

# Phylogeny and Phylogeography of the Genus *Geothelphusa* (Crustacea: Decapoda, Brachyura, Potamidae) in Southwestern Taiwan Based on Two Mitochondrial Genes

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Eleven species of *Geothelphusa* have been reported from southwestern Taiwan (Tainan, Kaohsiung and the northern part of Pingtung counties): *G. albogilva* Shy, Ng, and Yu, 1994; *G. ancylophallus* Shy, Ng, and Yu, 1994; *G. caesia* Shy, Ng, and Yu, 1994; *G. lili* Chen, Cheng, and Shy, 2005; *G. nanhsi* Shy, Ng, and Yu, 1994; *G. neipu* Chen, Cheng, and Shy, 1998; *G. olea* Shy, Ng, and Yu, 1994; *G. pingtung* Tan and Liu, 1998; *G. shernshan* Chen, Cheng, and Shy, 2005; *G. tsayae* Shy, Ng, and Yu, 1994 and *G. wutai* Shy, Ng, and Yu, 1994. Comparisons of DNA sequences encoding parts of the mitochondrial large subunit (16S) rRNA and cytochrome oxidase subunit I (COI) genes revealed three major clades, of which one is the species *G. ancylophallus*, and the other two are species groups here referred to as the *G. olea* and *G. pingtung* clades. *Geothelphusa ancylophallus* is geographically restricted and adapted to an ecologically challenging habitat with an unstable water supply and uneven topology. The *G. olea* clade (*G. olea*, *G. caesia*, *G. nanhsi*, *G. tsayae*, and *G. wutai*) is widely distributed throughout central-western and southwestern Taiwan. The *G. pingtung* clade (*G. pingtung*, *G. neipu* and *G. shernshan*) is confined to southwestern Taiwan between the previously defined southernmost clades of *G. tawu*, *G. albogilva*, and *G. ferruginea*, and the *G. olea* clade to the north. It includes an isolated population on distant Chaishan Mountain near Taiwan Strait, which probably dispersed from the peripheral hills of the Central Range during the early Pleistocene. The available genetic evidence indicates that the differential coloration observed in members of the *G. olea* and *G. pingtung* clades is not reflected in mtDNA, appears to be dependent on environmental conditions, food, etc., and has little value as a taxonomic character. Possible geological events and climatic factors responsible for the historic isolation of the different freshwater crab clades in southwestern Taiwan are discussed in detail.

**Key words:** phylogeography, mitochondrial genes, southwestern Taiwan, freshwater crabs

## INTRODUCTION

Freshwater crabs of the genus *Geothelphusa* are restricted to the East Asian islands of Taiwan, the Ryukyus and the rest of Japan (Shy and Yu, 1999; Yoshigou, 1999). More than 30 species of this genus are known from Taiwan, of which 11 (*G. albogilva* Shy, Ng and Yu, 1994; *G. ancylophallus* Shy, Ng and Yu, 1994; *G. caesia* Shy, Ng and Yu, 1994; *G. lili* Chen, Cheng and Shy, 2005; *G. nanhsi* Shy, Ng and Yu, 1994; *G. neipu* Chen, Cheng and Shy, 1998; *G.*

*olea* Shy, Ng and Yu, 1994; *G. pingtung* Tan and Liu, 1998; *G. shernshan* Chen, Cheng and Shy, 2005; *G. tsayae* Shy, Ng and Yu, 1994 and *G. wutai* Shy, Ng and Yu, 1994) have been reported from southwestern Taiwan (Tainan, Kaohsiung, and northern Pingtung counties) (Fig. 1) (Shy *et al.*, 1994, 1996, 2000; Tan and Liu, 1998; Chen *et al.*, 1998, 2001, 2003, 2005; Shy and Yu, 1999). Recently, Shih *et al.* (2004) analyzed the relationships of species of *Geothelphusa* from southern Taiwan based on mitochondrial 16S rRNA sequences and argued that *G. neipu* was in fact a junior synonym of *G. pingtung*. That study also indicated that two species from offshore islands, *G. lanyu* Shy, Ng, and Yu, 1994 (from Lanyu) and *G. lutao* Shy, Ng, and Yu, 1994 (from Lyudao) may be synonyms of the closely allied *G. tawu* Shy, Ng, and Yu, 1994, which is widely distributed bet-

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ween the Dawu River (Taitung County) and the Fenggang River (Pingtung County) (Fig. 1), based on insignificant differences in mitochondrial (mt) 16S rRNA sequences and minor differences in adult morphology. Recently, Chen *et al.* (2001, 2003) argued for the existence of several additional species of *Geothelphusa* in the northern and central regions of Pingtung County, but these assertions need to be carefully re-examined in light of the present work.

The present study builds on the study by Shih *et al.* (2004) and examines mitochondrial gene sequences of *Geothelphusa* species from southwestern Taiwan. Most of southwestern Taiwan is made up of lowland plains (*i.e.* Chianan, Kaohsiung, and Pingtung Plains) that are all less than 200 m above sea level. Only a small area near the Central Range consists of hills or mountains (Fig. 1). We compared DNA sequences of the mitochondrial large subunit (16S) rRNA and cytochrome oxidase I (COI) genes, which are the most commonly used genes for phylogenetic and population studies of arthropods (see reviews by Hwang and Kim, 1999; Schubart *et al.*, 2000; Cruickshank, 2002), to test the validity of putative species of *Geothelphusa* in southwestern Taiwan, to quantify the extent of genetic diversity within the group, and to determine ecological characters and possible biogeographic boundaries.

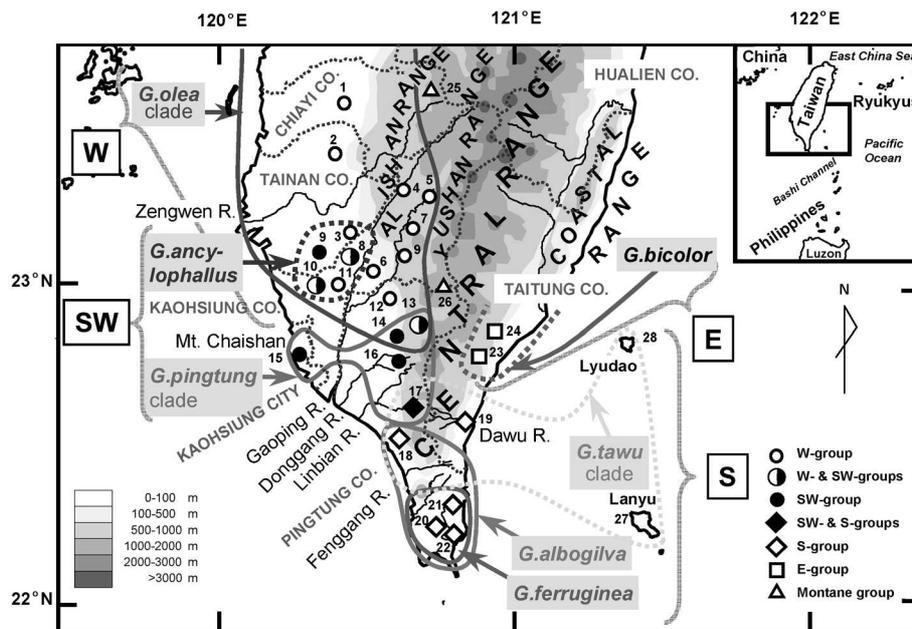
## MATERIALS AND METHODS

Between 1996 and 2004, 68 specimens of *Geothelphusa* were collected from streams in Chiayi County, Tainan County, Kaohsiung County, Kaohsiung City and the northern part of Pingtung County (Fig. 1). The material included all 11 described species from the area, namely, *G. albogilva*, *G. ancylophallus*, *G. caesia*, *G. lili*, *G. nanhsi*, *G. neipu*, *G. olea*, *G. pingtung*, *G. shernshan*, *G. tsayae*, and *G. wutai* (see Shy *et al.*, 1994; Shy and Yu, 1999) (Table 1). Specimens of *G. ancylophallus*, *G. lili*, *G. pingtung*, *G. shernshan*, and *G. wutai* were collected from their type localities, while speci-

mens of *G. caesia*, *G. nanhsi*, *G. neipu*, *G. olea*, and *G. tsayae* were collected from the vicinity of their type localities. In addition, specimens of *G. albogilva*, *G. bicolor*, *G. ferruginea*, *G. lanyu*, *G. lutao*, *G. tawu* (all from, or from the vicinity of, the type localities), and a number of undetermined specimens (from Chiayi, Kaohsiung, and Taitung counties) were included for comparison. *Geothelphusa miyazakii* Miyake and Chiu, 1965 from northern Taiwan (Yangmingshan, Taipei City), as well as *G. aramotoi* Minei, 1973 and *G. sakamotoana* (Rathbun, 1905) from the Ryukyus (Okinawa), served as outgroups. All specimens were preserved in 75–95% ethanol after collection and deposited in the Zoological Collections of the Department of Life Science, National Chung Hsing University (NCHU-ZOOL; see Table 1 for catalog numbers).

Genomic DNA was isolated from the muscle tissue of legs by phenol-chloroform extraction (Kocher *et al.*, 1989) or by using the Sigma Mammalian Genomic DNA Miniprep Kit. A region of approximately 550 bp of the 5' end of the 16S rRNA gene was selected for amplification by polymerase chain reaction (PCR) using primers 1471 (5'-CCTGTTTANCAAAAACAT-3') and 1472 (5'-AGATAGAA-ACCAACTGG-3') (Crandall and Fitzpatrick, 1996). A portion of the mitochondrial COI gene was amplified by PCR using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer *et al.*, 1994). An internal primer designed by Roman and Palumbi (2004) for *Carcinus maenas* (5'-GCTTGAGCTGGCATTAGTAGG-3') was also used. The PCR conditions for the above primers were 50 s at 94°C / 70 s at 45°C / 60 s at 72°C (40 cycles), followed by a 72°C extension for 10 minutes. Sequences were obtained by automated sequencing (ABI PRISM 377 Sequencer and MegaBACE DNA Analysis System 500) and were aligned with the aid of Clustal W version 1.4 (Thompson *et al.*, 1994) and BioEdit version 5.09 (Hall, 2001), after verification with the complimentary strand. Sequences of the different haplotypes have been deposited in the DDBJ databases (accession nos. are shown in Table 1).

The best-fitting model for sequence evolution of the combined 16S and COI data set was determined by MrModeltest version 2.2 (Nylander, 2005), selected by hLRT (hierarchical likelihood ratio test), and was subsequently applied for the analyses of minimum



**Fig. 1.** Collection sites for species of *Geothelphusa* in southwestern and southern Taiwan. For locality names and haplotypes, see Table 1. The differently grayed lines indicate the possible biogeographic boundaries for each of the main clades based on the molecular results of our study. W, western group; SW, southwestern group; S, southern group; E, eastern group; Co, County; R, River.

**Table 1.** Forty-three haplotypes of the 16S rRNA gene and 52 haplotypes of the COI gene of *Geothelphusa*, corresponding to specimens collected from southwestern Taiwan and adjacent areas (Fig. 1). Most species were identified based on Shy *et al.* (1994) and Shy and Yu (1999). If the 16S sequence of the species identified did not correspond to the actual species, the species name is given in quotation marks. Co., County. Specimens collected from, or from the vicinity of, the type locality are marked with “\*” or “†” following the species name, respectively.

Regions	Species identified	Localities	No. of localities in Fig. 1	Catalogue no. of museum (NCHUZOO)	Sample size	Haplotypes of 16S	DBJ Accession no.	Haplotypes of COI	DBJ Accession no.
<b>Western region (W)</b>					<b>22</b>				
	<i>G. olea</i>	Chiayi City (Botanical Garden)	1	13009	2	Go-01, 02	AB266149, 266150	Go-C01, C02	AB266262, 266263
	<i>G. olea</i> <sup>†</sup>	Tainan Co. (Dakeng, Dongshan)	2	13010	1	Go-03	AB266151	Go-C02	AB266263
	<i>G. nanhsi</i> <sup>†</sup>	Tainan Co. (Jingmian, Nanhua)	3	13011	1	Go-04	AB266152	Go-C04	AB266264
	<i>G. olea</i> <sup>†</sup>	Kaohsiung Co. (Mincyuan, Sanmin)	4	13012	1	Go-05	AB266153	Go-C05	AB266265
	<i>G. caesia</i> <sup>†</sup>	Kaohsiung Co. (Meinong)	6	13013	1	Go-05	AB266153	Go-C05a	AB266266
	<i>G. tsayae</i>	Kaohsiung Co. (Mujha, Neimen)	8	13014	1	Go-05	AB266153	Go-C05b	AB266267
	<i>G. tsayae</i>	Kaohsiung Co. (Dagangshan, Alian)	10	13015	1	Go-05	AB266153	Go-C05c	AB266268
	<i>G. caesia</i> <sup>†</sup> , <i>G. tsayae</i> <sup>†</sup>	Kaohsiung Co. (Baolai, Liouguei)	7	13016, 13017	2	Go-06	AB266154	Go-C06, C06a	AB266269, 266270
	<i>G. caesia</i> <sup>†</sup>	Kaohsiung Co. (Meinong)	6	13018	1	Go-07	AB266155	Go-C07	AB266271
	<i>G. tsayae</i> <sup>†</sup>	Kaohsiung Co. (Fusing)	5	13019	2	Go-08, 09	AB266156, 266157	Go-C08, C09	AB266272, 266273
	<i>G. olea</i>	Kaohsiung Co. (Tianliao)	11	13020	1	Go-10	AB266158	Go-C10	AB266274
	<i>G. tsayae</i>	Kaohsiung Co. (Tianliao)	11	13021	1	Go-11	AB266159	Go-C05c	AB266268
	<i>G. tsayae</i>	Kaohsiung Co. (Meinong)	6	13022, 13023	2	Go-12	AB266160	Go-C12, C12a	AB266275, 266276
	<i>G. tsayae</i>	Pingtung Co. (Cingshan, Sandimen)	12	13024	1	Go-12	AB266160	C12b	AB266277
	<i>G. tsayae</i>	Pingtung Co. (Cingshan, Sandimen)	12	13025	1	Go-13	AB266161	Go-C13	AB266278
	<i>G. wutai</i> *	Pingtung Co. (Wutai)	13	13026, 13027, 13028	3	Go-14	AB266162	Go-C14	AB266279
<b>Southwestern region (SW)</b>					<b>23</b>				
	<i>G. ancylophallus</i> <sup>†</sup>	Tainan Co. (Longchuan, Longci)	9	13029	1	Gan-1	AB266163	Gan-C1	AB266280
	<i>G. ancylophallus</i> *	Kaohsiung Co. (Mujha, Neimen)	8	13030	1	Gan-2	AB266164	Gan-C2	AB266281
	<i>G. ancylophallus</i> <sup>†</sup>	Kaohsiung Co. (Dagangshan, Alian)	10	13031	1	Gan-2	AB266164	Gan-C2a	AB266282
	<i>G. albogilva</i>	Kaohsiung City (Chaishan (=Shoushan), Gushan)	15	13032	2	Gp-1	AB266165	Gp-C1	AB266283
	<i>G. albogilva</i>	Kaohsiung City (Chaishan, Gushan)	15	13033	1	Gp-1	AB266165	Gp-C1	AB266283
	<i>G. albogilva</i>	Kaohsiung City (Chaishan, Gushan)	15	13034	1	Gp-2	AB266166	Gp-C2	AB266284
	<i>G. shernshan</i> *	Pingtung Co. (Shenshan, Wutai)	13	13035	3	Gp-3	AB127363	Gp-C3	AB266285
	<i>G. pingtung</i> *	Pingtung Co. (Taiwu)	16	13036	1	Gp-4	AB127365	Gp-C4	AB266286
	<i>G. albogilva</i>	Pingtung Co. (Lili, Chunrih)	17	13037	1	Gp-4	AB127365	Gp-C4	AB266286
	<i>G. olea</i> , <i>G. pingtung</i> *	Pingtung Co. (Taiwu)	16	13038, 13039	2	Gp-4	AB127365	Gp-C4a	AB266287
	<i>G. neipu</i> <sup>†</sup> , “ <i>G. caesia</i> ”	Pingtung Co. (Liangshan, Majia)	14	13040, 13041	2	Gp-5	AB127364	Gp-C5, Gp-C5a	AB266288, 66289
	<i>G. pingtung</i> <sup>†</sup>	Pingtung Co. (Liangshan, Majia)	14	13042	2	Gp-5	AB127364	Gp-C5b	AB266290
	<i>G. pingtung</i> <sup>†</sup>	Pingtung Co. (Liangshan, Majia)	14	13043	1	Gp-5	AB127364	Gp-C5b	AB266290
	<i>G. pingtung</i> <sup>†</sup>	Pingtung Co. (Liangshan, Majia)	14	13044, 13045	2	Gp-5	AB127364	Gp-C5c	AB266291
	<i>G. pingtung</i> <sup>†</sup>	Pingtung Co. (Liangshan, Majia)	14	13046, 13047	2	Gp-6,7	AB266167, 266168	Gp-C5c	AB266291
<b>Southern region (S)</b>					<b>15</b>				
	<i>G. albogilva</i>	Pingtung Co. (Shihwen, Chunrih)	18	13048	1	Ga-1	AB127373	Ga-C1	AB266292
	<i>G. albogilva</i>	Pingtung Co. (Shihwen, Chunrih)	18	13049, 13050	2	Ga-2,3	AB127371, 127372	Ga-C2	AB266293
	<i>G. albogilva</i>	Pingtung Co. (Shihwen, Chunrih)	18	13051	1	Ga-4	AB127370	Ga-C2	AB266293
	<i>G. albogilva</i> <sup>†</sup>	Pingtung Co. (Nanrenshan, Manjhou)	21	13052	1	Ga-5	AB127366	Ga-C5	AB266294
	<i>G. albogilva</i> <sup>†</sup>	Pingtung Co. (Maozaikengnei, Hengchun)	20	13053	1	Ga-5	AB127366	Ga-C5a	AB266295
	<i>G. albogilva</i> <sup>†</sup>	Pingtung Co. (Gangko R., Manjhou)	22	13054	1	Ga-5	AB127366	Ga-C5b	AB266296
	<i>G. ferruginea</i>	Pingtung Co. (Shihwen, Chunrih)	18	13055	1	Gtw-1	AB127375	Gtw-C1	AB266297
	<i>G. lili</i> *	Pingtung Co. (Cijia (Lili), Chunrih)	17	13056, 13057	2	Gtw-2,3	AB127377, 127376	Gtw-C2,3	AB266298, 266299
	<i>G. tawu</i> *	Taitung Co. (Dawu R., Dawu)	19	13058	1	Gtw-4	AB127381	Gtw-C4	AB266300
	<i>G. lanyu</i> *	Taitung Co. (Lanyu)	27	13059	1	Gtw-5	AB127380	Gtw-C5	AB266301
	<i>G. luto</i> *	Taitung Co. (Lyudao)	28	13060, 13061	2	Gtw-6	AB127382	Gtw-C6, C6a	AB266302, 266303
	<i>G. ferruginea</i> <sup>†</sup>	Pingtung Co. (Maozaikengnei, Hengchun)	20	13062	1	Gf-1	AB127383	Gf-C1	AB266304
<b>Eastern region (E)</b>					<b>2</b>				
	<i>G. bicolor</i> *	Taitung Co. (Jihben, Jinfeng)	24	13064	1	Gb-1	AB127384	Gb-C1	AB266306
	<i>G. sp. 4</i>	Taitung Co. (Taimali)	23	13063	1	Gsp-4	AB127385	Gsp-C4	AB266305
<b>Montane region (M)</b>					<b>6</b>				
	<i>G. sp. 1</i>	Chiayi Co. (Alishan R., Alishan)	25	13065	1	Gsp-1	AB266169	Gsp-C1	AB266307
	<i>G. sp. 1</i>	Chiayi Co. (Alishan R., Alishan)	25	13066	2	Gsp-1	AB266169	Gsp-C1	AB266307
	<i>G. sp. 1</i>	Chiayi Co. (Alishan R., Alishan)	25	13067	1	Gsp-1	AB266169	Gsp-C1a	AB266308
	<i>G. sp. 2</i>	Kaohsiung Co. (Yuguting, Daguehu, Maolin)	26	13068	1	Gsp-2	AB266170	Gsp-C2	AB266309
	<i>G. sp. 3</i>	Kaohsiung Co. (Yuguting, Daguehu, Maolin)	26	13069	1	Gsp-3	AB266171	Gsp-C3	AB266310
<b>All localities</b>					<b>68</b>				
<b>Outgroups</b>									
	<i>G. miyazakii</i>	Yangmingshan, Taipei City		13070	1	Gmy-1	AB266172	Gmy-C1	AB266311
	<i>G. aramotoi</i>	Northern Okinawa, the Ryukyus		13071	1	Gar-1	AB266173	Gar-C1	AB266312
	<i>G. sakamotoana</i>	Southern Okinawa, the Ryukyus		13072	1	Gsk-1	AB266174	Gsk-C1	AB266313

evolution (ME) and Bayesian inference (BI). The ME tree was constructed with the program MEGA2 version 2.1 (Kumar *et al.*, 2001) with the gamma correction obtained from MrModeltest, the Kimura (1980) two-parameter model, and 2,000 bootstrap reiterations (CNI level=2, initial tree=NJ, and maximum number of trees to retain=1). A maximum parsimony (MP) tree was constructed using the program PAUP\* version 4.0b10 (Swofford, 2001) with 2,000 bootstrap reiterations of a simple heuristic search, TBR branch-swapping (tree bisection-reconnection), and 100 random-addition sequence replications. All characters were equally weighted. Gaps in MP tree construction were treated as missing data. In order to avoid excessive computation time, the total number of rearrangements for each search was limited to 500,000 for the MP analysis. Bayesian analyses (BI) were performed with MrBayes version 3.1.1 (Ronquist and Huelsenbeck, 2003) using the model selected by MrModeltest. The search was run with four chains for one million generations, with trees sampled every 100 generations (the first 500 trees were later discarded as "burn-in"). The best-fitting model determined by MrModeltest was also applied to estimate inter- and intrapopulation genetic diversity with PAUP\*.

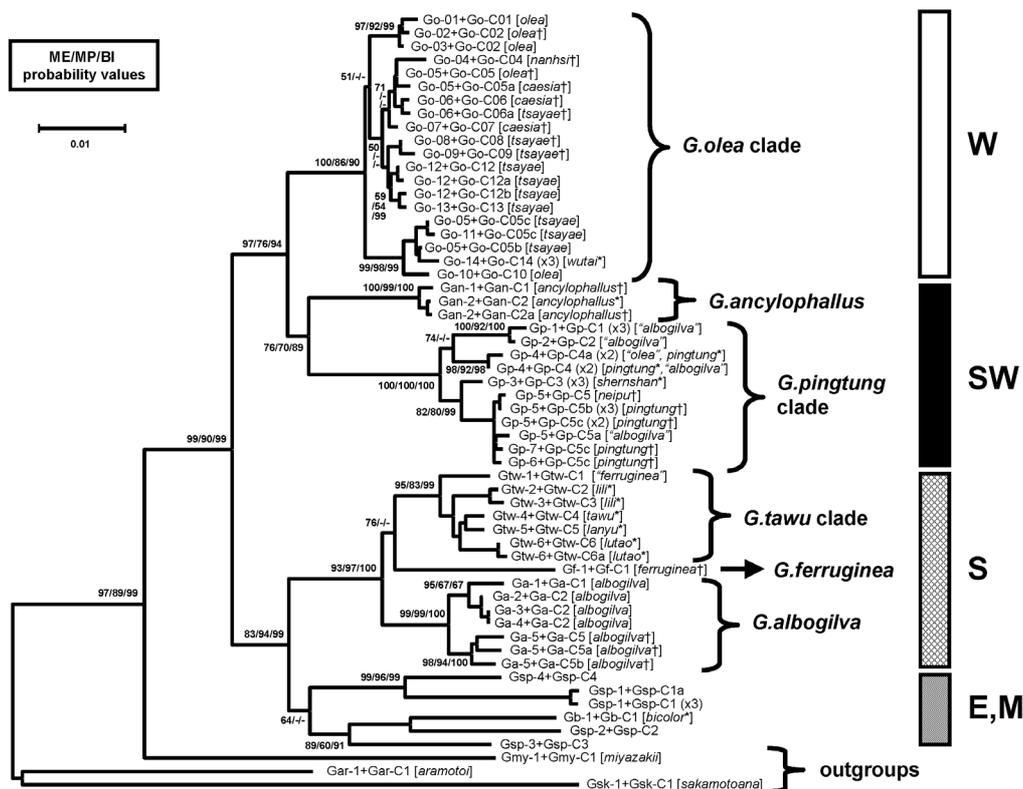
Phylogenetic reconstructions identified a clade around *Geothelphusa pingtung* composed of very closely related haplotypes, which included individuals collected from Chaishan (=Shoushan), Kaohsiung City, and the southwestern side of the Central Range. To examine the relationships of these haplotypes in detail, a gene genealogy of combined 16S rRNA and COI was constructed using the program TCS version 1.20 (Clement *et al.*, 2000) with gaps treated as a missing data.

## RESULTS

A 557-bp segment (excluding the primer regions) of the 16S rRNA gene was amplified from all 68 specimens and aligned; 99 positions were variable and 57 parsimony informative. Among the total number of sequences, 43 different haplotypes were distinguished (Table 1). The studied segment of the 16S sequences was AT rich (72.6%) (T, 36.9%; A, 35.7%; G, 17.1%; C, 10.3%). For the COI gene, a 616-bp segment was compared, resulting in 52 different haplotypes. The studied segment of the COI sequences was also AT rich (66.2%) (T, 37.4%; A, 28.8%; G, 15.4%; C, 18.4%). In this gene, 177 positions were variable and 140 parsimony informative.

The best model selected by MrModeltest was the GTR+I+G model (Rodriguez *et al.*, 1990) for the combined 16S and COI segment of 1,173 bp (proportion of invariable sites=0.6796, gamma distribution shape parameter=1.4465). The phylogenetic tree constructed by ME analysis, with the respective confidence values from the ME, MP, and BI analyses, is shown in Fig. 2. Only confidence values larger than 50% are shown. For MP, a single tree was recovered with a tree length of 614 steps, a consistency index of 0.54, and a retention index of 0.84.

Based on Fig. 2, three groups (W, SW, and S) corre-



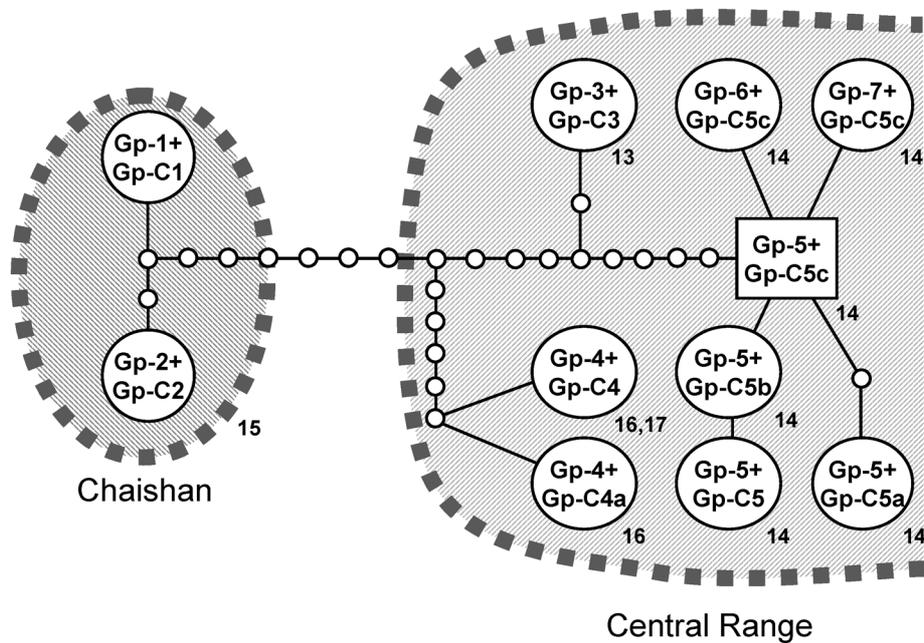
**Fig. 2.** Minimum evolution (ME) tree of the species of *Geothelphusa* in southwestern and southern Taiwan based on 1,173 bp of the combined 16S rRNA and cytochrome oxidase I genes. Probability values at the nodes represent bootstrap values for ME and maximum parsimony (MP), and posterior probability for Bayesian inference (BI). Species names following the haplotypes are the species identified according to morphology and coloration. Specimens collected from, or from the vicinity of, the type locality are indicated by "\*" or "+" following the species name, respectively. For abbreviations and haplotypes, see Table 1.

sponding to distinct geographic regions (Fig. 1) could be separated with high support. One monophyletic group ranges from Chiayi, Tainan, Kaohsiung to Wutai of Pingtung County and is here labeled as group W (western Taiwan). Five species (*G. caesia*, *G. nanhsi*, *G. olea*, *G. tsayae*, *G. wutai*) belong to this clade, which is here referred to as the *G. olea* clade (see Discussion for details). Similarly, the sister clades corresponding to *G. ancylorhynchus* and species around *G. pingtung* are here assigned to the SW (southwestern Taiwan) group. The clades of *G. tawu* (including *G. lanyu* and *G. lutao*), *G. albogilva*, and *G. ferruginea* were previously treated in Shih *et al.* (2004) with 16S and are here further supported by the combined 16S-COI tree. They represent the southern (S) Taiwanese group. In Fig. 2, the W and SW groups are more closely related to each other than to the S group. In contrast, the E (eastern) and M (montane) forms are related to the S group, with relatively high

confidence values. However, the proposed relationships between the E and M groups are supported by low confidence values.

The haplotype network constructed to further depict the relationships among the combined 16S and COI haplotypes of the *G. pingtung* clade is shown in Fig. 3. The combined haplotype Gp-5+Gp-C5c is central relative to all other haplotypes and is therefore assumed to represent the ancestral haplotype of the combined 16S and COI (*cf.* Clement *et al.*, 2000).

For the six main species clades shown in Fig. 2, the pairwise nucleotide divergences under the GTR+I+G model, and the mean number of intra- and interclade differences, are shown for 16S and COI in Tables 2 and 3, respectively. For the 16S gene, the genetic distance within each clade (ranging from 0.23% to 0.64%, average=0.47%) is significantly lower and non-overlapping in comparison to the dis-



**Fig. 3.** Genealogical network for the combined 16S rRNA and COI haplotypes observed within the *Geothelphusa pingtung* clade, which included specimens collected from Chaishan, Kaohsiung City, and the Central Range, Taiwan (Fig. 1). The ancestral haplotype, or root of the network, is indicated by a square. Unlabelled nodes indicate inferred haplotypes not found in the sampled populations. The numbers beside nodes correspond to the collection sites in Table 1 and Fig. 1.

**Table 2.** Matrix of percent pairwise nucleotide divergence (lower left) and mean number of differences (upper right) among the main species clades of *Geothelphusa* collected from southwestern Taiwan and adjacent areas, based on 557 bp of the 16S rRNA gene. Nucleotide divergence was estimated from pairwise distances of nucleotides based on the GTR+I+G model of nucleotide substitution. Abbreviations for clades: Go, *G. olea*; Gan, *G. ancylorhynchus*; Gp, *G. pingtung*; Ga, *G. albogilva*; Gtw, *G. tawu*; Gf, *G. ferruginea*.

clades	withinclade		between clades					
	nucleotide divergence	mean nucleotide difference	Go	Gan	Gp	Ga	Gtw	Gf
Go	0.6	3	–	11.1	12.6	15.2	16.5	16.5
Gan	0.39	2	2.44	–	9.5	10.9	12.3	13.5
Gp	0.64	3.2	2.78	2.05	–	14.2	15.8	17
Ga	0.23	1.2	3.5	2.49	3.16	–	5	9.6
Gtw	0.47	2.4	3.83	2.86	3.54	1.01	–	9.3
Gf	–	–	3.87	3.2	3.92	2.05	1.98	–

**Table 3.** Matrix of percentage pairwise nucleotide divergence (lower left) and mean number of differences (upper right) among the main species clades of *Geothelphusa* collected from southwestern Taiwan and adjacent areas, based on 616 bp of the cytochrome oxidase I gene. Nucleotide divergence was estimated from pairwise distances of nucleotides based on the GTR+I+G model of nucleotide substitution. The abbreviations for clades: Go, *G. olea*; Gp, *G. pingtung*; Ga, *G. albogilva*; Gtw, *G. tawu*; Gf, *G. ferruginea*.

clades	within clade		between clades					
	nucleotide divergence	mean nucleotide difference	Go	Gan	Gp	Ga	Gtw	Gf
<b>Go</b>	1.23	7.1	–	22.8	33.7	43	43.4	47.5
<b>Gan</b>	0.21	1.3	4.26	–	33.4	51.4	47.8	53.3
<b>Gp</b>	1.68	9.8	7.22	7.11	–	55.4	51.7	57.6
<b>Ga</b>	1.78	10.6	9.97	12.26	14.43	–	25.9	30.6
<b>Gtw</b>	1.43	8.4	10.32	11.53	13.43	4.89	–	25.1
<b>Gf</b>	–	–	12.13	13.78	15.99	6.36	4.78	–

tances between clades (ranging from 1.01% to 3.92%, average=2.85%) ( $P<0.001$ , Mann-Whitney U test). The mean number of differences within species (1.2 to 3.2, average=2.4) is also significantly lower than that between species (5 to 17, average=12.6) ( $P<0.001$ , Mann-Whitney U test). The same is true for the COI gene, where the genetic distance within each clade (0.21% to 1.78%, average=1.27%) is significantly lower in comparison to the distances between clades (4.26% to 15.99%, average=9.90%) ( $P<0.001$ , Mann-Whitney U test), and the mean number of differences within species (1.3 to 10.6, average=7.4) is also significantly lower than that between species (22.8 to 57.6, average=41.5) ( $P<0.001$ , Mann-Whitney U test).

### DISCUSSION

No significant difference was found between the combined 16S rRNA and COI sequences among the specimens of five species of *Geothelphusa* (*G. caesia*, *G. nanhsi*, *G. olea*, *G. tsayae* and *G. wutai*) collected from their type localities or the immediate vicinity (Table 1, Fig. 1). Previous studies have reported at least a 5-bp difference along the same fragment of 16S mtDNA between different species of *Geothelphusa* (see Shih *et al.*, 2004) and *Nanhaipotamon* (see Shih *et al.*, 2005). In our results, the difference in 16S within the *G. olea* clade ranges from 0 to 5 bp (average=3) and therefore we did not subdivide this clade, although two subclades are supported by high probability values. Schubarth *et al.* (1998) and Tong *et al.* (2000) argued that the average sequence divergence of the COI gene is about 2.5 times that of the 16S gene. In our study, the divergences between COI and 16S for the *G. olea* and *G. pingtung* clades correspond to this ratio (2.4 times and 3.1 times, respectively; Tables 2 and 3), but the divergences cannot distinguish consistently between described species within these two clades.

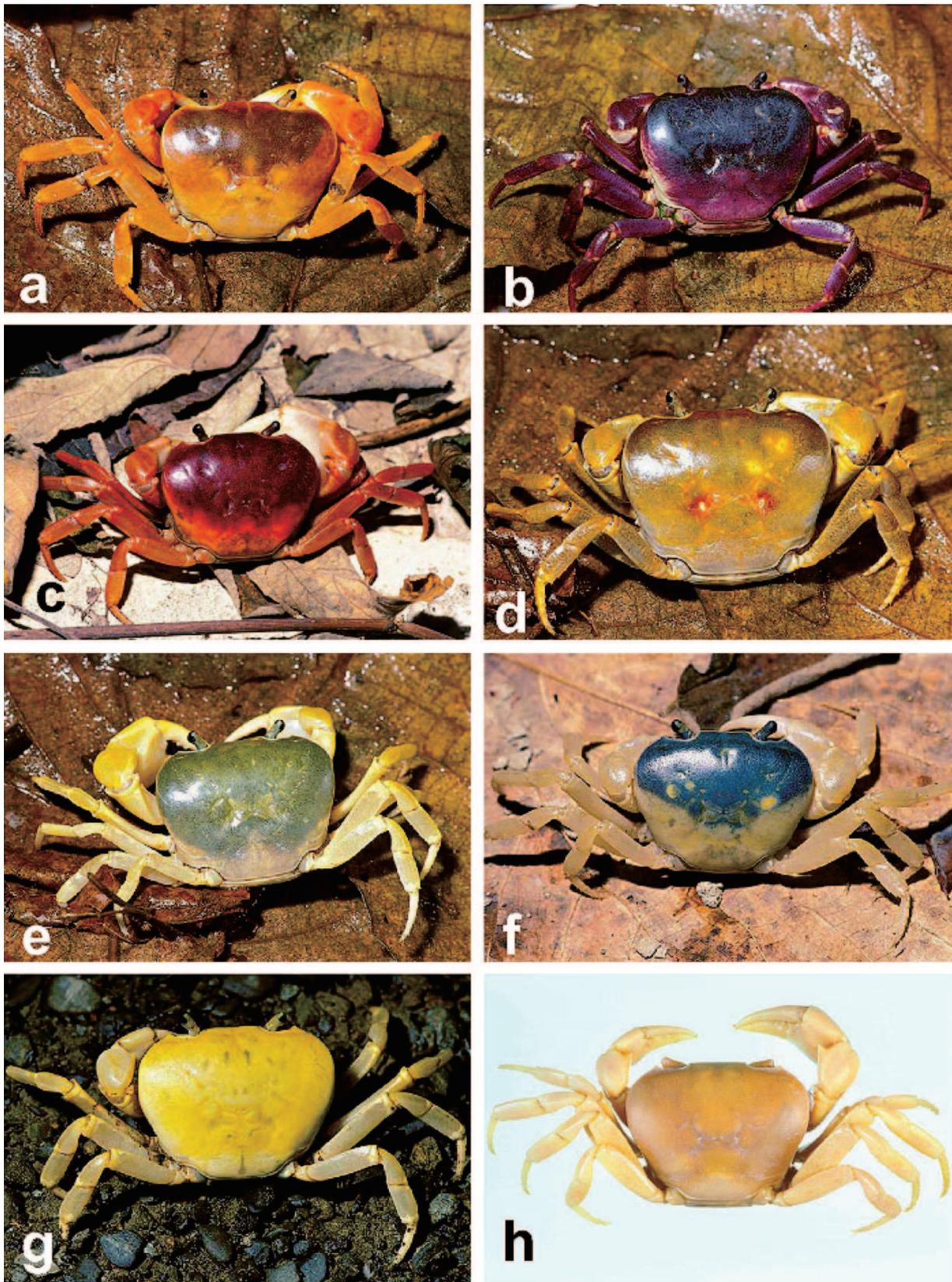
While this suggests that all five species of that all five species of the *G. olea* clade may be considered a single species or that the species need to be redefined, any formal taxonomic action would be premature, because while some of these taxa are superficially morphologically similar, they can still be clearly separated by discrete gonopodal and carapace features. Additional analyses using discrete morphological and morphometric characters may be necessary to distinguish between taxa (as in Harrison and Hanley, 2005).

It is useful to note that the specimens used to describe

*G. nanhsi*, *G. tsayae*, and *G. wutai* are relatively small (carapace width (CW) of the largest males: 23.3, 29.5 and 26.0 mm, respectively, *cf.* Shy *et al.*, 1994), while those of *G. olea* are larger (CW of the largest male, 35.4 mm; Shy *et al.*, 1994). Although the type series of *G. nanhsi*, *G. tsayae*, and *G. wutai* are clearly mature specimens, this does not preclude the possibility that environmental conditions affect the adult size and / or maturity of a given species. The distinct bluish-gray coloration of *G. caesia* may be the result of an unusual diet and / or substrate, and it is also possible that some (or all) of the species in group W are phylogenetically very “young”, having evolved only recently, without complete lineage sorting (see Neigel & Avise, 1986). Synonymization of species based on mtDNA alone and irrespective of studies on mitochondrial introgression, reproductive isolation, and / or morphological distinctiveness is not always appropriate (see Lee, 2004; Moritz and Cicero, 2004; Meier *et al.*, 2006).

The southernmost limit of the distributional range of the *G. olea* clade is near Wutai, Pingtung County (Fig. 1). According to Shy *et al.* (1994) and Shy and Yu (1999), *G. olea* is distributed throughout the western part of the Central Range from Sanjhih, Taipei County to Yujing, Tainan County. However, the northernmost distribution of the *G. olea* s. str. is actually near Dongshih, Taichung County, at the boundary of Taichung and Miaoli counties (Shih *et al.*, unpublished), and specimens from the northern part of Taiwan that are morphologically very similar to *G. olea* probably belong to another species. A study of this material is currently underway.

The distribution of the relatively large-sized *G. ancylophallus* (CW 32.4 mm for the largest male) is more restricted compared with other similarly sized species, *e.g.*, *G. olea*, *G. pingtung*, and *G. albogilva*. *Geothelphusa ancylophallus* occurs in the foothills of the mountains along the western coastal plains between the Zengwen and Kaoping Rivers (Fig. 1), *i.e.*, the Erren (=Erhjen) River system, where the habitat is composed mainly of Plio-Pleistocene mudstones. Loose structures resulting from strong river erosion have given it an appearance of “badlands,” and erosion and high salt content allow only weeds and shrubs to grow in this area (Shih, 1967; Hsieh and Knuepfer, 2001). Active mud volcanoes present along some of the major faults indicate that there is still high fluid pressure underlying this region. During most of the year this region suffers drought, except



**Fig. 4.** Dorsal views of specimens of *Geothelphusa pingtung* Tan and Liu, 1998. (**a–g**) Variation in color of different individuals. (**a, b, d, e**) Sandimen, Pingtung County, Taiwan, 16 May 2002; (**c, f**) Taiwu, Pingtung County, Taiwan, 12 Nov. 2000; (**g**) Lili, Chunrih, Pingtung County, Taiwan, 10 Sep. 2002; (**h**) male specimen collected by Hans Sauter at Yentempo (=Yancheng, Kaohsiung City, Taiwan), 1906 (catalog no. Staatssammlung München 1147/8).

in the summer, when most of the rain falls (Wu, 1992). The habitats from which *G. ancylophallus* were collected are drained by small springs or are in the vicinity of artificial reservoirs. In a few cases, *G. ancylophallus* was found sympatrically with *G. olea* and *Candidiopotamon rathbunae*, in larger water bodies with rich vegetation (H.-T. Shih, pers. obs.). It is likely that *G. ancylophallus* has become adapted to habitats with an unstable water supply and topology, which might have excluded most other species.

Chen *et al.* (1998) described a new large-sized, purple-colored species, *G. neipu*, from Neipu, Pingtung County. Earlier the same year, Tan and Liu (1998) described a purple and orange species, *G. pingtung*, from Taiwu, a site near Neipu in Pingtung County. Chen *et al.* (2001, 2003) indicated that two new species of *Geothelphusa* from southwestern Taiwan were being described, and that several other possible new species, distinguished primarily by their color patterns, would also be recognized. We have collected specimens of *Geothelphusa* with the color forms shown in Chen *et al.* (2003) from the upstream areas of the Donggang and Linbian Rivers (Fig. 1), including orange, purple, green, pale brown, blue, and several intermediate color forms (Fig. 4a–g). The specimens of *G. shernshan* used for the recent publication by Chen *et al.* (2005) were also included (Fig. 4b).

The combined 16S rRNA and COI sequences of these color forms presented here are identical or very close (Tables 2, 3), and all belong to a monophyletic group (Fig. 2). This indicates that all of the color forms of *Geothelphusa* in this area, in the absence of other morphological differences, should be regarded as a single species, *G. pingtung*, but more morphological studies are needed. As Shih *et al.* (2004) argued, there are neither genetic nor morphological grounds to separate *G. neipu* and *G. pingtung*, with the latter name having priority. Interestingly, the yellow form of *G. pingtung* (Fig. 5g) is found only near the boundary between SW and S Taiwan (Lili River, a tributary of the Linbian and Shihwen Rivers), and at Chaishan, where it has been apparently misidentified as *G. albogilva* (see Chen *et al.*, 2001, 2003).

*Geothelphusa pingtung* has a wide distribution around the hills of the Central Range, but a small population of *G. pingtung* was also found at Chaishan near Taiwan Strait (Fig. 1). Chaishan belongs to an uplifted mountain of coral reefs with a maximum altitude of 330 m and has no permanent running streams. Its aquatic habitats depend on small springs and periodic rainfall. There are no mountains or hills on the 40 km-wide plain between Chaishan and the Central Range. Although the Chaishan reefs were uplifted above sea level during the Pleistocene (Gong *et al.*, 1996, 1998), the other parts of the Kaohsiung area were covered by seawater during two interglaciations and glaciations since the late Pleistocene (Sun, 1964). The most recent transgression-regression cycle happened during the Holocene (Chen *et al.*, 1994), and the Kaohsiung area was reexposed as dry land between 8000 and 1000 years b.p. (Su, 1999).

Genetically, the populations of *G. pingtung* from the Central Range and Chaishan are separated by an average of 4.3 bp for 16S and 13 bp for COI, and the Chaishan haplotypes form a single clade compared to other populations. The nucleotide divergence between the populations of the

Central Range and Chaishan is 0.86% for 16S and 2.27% for COI. Based on a substitution rate of 0.88% per million years for 16S (see Schubart *et al.*, 1998), these populations of *G. pingtung* are estimated to have separated about 1 mya (million years ago) during the early Pleistocene. In addition, there are 2 unique bp for 16S and 5 unique bp for COI in the Chaishan population that distinguish it from the Central Range population.

In the haplotype network of the *G. pingtung* clade (Fig. 3), the combined haplotypes Gp-5+Gp-C5c (Liangshan, Majia, Pingtung County) in the Central Range are central relative to other haplotypes, and we assume them to represent the ancestral haplotype. Therefore, we here suggest that the present population of *G. pingtung* at Chaishan arrived through dispersal from the peripheral hills of the Central Range during the early Pleistocene and subsequently was isolated. The Chaishan population of *G. pingtung* should therefore be recognized at least as a geographic subspecies, on the basis of the above evidence. Clearly, further morphological studies are needed, and these are currently being carried out.

The distribution of *G. pingtung* overlaps partially with *G. olea* s. str., and the two species are sympatric at Wutai, Pingtung County (Fig. 1, locality 13). The southernmost distribution of *G. pingtung* is near Lili, Chunrih, Pingtung County (Fig. 1, locality 17). Shih *et al.* (2004, 2006) noted that large-sized freshwater crabs (e.g., *G. albogilva* and *Candidiopotamon rathbunae*) inhabit low mountains and were likely to have been more easily isolated by large mountains compared to small-sized species (e.g., *G. tawu*). The dispersal of *G. pingtung* southward appears to have been blocked by the mountains of the southwestern tip of the Central Range.

Bott (1967: 212) reported *Geothelphusa dehaani* (White, 1847) from "China, Yentempo". In fact, the locality "Yentempo" is the old name for Yancheng, Kaohsiung City, Taiwan (Chu and Yamanaka, 1973) and is located at the foot of Chaishan. The specimens of *G. dehaani* used by Bott (Fig. 4h), from the Zoologische Staatssammlung München, Germany, were examined (catalog no. 1147/8) and comprised two males, collected by Hans Sauter in 1906. The morphology of these specimens agrees well with the Chaishan population of *G. pingtung* (identified by Tohru Naruse).

The variable coloration of crabs within the *G. pingtung* and *G. olea* clades may result from selection pressures posed by the immediate habitat (Kent, 1901; Bedini, 2002), with the background colors of the substrate (e.g., differently colored leaves and / or soils) influencing the natural color of the crabs. The bluish-gray color of *G. caesia* (in the *G. olea* clade) near Jiasian and Meinong, Kaohsiung County, and the yellow form of *G. pingtung* at Chaishan and in the south of Lili River, may simply be the result of available food sources or of substrate coloration (Suzuki and Tsuda, 1991). The inheritance of an autosomal recessive color pattern as found in freshwater crayfish may be another possible explanation (see review by Walker, 2000).

In their genetic study, Shih *et al.* (2004) proposed *G. lanyu* and *G. lutao* to represent possible synonyms of *G. tawu*, considering the minor differences in gonopodal morphology to be intraspecific variation. This view is further supported by the present study, which adds the COI gene to the

data set. However, it is noteworthy that representatives from Lyudao Island, formerly classified as *G. lutao*, form a monophyletic group in our tree (Fig. 2) and have diagnostic genetic differences. The recently described species, *G. lili* Chen, Cheng, and Shy, 2005, included in our analyses likewise forms part of the *G. tawu* clade (Fig. 2), suggesting that it may also prove to be a synonym of *G. tawu*.

Some specimens belonging to three apparently undescribed species of *Geothelphusa* (M group) collected from the high mountain areas (in excess of 1,500 m altitude) near the Central Range in Chiayi and Kaohsiung County (*G. sp. 1*, *G. sp. 2*, and *G. sp. 3* in Table 1) show a distant relationship to the crabs distributed at low altitudes (W group). However, they tend to be closer to the S and E groups in the eastern part of the Central Range (Fig. 2). One specimen identified as "*G. bicolor*" (haplotype TT1) in Shih *et al.* (2004) was very different from true *G. bicolor* from the type locality in both 16S and COI, and is here considered an undescribed species (*G. sp. 4*). The M group may be a relict species group isolated by past glaciations, or it may be a species that is adapted to a montane environment that has evolved from lowland relatives. This can only be elucidated after further and more extensive sampling in Taiwan's montane areas.

Shih *et al.* (2006) studied the phylogeography of *Candidiopotamon rathbunae* from Taiwan and obtained similar results with respect to a clear-cut division of freshwater crabs from southwestern Taiwan (SW group). The nucleotide divergence between the SW group of *C. rathbunae* and other groups of Taiwan is high, and the SW group separated from NW and W groups at about  $3.3 \pm 0.8$  mya based on a substitution rate of 0.88% per million years (see Schubart *et al.*, 1998). If the same rate is applied to the SW clade of *Geothelphusa*, a separation from the W and SW clades is estimated to have occurred at about 3.1 mya (nucleotide divergence is 2.71%).

The striking similarity in the molecular dating of the origin of freshwater crabs from SW Taiwan in both dominant genera supports the present hypothesis of a shared biogeographic history and the same underlying mechanisms of isolation in this part of the island. The uplifting of the Yushan Range and / or Alishan Range must have separated the Donggang and Linbian Rivers from more northern river systems. In addition, the formation of foreland basins in the Chianan and Kaoping areas, which is estimated to have taken place about 3 mya, may have played an important role during the isolation of *Candidiopotamon* (see Shih *et al.*, 2006) and *Geothelphusa* in SW Taiwan from adjacent areas. Except for the overlap area with *G. olea* (Wutai, Pingtung County, locality 13 in Fig. 1) and *G. tawu* (Lili, Chunrih, Pingtung County, locality 17 in Fig. 1), there is no small-sized species of *Geothelphusa* between Chaishan and the Linbian River (Fig. 1). This region has a tropical monsoon climate with dry winters, and the winter rainfall is the one of the lowest in all of Taiwan (Wan, 1973, 1974; Chen and Huang, 1999; Hsieh and Knuepfer, 2001). During most seasons, the habitats of the crabs in this region are characterized by drought, and it is possible that only large-sized species can survive these arid conditions. This relatively extreme environment may also have played an important role as an isolating mechanism preventing introgression

from other species.

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