Zoological Studies **47**(1): 114-125 (2008)

Zoological Studies

Taxonomy, Phylogeny, and Biogeography of the Endemic Mudflat Crab *Helice*/*Chasmagnathus* Complex (Crustacea: Brachyura: Varunidae) from East Asia

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(Accepted August 1, 2007)

Hsi-Te Shih and Hiroshi Suzuki (2008) Taxonomy, phylogeny and biogeography of the endemic mudflat crab *Helice*/*Chasmagnathus* complex (Crustacea: Brachyura: Varunidae) from East Asia. *Zoological Studies* **47**(1): 114-125. The taxonomy, phylogeny, and biogeography of the endemic genera *Helice*, *Helicana*, and *Chasmagnathus* from East Asia were studied using 2 mitochondrial genes: the large subunit (16S) ribosomal (r)RNA and cytochrome oxidase subunit I (COI). *Helice* and *Helicana* were each shown to be monophyletic genera, while 3 species of the genus *Helice*, *H. latimera*, *H. formosensis*, and *H. tientsinensis*, formed an unresolved "*H. latimera* clade", implying that they may represent the same species with an intraspecific variable number of suborbital tubercles. *Helice tridens* is predominantly distributed Japan and might be adapted to a more-temperate (northern) climate with cooler temperatures. The 3 species of *Helicana* can very clearly be genetically distinguished from one another. The sympatric purple and olive forms of *Chasmagnathus convexus* appear to belong to the same species, and the color differences are probably caused by variable food sources or the substrate on which it lives. http://zoolstud.sinica.edu.tw/Journals/47.1/114.pdf

Key words: *Helicana*, 16S rRNA, Cytochrome oxidase I, Morphology, Distribution.

The genera *Helice* sensu lato and *Chasmagnathus*, belonging to the family Varunidae sensu Schubart et al. (2002), are common crabs which burrow in intertidal mudflats, swamps, salt marshes and estuaries, especially in the high intertidal and supralittoral zones (Tune Sakai 1976, Dai et al. 1984 1986, Dai and Yang 1991, Shih 1998 2007, Ng et al. 2001, Kwok and Tang 2005). Katsushi Sakai and Yatsuzuka (1980) erected a new subgenus, *Helicana*, within the genus *Helice* to include *H. wuana* and *H. japonica*. Recently, K. Sakai et al. (2006) revised the *Helice*/*Chasmagnathus* group, and 6 genera were recognized. Three genera, *Chasmagnathus* (*C. convexus* de Haan, 1833), *Helice* (*H. tridens* de Haan, 1835, *H. latimera* Parisi, 1918, *H. formosensis* Rathbun, 1931, and *H. tientsinensis* Rathbun, 1931) (Fig. 1), and *Helicana* (*H. wuana* (Rathbun, 1931), *H. japonica* Sakai and Yatsuzuka, 1980, and *H. doerjesi* Sakai, Türkay and Yang, 2006) (Fig. 2), are endemic to East Asia (Korea, Japan, China, the Ryukyus, Taiwan, and northern Vietnam) (T. Sakai 1976, Kosuge et al. 1997, K. Sakai et al. 2006, Shih 2007), this being the area of highest diversity of mudflat crabs. *Pseudohelice subquadrata* (Dana 1851) (= *Helice Leachii* Hess, 1865) is widely distributed throughout the Indo-West Pacific, but is more abundant in the tropics. The remaining 2 genera are restricted to the Southern Hemisphere: *Austrohelice crassa* (= *Helice crassa* Dana 1851) from New Zealand and *Neohelice granulata* (= *Chasmagnathus granulatus* Dana 1851) from South America (see Shih 2007).

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Ecologically, most species of mudflat crabs seem to be more active during nighttime, although some species can be found during the day. In Japan, *Helice tridens* and *Helicana japonica* can be active in daytime (Henmi and Murai 1999), and the levels of activity between daytime and night are the same in *H. tridens* (Kuroda et al. 2005). However, tolerance of salinity and dryness by *H. japonica* is lower than that by *H. tridens* (Omori et al. 1998). In Taiwan, *Helice formosensis* and *Helicana doerjesi* can also be seen in the day, but their activity levels at night are unknown (Shih 2007). Both species are reported to attack other crabs at dusk when the light is weak (Shih 1997, Shih et al. 2005b). The large-sized *Chasmagnathus convexus* (with a carapace which can attain a width of 45-50 mm; Yamaguchi 2002) is more active at night than during the daytime (Nakasone et al. 1983, Yamaguchi 2002). Ecological studies of *Helice*/*Chasmagnathus* crabs of the world were recently reviewed by Shih (2007).

In parts of Taiwan, Korea, and China, these crabs are eaten by local people (Maki and Tsuchiya 1923, Kamita 1941, Dai et al. 1984, Wei and Chen 1991), but there have also been some infective cases of lung fluke, *Paragonimus* spp., on *Helice tientsinensis* and *Chasmagnathus convexus* (Li et al. 1966, Dai et al. 1984, Chen et al. 1985, Wei and Chen 1991). However, after an extensive examination, no lung flukes were found in mudflat crabs from Taiwan (Chiu 1964). The rodent fluke, *Microphalloides japonicus*, which naturally infects rats and birds, was also found in mudflat crabs from Japan, Taiwan, Korea, and China (Yoshida 1938, Chiu 1964, Seo et al. 1964, Chen et al. 1985, Kifune and Koga 1999, Liu et al. 2000, Shigi et al. 2005).

The taxonomy of the genera *Helice* sensu stricto (s. str.) and *Helicana* in East Asia has been unsatisfactory for a long time, especially for *Helice* s. str., as illustrated by the long list of synonyms under each species (see K. Sakai et al. 2006). Presently, 4 species are included within the genus *Helice* s. str. and the key interspecific differences mostly rely on the size, shape, and number of tubercles on the suborbital ridge. However, the key morphological differences are variable within species, and greatly differ between sexes. In addition, the number of suborbital tubercles of females interspecifically overlap to a certain degree. Although this structure is believed to be a kind of stridulating apparatus (it has been proposed to

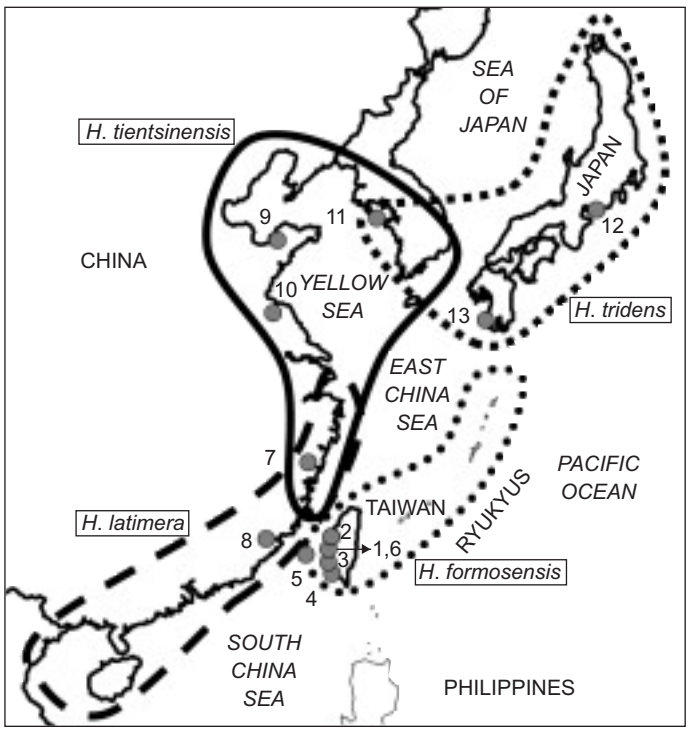

Fig. 1. Collection sites (gray circles) for the genus *Helice* sensu stricto from Japan, Korea, China, and Taiwan used in this study. Numbers beside the circles indicate collection sites given in table 1. The different lines indicate the biogeographic boundaries for *Helice* spp. (Kosuge et al. 1997, K. Sakai et al. 2006, Shih 2007).

produce sound for individual communication), there is still no study which demonstrates such a function (see K. Sakai et al. 2006).

The high diversity of mudflat crabs in East Asia was probably caused by some geological events around the Yellow Sea and East China Sea (Figs. 1, 2). Before the opening of the Okinawa Trough due to the collision of tectonic plates, East Asian islands were connected to the Asian continent (Kimura 2002). The opening of the Okinawa Trough might have formed a large lagoon or inner sea during some geological periods, which increased the complexity of the geographical barriers and might have been a major isolating mechanism for certain intertidal organisms. In addition, habitats of intertidal organisms might become isolated when land bridges are formed during glaciations, and those barriers might disappear when the seawater returned during interglacial periods. The Yellow Sea and East China Sea are shallow seas with average depths of 50 and 100 m, respectively, and are located on the continental shelf along the coasts of China, Korea, Japan, the Ryukyus, and Taiwan. When global sea levels fell, the 2 seas might have become more or less continuous land. Both the collision of tectonic plates and sea level fluctuations may have formed certain geographic barriers which definitely restricted gene flow to some degree, and speciation may have occurred in certain intertidal organisms. The divergence and speciation of East Asian mudflat crabs were very likely caused by the above isolation mechanisms.

Molecular markers, large subunit (16S) ribosomal (r)RNA and cytochrome oxidase I (COI), have been proven to be useful markers for establishing species boundaries of decapod crustaceans (Geller et al. 1997, Sarver et al. 1998, Schneider-Broussard et al. 1998, Schubart et al. 1998b 2001, Shih et al. 2004 2005a 2006 2007a b, Shih and Cai 2007, Yeo et al. 2007). The purpose of this study was to analyze endemic mudflat crabs of East Asia using the above molecular characters in order to discuss their species boundaries, and possible related geological events are proposed to explain their phylogeny and biogeography.

MATERIALS AND METHODS

The material included 3 genera, *Helice* (*H. tridens*, *H. latimera*, *H. formosensis*, and *H. tientsi-*

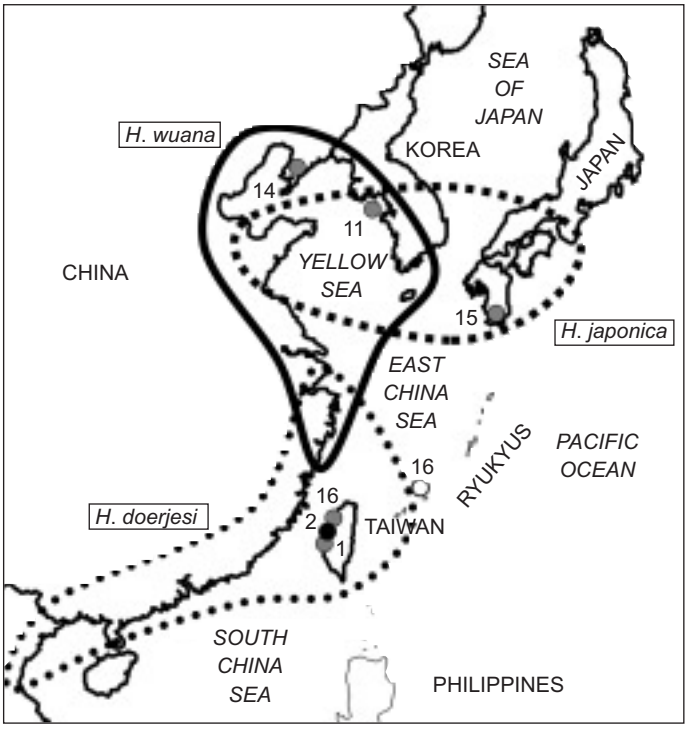

Fig. 2. Collection sites (gray circles) for the genus *Helicana* from Japan, Korea, China, and Taiwan used in this study, with collection sites for *Chasmagnathus convexus* (black circle) and *Pseudohelice subquadrata* (white circle). Numbers beside the circles indicate collection sites given in table 1. Different lines indicate the biogeographic boundaries for *Helicana* spp. (T. Sakai, 1976, K. Sakai et al. 2006, Shih 2007).

nensis), *Helicana* (*H. wuana*, *H. japonica*, and *H. doerjesi*) and *Chasmagnathus* (*C. convexus*). They were collected on mudflats from Japan (Kagoshima Prefecture (Pref.) and Aichi Pref.), South Korea (Ganghwa I.), China (Liaoning Province (Prov.), Hebei Prov., Shandong Prov., and Fujian Prov.), and Taiwan (Hsinchu County (Co.), Taichung Co., Changhua Co., Tainan Co., and Kaohsiung Co.) (Figs. 1, 2, Table 1). *Pseudohelice subquadrata* was used as an outgroup in the phylogenetic analysis, because of its exclusive adult and larval morphology, and allozyme differences (see K. Sakai et al. 2006). 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 (NCHUZOOL; see table 1 for catalog numbers).

Genomic DNA was isolated from the muscle tissue of legs using the Sigma mammalian genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). A region of ~550 base pairs (bp) at the 5'-end of the mitochondrial 16S rRNA gene was selected for amplification by a polymerase chain reaction (PCR) using the primers, 1471 (5'-CCT GTTTANCAAAAACAT-3') and 1472 (5'-AGATA GAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996). A portion of the COI gene was amplified by PCR using the primers, LCO1490 (5'-GGTCAA CAAATCATAAAGATATTGG- 3') and HCO2198

Table 1. Twelve haplotypes of 16S ribosomal (r)RNA and 15 haplotypes of cytochrome oxidase subunit I (COI) genes of the genera *Helice*, *Helicana*, *Chasmagnathus*, and *Pseudohelice* from East Asia used in this study. Species were identified based on K. Sakai et al. (2006). The numbers within brackets after the localities correspond to those in figures 1 and 2. Co., county; Pref., prefecture; Prov., province; NCHUZOOL, the Zoological Collections of the Department of Life Science, National Chung Hsing Univ.; DDBJ, the DNA Data Bank of Japan; [a]legs provided by S. L. Yang, Beijing, China; [b]leg provided by N. K. Ng, National Univ. of Singapore, Singapore

Species	Localities	NCHUZOOL catalog no.	Sample size	Haplotypes of 16S	DDBJ access. no.	Haplotypes of COI	DDBJ access. no.
genus *Helice*							
H. formosensis	Shengang, Changhua Co., Taiwan [1]	13083	1	HLT1	AB334531	HLT-C1	AB334543
	Wenliao, Taichung Co., Taiwan [2]	13084	1	HLT1	AB334531	HLT-C1	AB334543
	Anping, Tainan City, Taiwan [3]	13085	1	HLT1	AB334531	HLT-C1	AB334543
	Linyuan, Kaohsiung Co., Taiwan [4]	13086	1	HLT1	AB334531	HLT-C1	AB334543
	Cingluo, Penghu Co., Taiwan [5]	13087	1	HLT1	AB334531	HLT-C2	AB334544
	Gaomei, Taichung Co., Taiwan [6]	13088	1	HLT1	AB334531	HLT-C1	AB334543
H. latimera	Fuzhou City, Fujian Prov., China [7]	-[a]	1	HLT1	AB334531	HLT-C1	AB334543
	Xiamen City, Fujian Prov., China [8]	13089	1	HLT2	AB334532	HLT-C3	AB334545
H. tientsinensis	Qingdao City, Shandong Prov., China [9]	-[b]	1	HLT3	AB334533	HLT-C4	AB334546
	Beidaihe, Hebei Prov., China [10]	-[a]	1	HLT4	AB334534	HLT-C3	AB334545
	Ganghwa I., Incheon, South Korea [11]	13090	1	HLT5	AB334535	HLT-C5	AB334547
	Ganghwa I., Incheon, South Korea [11]	13091	1	HLT5	AB334535	HLT-C1	AB334543
H. tridens	Aichi Pref., Japan [12]	13092	1	HTR1	AB334536	HTR-C1	AB334548
	Fukiage, Kagoshima Pref., Japan [13]	13093	1	HTR1	AB334536	HTR-C2	AB334549
	Fukiage, Kagoshima Pref., Japan [13]	13094	1	HTR2	AB334537	HTR-C1	AB334548
genus *Helicana*							
H. wuana	Dalian, Liaoning Prov., China [14]	-[a]	1	HW1	AB334538	HW-C1	AB334550
	Ganghwa Island, Incheon, South Korea [11]	13095	2	HW1	AB334538	HW-C2	AB334551
H. japonica	Obama, Kagoshima Pref., Japan [15]	13096	1	HJ1	AB334539	HJ-C1	AB334552
	Obama, Kagoshima Pref., Japan [15]	13097	1	HJ1	AB334539	HJ-C1A	AB334553
H. doerjesi	Shengang, Changhua Co., Taiwan [1]	13098	1	HD1	AB334540	HD-C1	AB334554
	Haishangu, Hsinchu City, Taiwan [15]	13099	1	HD1	AB334540	HD-C1	AB334554
Chasmagnathus convexus	Wenliao, Taichung Co., Taiwan [2] (purple form)	13100	1	CC1	AB334541	CC-C1	AB334555
	Wenliao, Taichung Co., Taiwan [2] (yellow form)	13101	1	CC1	AB334541	CC-C1A	AB334556
Outgroup							
Pseudohelice subquadrata	Miyako I., the Ryukyus [16]	13102	1	HSQ1	AB334542	HSQ-C1	AB334557
Total			25				

(5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). PCR conditions for the above primers were denaturation for 50 s at 94°C, annealing for 70 s at 45°C, and extension for 60 s at 72°C, followed by a final extension for 10 min at 72°C. Sequences were obtained by automated sequencing (ABI PRISM 377 Sequencer and MegaBACE DNA Analysis System 500, Amersham, UK) and were aligned with the aid of CLUSTAL W (vers. 1.4, Thompson et al. 1994) and BIOEDIT (vers. 5.09, Hall 2001), after verification with the complementary strand. Sequences of the different haplotypes were deposited in the DNA Data Bank of Japan (DDBJ), and the accession numbers are given in table 1.

The best-fitting model for sequence evolution of the combined 16S and COI datasets was determined by MrModeltest (vers. 2.2, Nylander 2005), selected by the hLRT (hierarchical likelihood ratio test), and was subsequently applied to the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) analyses. The NJ tree was constructed with the PAUP* program (vers. 4.0b10, Swofford 2003) with 2000 bootstrap reiterations. A maximum parsimony (MP) tree was constructed using the PAUP* program with 2000 bootstrap reiterations of a simple heuristic search, TBR (tree bisection-reconnection) branch-swapping, and 100 randomly added sequence replications. All characters were equally weighted. Gaps in MP tree construction were treated as missing data. The ML analysis was also calculated by PAUP* with 200 bootstrap replications and 20 randomly added 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 2000 for the ML analysis. The BI analysis was performed with MrBayes (vers. 3.1.1, Ronquist and Huelsenbeck 2003) using the model selected by MrModeltest. The search was run with 4 chains for 2 x 10^6 generations, with trees being sampled every 100 generations (the 1st 500 trees were later discarded as the burn-in).

Phylogenetic reconstructions identified a clade containing *Helice latimera*, *H. formosensis*, and *H. tientsinensis* composed of very closely related haplotypes, which included individuals collected from Taiwan, coastal China, and Korea (Fig. 1). To examine the relationships of these haplotypes in detail, a gene genealogy of the combined 16S rRNA and COI data was constructed using the TCS program (vers. 1.21, Clement et al. 2000) with gaps treated as missing data.

RESULTS

A ~560 bp segment (excluding the primer regions) of the 16S rRNA from all 25 specimens was amplified and aligned; 83 positions were variable and 41 parsimoniously informative. Among the total number of sequences, 12 different haplotypes (excluding the outgroup) were distinguished (Table 1). The studied segment of 16S rRNA sequences was AT rich (71.3%) (T, 36.7%; A, 34.6%; G, 18.0%; and C, 10.6%). For the COI gene, a 658 bp segment was compared, resulting in 15 different haplotypes (excluding the outgroup) (Table 1). The studied segment of the COI sequence was also AT rich (60.2%) (T, 33.5%; A, 26.7%; G, 17.6%; and C, 22.2%). In this gene, 192 positions were variable and 167 parsimoniously informative.

The best model selected by MrModeltest for the combined 16S rRNA and COI segment of 1218 bp was the GTR + I + G model (with a proportion of invariable sites of 0.6001 and a gamma distribution shape parameter of 0.5095). The tree of the phylogram constructed from the NJ analysis, with the respective confidence values from the MP, ML, and BI analyses, is shown in figure 3. Only confidence values > 50% are shown. For the MP analysis, a single tree was recovered with a tree length of 457 steps, a consistency index of 0.76, and a retention index of 0.87.

Based on figure 3 of the combined dataset, *Helice* s. str. formed a monophyletic clade with high confidence values in all 4 methods. Only 2 groups were revealed within the genus *Helice*. Three species, *H. latimera*, *H. formosensis*, and *H. tientsinensis*, were mixed and formed a clade, herein termed the "*H. latimera* clade" for convenience, with high confidence values (except for the ML and BI methods). The remaining species formed a highly supported clade which contained only *H. tridens*. Pairwise base-pair differences and nucleotide divergence with the uncorrected p-distance between those haplotypes of 16S rRNA and COI in this study are shown in tables 2 and 3, respectively. The base pair differences and nucleotide divergence of 16S rRNA (and COI) within the *H. latimera* clade was ≤ 6 (4) bp and 1.08% (0.61%), respectively, whereas pairwise differences between other species in our study were ≥ 9 (30) bp and 1.46% (4.56%), respectively.

Helicana was also monophyletic with high support in all 4 methods (Fig. 3), and the 3 species, *H. wuana*, *H. japonica*, and *H. doerjesi*, could very clearly be genetically distinguished from

one another. The base-pair difference and nucleotide divergence of 16S rRNA (and COI) among the 3 species were 9-16 (36-74) bp and 1.46%-2.36% (5.47%-11.25%) (Tables 2, 3), respectively. *Chasmagnathus* formed a sister group to *Helicana*, but the confidence support was not high.

The haplotype network constructed to further depict the relationship within the combined 16S and COI haplotypes of the *Helice latimera* clade is shown in figure 4. The combined haplotype HLT1 + HLT - C1 is central relative to all other haplotypes and was therefore assumed to represent the ancestral haplotype of the combined 16S and COI data (cf. Clement et al. 2000).

DISCUSSION

Taxonomy and systematics

In our study, specimens of *H. latimera*, *H. formosensis*, and *H. tientsinensis* of the genus *Helice* s. str. were indistinguishable using 16S rRNA and COI. Apparently, the "*H. latimera* clade" defined herein is a species complex, and the species validity cannot be assured. In contrast, the 3 species, *H. wuana*, *H. japonica*, and *H. doerjesi* of the genus *Helicana*, were genetically distinct, with the latter 2 species being more closely related (Fig. 3, Tables 2, 3).

The taxonomy of the *H. latimera* species complex has been confusing for a long time (see K. Sakai et al. 2006). For example, Rathbun (1931) reported *H. tridens pingi* as a new subspecies, followed by Dai et al. (1986), but it was regarded as a synonym of *H. latimera* by K. Sakai and Yatsuzuka (1980) and Dai and Yang (1991). In addition, T. Sakai (1939 1976) invalidated *H. tridens formosensis* Rathbun, 1931 as a junior synonym of *H. tridens latimera* Parisi, 1918, followed by Dai et al. (1986), but it was later considered to be the valid species, *H. formosensis* (K. Sakai and Yatsuzuka 1980, Dai and Yang 1991, K. Sakai et al. 2006). Moreover, specimens from the Ryukyus were first identified as *Helice* (*Helice*) *tridens tridens* de Haan, 1835 by K. Sakai and Yatsuzuka (1980), but later as *H. formosensis* by K. Sakai et al. (2006).

Such phenomena imply that the taxonomy of this group is problematic and should be revised.

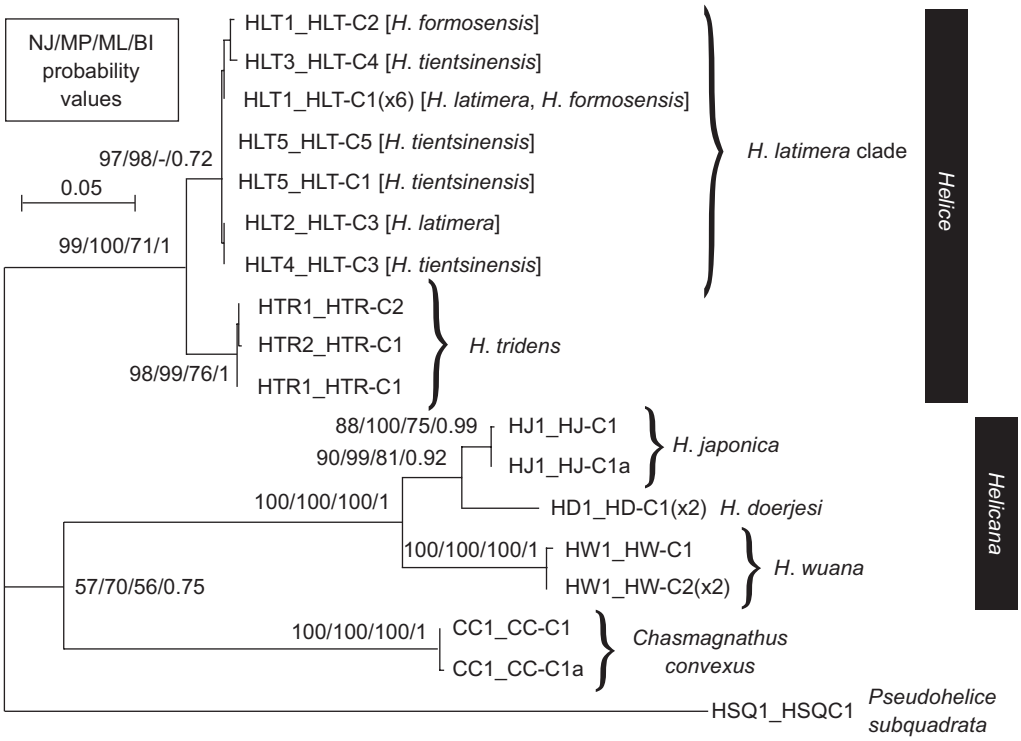

Fig. 3. A neighbor-joining (NJ) tree of species of the *Helice*/*Chasmagnathus* complex from Japan, Korea, China, and Taiwan, based on 1218 bp of the combined 16S ribosomal (r)RNA and cytochrome oxidase subunit I genes. Probability values at the nodes represent bootstrap values for NJ, maximum parsimony (MP), and maximum likelihood (ML), and posterior probability for Bayesian inference (BI). For haplotype names see table 1.

Although several reports diagnosed these species (e.g., the suborbital crenulation was distinct for typical specimens of these species), the characters are not always reliable. For the same species, males and females sometimes show a large degree of difference. For example, K. Sakai et al. (2006) concluded that the numbers of granules on the suborbital ridge of males of *H. tridens*, *H. formosensis*, *H. tientsinensis*, and *H. latimera* are 9-16, 15-22, 33-37, and 64-67; and are 15-28, 24-29, 26-37, and 37-55 for females, respectively. In particular, the numbers of granules of females of different *Helice* s. str. species overlap from 1 to 5. A female may be identified as *H. tridens*, *H. formosensis*, or *H. tientsinensis*, if it has 26, 27, or 28 granules on the suborbital ridge.

Shih et al. (2004 2005a 2006 2007a b) and Yeo et al. (2007) proposed at least a 6-7 bp differ-

Table 2. Pairwise nucleotide percentage divergence (p-distance) matrix (lower-left) and the base-pair (bp) differences (upper right) based on 560 bp of the 16S ribosomal (r)RNA gene between haplotypes of the *Helice*/*Chasmagnathus* complex from East Asia (Table 1). For abbreviations of haplotypes see table 1

	Helice							*Helicana*			*Chasmagnathus*	*Pseudohelice*
	HLT1	HLT2	HLT3	HLT4	HLT5	HTR1	HTR2	HW1	HJ1	HD1	CC1	HSQ1
HLT1	-	2	3	3	3	12	10	29	34	34	37	53
HLT2	0.36	-	5	1	3	14	12	30	35	35	36	54
HLT3	0.54	0.90	-	6	6	15	13	30	35	33	38	56
HLT4	0.54	0.18	1.08	-	4	15	13	30	35	35	35	53
HLT5	0.36	0.36	0.90	0.54	-	13	11	29	34	34	37	55
HTR1	1.26	1.62	1.80	1.80	1.62	-	4	38	37	41	47	61
HTR2	1.44	1.80	1.99	1.99	1.80	0.18	-	36	35	39	45	59
HW1	5.24	5.42	5.42	5.42	5.06	5.95	6.14	-	14	16	31	49
HJ1	5.80	5.98	5.98	5.98	5.62	5.43	5.61	2.17	-	9	38	53
HD1	5.63	5.81	5.44	5.81	5.45	5.98	6.17	2.36	1.46	-	40	55
CC1	6.52	6.34	6.70	6.16	6.34	7.42	7.60	5.44	6.36	6.55	-	56
HSQ1	8.70	8.88	9.24	8.70	8.89	9.25	9.42	7.98	9.08	9.27	9.08	-

Table 3. Pairwise nucleotide percentage divergence (p-distance) matrix (lower-left) and the base-pair (bp) differences (upper right) based on 658 bp of the cytochrome oxidase subunit I (COI) gene between haplotypes of the *Helice*/*Chasmagnathus* complex from East Asia (Table 1). For abbreviations of haplotypes see table 1

	Helice							*Helicana*					*Chasmagnathus*		*Pseudohelice*
	HLT-C1	HLT-C2	HLT-C3	HLT-C4	HLT-C5	HTR-C1	HTR-C2	HW-C1	HW-C2	HJ-C1	HJ-C1a	HD-C1	CC-C1	CC-C1a	HSQ-C1
HLT-C1	-	2	2	4	1	31	32	107	108	103	102	107	89	89	104
HLT-C2	0.30	-	2	4	1	31	32	107	108	103	102	107	91	91	105
HLT-C3	0.30	0.30	-	4	1	31	32	107	108	103	102	107	89	89	103
HLT-C4	0.61	0.61	0.61	-	3	33	34	108	109	102	101	108	92	92	105
HLT-C5	0.15	0.15	0.15	0.46	-	30	31	106	107	102	101	106	90	90	104
HTR-C1	4.71	4.71	4.71	5.02	4.56	-	1	108	108	98	97	103	96	96	100
HTR-C2	4.86	4.86	4.86	5.17	4.71	0.15	-	109	109	99	98	104	95	95	99
HW-C1	16.26	16.26	16.26	16.41	16.11	16.41	16.57	-	4	71	70	74	116	117	121
HW-C2	16.41	16.41	16.41	16.57	16.26	16.41	16.57	0.61	-	69	68	70	115	116	121
HJ-C1	15.65	15.65	15.65	15.50	15.50	14.89	15.05	10.79	10.49	-	1	36	112	111	109
HJ-C1a	15.50	15.50	15.50	15.35	15.35	14.74	14.89	10.64	10.33	0.15	-	37	111	110	108
HD-C1	16.26	16.26	16.26	16.41	16.11	15.65	15.81	11.25	10.64	5.47	5.62	-	115	114	109
CC-C1	13.53	13.83	13.53	13.98	13.68	14.59	14.44	17.63	17.48	17.02	16.87	17.48	-	2	119
CC-C1a	13.53	13.83	13.53	13.98	13.68	14.59	14.44	17.78	17.63	16.87	16.72	17.33	0.30	-	118
HSQ-C1	15.81	15.96	15.65	15.96	15.81	15.20	15.05	18.39	18.39	16.57	16.41	16.57	18.09	17.93	-

ence (~1%) of the ~560 bp 16S rRNA between different freshwater crab species. If this criterion was applied to the *Helice*/*Chasmagnathus* complex from East Asia, and considering the relationship given in the phylogenetic tree, most species would be successfully supported, except for the *H. latimera* clade. The base-pair differences of 16S rRNA within the *H. latimera* clade are ≤ 6 bp, whereas pairwise differences between other species in our study are ≥ 9 bp (Table 2). In addition, COI is considered to be more than 2 times more variable than 16S rRNA (Schubart et al. 1998a, Tong et al. 2000, Shih et al. 2007b). In our study, the average ratio of divergence of COI and 16S rRNA between species was 2.40 (range, 1.62-4.86) which fits the above estimation, but the divergence of COI was lower than that of 16S rRNA (with a ratio of 0.63) within the *H. latimera* clade (Tables 2, 3). More studies in the future may provide a suitable explanation for this phenomenon. The haplotype network of the *H. latimera* clade (Fig. 4) showed that the combined haplotype HLT1+HLT-C1 (from Fujian, China and western Taiwan) are central relative to other haplotypes and are the assumed ancestral haplotype, but apparently there is no tendency for clustering within this clade.

Therefore, it is reasonable to consider the *H. latimera* clade as a single species, and the species name, *Helice latimera* Parisi, 1918, has the priority. The different morphological characters among these 3 "species" might be attributable to intraspecific variations. Variations of this character are probably due to individual differences, growth, environmental adaptations, or the product of female choice, but more studies are necessary to clarify this issue. Some other characters were used to morphologically separate species of *Helice* s. str. (see K. Sakai et al. 2006), but they are not satisfactory. For example, although drawings of the male gonopods and female gonopores for each species of *Helice* s. str. have been provided (K. Sakai et al. 2006), the differences are still minor and not very helpful. In K. Sakai et al.

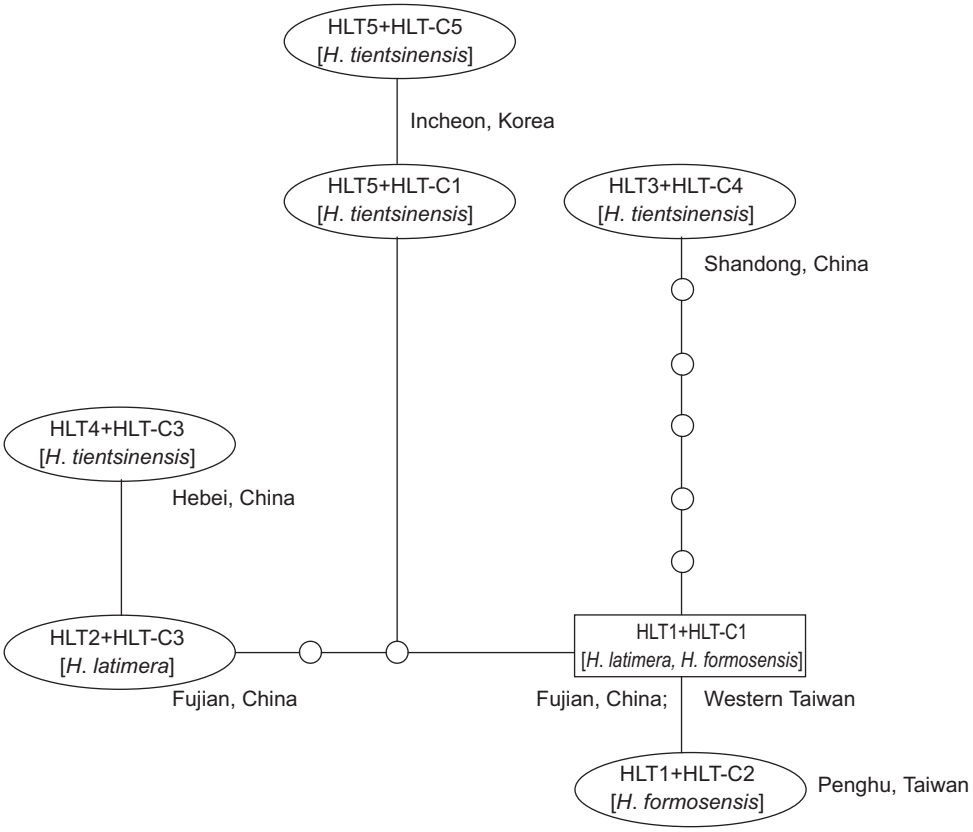

Fig. 4. Genealogical network for the combined 16S ribosomal (r)RNA and cytochrome oxidase subunit I (COI) haplotypes observed within the *H. latimera* clade of the genus *Helice*, which includes specimens collected from Taiwan, coastal China, and Korea (Fig. 1). The ancestral haplotype, or root of the network, is indicated by a square. Unlabeled nodes indicate inferred haplotypes not found in the sampled populations.

(2006), the picture of the gastric mill was only provided for each genus of *Helice*/*Chasmagnathus* crabs, because the authors mentioned that the gastric mill teeth significantly differ between genera but are relatively homogenous within a genus. A similar problem of species boundaries also occurs in other varunid crabs, e.g., the high genetic similarity among *Eriocheir japonica*, *E. sinensis*, and *E. hepuensis*. Neither mitochondrial 16S rRNA and COI, nor nuclear ITS, can be used as a reliable genetic marker to satisfactorily distinguish among members of this group, and the subspecies status has been proposed to conform to this relationship (Chu et al. 2003, Tang et al. 2003). Within *Helice* s. str., *H. tridens* forms a sister group to the *H. latimera* clade, with satisfying relationship and molecular difference (Fig. 3, Tables 2, 3).

Specimens (2 males and 1 female) collected from Deogjeog-do I., an island offshore of Inchon in central-western Korea and deposited in the Museum national d'Histoire Naturelle (Paris), were identified as *Helice tridens* (see K. Sakai et al. 2006: 24). While *H. tientsinensis* and *H. wuana* are the most commonly recorded species in Korea (see Kamita 1941, K. Sakai et al. 2006), the material of *H. tridens* from Korea should be reexamined. Otherwise, the distribution of *H. tridens* is almost exclusively restricted to Japan, and the species shows a northerly distribution, from Kagoshima and Kyushu to Aomori, the northernmost prefecture of Honshu (from 30° to 41°N) (K. Sakai et al. 2006).

The species assigned to *Helicana* by K. Sakai et al. (2006) including *H. wuana*, *H. japonica*, and *H. doerjesi*, all appeared in 1 clade. This genus is monophyletic in our molecular study and endorses the initial decision by K. Sakai and Yatsuzuka (1980) to assign *H. wuana* and *H. japonica* to a separate subgenus *Helice* (*Helicana*) and later to give this taxon full generic status (K. Sakai et al. 2006). In contrast to *Helice*, *Helicana* shows more-stable interspecific characters at least in male suborbital tubercles and male gonopods, although identifying females is still difficult (see K. Sakai et al. 2006). The 3 species comprising this genus could also successfully be distinguished by molecular characters (Fig. 3). The base-pair difference of 16S rRNA (and COI) among the 3 species was 9-16 (36-74) bp (Tables 2, 3), which shows a satisfactory species boundary if the freshwater crab criterion is applied (Shih et al. 2004 2005a 2006 2007a b, Yeo et al. 2007).

K. Sakai et al. (2006) considered some specimens from Korea (of Deogjeog-do I.) and China (of Shandong Prov.) as *Helicana japonica*. Because *H. wuana* is the most common *Helicana* in the above area of the Yellow Sea (see Kamita 1941, K. Sakai et al. 2006), and *H. wuana* and *H. japonica* were confused by Dai et al. (1986) and Dai and Yang (1991), these materials should also be reex-

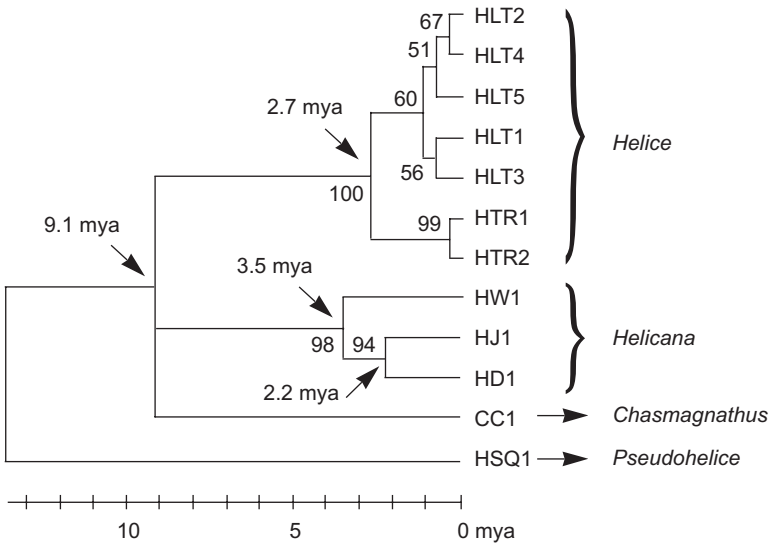

Fig. 5. A linearized neighbor-joining (NJ) tree of species of the *Helice*/*Chasmagnathus* complex from Japan, Korea, China, and Taiwan, based on 560 bp of the 16S ribosomal (r)RNA gene, with the estimated time of divergence (at the bottom) by the molecular clock calibrations of 0.65%/10^6 yr. Probability values at the nodes represent values after 2000 bootstrap reiterations with the uncorrected p-distance. For haplotype names see table 1.

amined. Otherwise, *H. japonica* is endemic to the southern part of Japan (the Pacific side of Shikoku and Kyushu) (K. Sakai et al. 2006).

There are 2 color forms, purple and olive, of *Chasmagnathus convexus* (see Shih 1998 2007) which occur sympatrically at our collection site. The 2 color forms have identical 16S rRNA sequences, and only exhibit a 2 bp difference in COI with haplotype CC-C1 for the purple and CC-C1a for the olive form (Tables 2, 3). The different color forms of this species may simply be the result of available food sources or substrate coloration, but appear to have no genetic basis (see Shih et al. 2007b).

Biogeography

The substitution rates of 16S rRNA, at 0.65%/10^6 yr for marine sesarmids (Schubart et al. 1998a) were applied to our data to construct a linearized NJ tree (Fig. 5), using the MEGA4 program (vers. 4.0, Tamura et al. 2007) with an uncorrected p-distance and 2000 bootstrap reiterations. The genera *Helice*, *Helicana*, and *Chasmagnathus* appeared to separate at nearly the same time, 9.1 million yr ago (mya). The estimated divergence times of the 3 genera of East Asian mudflat crabs are close to the beginning date of the opening of the Okinawa Trough, at 6-10 mya (6 mya from Sibuet and Hsu 2004; 7 mya from Kimura 2002; 10 mya from Miki et al. 1993, Kimura 2000). We propose that this geological event might have been the major isolating mechanism for the divergence of these 3 genera of mudflat crabs in East Asia.

In addition, global sea level changes corresponding to interglacial periods and glaciations may have been another isolating mechanism. From eustatic changes in global sea levels (see Woodruff 2003), sea levels fell rapidly from +40 m (above the present day) to below -50 m at 6 mya, then rose to +90 and +100 m during 5.5-4.2 mya. When the global sea levels fell, the shallow Yellow Sea and East China Sea might have more or less become continuous land. The intertidal organisms should have moved further eastward to the margins of the continent during the glaciations. The margins might have been the Okinawa Trough, or the more-easterly Ryukyu Trench (Kimura 2002). After the end of the glaciations, the intertidal organisms might have remained around the East Asian islands, or moved to the margin of the present Asian continent. Such changes in the positions of the coastal areas might have forced some intertidal organisms to have dispersed several times during these geological periods.

Subsequent repeated global changes in sea level, from +100 m to -100 m, might have provided sufficient isolating barriers to explain speciation within each genus of East Asian mudflat crabs. For instance, the genus *Helicana* began to diverge about 3.5 mya (Fig. 5), which might have been related with sea levels falling to -20 m at 3.5 mya. Later, the times of separation of *H. tridens* and the *H. latimera* clade, and *H. japonica* and *H. doerjesi*, are 2.7 and 2.2 mya (Fig. 5), respectively, which might be related to sea levels falling to -100 m at 2.75 mya and the later cyclic falling until 2 mya (see Woodruff 2003). The speciation of both the ancestral *Helice tridens* and *Helicana japonica* within Japan is suggested as being due to an isolating effect of glaciations from 2.75 to 2 mya. Apparently, *H. tridens* tolerates cooler (and more-northerly) habitats and is likely limited to Japan at present.

Acknowledgments: This study was supported by a grant from the National Science Council, Taiwan (NSC95-2621-B-005-004-) to H.T. Shih. We wish to express our thanks to Taewon Kim, Tohru Naruse, Si-Liang Yang, Ho-Tien Hung, Jung-Hsiang Lee, and Ngan Kee Ng for specimen collection. Special thanks go to Christoph D. Schubart, Universität Regensburg, Regensburg, Germany and Si-Liang Yang, Beijing Natural History Museum, Beijing, China, for providing useful comments. We also acknowledge 2 anonymous reviewers who greatly improved this manuscript.

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