow}$ (12.9 \times 10.2 mm), NCHUZOOL 17158, Longmen R., Lanyu, 2 Apr. 2008; 10.7, 12.9 × 9.8 mm), NCHUZOOL 14327, Longmen R., Lanyu, 3 Apr. 2015; 1 \diamond (13.5 × 10.5 mm), 2 \Diamond \Diamond $(17.4 \times 13.2, 16.3 \times 12.6 \text{ mm})$, NCHUZOOL 17142, Longmen R., Lanyu, coll. K. Chang et al., 2 Oct. 2022; 1 $(16.3 \times 12.3 \text{ mm})$, 2 $2 \neq 4$ (16.5 \times 13.0, 11.3 \times 9.0 mm), 1 ovig. ♀ (16.4 × 12.6 mm), NCHUZOOL 13059, Longmen R., Lanyu, 5 Jun. 1993; 5 👌 👌 (17.2 × 13.2, 15.2 × 11.7, 14.7 × 11.3, 14.4 × 11.1, 7.7 × 5.9 mm), $1 \stackrel{\circ}{\rightarrow} (14.0 \times 11.0 \text{ mm})$, NCHUZOOL 17143, Langdao R., Lanyu, 1 Apr. 2008.

RESULTS

Taxonomic identity of the *G. tawu* species complex

The CWs of male adult specimens used for comparison of legs and G1s (13.3–19.3 mm for *G. tawu*; 12.6–21.4 mm for *G. lutao*; and 12.0–19.7 mm for *G. lanyu*), as well as the ratios of DL/PL of P3–P5, ML/MW of P3, TLG1/TSL and SML/SMW of G1 are shown in table 2. From the scatter plots of the DL/PL ratios of P3–P5, as well as the TLG1/TSL (Fig. 3A–C) and SML/SMW ratios of G1 (Fig. 4), no clear patterns

can be found among the three species. However, the ML/MW ratios of P3 in *G. lanyu* tend to be higher (*i.e.*, more slender) than the other two species (Fig. 3D), but some specimens of the three species have very close values.

With regard to the terminal segment curvature of G1, all three species have specimens with straighter (Fig. 5A, C, E) and slightly curved (Fig. 5B, D, F) forms. Regarding the tooth on the outer proximal margin of the subterminal segment, both *G. tawu* and *G. lutao* have specimens with the tooth or without (Fig. 6A–D), but all specimens of *G. lanyu* have the tooth (Fig. 6E).



Fig. 3. (A–C) Ratios of dactyl length (DL) to propodus length (PL) of P3 (A), P4 (B) and P5 (C) plotted as a function of carapace width (CW) in male *G. tawu* (gray triangles), *G. lutao* (green squares) and *G. lanyu* (blue diamonds). (D) Ratios of merus length (ML) to merus width (MW) of P3 as a function of CW in males of three species.

Molecular analyses

A 550 bp segment of the 16S rDNA from 67 specimens and a 616–658 bp segment of *COI* from 57 specimens of *G. tawu*, *G. lutao* and *G. lanyu* were amplified and aligned. A total of 19 haplotypes of

16S gene were found for the three species, with one haplotype ("Gtw2") shared by *G. tawu* and *G. lanyu*; and only one haplotype ("Gtw16") found in *G. lutao* (Table 1). There were 33 haplotypes of *COI*, with 20 for *G. tawu*, six for *G. lutao* and seven for *G. lanyu*. Five shorter segments of *COI* using the primer COL14



Fig. 4. (A) Ratios of total length of G1 (TLG1) to terminal segment length of G1 (TSL) as a function of carapace width (CW) in *G. tawu* (gray triangles), *G. lutao* (green squares) and *G. lanyu* (blue diamonds). (B) Ratios of synovial membrane length (SML) to synovial membrane width (SMW) as a function of CW in three species. Empty symbols are for holotypes.



Fig. 5. Right G1s showing the degree of terminal segment curvature. (A) *G. tawu* (CW 17.9 mm, NCHUZOOL 17152). (B) *G. tawu* (CW 19.0 mm, NCHUZOOL 17163). (C) *G. lutao* (CW 18.2 mm, NCHUZOOL 13061). (D) *G. lutao* (CW 18.9 mm, NCHUZOOL 14992). (E) *G. lanyu* (CW 17.2 mm, NCHUZOOL 14327). (F) *G. lanyu* (CW 17.2 mm, NCHUZOOL 17143). Scale bars = 1 mm.

shared the same sequences with longer segments, viz. Gtw-C12=Gtw-C9, Gtw-C18=Gtw-C16, Gtw-C17=Gtw-C19, Glt-C6=Glt-C1 and Gly-C6=Gly-C1 (Table 1). Phylogenetic analysis based on the combined 16S and COI database with 1208 bp from 70 specimens showed one main clade with high supports by BI and ML methods (Fig. 7). However, even though the clades of G. lutao and G. lanvu are supported, haplotypes of G. tawu from the Taiwan main island do not form a clade or any clear groupings, even when excluding the more different Taimali haplotype ("Gtw1+Gtw-C1" in Figs. 7, 8; Fig. 1: no. 1). The haplotypes of G. lutao and those from Alangyi (Fig. 1: no. 4) and Shuanliou (Fig. 1: no. 5) from the Taiwan main island are very closely related and form another larger clade, named as "large G. lutao clade".

Based on the substitution rates of 16S and *COI*, the divergence time estimation for the main nodes (Fig. 7) showed the *G. tawu* species complex separated from related species about 1.16 mya (million years ago), and the divergence time between the Taimali haplotype and others is about 0.7 mya. The divergence times of *G. lutao* and *G. lanyu* from others are estimated as 0.2 and 0.33 mya, respectively. In the network of 16S+COI haplotypes (Fig. 8), haplotypes of *G. lutao* and *G. lanyu* are only separated from those from the Taiwan main island by ≥ 2 bp and ≥ 3 bp , respectively.

From the pairwise nucleotide divergences of K2P

distances and bp differences among *COI* haplotypes of the *G. tawu* species complex (Table 3), the maximum intraspecific nucleotide divergences (and bp differences) of *G. lanyu*, *G. lutao*, *G. tawu* and *G. tawu* excluding distinct haplotypes (from Shihwen and Taimali; Fig. 1: no. 9, 1, respectively; Figs. 7, 8) are $\leq 0.49\%$ (≤ 3 bp), $\leq 0.65\%$ (≤ 4 bp), $\leq 2.5\%$ (≤ 15 bp) and $\leq 1.48\%$ (≤ 9 bp), respectively. The minimum interspecific divergence (and bp difference) of the species complex are as small as 0.16-0.65% (1–4 bp).

DISCUSSION

Morphological comparison

According to Shy et al. (1994 2020) and Shy and Lee (2009), the main characters of ambulatory legs and G1 were useful to distinguish *G. tawu*, *G. lutao* and *G. lanyu*. For example, the merus of ambulatory legs are slender in *G. lanyu* (vs. stout in other two species) (Shy et al. 2020: 16). In our study, most *G. lanyu* specimens tend to have more slender ambulatory merus, but some are still close to the other two species (Table 2; Fig. 3D), which shows that this character is not very reliable. *Geothelphusa lanyu* also was thought to have longer dactyl to propodus ratios than that in *G. lutao* (Shy et al. 2020: 95), but the ratios of DL/PL of P3–P5 in the three



Fig. 6. Right G1s showing the outer proximal margin of subterminal segment with or without a tooth. (A) *G. tawu* (CW 17.8 mm, NCHUZOOL 17152). (B) *G. tawu* (CW 19.3 mm, NCHUZOOL 17163). (C) *G. lutao* (CW 15.7 mm, NCHUZOOL 14992). (D) *G. lutao* (CW 18.2 mm, NCHUZOOL 13061). (E) *G. lanyu* (CW 17.2 mm, NCHUZOOL 14327). Scale bars = 1 mm.

species are variable (Table 2; Fig. 3A–C) which shows that this character is not useful as well.

The characters of the G1 are generally used to separate species in freshwater crabs and do not vary very much between adult and subadult males (Shy et al. 2020: xxiv). Shy et al. (1994 2020) and WJ Chen et al. (2007) showed that *G. lanyu* has a more curved terminal segment and a larger ratio of TLG1/TSL than *G. lutao*, but the terminal segment curvature (Fig. 5) and ratios in G1 (Fig. 4) are also variable in the three species.

Shy et al. (1994 2020) and Shy and Lee (2009) showed *G. tawu* and *G. lanyu* have a tooth on the outer proximal margin of the subterminal segment, whereas *G. lutao* is toothless. However, all three species examined may have such a tooth (Fig. 6B, D, E), while some specimens of *G. tawu* and *G. lutao* are toothless (Fig. 6A, C), which shows that this character is not reliable.

Molecular analyses

In the phylogenetic tree based on 16S and *COI* constructed by BI and ML methods, only one main clade is found (Fig. 7). Although haplotypes from Lyudao and Lanyu form two clades, corresponding to *G. lutao* and *G. lanyu*, respectively, haplotypes of *G. tawu* from southern Taiwan do not form any clear groupings (Figs. 7, 8). In such case, only one taxa is supported genetically. A similar case has been found in the classification of fiddler crabs. In Shih et al. (2016b), although the haplotypes of *"Australuca* Crane, 1975" form a clade, others of *Tubuca* Bott, 1973 do not form another clade. As a result, the study concluded that only one large clade of *Tubuca* could be recognized, and *Australuca* was synonymized with *Tubuca*.

Regarding the distances within and between species, the minimum distances of 16S and COI of



Fig. 7. A Bayesian inference (BI) tree of the *Geothelphusa tawu* species complex and the related species, based on the combined 16S and *COI* markers. Values at the nodes are the support values for BI and maximum likelihood (ML) analyses. Only values > 50% are shown. The divergence times estimated are shown in reverse color beside the main nodes. For haplotype names, see table 1.

G. lutao and G. lanyu from G. tawu are only 2 bp and 3 bp, respectively (Fig. 8). For the 16S marker, our study found a similar result to Shih et al. (2004): the haplotype "Gtw2" is commonly shared between G. tawu and G. lanyu, but the haplotype "Gtw16" is unique in G. lutao (Table 1; Figs. 7, 8). For the intraspecific and interspecific distances of the barcoding COI marker (Table 3), the maximum intraspecific distances are equal or larger than the minimum interspecific distances, even when excluding the more distinct haplotypes from Shihwen and Taimali. For example, the maximum intraspecific distances of G. lutao and G. lanyu are 0.65% and 0.49%, but the minimum interspecific distances of the two species with G. tawu are 0.16% and 0.49% (Table 3). In addition, the minimum interspecific distance between G. tawu and G. lutao is only 0.16% (1 bp difference). As a result, the species delimitation of the three species can not be supported by 16S and *COI* distances. However, one specimen from the northern area (no. 1 on Fig. 1) has more different haplotypes (Figs. 7, 8) which might represent a cryptic species which should be studied by including more specimens.

Due to isolation by barriers such as mountains and oceans, freshwater crabs with weak dispersal ability may easily form new species due to the founder effect (Shih and Shy 2009; Schubart and Santl 2014). However, the isolated population is not necessarily a different species if the morphological and molecular evidence is not strong enough, *e.g.*, the freshwater crabs *Potamon elbursi* Pretzmann, 1962 in Iran (Keikhosravi et al. 2015), the crayfish *Astacus astacus* (Linnaeus, 1758) in Europe (Schrimpf et al. 2014), and the beetle



Fig. 8. Genealogical TCS network for the *COI* haplotypes observed from the *Geothelphusa tawu* species complex and other related congeneric species. Unlabelled nodes indicate inferred haplotypes not found in the sampled populations. For haplotype names, see table 1.

Osmoderma barnabita Motschulsky, 1845 in eastern Europe (Landvik et al. 2017). Based on the available morphological and molecular evidence, *G. tawu*, *G. lutao* and *G. lanyu* should be treated as a single species, with *G. lutao* and *G. lanyu* being synonymized under *G. tawu* (also see comments in Shih et al. 2004).

Explanation for the freshwater crab distributions in offshore islets

Because the specimens from Lyudao and Lanyu have unique *COI* haplotypes (Table 1, Figs. 7, 8), the populations in the two islets could be considered as two founder populations that may still be in the speciation processes. The divergence times of the populations in Lyudao and Lanyu are estimated as 0.2 and 0.33 mya, respectively (Fig. 7), which are very recent compared with the divergence time of > 5 mya of *Candidiopotamon rathbuni* in Taiwan main island (Shih et al. 2006) caused by tectonic events. In addition, the divergence time of *G. lutao* is more recent because three more basal haplotypes (Fig. 7) found in the Taiwan main island (nos. 4 and 5 on Fig. 1) are within the "large *G. lutao* clade." It is suggested that the cladogenesis event of this clade occurred on the Taiwan main island at 0.33 mya with a branch that colonized Lyudao islet

Table 2. The ratios of different segments of P3-P5 and G1 (Fig. 2) in the Geothelphusa tawu species complex

Species	CW (mm)	DL/PL of P3	DL/PL of P4	DL/PL of P5	ML/MW of P3	TLG1/TSL of G1	SML/SMW of G1	NCHUZOOL
G. tawu	18.4					5.1	3.4	(holotype*)
	19.3	1.1	1.1	1.0	3.1	6.6	3.3	17163
	19.0	1.1	1.2	1.1	3.5	5.8	2.5	17163
	18.8	1.1	1.1	1.1	3.4	5.7	3.5	13057
	17.9	1.2	1.2	1.2	3.5	6.1	2.8	17152
	17.8	1.1	1.2	1.1	3.4	6.5	3.4	17152
	17.5	1.2	1.2	1.2	3.2	5.8	3.8	17144
	16.6	1.3	1.3	1.1	3.2	-	-	13057
	15.6	1.1	1.1	1.1	3.1	-	-	17164
	14.5	1.2	1.2	1.2	3.2	5.5	-	13155
	13.3	1.0	1.0	1.0	3.4	5.3	3.2	13283
G. lutao	21.4					5.7	2.7	(holotype*)
	21.4	1.1	1.1	1.0	3.5	6.7	3.3	14992
	19.2	1.2	1.2	1.1	3.5	5.7	3.7	14992
	18.9	1.1	1.1	1.2	3.4	6.4	3.5	14992
	18.5	1.2	1.2	1.2	3.6	5.6	-	13060
	18.2	1.2	1.2	1.2	3.6	5.5	3.2	13061
	16.9	1.2	1.2	1.1	3.0	5.9	3.2	14992
	15.7	1.1	1.2	1.2	3.2	5.9	-	14992
	15.0	1.0	1.2	1.2	3.1	5.5	2.5	14993
	13.7	1.1	1.2	1.2	3.3	5.4	3.2	14993
	12.6	1.2	1.2	1.1	3.2	-	-	17145
G. lanyu	20.2					7.2	2.9	(holotype*)
	19.7	1.1	1.2	1.1	3.8	6.3		17162
	18.7	1.1	1.1	1.1	3.9	6.2	3.8	17141
	17.2	1.1	1.2	1.2	3.6	5.7	2.9	17143
	17.2	1.2	1.1	1.2	3.9	6.9	3.6	14327
	16.9	1.1	1.3	1.2	4.4	-	-	14327
	16.3	1.2	1.1	1.1	3.9	6.2	-	17149
	15.1	1.2	1.2	1.2	3.8	5.8	-	17158
	14.7	1.1	1.2	1.1	3.7	-	-	17143
	14.4	1.2	1.2	1.0	3.8	6.1	3.2	17143
	13.6	1.1	1.1	1.1	3.9	6.6	3.0	17158
	12.0	1.2	1.1	1.1	3.5	-	-	17141

DL, dactyl length; PL, propodus length; ML, merus length; MW, merus width; TLG1, total length of G1, TSL, terminal segment length; SML, synovial membrane length; SMW, synovial membrane width. *, ratios of the G1s of holotypes are based on the drawings in Shy et al. (2020).

at 0.2 mya. The more plausible explanation for the colonization in Lyudao and Lanyu is hypothesized as rafting dispersal during glacial maxima.

The glacial maxima events that occurred at about 0.15, 0.25 and 0.35 mya (Compton 2011) more or less agree with the divergence times estimated to be 0.2 and 0.33 mya (Fig. 7). During the stages of glacial maxima, the sea level was much lower, which might have changed the flow of the Paleo-Kuroshio Current to the east of the Ryukyu Arc (see Shih et al. 2022a), with reduced flow in the east of southern Taiwan. Another hypothesis argues that the Paleo-Kuroshio Current might still have flowed into the Okinawa Trough, but the volume transport was significantly reduced (Kao et al. 2006).

The transoceanic dispersal of freshwater crabs has been proposed for their colonization in Madagascar from the African continent (Daniels et al. 2006). An experimental study has also supported that some species of *Esanthelphusa* Naiyanetr, 1994 (Gecarcinucidae) and *Eosamon* Yeo & Ng, 2007 (Potamidae) can stay totally within sea water for > 2 weeks (Esser and Cumberlidge 2011). In an unpublished study (MW Chen 2008: table 15), many individuals of *G. ferruginea* and *G. pingtung* Tan & Liu, 1998 from southern Taiwan survived in a seawater environment for > 20 days and 30 days, respectively. Therefore, the founder populations of freshwater crabs in Lyudao and Lanyu are hypothesized as the result of dispersal by rafting of floating vegetation from the Taiwan main island when the flow of the Paleo-Kuroshio Current was weaker during glacial maxima at 0.2–0.33 mya (Fig. 7).

Because of the shared 16S haplotype of specimens from Lanyu and the Taiwan main island, Shih et al. (2004) have hypothesized that modern typhoons, migratory birds, and human introduction enabled the colonization of *Geothelphusa* in Lyudao and Lanyu. However, these hypotheses should be rejected given our results from higher resolution 16S and *COI*, which estimate that the dispersal events occurred during the Middle Pleistocene.

CONCLUSIONS

The three species can not be distinguished by the key characters used previously, including the ratios of different segments of P3–P5 and G1, as well as the curvature of the G1 terminal segment and the existence of a tooth on the outer margin of the G1 subterminal segment. The molecular analyses based on the phylogenetic tree and TCS network of the 16S and *COI*, as well as the barcoding distances, also can not support the species delimitation of the three species. However, our results suggest two founder populations in Lyudao and Lanyu, as they have unique haplotypes of *COI*. The colonizations in the two offshore islets are hypothesized as transoceanic dispersal by rafting from the Taiwan main island during glacial maxima when the Paleo-Kuroshio Current was weak in this region.

Table 3. Matrix of percentage of pairwise nucleotide divergences with Kimura 2-parameter (K2P) distances and number of basepair (bp) differences based on the cytochrome *c* oxidase subunit I (*COI*) gene within and between species of the *Geothelphusa tawu* species complex. In the right half, lower-left values are K2P distances and upper-right ones are bp differences. Range of values is given in parentheses

	Intraspecific			Interspecific						
	nucleotide divergence	bp difference	G. lanyu	G. lutao	G. tawu	G. tawu*	Shihwen haplotype	Taimali haplotype		
G. lanyu	0.1 (0–0.49)	0.6 (0-3)		6.57 (4–10)	5.54 (3–13)	5.11 (3–9)	9.3 (9–11)	12.2 (12–13)		
G. lutao	0.26 (0-0.65)	1.6 (0-4)	1.08 (0.65–1.65)		7.3 (1–16)	6.81 (1–11)	12.27 (10–14)	14.27 (12–16)		
G. tawu	1.02	6.22 (0–15)	0.91 (0.49–2.15)	1.2 (0.16–2.67)						
G. tawu*	0.86 (0-1.48)	5.24	0.83	1.12 (0.16–1.82)			10.63 (8–13)	12.88 (11–15)		
Shihwen haplotype	· /		1.53	2.03 (1.65–2.32)		1.75 (1.31-2.15)		12 (12–12)		
Taimali haplotype			2.01 (1.98–2.15)	2.37 (1.99–2.67)		2.13 (1.82–2.5)	1.98 (1.98–1.98)	()		

*, excluding the more distinct haplotypes from Shihwen and Taimali (Fig. 1: no. 9 and no. 1, respectively; Figs. 7, 8).

Acknowledgments: This study was supported by grants from the Ministry of Science and Technology (MOST 105-2621-B-005-002-MY3; 111-2621-B-005-003), Executive Yuan, Taiwan, to HTS. Thanks are also due to Hung-Chang Liu, Chia-Hsiang Wang, Jhy-Yun Shy, Yin-Ju Yang, Jheng-Jhang Li and the members of HTS's lab for providing samples used in this study. We acknowledge Chao Huang and one anonymous referee who greatly improved the manuscript, as well as Peter K. L. Ng for providing comments.

Authors' contributions: HTS conceived this study, performed the molecular genetic analysis, and drafted the manuscript. JWH performed the morphological comparison and discussion, and drafted the manuscript. KC collected specimens, performed the morphometric analyses and drafted the manuscript. MWC performed the molecular work and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests: HTS, JWH, KC and MWC declare that they have no conflict of interest.

Availability of data and materials: Sequences generated in the study were deposited into the GenBank database (accession numbers in Table 1).

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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