



Intraspecific genetic diversity of the endemic freshwater crab *Candidiopotamon rathbunae* (Decapoda, Brachyura, Potamidae) reflects five million years of the geological history of Taiwan

Hsi-Te Shih¹, Huei-Chuan Hung², Christoph D. Schubart³, Chaolun Allen Chen⁴ and Hsueh-Wen Chang^{5*}

¹Department of Life Science, National Chung-Hsing University, Taichung, Taiwan, ²National Taichung Nursing College, Taichung, Taiwan, ³Biologie I, Universität Regensburg, Regensburg, Germany, ⁴Research Center of Biodiversity, Academia Sinica, Nangang, Taipei, Taiwan and ⁵Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan

ABSTRACT

Aim *Candidiopotamon rathbunae* (Crustacea: Brachyura: Potamidae) is a freshwater crab endemic to Taiwan, with a distribution covering almost the entire island. Crab populations from different river systems cannot be distinguished morphologically. In this study, we investigate the phylogeography of *C. rathbunae* from 18 of the main river systems of Taiwan, in order to reveal geographical differentiation and cryptic endemism. We used specimens of two congeners (*C. okinawense* and *C. kumejimense*) and the closely related *Amamiku amamensis* from the Ryukyu Islands as outgroups.

Location Taiwan, with the Ryukyu Islands (Japan) as the outgroup locality.

Methods Mitochondrial DNA sequences encoding 553 basepairs of the large subunit rRNA (16S rRNA) gene were obtained from 96 specimens throughout Taiwan and the Ryukyus. We compared these sequences by means of phylogenetic analyses (minimum evolution, maximum likelihood, maximum parsimony, and a genealogical parsimony network) and molecular-clock time estimates.

Results The estimated timing of the corresponding separations clearly correlates with geological events during the Penglai Orogeny of Taiwan (c. 5 Ma) following the collision of the Philippine Sea plate with the Eurasian continental plate. A deep split of 6.29% sequence divergence was found between the eastern clade (S, SE, and E groups) and the western clade (NW, W, and SW groups) of *C. rathbunae*. Separation of these groups reflects the isolating effect of the uplift of the Taiwan Central Range (c. 5 Ma). The separation of the SW group from the W group, and of the E group from the SE group can also be explained by geological events during the Taiwanese orogeny, the estimated geological timing being in close accordance with our molecular dating.

Main conclusions According to the molecular results, the ancestral *Candidiopotamon* was among the earliest non-marine colonizers of Taiwan, settling on this island no later than the early Pliocene. Our molecular data reveal that *C. rathbunae* can be subdivided into six groups in accordance with geography.

Keywords

16S rRNA gene, allopatric divergence, *Candidiopotamon* molecular clock, mtDNA sequence, phylogeography, Potamidae, Taiwanese orogeny.

*Correspondence: Hsueh-Wen Chang, Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan. E-mail: hwchang@mail.nsysu.edu.tw

INTRODUCTION

True freshwater crabs are by definition those crabs that spend their entire life cycle within freshwater environments. Their larvae are not released or transported into the sea; rather, the fertilized eggs develop directly into juvenile crabs and the mother crabs exert brood care behaviour towards the early juveniles (Ng, 1988). The dispersal abilities of freshwater crabs are limited (Kobayashi, 2000; Daniels *et al.*, 2002). During the Japanese winter, when the water temperature is low and the streams are often dry, freshwater crabs generally hibernate inside their burrows (Minei, 1968; Shimotsukasa & Wada, 1995). Because of these habits, gene flow among different river systems is reduced. If such isolating conditions endure, genetic differentiation, and eventually speciation, may take place.

The genera *Geothelphusa* and *Candidiopotamon* are the dominant freshwater crabs of the East Asian islands, including Taiwan, the Ryukyus, and Japan. While there are 28 species of *Geothelphusa* in Taiwan, one in Diaoyutai (= Sentaku) Islands, 10 in the Ryukyus, and two in Japan (Shy *et al.*, 1994; Shy & Ng, 1998; Tan & Liu, 1998; Shy & Yu, 1999; Yoshigou, 1999; Suzuki & Okano, 2000; Ng *et al.*, 2001; Naruse *et al.*, 2004c), only a single species of *Candidiopotamon* occurs in Taiwan, and, until recently, three species were recognized from the central Ryukyus, and one from Guangdong, China (de Man, 1914; Dai, 1999; Shy & Yu, 1999; Yoshigou, 1999; Ng *et al.*, 2001). After Naruse *et al.* (2004b) erected the new genus *Amamiku* for the Ryukyuan *Candidiopotamon amamense* Minei, 1973, a total of four species were left in the genus *Candidiopotamon*. The only species of *Candidiopotamon* from Taiwan, *Candidiopotamon rathbunae* (de Man, 1914), is distributed throughout the island, with the exception of the north-east. It occurs from sea level to mountain creeks at altitudes of about 2000 m (Shy & Yu, 1999). These crabs are important by virtue of their being a second intermediate host of the human lung fluke *Paragonimus westermani* (Kerbert) in Taiwan, which was especially common before the 1960s (Koba, 1936; Chiu, 1964). The reproductive behaviour and the ecology of *C. rathbunae* in central Taiwan have been described by Liu & Li (2000).

The island of Taiwan emerged above sea level as a result of the collision of the Philippine Sea plate and Eurasian continental plate *c.* 5 Ma (the Penglai Orogeny, Teng, 1990; Liu *et al.*, 2000). The orogeny also uplifted the longitudinal Central Range of Taiwan to heights of almost 4000 m, an event that may have played an important role in isolating flightless animals that are not able to migrate across the mountains. In a study concerning *Geothelphusa* in southern Taiwan, Shih *et al.* (2004) showed that the south-western extension of the Central Range, although much lower than the rest of the range, still prevented gene flow for the lowland species of this genus.

In the present study, we compare DNA sequences of the mitochondrial 16S rRNA gene of *C. rathbunae* among several geographically separated populations from Taiwan in order to

study the extent of genetic diversity among them and the occurrence of regional endemism.

MATERIALS AND METHODS

Specimen collection

The Central Range extends from north-eastern to south-western Taiwan, with Mt Yushan (Mt Jade) its highest peak (3952 m) in the centre (Fig. 1) dividing eastern from western Taiwan. From 1994 to 2003, a thorough survey and collection of *C. rathbunae* from all the streams in mountains and coastal plains, throughout the island, was carried out. In total, 88 specimens from 18 main river systems (Fig. 1, Table 1) were sampled for molecular analysis. Specimens of the congeneric and closely related species *C. okinawense* Minei, 1973, *C. kumejimense* Minei, 1973, and *Amamiku amamensis* (Minei, 1973) were collected from Okinawa, Kumejima, and Amami, respectively, to serve as outgroups (Table 1). After collection, specimens were preserved in 75–95% ethanol.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from leg-muscle tissue by phenol–chloroform extraction (Kocher *et al.*, 1989) or the Sigma mammalian genomic DNA miniprep kit. A region of approximately 550 basepairs of the 5′-end of the 16S rRNA gene was selected for amplification with polymerase chain reaction (PCR) using the universal primers 1471 (5′-CCTGTTTANCAAAA-CAT-3′) and 1472 (5′-AGATAGAAACCAACCTGG-3′) (Crandall & Fitzpatrick, 1996). The PCR conditions were 35–40 cycles of 50 s at 94 °C, 70 s at 45 °C, 60 s at 72 °C (denaturation/annealing/extension), followed by a final extension for 10 min at 72 °C and storage at 4 °C. 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 complementary strand. Sequences of the haplotypes have been deposited in the DNA Data Bank of Japan (DDBJ) data base (accession numbers AB208589–208599, 208601–208612, 208614–208630).

Phylogenetic analyses

The best-fitting model for sequence evolution was determined by MODELTEST (version 3.6, Posada & Crandall, 1998), and was subsequently applied for a maximum likelihood (ML) and in part for a minimum evolution (ME) analysis. The ME tree was constructed with the program MEGA2 (version 2.1, Kumar *et al.*, 2001) with the gamma correction obtained from MODELTEST, the Kimura (1980) two-parameter model, and 2000 bootstrap reiterations (CNI level = 2, initial tree = NJ, and max. number of trees to retain = 1). A maximum parsimony (MP) tree was constructed using the program PAUP* (version 4.0b8, Swofford, 2001) with 2000 bootstrap

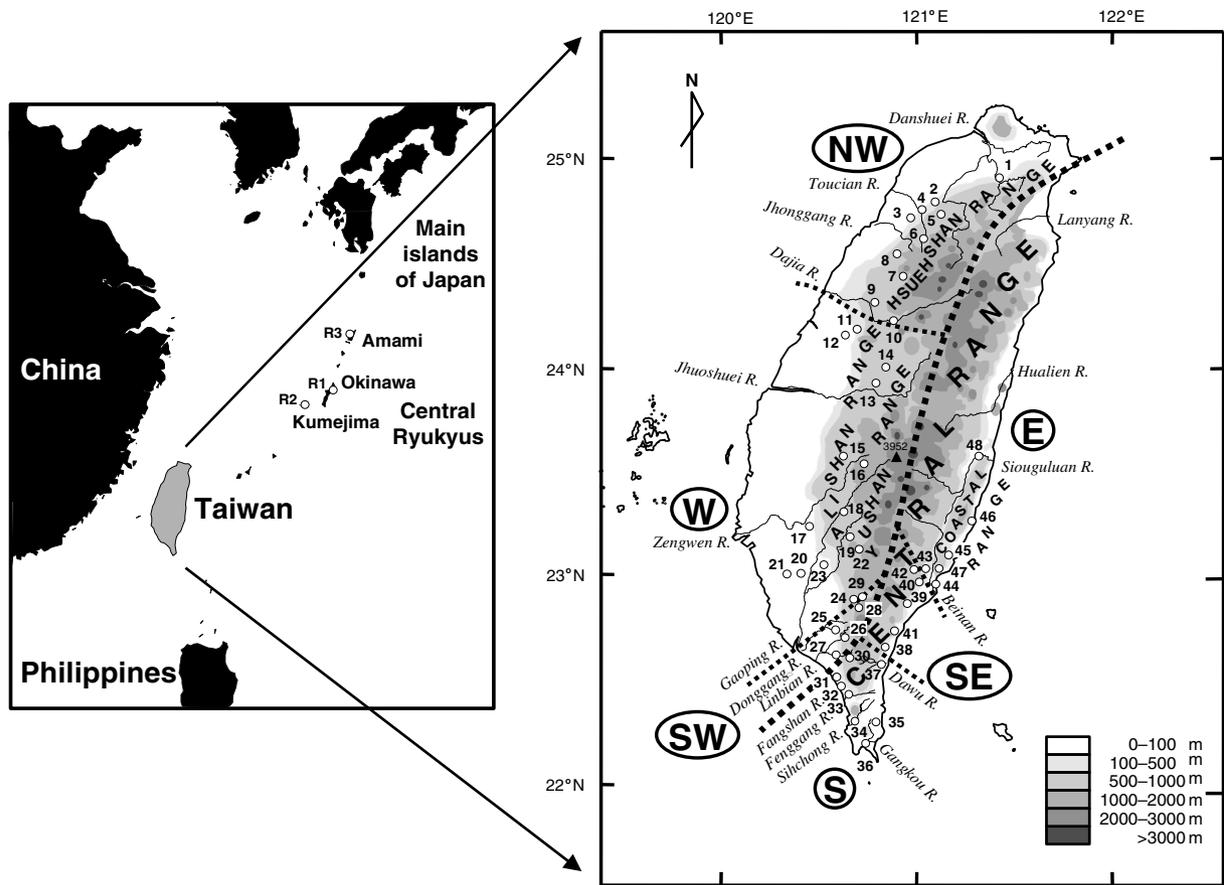


Figure 1 Collection sites (open circles) for *Candidiopotamon rathbunae* in Taiwan, and for the congeneric and closely related species from the Ryukyus. Dotted lines indicate the possible biogeographical boundaries among them. For abbreviations of regions see Table 1.

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. The ML analysis was also calculated by PAUP* with 100 bootstrap replications and 20 random-addition sequence replications. The other parameters were the same as in the MP analysis. In order to avoid excessive computation time, the total number of rearrangements for each search was limited to 500,000 for MP analysis and 1000 for ML analysis. The best-fitting model determined by MODELTEST was then applied to estimate inter- and intra-population genetic diversity with PAUP*. The diagnostic genetic distances, i.e. the percentage of the nucleotide differences that always separate two populations, were calculated among megapopulations (i.e. those belonging to the same watershed). To examine the relationships of these haplotypes of *C. rathbunae* in detail, a gene genealogy network of 16S rRNA was constructed using the program TCS (version 1.20, Clement *et al.*, 2000) with the gaps treated as missing.

Molecular dating

Because of the limited records of freshwater crab fossils from eastern Asian islands, with the exception of the fossil records

of Holocene *Geothelphusa dehaani* (White) from the main islands of Japan (Karasawa, 1997) and late Pleistocene *G. tenuimanus* (Miyake & Minei) from Okinawa, central Ryukyus (Naruse *et al.*, 2004a), dating of divergence events is difficult. Divergence rates of the 16S-br region of the 16S rRNA gene in brachyuran crabs (see Schubart *et al.*, 2000) have been estimated for *Uca* (see Sturmbauer *et al.*, 1996) and *Sesarma* (marine and non-marine representatives, Schubart *et al.*, 1998a). Both calibrations were based on the closure time of the Panama Canal and rendered similar results. Here, we used the substitution rates of 0.88% per million years established for terrestrial *Sesarma* (see Schubart *et al.*, 1998a) for application to the dating of *C. rathbunae* divergence events. Among the known calibrations for decapod crustaceans, this is the only available rate established for terrestrial or limnic crabs. For molecular dating, substitution rates between sequences were estimated according to the Jukes–Cantor model, with the gamma correction obtained from MODELTEST. Rate constancy was tested using the branch-length test of LINTREE (Takezaki *et al.*, 1995). A linearized ME tree reflecting the estimated time intervals of population divergences in *C. rathbunae* was constructed using the program MEGA2 with the same parameters as the ME analysis.

Table 1 Thirty-six haplotypes of *Candidiopotamon rathbunae* (de Man, 1914) collected from 18 different populations of Taiwan, and four haplotypes of the congeneric species and closely related species from the Ryukyus (Fig. 1). Numbers within brackets correspond to localities in Fig. 1. R., river; Co., county. The abbreviations for haplotypes of outgroups are CO, *C. okinawense*; CK, *C. kumejimensis*; and CA, *C. amamense* (now *Amamiku amamensis*)

Region and haplotype	Sample size	Localities	DDBJ Accession no.
North-western region (NW)	18		
TPWL1, 2	2	Taipei Co. (Wulai [1])	AB208589–AB208590
PCM	10	Taipei Co. (Wulai [1]); Hsinchu Co. (Guansi [2]; Beipu [3]; Hengshan [4]; Jianshih [5]; Wufong [6]); Miaoli Co. (Erbensong, Tai-an [7]; Baguali, Tai-an [8])	AB208591
HCJS	1	Hsinchu Co. (Jianshih [5])	AB208592
MLTA	1	Miaoli Co. (Baguali, Tai-an [8])	AB208593
TCDS1, 2, 3	3	Taichung Co. (Dongshih [9])	AB208594–AB208596
TCGG	1	Taichung Co. (Guguan [10])	AB208597
Western region (W)	21		
TCNT	3	Taichung Co. (Taiping [11]; Caohu, Dali [12]); Nantou Co. (Shueili R., Shueili [13])	AB208598
NT1	3	Nantou Co. (Lianhuachih, Yuchih [14]; Shueili R., Shueili [13])	AB208599
TNKH	11	Chiayi Co. (Yimasi, Jhuci [15]; Banta, Alishan [16]); Tainan Co. (Gueidan, Nansi [17]); Kaohsiung Co. (Mincyuan, Sanmin [18]; Baolai, Liouguei [19]; Tianliao [20]; Daganshan, Alian [21])	AB208601
KH1	1	Kaohsiung Co. (Chuyunshan, Taoyuan [22])	AB208602
KH2	1	Kaohsiung Co. (Meinong [23])	AB208603
PTWT	2	Pingtung Co. (Yila, Wutai [24]; Jiamu, Wutai [24])	AB208604
South-western region (SW)	12		
PTTL	8	Pingtung Co. (Taiwu [25]; Laiyi [26]; Lili, Chunrih [27])	AB208605
PTSD1, 2, 3	4	Pingtung Co. (Haocha, Sandimen [28]; Wutai [29])	AB208606–AB208608
Southern region (S)	16		
PTFS1	3	Pingtung Co. (Jioucijia, Chunrih [30]; Cili R., Shihzih [31])	AB208609
PTFS2	1	Pingtung Co. (Nanshihhu R., Shihzih [31])	AB208610
PTFS3,4	2	Pingtung Co. (Fangshan R., Shihzih [32])	AB208611–AB208612
PTHC1	6	Pingtung Co. (Nanshihhu R., Shihzih [31]; Fangshan R. [32], Fenggang R. [33], Shihzih; Sihchong R., Checheng [34]; Nanrenshan, Manzhou [35])	AB208614
PTHC2	2	Pingtung Co. (Maozaikengnei, Hengchun [36]; Nanrenshan, Manzhou [35])	AB208615
TTDW	2	Taitung Co. (Dawu [37])	AB208616
South-eastern region (SE)	12		
TTS	7	Taitung Co. (Taiban, Daren [38]; Jihben [39]; Lijia, Beinan [40])	AB208617
TTTM	2	Taitung Co. (Taimali [41])	AB208618
TTYP1, 2, 3	3	Taitung Co. (Hongye, Yanping [42])	AB208619–AB208621
Eastern region (E)	9		
TTN	3	Taitung Co. (Luming R., Yanping [43]; Fuyuan, Beinan [44]; Taiyuan, Donghe [45])	AB208622
TTCG	1	Taitung Co. (Sansiantai, Chenggong [46])	AB208623
TTLS	1	Taitung Co. (Luanshan, Yanping [47])	AB208624
TTJF	1	Taitung Co. (Jiafeng, Beinan [43])	AB208625
HLJP	3	Hualien Co. (Jingpu, Fengbin [48])	AB208626
All localities	88		
Outgroups	8		
CO	3	Ryukyus (Okinawa [R1])	AB208627
CK1	2	Ryukyus (Kumejima [R2])	AB208628
CK2	1	Ryukyus (Kumejima [R2])	AB208629
CA	2	Ryukyus (Amami [R3])	AB208630

RESULTS

Sequence diversity

A 550-basepair (bp) segment (excluding the primer regions) of the 16S mtDNA gene from 88 specimens of *C. rathbunae* (Table 1) was amplified and aligned. The data set included 44 variable positions and 37 parsimony-informative ones. Among the 88 sequences corresponding to *C. rathbunae*, we found 36 different haplotypes. These sequences are AT-rich (71.2%) (T: 37.0%, A: 34.2%, G: 19.1%, C: 9.7%). The sequences of the congeneric species and closely related species, *C. okinawense*, *C. kumejimense* and *A. amamensis*, were also amplified as outgroups and aligned with four haplotypes from eight specimens (Table 1).

Phylogenetic analyses

The model explaining our data best, as calculated by MODELTEST, was TrN + I + G (proportion of invariable

sites = 0.5864, gamma distribution shape parameter = 0.5189). The phylogenetic tree constructed with three different reconstruction methods subdivides *C. rathbunae* into eastern and western clades. In total, six groups, NW, W, SW, S, SE, and E can be recognized based on the ME tree (Fig. 2). However, the SE group is supported by low bootstrap values. Both the MP and ML analyses support these groups, except for the W and SE groups. The linearized ME tree plotted on the molecular clock calibration is also shown in Fig. 2. The six main groupings of Taiwanese *C. rathbunae* obtained from the 16S rRNA gene are in accordance with their geographical distribution (Figs 1 & 2), with the exception of nine haplotypes that were found to be near the here-defined geographical boundaries (e.g. W-SW, SW-S, and SE-E groups in Fig. 1) and which could have been translocated by rare secondary contact or by human activities. The genetic distances as a percentage of only those positions always separating the six main groups (diagnostic distances) and the corrected nucleotide divergences (based on TrN + I + G model) are shown in Table 2 (upper right and lower left, respectively).

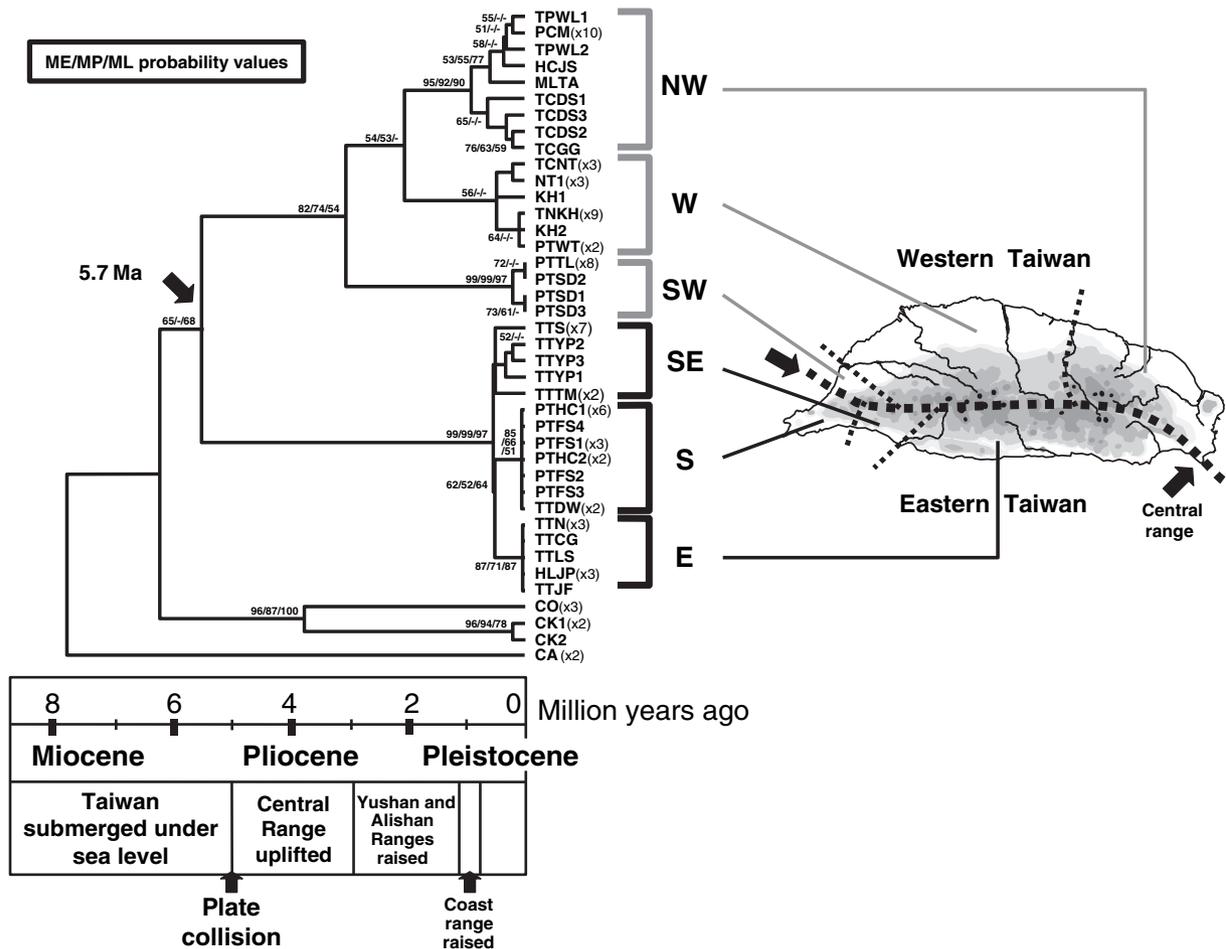


Figure 2 A linearized minimum evolution tree of six megapopulations of *Candidiopotamon rathbunae*, and the congeneric and closely related species from the Ryukyus based on 553 basepairs of the 16S rRNA gene, with the estimated times of divergence based on molecular-clock calibrations and the distribution of haplotypes in each region. Probability values at the nodes represent confidence after 2000 (ME, MP) and 100 (ML) bootstrap reiterations in the analysis of minimum evolution, maximum parsimony, and maximum likelihood, respectively. The main geological events in Taiwan are shown below. For abbreviations of haplotypes and regions see Fig. 1 and Table 1.

Table 2 Pairwise nucleotide divergence matrix (lower left) and diagnostic genetic distances (upper right) between 553 basepairs of the 16S rRNA gene corresponding to population groups of *Candidiopotamon rathbunae*, and the Ryukyuan congeners and closely related species. Nucleotide divergence is estimated from pairwise distance of nucleotides based on the calculated TrN + I + G model of nucleotide substitution. The abbreviations correspond to those in Table 1. All values are shown as percentages

	Within group	Between groups									
		Nucleotide divergence									
		NW	W	SW	S	SE	E	CO	CK	CA	
NW	0.64	–	1.08	2.71	4.52	4.16	4.16	4.52	4.34	5.79	
W	0.41	2.11	–	1.99	4.16	3.80	3.98	4.88	4.16	6.51	
SW	0.12	4.02	2.27	–	5.24	4.88	5.24	5.42	4.88	6.33	
S	0.11	6.90	5.19	6.71	–	0.36	0.72	6.33	5.24	7.78	
SE	0.30	6.60	4.90	6.40	0.63	–	0.72	5.97	4.88	7.23	
E	0.08	7.08	5.38	7.02	0.67	0.82	–	5.61	4.52	7.41	
CO	–	9.04	7.90	8.86	10.63	10.31	9.58	–	3.07	7.78	
CK	0.19	7.59	5.99	7.38	7.79	7.47	6.81	3.86	–	7.23	
CA	–	9.49	9.46	9.43	10.69	9.90	10.36	11.83	9.90	–	

Among these groups, the eastern clade (including S, SE, and E groups) is always separated from the western clade (including NW, W, and SW groups) by at least 2.17% diagnostic distance (6.29% corrected divergence). Within the western clade, the NW group can be separated from the W group with a distance of 1.08% (2.11% corrected divergence). The SW group is isolated from the W + NW groups by 1.81% diagnostic distance (3.32% corrected divergence). Within the eastern clade, the E group is isolated from the S + SE group, although the diagnostic distance between them is comparatively low (0.54%) (0.73% corrected divergence). The corrected nucleotide divergences within geographical groups (0.08–0.64%, average = 0.28%) are significantly lower than the divergences between regions (0.63–7.08%, average = 4.45%, see Table 2) ($P < 0.001$, Mann–Whitney U -test). For comparison, the divergence between all populations of *C. rathbunae* and the congeneric species from the Ryukyus (*C. okinawense* and *C. kumejimense*) is 1.08% diagnostic distance (7.97% corrected divergence). Within all *C. rathbunae*, the S and SE groups are most distant from the Ryukyuan congeneric species (3.98% and 3.80% diagnostic distance, and 8.74% and 8.42% corrected divergence, respectively), thereby reflecting geographical distance.

The parsimony network constructed to depict the relationships among the haplotypes of *C. rathbunae* is shown in Fig. 3. The six distinct lineages correspond to the six megapopulations recovered, which belong to the eastern and western clades. The haplotypes separated by less than 10 steps have a probability $\geq 0.95\%$ of being parsimony-connected (Templeton *et al.*, 1992). The connections between the eastern and western clades consist of 16 steps, and the connections within the clades are less than nine steps.

Molecular dating

The divergence (Kimura two-parameter model with gamma correction obtained from MODELTEST) between all Taiwanese *C. rathbunae* and two Ryukyuan congeners (*C. okinawense* and *C. kumejimense*) is $5.58 \pm 0.89\%$. It is thus estimated (based on the substitution rate of 0.88% per million years) that they diverged 6.3 ± 1.0 Ma, which could be considered the

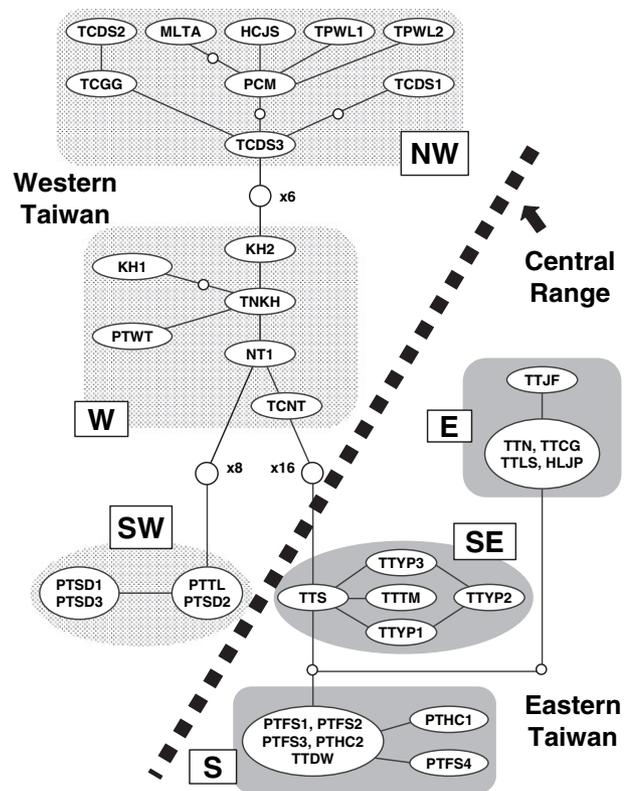


Figure 3 A genealogical parsimony network for the 16S mtDNA haplotypes of the populations of *Candidiopotamon rathbunae*. Unlabelled nodes indicate inferred steps not found in the sampled populations, and the numbers beside nodes indicate the number of substitutional changes between haplotypes. For abbreviations of haplotypes and regions see Fig. 1 and Table 1.

minimum time that Taiwan must have been populated by an ancestral *Candidiopotamon*. The divergence between the eastern and western clades of *C. rathbunae* is $5.03 \pm 0.96\%$, and thus the separation is estimated as 5.7 ± 1.1 Ma. The isolation between the NW and W groups was dated to about 2.2 ± 0.7 Ma (divergence = $1.96 \pm 0.58\%$). The NW and W groups separated from the SW group about 3.3 ± 0.8 Ma ($2.89 \pm 0.69\%$). The divergence between the E and S + SE

groups is comparatively recent, namely 0.8 ± 0.4 Ma ($0.71 \pm 0.32\%$). Tests of rate constancy were not rejected (chi-square test, $P = 0.84$), with some haplotypes in the W group (TCNT, KH2, TNKH, and NT1) being slightly slower in their substitution rate ($P < 0.05$).

DISCUSSION

Most older phylogeographic studies on the Taiwanese fauna dated first colonization events back to the existence of a land bridge to continental China during Pleistocene glaciations. This has been postulated for freshwater fishes (0.055 Ma for *Zacco pachycephalus*, see Wang *et al.*, 1999; 0.1 – 1 Ma for *Acrossocheilus paradoxus*, see Wang *et al.*, 2000; general discussion in Tzeng, 1986) and frogs (0.15 – 0.24 Ma for *Rhacophorus taipeianus* and 0.38 – 0.45 Ma for *R. moltrrehti*, see Yang *et al.*, 1994; early Pleistocene for *Rana limnocharis*, suggested by Toda, 1999). On the other hand, Creer *et al.* (2001) estimated that isolation of Taiwanese populations of the bamboo viper (*Trimeresurus stejnegeri*) from continental China occurred between the middle Pliocene (*c.* 3 Ma) and the late Pliocene (*c.* 2 Ma), which would agree with Hsu's (1990) description of Taiwanese orogeny and with Liu & Ding's (1984) estimate of isolation time between Taiwan and China. More recently, however, Taiwanese geological events have been recalculated, resulting in an estimate of *c.* 5 Ma for the emergence above sea level of the northern and central parts of the Central Range (Teng, 1990; Liu *et al.*, 2000).

The colonization by terrestrial biota of all East Asian islands probably occurred from continental Asia. The Taiwan Strait, with an average depth of 70 – 100 m and an average width of 130 – 250 km, separates the island of Taiwan from continental China. Taiwan may have played an important role as a dispersal centre for other western Pacific islands, as suggested by other studies of terrestrial organisms (Lue & Chen, 1997; Shen, 1997; Ota, 1999). During Pliocene and Pleistocene glaciation peaks, it is possible that most terrestrial animals may have been able to migrate easily from China to Taiwan as a result of the lowered sea level. Nevertheless, we do not find evidence for repeated exchange between Taiwan and China in the distribution of freshwater crabs (Dai, 1999), probably owing to their preference for mountain streams, warm and humid climates, and their low dispersal capabilities.

If we assume a Pleistocene colonization of Taiwan by *C. rathbunae*, the substitution rate for the 16S rRNA gene would be five-fold higher than the values obtained in other studies of crustaceans (reviewed by Schubart *et al.*, 2000). Based on new molecular findings (mtDNA sequences of the 12S rRNA gene), Lin *et al.* (2002) discussed whether the grass lizard *Takydromus sauteri* and its sister taxa diverged either 5.8 Ma (Tertiary-origin model) or 0.72 Ma (Quaternary-origin model). They favour the Tertiary-origin model, because their data fit well with the mutation rate of vertebrate mitochondrial DNA. This new dating of grass lizard divergence, 5.8 Ma, is thus very similar to the 6.3 (± 1.0) Ma estimated for *Candidiopotamon* in our study, when applying the substitution

rate of 0.88% per million years (see Schubart *et al.*, 1998a) for 16S rRNA sequence divergence of *C. rathbunae*. This suggests that Taiwan has been populated by an ancestral *Candidiopotamon* for more than five million years, which matches well with the age of the uplift of the island. Shen (1994, 1996) suggested that Taiwan Island has a biological history of approximately 5 Ma. Thus, the present postulated time of colonization by *Candidiopotamon* and the grass lizard *T. sauteri* would seem to represent some of the earliest records of non-volant life on the new island or its precursors.

After the initial colonization of Taiwan, which occurred not later than the early Pliocene, *Candidiopotamon* probably distributed rapidly across the island. Later geological events of the Taiwanese orogeny are reflected in the genetic divergence of the 16S rRNA gene of *C. rathbunae*. The eastern and western clades are each monophyletic and are well supported by all three phylogenetic methods (Fig. 2). The divergence time between the eastern and western clades is estimated to be 5.7 ± 1.1 Ma. The arc-continent collision of the Philippine Sea plate and Eurasian continental plate occurred about 5 Ma, and prompted the rapid uplift of the Central Range and its emergence above sea level (Teng, 1990; Liu *et al.*, 2000). Therefore, our estimate of the early divergence between eastern and western clades may reflect the isolating effect of the Central Range.

Two million years later, *i.e.* 3.3 ± 0.8 Ma, the NW + W groups separated from the SW group (Fig. 2). The isolation of the range-restricted SW group might have been caused by the uplift of the Yushan Range and/or Alishan Range, which acted as watersheds to separate the Donggang River and Linbian River from more northern river systems (Fig. 1). A recent study on the evolution of foreland basins (Chen *et al.*, 2000) has suggested that the formation of foreland basins (such as the Jianan foreland basins and Gaoping foreland basins) took place about 3 Ma, perfectly corresponding to the estimated timing of the separation of the SW group. The NW and W groups are isolated by the Dajia River: their divergence was dated to 2.2 ± 0.7 Ma (Fig. 2), and might have been caused by the westward protrusion of the Hsuehshan Range (Fig. 1) during the early Pleistocene (Ho, 1974).

Although the altitude of the south-western extension of the Central Range, which separates the SW and S groups of *C. rathbunae*, is not as high as other parts of the range, the mountains evidently play an important role in isolating the two groups. Shih *et al.* (2004) showed that the isolating effect of the mountains in this region on large-sized lowland species of *Geothelphusa*, *G. albogilva* and *G. pingtung*, was conspicuous compared with the effect on the small-sized highland species *G. tawu*. Similarly, restricted gene flow in *C. rathbunae*, a large species of freshwater crab that is abundant in lowland areas, also appears to have taken place in the south-western extension of the Central Range.

The divergence time between the E, S and SE groups is more recent (0.8 ± 0.4 Ma) and may have resulted from the formation of the Coastal Range (Fig. 1), which occurred between 1 and 0.5 Ma (Chen & Wang, 1988, 1996; Dorsey &

Lundberg, 1988). Although the divergence level between these groups is low (0.54% diagnostic distance and 0.73% corrected divergence), the isolation of the E group is well supported by all three phylogenetic methods (Fig. 2). The E and SE groups are geographically separated by the Beinan River system. In their phylogeographic study of bamboo vipers, Creer *et al.* (2001) detected similar allopatric fragmentation events spanning the southern tip of the Coastal Range. This isolation is considered to be caused by a shallow hydrographic barrier (Chai, 1972). The boundary between the S and SE groups is located between Dawu and Taimali, Taitung County, where a series of mountains oriented from west to east, Mt Dahan (1688 m above sea level), Mt Guzhilun (1631 m), Mt Shenglin (907 m), and Mt Jianaimai (781 m) (all from the Central Range), extends eastwards to c. 2.5 km from the sea shore. The mountains in this area must have been serious impediments to gene flow northwards or southwards. Similar findings in this region were also shown recently for *Geothelphusa* (Shih *et al.*, 2004). Our study concurs with results obtained by Daniels (2003), who mentions high levels of dispersal occurring within and between drainages, but points out that high mountain barriers will impede gene flow between drainage basins (geographical regions).

Despite the relatively large genetic differentiation among populations of Taiwanese *C. rathbunae* (almost 7% corrected divergence, Table 2), crabs from different geographical regions are still considered as a single species. Studies on the genetic structure and morphological differences of freshwater crabs from South Africa also showed a similar phenomenon of cryptic speciation (Daniels *et al.*, 2003; Daniels, 2003). Kitaura *et al.* (2002) reviewed the 16S rRNA gene sequence divergence between crustacean species and showed that there is often 6–8% divergence between two species and up to 3% within species. Schubart *et al.* (1998b, 1999) found 11-bp to 14-bp difference within the same gene region (2–3%) between two closely related freshwater crabs *Sesarma ayatum* and *S. bidentatum* from Jamaica. In our study, the diagnostic genetic distance within the 16S mtDNA region among the six main populations ranged from 0.36% to 5.24%, and the corrected divergence ranged from 0.63% to 7.08% (Table 2). The fact that there are fewer than nine steps within each clade shows that the haplotypes within each clade are parsimony-connected ($\geq 0.95\%$, Templeton *et al.*, 1992). In contrast, there are 16 steps between clades, and therefore these could be considered as not connected. Our data suggest that at least the monophyletic eastern and western clades, separated by about 5 Ma of evolutionary history, should constitute two different species. Future detailed morphological comparisons may provide evidence for such a species separation.

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BIOSKETCHES

Hsi-Te Shih is an assistant professor at the Department of Life Science, National Chung-Hsing University. His interests are systematic and phylogeographic studies of crabs and other terrestrial invertebrates.

Huei-Chuan Hung is an associate professor at the National Taichung Nursing College, and studies human diseases using molecular tools.

Christoph D. Schubart is an assistant professor at the University of Regensburg in Germany. His current scientific interests are focused on phylogeny and population genetics of decapod crustaceans, with emphasis on radiations of freshwater and terrestrial forms.

Chaolun Allen Chen is an associate research fellow of the Research Center of Biodiversity, Academia Sinica. He has been studying the evolution of corals and other animals for several years.

Hsueh-Wen Chang is an associate professor at the Department of Biological Sciences, National Sun Yat-Sen University, and specializes in the ecology and evolution of terrestrial animals.

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