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# Phylogeography of the Fiddler Crab *Tubuca arcuata* (Crustacea: Brachyura: Ocypodidae) in East Asia and Northern Vietnam

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The fiddler crab *Tubuca arcuata* (Crustacea: Ocypodidae: Gelasiminae) is widely distributed across East Asia and northern Vietnam. Particularly, this species inhabits estuarine mangroves and mudflats of the East Asian continent, as well as the main islands of Japan and the Ryukyus. By comparing sequences of the mitochondrial 16S, cytochrome oxidase subunit I (*COI*), and control region genes, two main clades of this species were elucidated. The northern (N) clade was mostly restricted to the region north of the Taiwan Strait, whereas the wide (W) clade could be found throughout the entire range inhabited by this species. Based on the distribution of the ancestral haplotypes of *COI* and the divergence time of the two clades, our findings suggest that the land bridge barrier present in the Taiwan Strait during glaciations might have played an important role in their cladogenesis, approximately 0.93 million years ago. Here, we propose that the distribution of the ancestral haplotype of the W clade in the Ryukyus and the main islands of Japan was determined by the Paleo-Kuroshio Current, whereas the modern distribution pattern is shaped by the modern Kuroshio Current and other warm currents during the crab reproduction season in the summer.

**Key words:** Mitochondrial genes, 16S rDNA, Cytochrome oxidase subunit I, Control region gene, Glaciation, Land bridge, Taiwan Strait.

## BACKGROUND

The Taiwan Strait is a shallow and narrow watergate located in East Asia, close to the Tropic of Cancer (23.5°N) and between the island of Taiwan and the East Asian continent (Fig. 1A). This strait has been hypothesized to play a key role in the dispersal of continental coastal organisms between subtropical and tropical regions by acting as a barrier. During the glacial period, when the sea level was 140 m below the present level (shaded areas in Fig. 1A), the Taiwan Strait became a land bridge (Boggs et al. 1979; Voris 2000; Xu et al. 2009) that presumably allowed some terrestrial animals to migrate from continental China to Taiwan and even to the Ryukyu Islands (*e.g.*, mammals, Ho et al. 1997 2000; Cai 1999; reptiles, Lin et al. 2002; frogs, YH Wang et al. 2017; freshwater crabs, Shih et al. 2007 2011). However, the glacial land bridge of the Taiwan Strait also acted as a geographical barrier that impeded the dispersal of marine coastal organisms between the East China Sea (ECS) and the South China Sea (SCS) (see below).

Several hypotheses for the phylogeographic patterns of marine organisms in East Asia have been proposed (see Fig. 1A). For example, some studies have indicated that (1) there is little divergence along the coastal regions of the East Asian continent (*e.g.*, mudskippers, He et al. 2015; filefish, ZY Wang et al. 2016; flathead fish, Cheng et al. 2019); (2) the Yellow Sea (YS) clades are unique, due to the occurrence of local ocean gyres (*e.g.*, marine snails, Ni et al. 2015; Zhao et al. 2017; swimming crabs, Han et al. 2015); (3) the northern and southern clades in both continental and insular regions are separated by the Yangtze River, due to freshwater discharge (*e.g.*, marine snails, Dong et al. 2012; Yu et al. 2014; Ni et al. 2015 2017; J Wang et al. 2015; algae, Cheang et al. 2010; J Wang et al. 2015); (4) the northern and southern clades in the continental region were separated by the Taiwan Strait during glaciations (*e.g.*, mullets, Liu et al. 2007b; mitten crabs, Xu et al. 2009; bivalves, XX Wang et al. 2017); and (5) the Okinawa (or the Ryukyus) clade is unique (*e.g.*, barnacles, Chang et al. 2017; intertidal crabs including *Tmethypocoelis*, *Mictyris*, and *Scopimera*, Davie and Kosuge 1995; Davie et al. 2010; Wong et al. 2010).

*Tubuca arcuata* (De Haan, 1835), one of the most common species of fiddler crabs in East Asia, is widely distributed across mudflats and estuaries in the subtropical to tropical regions of East Asia, Korea, Japan (including the Ryukyus), Taiwan (including Penghu), China (including Hainan and Hong Kong), and northern Vietnam (Crane 1975; Yoshigou 2001; Shih et al. 2010 2022). This species was grouped among the continental fiddler crabs in East Asia, by exhibiting a similar distribution to *Austruca lactea*, *Gelasimus borealis*, and *Paraleptuca splendida* (see Shih 2012). The general ecology, reproduction, growth and behavior of *T. arcuata* have been extensively studied (*e.g.*, JT Shih 1990 1992; JT Shih et al. 1991; Murai 1992; JH Shih and Lan 1993; JH Shih and Wang 1993; Jones and Morton 1994; Li 1996; Otani et al. 1997; Wada and Murata 2000; Aoki et al. 2010). During its early development, this species undergoes five zoeal stages within 18 days, followed by the megalopal stage (Ko and Kim 1989). Furthermore, this species has been included in various phylogenetic studies of fiddler crabs (Levinton et al. 1996; Sturmbauer et al. 1996; Shih et al. 2016b) and a study analyzing its full
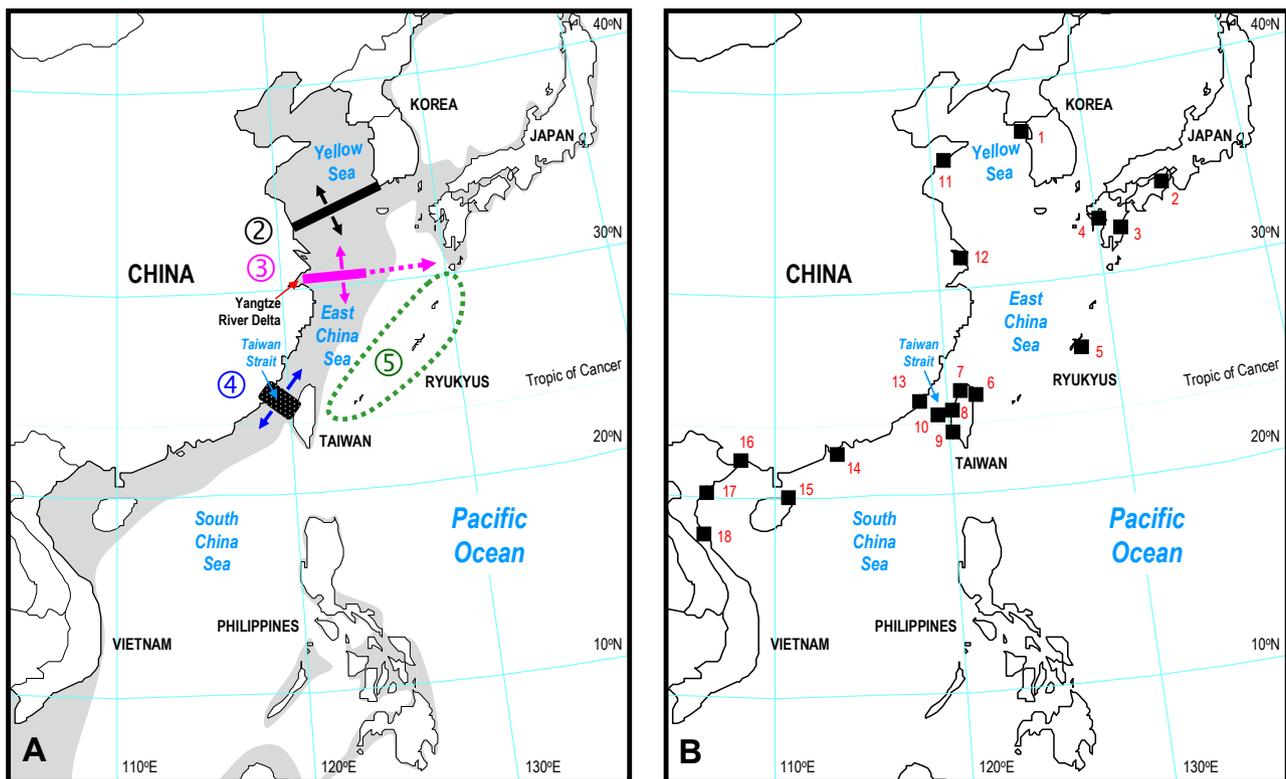


**Fig. 1.** A, Phylogeographic patterns of coastal organisms in continental and insular East Asia. (1) There is no divergence along the coastal regions of the East Asian continent (not shown); (2) the unique Yellow Sea clade; (3) northern and southern clades of the continental (and insular) regions separated by the Yangtze River; (4) the north and south clades along the continental region separated by the Taiwan Strait; (5) the unique Okinawa or Ryukyus clade. See the BACKGROUND section for more details. B, Collection sites (squares) for specimens of *Tubuca arcuata* from East Asia and northern Vietnam (the numbers next to the squares correspond to the localities in Table 1).

mitochondrial genome (Karagozlu et al. 2016), as well as two population genetics studies (*e.g.*, allozyme, Huang and JH Shih 1995; restriction fragment length polymorphism (RFLP), Aoki et al. 2008).

*Tubuca arcuata* is widely distributed throughout the continent and islands of East Asia and northern Vietnam, and the Okinawa population was considered to have minimal gene flow with other populations (Aoki et al. 2008). Therefore this species is a good candidate to test the above listed hypotheses regarding the phylogeographic patterns. Our study aimed to: (1) characterize a variety of mitochondrial genetic markers (16S rDNA, cytochrome oxidase subunit I [*COI*] and control region [CR]) in *T. arcuata* samples from various locations across their natural habitat range; (2) assess the uniqueness of the Okinawa population; and (3) explain the elucidated phylogeographic patterns in the context of historical glacial events and/or contemporary ocean currents.

## MATERIALS AND METHODS

*Tubuca arcuata* specimens collected from across East Asia, as well as the outgroup *T. forcipata* from Malaysia (Table 1; Fig. 1), were preserved in 70–95% ethanol and deposited into the Zoological Collections of the Department of Life Science, National Chung Hsing University, Taichung, Taiwan (NCHUZOOL); the Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan (NTOU) and the Zoological Reference Collection, Raffles Museum of Biodiversity Research, National University of Singapore (ZRC).

**Table 1.** Specimens and haplotypes of the 16S rRNA, *COI*, and CR genetic markers of *Tubuca arcuata* from East Asia and northern Vietnam, as well as the outgroup *T. forcipata* used in this study. The numbers within brackets following the localities correspond to those in figure 1B. For abbreviations of museums or universities see the MATERIALS AND METHODS

| | Locality | Catalogue no. of NCHUZOOL (unless indicated) | Haplotype of 16S | Access. no. of 16S | Haplotype of *COI* | Access. no. of *COI* | Haplotype of control region | Access. no. of control region |
|---|---|---|---|---|---|---|---|---|
| *T. arcuata* | North | | | | | | | |
| N clade | South Korea: Ganghwa Island, Incheon [1] | 13651 | S1 | LC053353 | C1 | LC053370 | | |
| | South Korea: Ganghwa Island, Incheon [1] | 13651 | S1 | OP167996 | C2 | OP153631 | | |
| | Japan: Yoshinogawa R., Tokushima [2] | —ᵃ | S1 | OP167997 | C3 | OP153632 | | |
| | Taiwan: Guandu, Taipei [7] | NTOU | S1 | OP167998 | C4 | OP153633 | D1 | OP183984 |
| | Taiwan: Guandu, Taipei [7] | NTOU | S1 | OP167999 | C5 | OP153634 | D2 | OP183985 |
| | Taiwan: Guandu, Taipei [7] | NTOU | S2 | OP168000 | C2 | OP153635 | D3 | OP183986 |
| | Taiwan: Houlong R., Miaoli [8] | 14614 | S3 | OP168001 | C2 | OP153636 | D4 | OP183987 |
| | Taiwan: Shengang, Changhua [9] | 14615 | S1 | OP168002 | C6 | OP153637 | D5 | OP183988 |
| | Taiwan: Dongshih, Chiayi County [9] | 14618 | S4 | OP168003 | C7 | OP153638 | D6 | OP183989 |
| | Taiwan: Cigu, Tainan [9] | 13260 | S1 | OP168004 | C1 | OP153639 | D7 | OP183990 |
| | Taiwan: Cigu, Tainan [9] | 13260 | S5 | AB491164 | C8 | AB491165 | D8 | OP183991 |
| | Taiwan: Cieding, Kaohsiung [9] | 15162 | S6 | OP168005 | C9 | OP153640 | D9 | OP183992 |
| | Taiwan: Caiyuan, Penghu [10] | 14628 | S7 | OP168006 | C2 | OP153641 | D10 | OP183993 |
| | Taiwan: Kinmen [13] | 14608 | S1 | OP168007 | C10 | OP153642 | D11 | OP183994 |
| | China: Qingdao [11] | 15163 | S1 | OP168008 | C2 | OP153643 | D12 | OP183995 |
| | China: Qingdao [11] | 15163 | S1 | OP168009 | C2 | OP153644 | D13 | OP183996 |
| | China: Qingdao [11] | 15163 | S1 | OP168010 | C11 | OP153645 | | |
| | China: Shanghai [12] | ZRC 1998.308 | S7 | OP168011 | C2 | OP153646 | | |
| | China: Xiamen, Fujian [13] | 13350 | S1 | OP168012 | C2 | OP153647 | D14 | OP183997 |
| W clade | South Korea: Ganghwa Island, Incheon [1] | 13651 | S1 | OP168013 | C12 | OP153648 | D15 | OP183998 |
| | South Korea: Ganghwa Island, Incheon [1] | 13651 | S1 | OP168014 | C12 | OP153649 | D16 | OP183999 |
| | Japan: Yoshinogawa R., Tokushima [2] | —ᵃ | S1 | OP168015 | C13 | OP153650 | D17 | OP184000 |
| | Japan: Yoshinogawa R., Tokushima [2] | —ᵃ | S8 | OP168016 | C14 | OP153651 | D18 | OP184001 |
| | Japan: Yoshinogawa R., Tokushima [2] | —ᵃ | S1 | OP168017 | C14 | AB535432 | D19 | OP184002 |
| | Japan: Yoshinogawa R., Tokushima [2] | —ᵃ | S1 | OP168018 | C15 | OP153652 | D20 | OP184003 |
| | Japan: Kaeda R., Miyazaki [3] | 14610 | S1 | LC097112 | C14 | AB535432 | D21 | OP184004 |
| | Japan: Kaeda R., Miyazaki [3] | 14610 | S1 | OP168019 | C14 | AB535432 | | |
| | Japan: Kaeda R., Miyazaki [3] | 14610 | S1 | OP168020 | C13 | OP153653 | D22 | OP184005 |
| | Japan: Kaeda R., Miyazaki [3] | 14610 | S1 | OP168021 | C16 | OP153654 | D23 | OP184006 |
| | Japan: Ohno R., Kumamoto [4] | —ᵃ | S1 | OP168022 | C17 | OP153655 | D24 | OP184007 |

**Table 1.**  (Continued)

| Locality | Catalogue no. of NCHUZOOL (unless indicated) | Haplotype of 16S | Access. no. of 16S | Haplotype of *COI* | Access. no. of *COI* | Haplotype of control region | Access. no. of control region |
|---|---|---|---|---|---|---|---|
| Japan: Nakagushuku Bay, Okinawa [5] | —ᵃ | S1 | OP168023 | C14 | AB535432 | D25 | OP184008 |
| Japan: Nakagushuku Bay, Okinawa [5] | —ᵃ | S9 | OP168024 | C18 | OP153656 | D26 | OP184009 |
| Japan: Nakagushuku Bay, Okinawa [5] | —ᵃ | S1 | OP168025 | C14 | AB535432 | D27 | OP184010 |
| Japan: Nakagushuku Bay, Okinawa [5] | —ᵃ | S9 | OP168026 | C19 | OP153657 | D28 | OP184011 |
| Japan: Nakagushuku Bay, Okinawa [5] | —ᵃ | S9 | OP168027 | C18 | OP153658 | D29 | OP184012 |
| Taiwan: Yilan [6] | 14625 | S10 | OP168028 | C14 | AB535432 | D30 | OP184013 |
| Taiwan: Yilan [6] | 14625 | S1 | OP168029 | C20 | OP153659 | D31 | OP184014 |
| Taiwan: Yilan [6] | 14620 | S11 | OP168030 | C14 | OP153660 | D32 | OP184015 |
| Taiwan: Jhuwei, New Taipei City [7] | 14627 | S1 | OP168031 | C21 | OP153661 | | |
| Taiwan: Jhuwei, New Taipei City [7] | 14627 | S1 | OP168032 | C12 | OP153662 | D33 | OP184016 |
| Taiwan: Guandu, Taipei [7] | NTOU | S1 | OP168033 | C22 | OP153663 | D34 | OP184017 |
| Taiwan: Guandu, Taipei [7] | NTOU | S1 | OP168034 | C23 | OP153664 | D35 | OP184018 |
| Taiwan: Guandu, Taipei [7] | NTOU | S12 | OP168035 | C24 | OP153665 | D36 | OP184019 |
| Taiwan: Haishangu, Hsinchu City [8] | 13305 | S13 | AB535406 | C14 | AB535432 | | |
| Taiwan: Yuanli, Miaoli [8] | 14616 | S14 | OP168036 | C25 | OP153666 | | |
| Taiwan: Wenliao, Taichung [8] | 14619 | S1 | OP168037 | C26 | OP153667 | D37 | OP184020 |
| Taiwan: Gaomei, Taichung [8] | 14629 | S15 | OP168038 | C27 | OP153668 | D38 | OP184021 |
| Taiwan: Shengang, Changhua [9] | 14624 | S1 | OP168039 | C14 | AB535432 | D39 | OP184022 |
| Taiwan: Shengang, Changhua [9] | 14615 | S16 | OP168040 | C28 | OP153669 | D40 | OP184023 |
| Taiwan: Shengang, Changhua [9] | 14611 | S17 | OP168041 | C29 | OP153670 | | |
| Taiwan: Hanbao, Changhua [9] | 14634 | S1 | OP168042 | C30 | OP153671 | | |
| Taiwan: Hanbao, Changhua [9] | 15164 | S18 | OP168043 | C31 | OP153672 | D41 | OP184024 |
| Taiwan: Cigu, Tainan [9] | 14612 | S1 | OP168044 | C32 | OP153673 | xxx | |
| Taiwan: Cigu, Tainan [9] | 14612 | S1 | OP168045 | C33 | OP153674 | D42 | OP184025 |
| Taiwan: Cigu, Tainan [9] | 14630 | S19 | OP168046 | C25 | OP153675 | D43 | OP184026 |
| Taiwan: Cieding, Kaohsiung [9] | 15162 | S1 | OP168047 | C14 | OP153676 | D44 | OP184027 |
| Taiwan: Linyuan, Kaohsiung [9] | 15165 | S3 | OP168048 | C34 | OP153677 | D45 | OP184028 |
| Taiwan: Cingluo, Penghu [10] | 14631 | S20 | OP168049 | C35 | OP153678 | D46 | OP184029 |
| Taiwan: Shuangyuan, Penghu [10] | 14663 | S21 | OP168050 | C33 | OP153679 | D47 | OP184030 |
| Taiwan: Kinmen [13] | 14622 | S22 | OP168051 | C36 | OP153680 | | |
| Taiwan: Kinmen [13] | 14747 | S1 | OP168052 | C14 | OP153681 | | |
| China: Shanghai [12] | ZRC 1998.308 | S23 | OP168053 | C37 | OP153682 | D48 | OP184031 |
| China: Xiamen, Fujian [13] | 13350 | S24 | OP168054 | C14 | AB535432 | | |
| China: Xiamen, Fujian [13] | 13350 | S1 | OP168055 | C14 | OP153683 | D49 | OP184032 |
| Hong Kong [14] | 13362 | S3 | OP168056 | C38 | OP153684 | D50 | OP184033 |
| Hong Kong [14] | 13362 | S1 | OP168057 | C39 | OP153685 | D51 | OP184034 |
| Hong Kong [14] | 13362 | S1 | OP168058 | C40 | OP153686 | D52 | OP184035 |
| Hong Kong [14] | 13362 | S1 | OP168059 | C41 | OP153687 | | |
| China: Dongzhai, Hainan [15] | 13363 | S25 | OP168060 | C42 | OP153688 | D53 | OP184036 |
| China: Dongzhai, Hainan [15] | 13363 | S25 | OP168061 | C42 | OP153689 | D54 | OP184037 |
| China: Dongzhai, Hainan [15] | 13363 | S26 | AB813667 | C43 | AB813684 | | |
| China: Dongzhai, Hainan [15] | 13363 | S1 | OP168062 | C44 | OP153690 | | |
| China: Dongzhai, Hainan [15] | 13363 | S1 | OP168063 | C31 | OP153691 | | |
| China: Qinzhou, Guangxi [16] | 13362 | S3 | OP168064 | C45 | OP153692 | D55 | OP184038 |
| Vietnam: Cat Ba Island [17] | 15140 | S1 | OP168065 | C14 | ON193478 | | |
| Vietnam: Nam Dinh [17] | ZRC | S3 | OP168066 | C46 | OP153693 | | |
| Vietnam: Nam Dinh [17] | 15144 | S1 | OP168067 | C14 | ON193480 | D56 | OP184039 |
| Vietnam: Nam Dinh [17] | 15144 | S1 | OP168068 | C14 | ON193481 | D57 | OP184040 |
| Vietnam: Nam Dinh [17] | 15144 | S3 | OP168069 | C14 | ON193482 | D58 | OP184041 |
| Vietnam: Nam Dinh [17] | 15100 | S1 | OP168070 | C14 | ON193479 | D59 | OP184042 |
| Vietnam: Ha Tinh [18] | 15141 | S1 | OP168071 | C47 | ON193483 | D60 | OP184043 |
| **Outgroups** | | | | | | | |
| *T. forcipata*  Malaysia: Johore | NTOU | fc-S | LC053355 | fc-C | LC053372 | fc-D1 | OP184044 |
| Malaysia: Johore | NTOU | fc-S | OP168073 | fc-C | OP153695 | fc-D2 | OP184045 |
| Malaysia: Johore | NTOU | fc-S | OP168074 | fc-C | OP153696 | fc-D3 | OP184046 |

ᵃ Voucher specimen not preserved.

Genomic DNA was isolated from muscle tissues using commercial kits and the sequences of the mitochondrial 16S rDNA [~560 base pairs (bp)], *COI* (658 bp), and CR (~905 bp) markers were obtained as described by Shih et al. (2013 2016b) with verification with the complementary strand. The primers used were 1471 (5'-CCTGTTTANCAAAAACAT-3'), 1472 (5'-AGATAGAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996), 16L29 (5'-YGCCTGTTTATCAAAAACAT-3') and 16H10 (5'-AATCCTTTCGTACTAAA-3') (Schubart 2009) for 16S; LCO1490 (5'-GGTCAACAAAT CATAAAGATATTGG-3') and HCO2198 (5'-TA AACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) for *COI*; and ILEUCAR3 (5'-GCTAYCCTTTTAAATCAGGCAC-3'; Pie et al. 2008), CR-F4 (5'-TAAGTATAACCGCAGATG-3'; Shih et al. 2013), as well as the three newly designed CR-F2 (5'-TAATAAAACATGCTGAGAACTGAAAC-3'), CR-R1 (5'-GATGGGGGCTGATAGGGGCTGTAGTGGT AA-3') and CR-R2 (5'-ATAAATAATAAGGACAAA-3') for CR. The sequences of the different haplotypes were deposited into the NCBI GenBank database (accession numbers are summarized in Table 1).

For the combined 16S, *COI*, and CR dataset, the best fitting models for sequence evolution of individual datasets were determined independently using the jModelTest tool (vers. 2.1.4, Darriba et al. 2012) and selected based on the Bayesian information criterion (BIC). The best models were the HKY, HKY+G, and TIM3+I+G models, respectively, and were subsequently applied to the partitioned Bayesian inference (BI) analysis. BI analysis was performed using the MrBayes package (vers. 3.2.6, Ronquist et al. 2012). Searches were conducted with 4 chains for 10 million generations and 4 independent runs, with trees sampled every 1000 generations. The convergence of the chains was determined by the average standard deviation of the split frequency values below the recommended threshold of 0.01 (Ronquist et al. 2005) and the first 3100 trees were discarded as burnin. A maximum parsimony (MP) consensus tree was constructed using MEGA software (vers. 11, Tamura et al. 2021), with 2000 bootstrap reiterations of a simple heuristic search, TBR branch-swapping (tree bisection-reconnection) (100 random-addition sequence replications; max no. of trees to retain = 10000). The relationships of the 16S and *COI* haplotypes among the *T. arcuata* populations were presented separately using the program TCS (vers. 1.23, Clement et al. 2000) with the gaps treated as the fifth state in 16S. As the network of CR haplotypes contains a lot of unsampled sequences (Leigh and Bryant 2015), it was not used in this study. The bp difference, as well as the pairwise estimates of the

Kimura 2-parameter (K2P) distance (Kimura 1980) and the uncorrected *p*-distance for the genetic divergence between haplotypes were calculated using MEGA.

## RESULTS

A 557 bp segment of the 16S gene from 81 specimens, a 658 bp segment of *COI* from 81 specimens, and a 920 bp segment of CR from 60 specimens of *T. arcuata* were amplified and aligned. A total of 26 haplotypes of the 16S gene, 47 haplotypes of *COI* and 60 haplotypes of CR were found (Table 1). Phylogenetic analyses based on the combined 16S, *COI*, and CR database of 2135 bp from 63 specimens (including the outgroups) elucidated two clades that were highly supported by BI and MP analyses (Fig. 2). One clade was labeled as the "N clade", because it was mainly distributed in the north region of the habitat range (green line in Fig. 5A), whereas the other clade was labeled as the "W clade" because it was widely distributed across the entire habitat range (red line in Fig. 5A). Two haplotypes ( "S1" and "S3") of 16S were shared by the N and W clades shown in the 16S haplotype network (Fig. 3?). 11 and 36 haplotypes of *COI* were present in N and W clades, respectively, without a shared haplotype (Table 1). For the CR marker, there were 14 and 46 haplotypes for the N and W clades, respectively, without a shared haplotype (Table 1).

For the *COI* haplotype network analysis based on 81 specimens, the network (Fig. 4) indicated that there were two clades that were separated by at least eight steps, which is consistent with the clades revealed by the combined 16S, *COI*, and CR phylogeny (Fig. 2). The ancestral haplotype of the N clade is "C2," which was represented by eight specimens distributed in Korea, South and North China, and Taiwan. In contrast, the ancestral haplotype of the W clade is "C14", which was represented by 19 specimens distributed in Japan (including Okinawa), Taiwan, South China, and Vietnam (Table 1; Figs. 4, 5B).

The nucleotide divergence with the K2P distance and bp difference between the *COI* haplotypes of the N and W clades are shown in table 2. The average nucleotide divergences and bp differences within the N and W clades were 0.23% (1.53 bp) and 0.32% (2.09 bp), respectively, whereas the divergence between clades was 1.44% (9.32 bp).

## DISCUSSION

The distribution of fiddler crabs is known to

be greatly influenced by the dispersal of planktonic larvae via ocean current transport (Barnwell and Thurman 1984; Hopkins and Thurman 2010; López-Duarte et al. 2011). However, according to the study of López-Duarte et al. (2011), species in mesohaline or oligohaline habitats (*e.g.*, the estuaries) exhibit narrower distributional ranges than species in euhaline habitats (*e.g.*, the open coasts) because larvae migrate vertically in estuaries to avoid currents and ensure their retention (Shih et al. 2015 2019). The distribution of *T. arcuata* is consistent with the above described estuarine pattern, with a narrower distribution compared to other open coastal species (*e.g.*, *Austruca annulipes*, *A. perplexa*, *Gelasimus tetragonon*, *Paraleptuca crassipes*; Crane 1975; Shih et al. 2009 2013; Shih and Poupin 2020). Our study identified two clades within *T. arcuata*: the N clade distributed in the northern region and the W clade distributed widely throughout the entire habitat range

(Figs. 2, 5A). However, only one unresolved clade in the network of 16S haplotypes (Fig. 3) and some shared 16S haplotypes in N and W clades were found, which implies a lower resolution of 16S compared with *COI* (Chu et al. 2015).

Based on the pairwise divergence rates of 1.66% per million years for *COI* in marine coastal crabs of the family Sesarmidae (Schubart et al. 1998), the N and W clades of *T. arcuata* possibly diverged approximately $0.93 \pm 0.3$ million years ago (mya) (with *p*-distance divergences of $1.54\% \pm 0.47\%$) during the Pleistocene. The divergence between these clades was probably caused by glaciation events, with fluctuations in the global sea levels occurring in cycles of 100,000 years during the Pleistocene (Haq et al. 1987; Woodruff 2003; Shih et al. 2011).

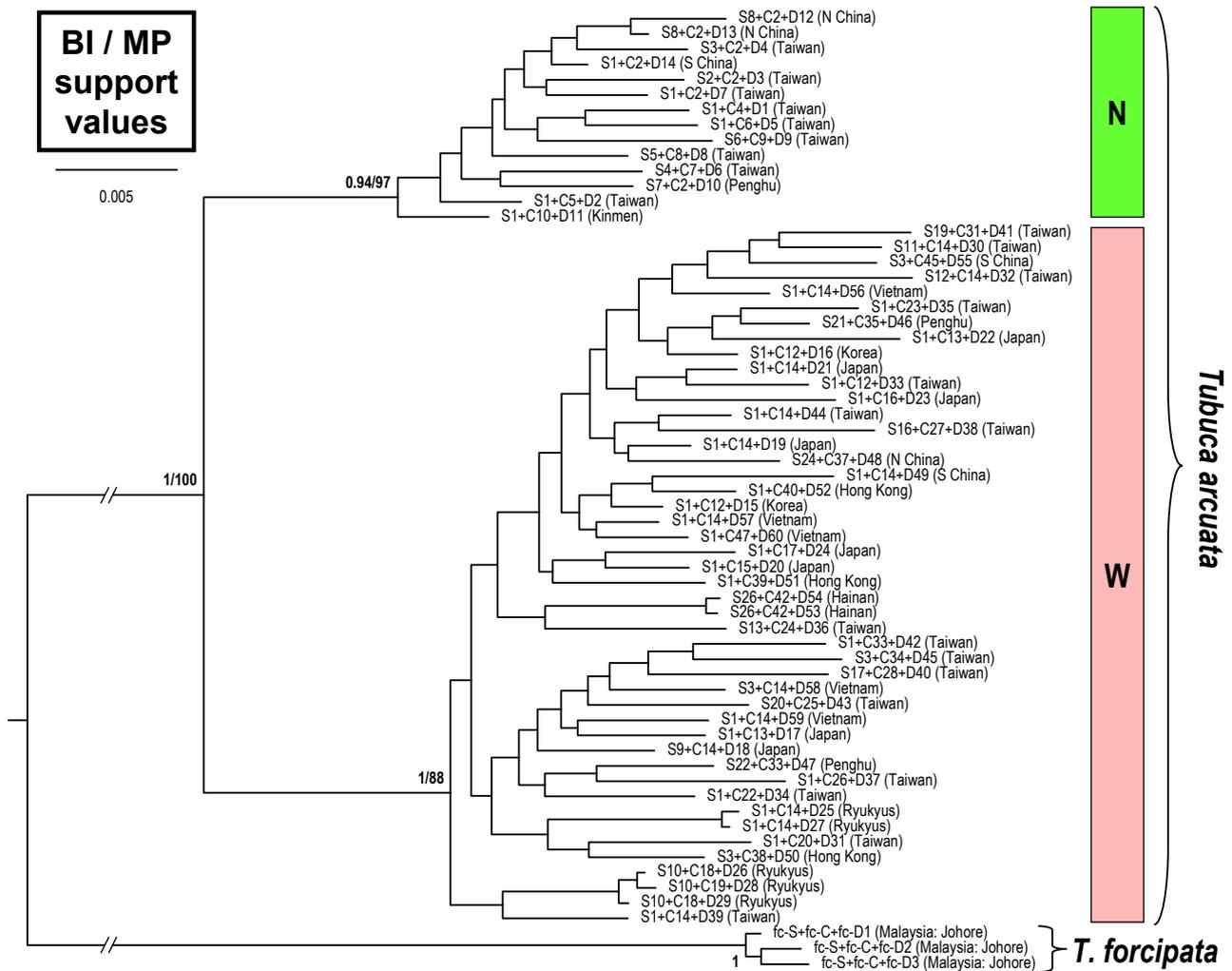During these glaciation periods, the main Paleo-Kuroshio Current was hypothesized to flow along the



**Fig. 2.** Bayesian inference (BI) tree for *Tubuca arcuata* based on the combined mitochondrial 16S, *COI*, and control region markers. The probability values at the nodes represent support values (> 50%) for BI and maximum parsimony (MP), respectively. See table 1 for haplotype names.

eastern side of the Ryukyus (Ujiié et al. 2003), with branches extending to areas further north (Ujiié et al. 1991) (Fig. 5B). This differs from the modern pattern of the Kuroshio Current and its branches (Fig. 5A). We hypothesize that the ancestral distributions of the N and W clades could be determined from the current distributions based on their ancestral haplotypes. That is, the ancestral N haplotype was likely distributed across the coastal regions of the ECS and the YS (green line in Fig. 5B). In contrast, the distribution of the ancestral W haplotype includes the northern coastal region of the SCS, Taiwan, Okinawa, and the main islands of Japan. Therefore, we hypothesize that the original ancestral distribution of the latter during the glaciation period was the northern SCS, but was further dispersed northwardly to Okinawa and the main islands of Japan by the Paleo-Kuroshio Current (Fig. 5B).

After the glaciation period, due to the emergence of modern warm currents (including the Kuroshio Current and its branches, the South China Sea Warm Current and the Taiwan Worm Current; blue arrows in Fig. 5A), the ancestral haplotypes of both clades were dispersed to their current habitat range in coastal regions (green and red arrows in Fig. 5B), and their descendant haplotypes also dispersed to more distant areas (green and red arrows in Fig. 5A). Based on the distribution of descendent W haplotypes in the coastal regions of the ECS and the YS as well as the presence of the descendent N haplotype in the main islands of Japan (Fig. 5A), we propose that the crabs' larvae were dispersed northwardly by warm currents during the reproduction seasons in the summer (blue solid arrows in Fig. 5A). However, the N clade does not extend its range to the SCS (only some extension in southwestern Taiwan). This may have been because the only southward current is the cold China Coastal Current in winter (blue dotted arrow in Fig. 5A; Kang et al. 2021), which would have impeded the southward dispersion of larvae. In other studies, the mixing between the N and W clades separated by the Taiwan Strait was more limited due to the low larvae dispersal (*e.g.*, mullets, Liu et al. 2007b; mitten crabs, Xu et al. 2009; bivalves, XX Wang et al. 2017).

In our study, the Okinawa population did not form an isolated clade and even shared an ancestral *COI* haplotype with others (Figs. 2, 4), which is different from a previous conclusion that the Okinawa population of *T. arcuata* was genetically distinct based on RFLP
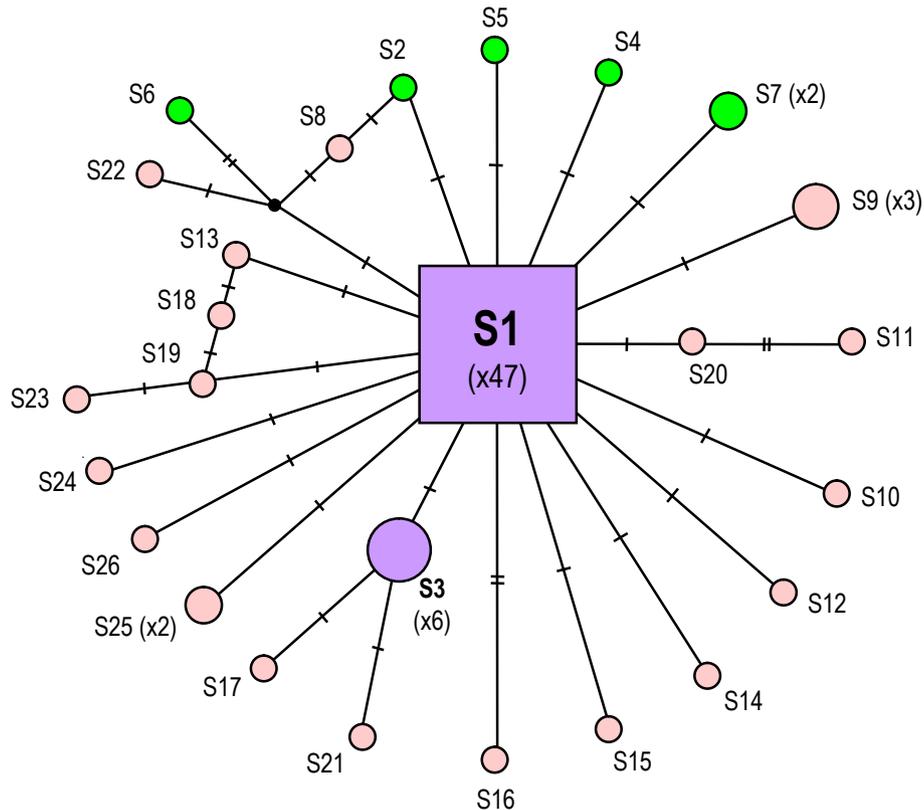


**Fig. 3.** Genealogical network for the 557-bp 16S haplotypes observed within *Tubuca arcuata*, constructed with TCS. The ancestral haplotype is indicated by square. Green and red circles mean the haplotypes of the N and W clades, respectively; and the square and circle in purple represent the haplotypes that are shared by N and W clades. Unlabelled hatches and node indicate inferred haplotypes not found in the sampled population.
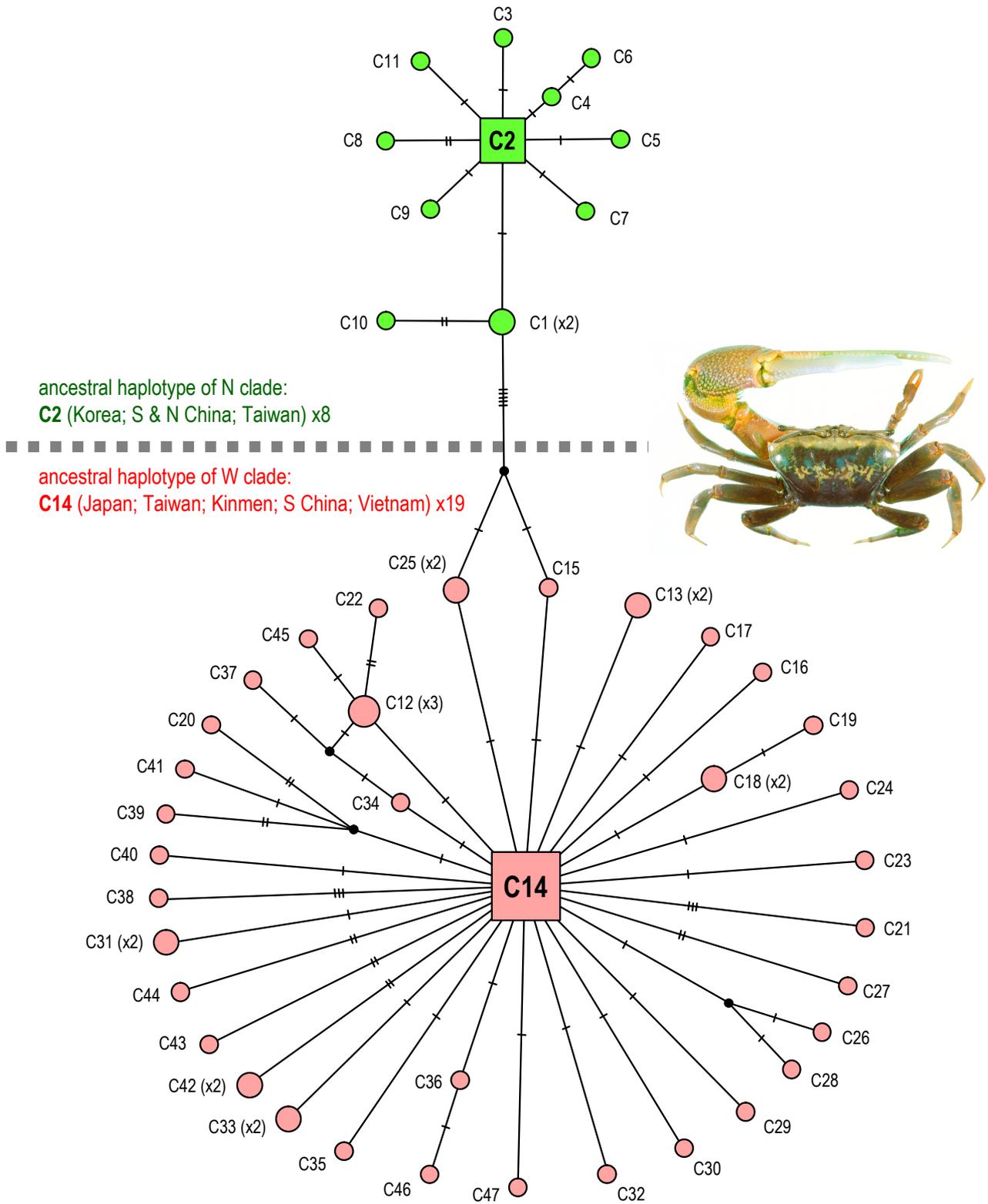
ancestral haplotype of N clade:
**C2** (Korea; S & N China; Taiwan) x8

ancestral haplotype of W clade:
**C14** (Japan; Taiwan; Kinmen; S China; Vietnam) x19

**Fig. 4.** Genealogical network for the 658-bp *COI* haplotypes observed within *Tubuca arcuata*, constructed with TCS. The ancestral haplotypes of the N and W clades are indicated by squares. Unlabelled hatches and nodes indicate inferred haplotypes not found in the sampled population.

analysis (Aoki et al. 2008). In fact, although one RFLP haplotype is dominant in the Okinawa group, all three haplotypes are shared with other populations (Aoki et al. 2008: table 2) and an alternative conclusion might be obtained by using a larger sample size. Another similar study on the phylogeography of the *Parasesarma bidens* complex (Sesarmidae) using 16S and *COI* (Shahdadi et al. 2022) showed one clade mainly distributed in the main islands of Japan and the Ryukyus, which might belong to the pattern of the unique Ryukyus clade (see BACKGROUND) (no. 5 in Fig. 1A), although the range also extended to the main islands of Japan and Taiwan.
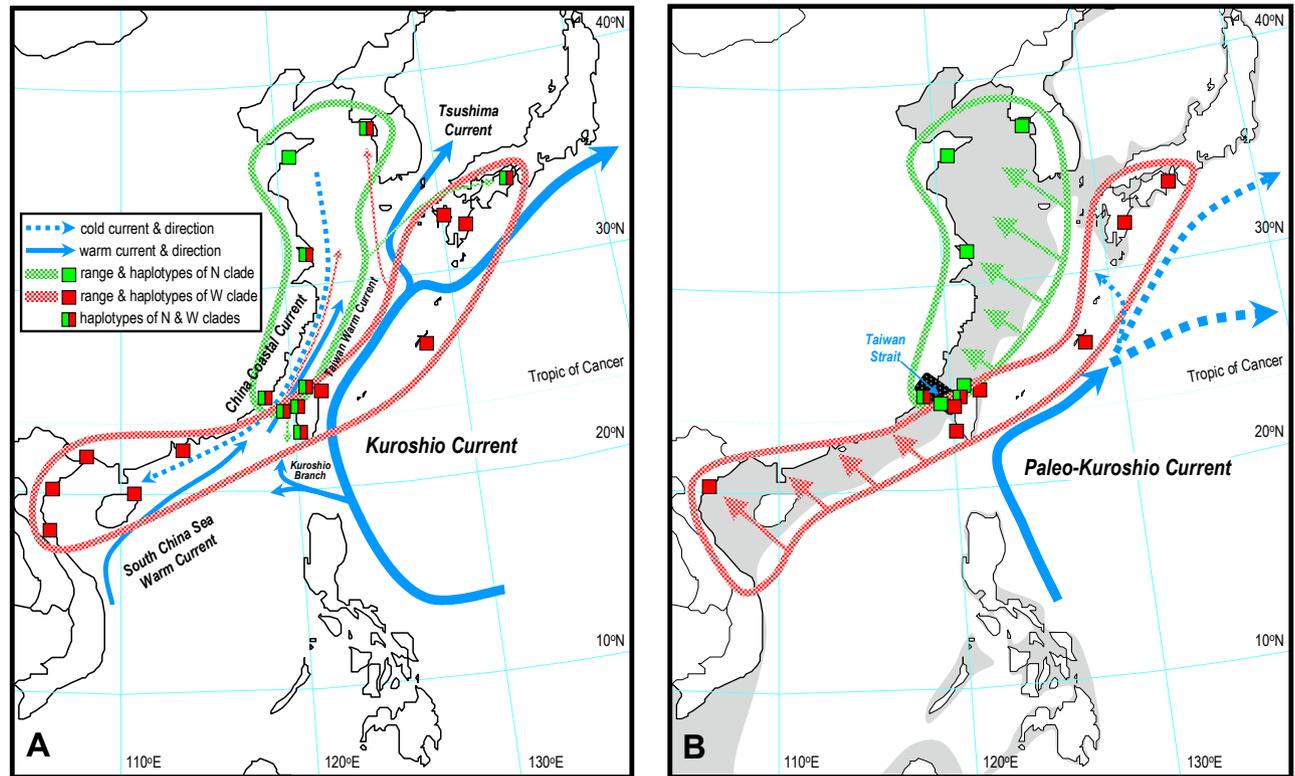


**Fig. 5.** A, Distributions of the N haplotypes (green squares) and the W haplotypes (red squares). The green and red lines and arrows indicate the possible biogeographical boundary and possible dispersal direction of the N and W clades after glaciation, respectively. The square with both red and green represents the locality with both N and W haplotypes. The blue solid and dotted lines and arrows represent the warm and cold currents in summer and winter, respectively. B, Distribution of the ancestral haplotypes of the N clade (green squares) and the W clade (red squares). The green and red lines and arrows indicate the possible biogeographical boundary and the assumed dispersal direction of the ancestral N and W clades after glaciation, respectively. The shaded sea areas represent continental shelves (< 120 m) that would have been dry during periods of low sea level (after Voris 2000; Xu et al. 2009). The blue solid line indicates the Paleo-Kuroshio Current (after Ujiié et al. 2003) and the dotted lines are the possible extended branches of the Paleo-Kuroshio Current (after Ujiié et al. 1991).

**Table 2.** Matrix of the percentage pairwise nucleotide divergences with K2P distance and mean number of bp differences based on *COI* within and between clades of *Tubuca arcuata* from across East Asia and northern Vietnam (see Table 1). In the right half, the lower-left values are K2P distance and the upper-right ones are mean bp differences. Ranges are indicated in parentheses

|  | Within clade | | Between clades | |
|---|---|---|---|---|
|  | nucleotide divergence | mean bp difference | N clade | W clade |
| N clade | 0.23 | 1.53 |  | 9.32 |
|  | (0–0.76) | (0–5) |  | (6–13) |
| W clade | 0.32 | 2.09 | 1.44 |  |
|  | (0–0.92) | (0–6) | (0.92–2.01) |  |

The estuarine crab species *Austruca lactea* has a similar distribution in East Asia and northern Vietnam, but there is no record of this species in the Ryukyus (Crane 1975; Shih et al. 2016a 2022). Based on the CR sequences of specimens from the main islands of Japan and Taiwan (including Penghu) (Tokuyama et al. 2020), three populations can be distinguished (Seto Inland Sea, Kumamoto in Kyushu, and Taiwan). However, these three putative clades could not be identified in their trees. Therefore, further analysis with different markers and more specimens covering the whole habitat range is necessary to clarify the genetic population structure of this species.

The phylogeographic pattern of *T. arcuata* elucidated herein was consistent with the hypothesis that the land bridge across the Taiwan Strait acted as a barrier for marine organisms during glaciations (see BACKGROUND) (no. 4 in Fig. 1A) and the Paleo-Kuroshio Current played an important role in larval dispersal to Okinawa and the main islands of Japan. Modern ocean currents in East Asia and the northern SCS promote the dispersal of larvae from both clades to farther areas. However, the N clade does not exist in the northern SCS due to the direction of warm currents in the summer (*i.e.*, reproduction season).

## CONCLUSIONS

Based on the combined mitochondrial 16S, *COI*, and CR markers, our findings suggest that the phylogenetic pattern of two distinct (N and W) clades in *Tubuca arcuata* originated from the separation by the land bridge in the Taiwan Strait during the glaciation period approximately 0.93 mya. For the W clade, the additional driving force of the Paleo-Kuroshio Current promoted larval dispersal northwardly from the SCS to Okinawa and the main islands of Japan. The modern distributional patterns of both clades are thus likely influenced by the Kuroshio Current and other warm currents in the summer. The Okinawa population does not form an isolated clade and shares the ancestral haplotype with others.

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