Zoological Studies

Integrative Taxonomy Reveals Freshwater Shrimp Diversity (Decapoda: Atyidae: *Neocaridina*) from Kyushu and Southern Honshu of Japan, with a Discussion on Introduced Species

Hsi-Te Shih[1,*,§] iD, **Yixiong Cai**[2,§] iD, **Nobuaki Niwa**[3,†], **Hidenori Yoshigou**[4], and **Yasuhiko Nakahara**[5]

[1]*Department of Life Science and Research Center for Global Change Biology, National Chung Hsing University, 250, Kuo Kuang Road, Taichung 402, Taiwan. *Correspondence: E-mail: htshih@dragon.nchu.edu.tw (Shih)*
[2]*National Biodiversity Centre, National Parks Board, 1 Cluny Road, Singapore 259569, Republic of Singapore. E-mail: cai_yixiong@nparks.gov.sg (Cai)*
[3]*Faculty of Science, Kyoto University, Kitashirakawa, Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan*
[4]*Chugai Technos Co. LTD, 9-12, Yokogawa-shin-machi, Nishi-ku, Hiroshima City 733-0013, Japan. E-mail: h.yoshigo@chugai-tec.co.jp (Yoshigou)*
[5]*SEIBU Environmental Research Co., LTD, Mikawachishin-machi, Sasebo-City, Nagasaki 859-3153, Japan. E-mail: caridina@nifty.com (Nakahara)*

Correct identification of species is crucial for invasion ecology and management, particularly in aquatic systems. In this study, specimens of the freshwater shrimp genus *Neocaridina* from Kyushu and southern Honshu of Japan were identified by using an integrative approach that combined DNA barcoding of mitochondrial cytochrome oxidase subunit I (*COI*) and morphological examination. Among the eight species detected, two are native, viz. *N. denticulata* and *N. ikiensis*. Four are regarded as non-indigenous, viz. *N. davidi*, *N. koreana*, *N. palmata*, *N.* aff. *palmata*, which are believed to have been introduced from other East Asian countries either by the aquarium trade or as live fish bait. The remaining two species are likely cryptic native species, which have either been mistaken for known species, *e.g.*, *N.* aff. *denticulata*, or species that have not been discovered before, *e.g.*, *N.* aff. *fukiensis.* While the four alien species have spread widely in central Honshu, northern Kyushu and Tsushima Island, their impacts on the native species and the overall ecology remain mostly unexplored. Problems associated with using DNA barcoding for species identification are highlighted for further research.

Key words: Japan, DNA barcoding, Cytochrome oxidase subunit I (*COI*), Morphology, Integrative taxonomy, *Neocaridina denticulata*, *N. davidi*, *N. ikiensis*, *N. koreana*, *N. palmata*

BACKGROUND

The impact of invasive species on ecology has been discussed extensively (Ehrenfeld 2010; Simberloff et al. 2013) and it has been suggested that the invasive pathways differ between terrestrial and aquatic ecosystems (Ehrenfeld 2010). Compared with terrestrial and marine systems, freshwater systems are more sensitive to introduced species due to the stronger impacts of introduced primary consumers on plant biomass, and a higher proportion of high-impact invaders. Additionally, freshwater systems exhibit similar degrees of extirpation of native species from either intra- or inter-continental invaders (Ricciardi and MacIsaac 2011).

Correct identification of species is important

for invasion ecology and biodiversity management (Darling and Blum 2007; Briski et al. 2016). In aquatic systems, species identification can be challenging due to difficult survey conditions, or because the life stages of organisms are poorly understood or not amenable to traditional identification methods. As a result, molecular detection methods using environmental samples often prove more successful (Darling and Mahon 2011; Darling 2015). DNA barcoding using the mitochondrial cytochrome *c* oxidase subunit I (*COI*) provides a reliable solution for species identification across all life stages (*e.g.*, eggs, larvae, and juveniles), sexually dimorphic species, and species with large morphological variations within or between populations (Hebert et al. 2003a b; Radulovici et al. 2010). DNA barcoding has been recommended as an effective tool to identify species correctly as a basis for invasion ecology research (Darling and Blum 2007; Briski et al. 2016).

The genus *Neocaridina* Kubo, 1938, native to freshwater habitats in East Asia, consists of over 30 recorded species (Liang 2004; De Grave and Fransen 2011; Shih et al. 2017 2019; Chen et al. 2018). While the species of both *Caridina* and *Macrobrachium* are either catadromous or land-locked, all known species of *Neocaridina* are land-locked with large eggs and abbreviated larval development (Shih and Cai 2007). Some species of atyid shrimps are popular in the aquarium trade due to their distinctive colors and land-locked habits. For example, *Neocaridina davidi* (Bouvier, 1904), with its varied coloration of red, yellow, blue, and black, is traded under names such as the Red Cherry Shrimp, Cherry Shrimp, and Fire Shrimp. Due to this trade, they have been introduced into regions like Hawaii, Japan, and Europe (Englund and Cai 1999; Klotz et al. 2013; Toyota et al. 2014; Hasegawa et al. 2015; Mitsugi et al. 2017; Shih et al. 2017; Jabłońska et al. 2018; Schoolmann and Arndt 2018). Species of *Neocaridina* are believed to have been imported into Japan from Korea since 1969 and from China since 1990 as live fish bait (Niwa 2010; Niwa and Ohtaka 2006; Shih et al. 2017). The first report of non-indigenous species of *Neocaridina* in Japan was in 2004, and they have since become widespread, ranging from Hokkaido to the Ryukyu Islands (*e.g.*, Nishino and Niwa 2004; Niwa 2010; Fujita et al. 2011; Nishino 2017 2020; Fuke et al. 2021; Nagai and Imai 2021; Onuki 2021; Kakui and Komai 2022; Onuki and Fuke 2022). Niwa (2010 2017) suggested that more than one species of *Neocaridina* has been introduced from China and Korea to Japan.

Morphological differences among *Neocaridina* species are subtle, with some species showing variations that complicate identification (Cai 1996; Liang 2004), especially among female specimens. Consequently,

molecular evidence has been increasingly employed for reliable species identification (Shih and Cai 2007; Shih et al. 2017 2019). Recent findings suggest that the species diversity of *Neocaridina* in the main islands of Japan exceeds prior estimates, encompassing native *N. denticulata* (De Haan, 1844) and *N. ikiensis* Shih, Cai, Niwa & Nakahara, 2017, and probably introduced species (Shih et al. 2017). In this study, we examine the species of *Neocaridina* from Kyushu and southern Honshu of Japan using an integrative approach combining DNA barcoding and morphology (Dayrat 2005). We also discuss species-level taxonomy and highlight problems associated with DNA barcoding for species identification.

MATERIALS AND METHODS

Specimens of the genus *Neocaridina* examined in this study were collected from the Kyushu and southern Honshu of Japan (Tables 1, 3; Fig. 1), and were preserved in 70–95% ethanol after collection. Specimens with typical characters were selected for illustration, using a drawing tube attached to a Nikon stereo microscope (model SMZ 1000). All specimens were subsequently deposited in 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 of the Lee Kong Chian Natural History Museum, National University of Singapore, Singapore (ZRC). The carapace length is abbreviated as "cl".

Following the recognition of several additional clades of *Neocaridina* from the main islands of Japan (Shih et al. 2017; Fuke et al. 2021), conflicting opinions have emerged regarding the valid names for these clades (*e.g.*, Fuke et al. 2021; Nagai and Imai 2021; Kakui and Komai 2022). In this study, we follow most of the names used by Shih and Cai (2007) and Shih et al. (2017 2019). To confirm the species of *Neocaridina* collected from Japan, the main morphological characters of representative specimens from each species were compared with either other specimens or taxonomic references from China, Japan, and Taiwan, including Cai (1996), Liang (2004), Shih and Cai (2007), and Shih et al. (2017 2019).

Genomic DNA was isolated from the muscle tissue of the abdomen by using the GeneMark tissue and cell genomic DNA purification kit (Taichung, Taiwan). A portion of the *COI* gene was amplified with PCR using the primers LCO1490 and HCO2198

(Folmer et al. 1994). The PCR conditions for these primers were denaturation for 50 s at 94°C, annealing for 70 s at 45–47°C, and extension for 60 s at 72°C (40 cycles), followed by extension for 10 min at 72°C. Sequences were obtained by automated sequencing (Applied Biosystems 3730), after verification with the complementary strand, with a 658-bp (base pair) segment of *COI*. Sequences of the different haplotypes have been deposited in the DNA Data Bank of Japan (DDBJ), along with other sequences published in Shih and Cai (2007) and Shih et al. (2017 2019) (Table 1). A neighbor-joining tree was constructed with the
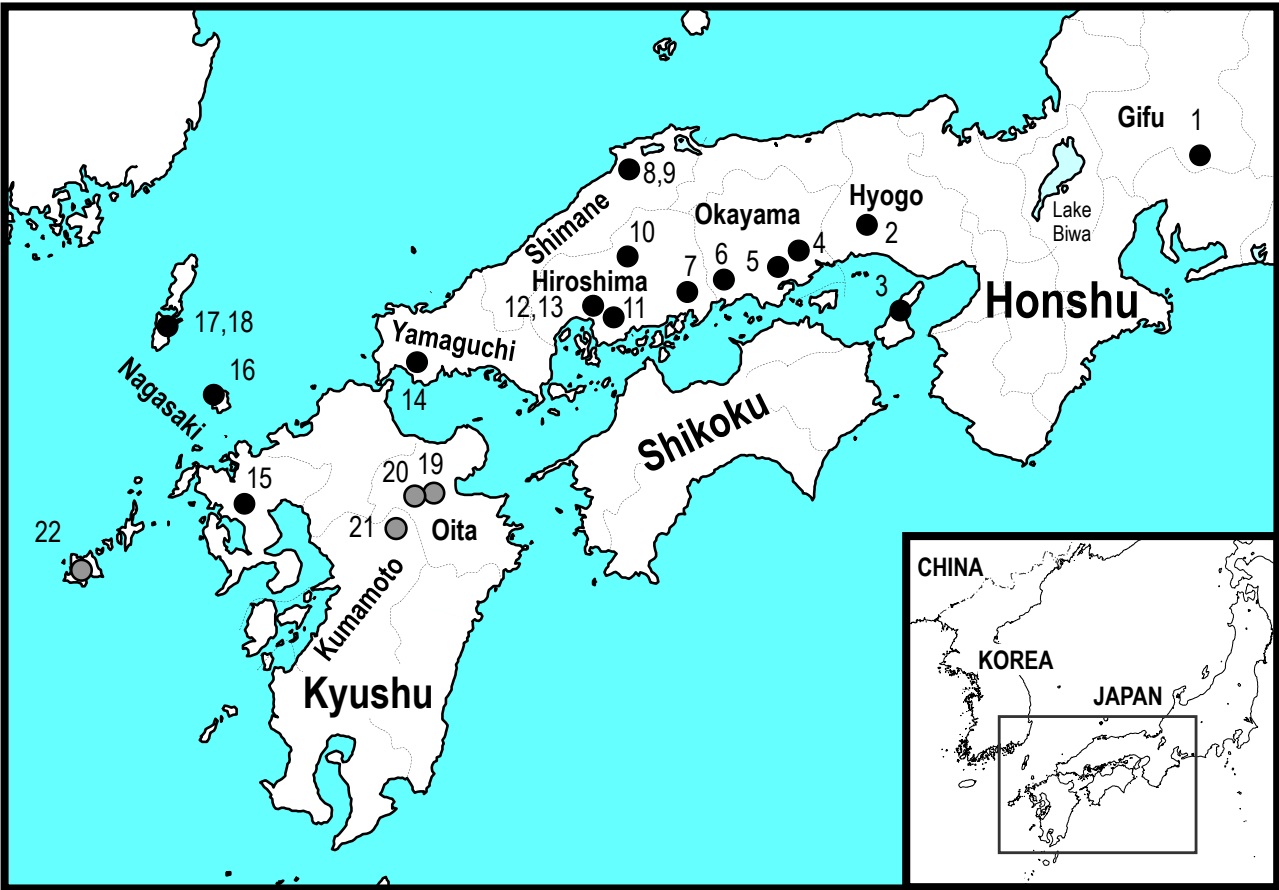
Fig. 1. Collection sites (nos. 1–18, black circles) for species of *Neocaridina* in the main islands of Japan (Honshu and Kyushu). For locality names and haplotypes, see table 1. Nos. 19–21 (gray circles) are the additional localities in Fuke et al. (2021).

Table 1. New *COI* haplotypes of specimens of *Neocaridina* species collected from the main islands of Japan and other localities used in this study. Those from main islands of Japan in Shih et al. (2017; marked with "*") are also included here. Numbers within brackets correspond to Japanese localities in figure 1. Accession numbers with identical haplotypes are parenthesized

Species	Locality	Catalogue no. of NCHUZOOL (unless indicated)	Sample size	Japanese sample size	Accession no.	Haplotypes (for new sequences)
N. davidi	China: Qingdao, Shandong	NTOU 20641	2	0	PP386817; PP386818	Ndv-1
	China: Putian, Fujian	13342	6	0	AB563166*	
	China: Jiangle, Fujian	13347; 14941; ZRC 2023.0214	4	3	AB563171*;	Ndv-2
	Japan: Himeji City, Hyogo [2]				PP386819–PP386821	
	Japan: Awaji Island, Hyogo [3]	14943	1	1	PP386822	Ndv-3
	Japan: Izumo City, Shimane [8]	14955	1	1	LC324766*	

Table 1. (Continued)

Species	Locality	Catalogue no. of NCHUZOOL (unless indicated)	Sample size	Japanese sample size	Accession no.	Haplotypes (for new sequences)
	Japan: Hiroshima City, Hiroshima [12]	14961	1	1	LC324767*	
	Japan: Ube City, Yamaguchi [14]	14952	1	1	PP386823	Ndv-4
	Japan: Ube City, Yamaguchi [14]	14952	1	1	PP386824	Ndv-5
N. denticulata	Japan: Mizunami City, Gifu [1]; Awaji Island, Hyogo [3]; Higashihiroshima City, Hiroshima [11]	14944, 14942, 14958	3	3	LC324765*	
	Akaiwa City, Okayama [4]; Ihara City, Okayama [6]	14948, 14949, 14968	3	3	LC324764*	
	Japan: Ihara City, Okayama [6]; Fukuyama City, Hiroshima [7]; Ube City, Yamaguchi [14]	14946; 14953; 14968; 14969; ZRC 2023.0211	6	6	PP386825–PP386830	Ndt-1
	Japan: Ihara City, Okayama [6]; Fukuyama City, Hiroshima [7]; Hiroshima City, Hiroshima [12]; Ube City, Yamaguchi [14]	14947; 14953; 14961; 14969; 15168	7	4	PP386831–PP386837	Ndt-2
	China: Pingyuan, Yunnan; Taihu Lake, Jiangsu					
	Japan: Izumo City, Shimane [9]	14956	1	1	PP386838	Ndt-3
	Japan: Miyoshi City, Hiroshima [10]	14965	2	2	PP386839; PP386840	Ndt-4
	Japan: Miyoshi City, Hiroshima [10]	14964	1	1	PP386841	Ndt-5
	Japan: Higashihiroshima City, Hiroshima [11]	14945	1	1	PP386842	Ndt-6
	Japan: Hiroshima City, Hiroshima [13]	14966	1	1	PP386843	Ndt-7
	Japan: Hiroshima City, Hiroshima [12, 13]	14960; 14967	3	3	PP386844–PP386846	Ndt-8
	Japan: Hiroshima City, Hiroshima [12]	ZRC 2024.0055	2	2	PP386847	Ndt-9
	China: Taihu Lake, Jiangsu	NCHUZOOL 15264	1	0	PP386848	Ndt-10
N. ikiensis	Japan: Iki Island, Nagasaki [16]	14935	2	2	LC324771*	
	Japan: Iki Island, Nagasaki [16]	14933 (paratype); 14932 (paratype)	2	2	LC324772*	
	Japan: Iki Island, Nagasaki [16]	14937; 14936	4	4	LC324773*	
	Japan: Iki Island, Nagasaki [16]	14933 (paratype)	1	1	LC324774*	
	Japan: Iki Island, Nagasaki [16]	14934; 14932 (paratype)	3	3	LC324775*	
N. koreana	Japan: Miyoshi City, Hiroshima [10]	14964; 14965	2	2	LC324777*	
N. palmata	China: Hanzhong, Shaanxi	NCHUZOOL 15265	2	0	PP386849; PP386850	Npm-1
	China: Nanping, Fujian	NCHUZOOL 15266	1	0	PP386851	Npm-2
	China: Changting, Fujian; Japan: Hyogo [2]	15269; 14940	2	1	PP386852; PP386853	Npm-3
	China: Leye, Guangxi; Vietnam: Hoa An, Cao Bang; Thanh Thuy, Vi Xuyen, Hai Giang; Hai Gang; Japan: Himeji City, Hyogo [2]	14975; 15174; 15175; 15176; 14940	5	1	LC324770*; PP386854–PP386857	Npm-4
N. aff. *denticulata*	Japan: Mizunami City, Gifu [1]	14959	1	1	PP386858	Nad-1
	Japan: Himeji City, Hyogo [2]	14940; ZRC 2023.0221	4	4	PP386859; PP386860–PP386862	Nad-2
	Japan: Himeji City, Hyogo [2]	ZRC 2023.0221	1	1	PP386863	Nad-3
	Japan: Himeji City, Hyogo [2]	14940; 14941	2	2	PP386864; PP386865	Nad-4
	Japan: Okayama City, Okayama [5]	14950; 14951	4	4	LC324778*	
	Japan: Izumo City, Shimane [9]; Sasebo City, Nagasaki [15]; Tsushima Island, Nagasaki [17, 18]	14957; 14962, 14963, 14971; 14970	11	11	LC324779*; PP386866–PP386875	Nad-5
N. aff. *fukiensis*	Japan: Fukuyama City, Hiroshima [7]	14947; 14946; ZRC 2023.0211	5	5	LC324776*	
N. aff. *palmata*	Japan: Himeji City, Hyogo [2]	14941	1	1	PP386876	Nap-1
	China: Yunnan; Japan: Himeji City, Hyogo [2]	15170; 15267; 14941; 14940	5	2	PP386877–PP386881	Nap-2
	China: Fujian: Jiangle; Nanping	15169; 15172	2	0	PP386882; PP386883	Nap-3
	Japan: Izumo City, Shimane [8]	14954	1	1	PP386884	Nap-4
	Japan: Miyoshi City, Hiroshima [10]	14965	1	1	PP386885	Nap-5
Total			128	85		

program MEGA (vers. 11, Tamura et al. 2021), with Kimura 2-parameter (K2P) distance (Kimura 1980) and 2000 bootstrap reiterations. Other analyses, including the nucleotide composition, variable and parsimony informative positions, and K2P distance between haplotypes were also calculated using MEGA.

COI sequences of *Neocaridina*, obtained from various studies conducted in Japan (Fuke et al. 2021; Nagai and Imai 2021; Kakui and Komai 2022), Taiwan (Han et al. 2019), China (Zhou et al. 2021) and Israel (Levitt-Barmats et al. 2019), as well as unpublished sequences in GenBank (from Canada and Hungary), were incorporated into the analysis if their sequence length was ≥ 495 bp. This inclusion aimed to elucidate the phylogenetic relationships among species within the East Asian Arc and to identify introduced species (Table 2). The best-fitting model for sequence evolution was determined by PartitionFinder (ver. 2.1.1, Lanfear et al. 2017) and selected based on the Bayesian information criterion (BIC). The obtained best model (HKY+I+G) was subsequently employed for Bayesian inference (BI) and maximum likelihood (ML) analyses. BI analysis was performed with MrBayes (ver. 3.2.3, Ronquist et al. 2012), running four chains for 10 million generations across four independent runs, with trees sampled every 1,000 generations. The convergence of chains was determined using the average standard deviation of split frequency values, which remained below the recommended threshold of 0.01 (Ronquist et al. 2020). The first 1200 trees were accordingly discarded as "burn-in". ML analysis was performed using IQ-TREE (vers. 2.2.0, Minh et al. 2020) with the best model, and 20,000 ultrafast bootstrap replicates were generated (Hoang et al. 2017). As the phylogeny of *Neocaridina* is still not clear, due to sequences of most species not being available in GenBank, a midpoint rooting method was employed (calculating tip to tip distances and then placing the root midway between the two longest tips) (Kinene et al. 2016).

RESULTS

Distribution of Japanese *Neocaridina* species

Specimens of eight species of *Neocaridina* (see "TAXONOMY") collected from the 18 collection sites in Kyushu and southern Honshu of Japan (Fig. 1), as well as other related species, are shown in table 3. Among these species, *N. denticulata* was found at 11 out of 18 localities, all in Honshu, sometimes sympatric with other species, *i.e.*, *N. davidi*, *N. koreana*, *N.* aff. *fukiensis*, *N.* aff. *palmata*, and *N.* aff. *denticulata*. The second most widespread species was *N.* aff. *denticulata*,

found at 7 localities in Honshu (4), Kyushu (1), and Tsushima (2). *N. davidi*, the next most widespread, was found at four localities in Honshu and one on Awaji Island, adjacent to Shikoku. *Neocaridina palmata* was collected from three localities in Honshu. *Neocaridina ikiensis* was only found at three localities on Iki Island. *Neocaridina koreana*, *N. palmata*, and *N.* aff. *fukiensis*, were only collected from one locality each in Honshu.

Half of the studied localities supported two or more species living sympatrically (Table 3). For example, Himeji City, Hyogo (Site 2) had four species occurring together, viz. *N. davidi*, *N. palmata*, *N.* aff. *palmata*, and *N.* aff. *denticulata*; Miyoshi City, Hiroshima (Site 10) had three species collected, viz. *N. denticulata*, *N.* aff. *palmata* and *N. koreana*. While Awaji Island (Site 3) had two species (*N. davidi* and *N. denticulata*), only a single species was found on the other two islands under study, viz. Iki Island (Site 16) with *N. ikiensis* and Tsushima Island (Sites 17, 18) with *N.* aff. *denticulata*.

Molecular analyses

A 658-bp (base pair) segment of *COI* from 115 specimens representing 12 *Neocaridina* species (with 84 specimens from Japan) was amplified, resulting in 53 different haplotypes (Table 1). The studied *COI* segment was AT rich (59.4%) (T, 32.5%; A, 26.8%; G, 19.8%; C, 20.9%). Within this gene fragment, 170 positions were variable and 143 were parsimoniously informative.

The neighbor-joining tree, based on 658 bp of *COI* (Fig. 2), reveals that the Japanese specimens collected in this study belong to eight clades, each corresponding to a specific species: *N. davidi*, *N. denticulata*, *N. ikiensis*, *N.* aff. *palmata*, *N. palmata*, *N.* aff. *fukiensis*, *N. koreana*, and *N.* aff. *denticulata*. These species were identified through morphological examination (see "TAXONOMY"). All clades are highly supported, except the *N. davidi* clade, which is moderately supported. In the *COI* tree (Fig. 2), *N. denticulata* and *N. davidi* appear as sister species, and they, along with *N. palmata* and *N.* aff. *palmata*, form a highly-supported large clade. *Neocaridina ikiensis* is closely related to *N.* aff. *fukiensis*, while *N. koreana* is sister to *N.* aff. *denticulata*.

The pairwise nucleotide divergences for *COI* with K2P distance (and differences in the total bp numbers) are shown in table 4. Most species are well separated from others by a minimum divergence of 3.77% (24 bp difference), except for a smaller divergence of 1.7% (11 bp difference) between *N. davidi* and *N. denticulata*.

By incorporating sequences from other studies, including those available in GenBank, the phylogeny of *Neocaridina* from East Asia is shown in figure 3,

with respective support values from BI and ML. The tree was rooted using midpoint rooting (see "Molecular analyses"), positioning *N. spinosa* and *N. hofendopoda* at the basal positions, followed by *N. iriomotensis* and *N. ishigakiensis* from the southern Ryukyus. The remaining species form a major group with two large clades. One large clade with high support is composed of four species: two sister species, *N. davidi* and *N. denticulata*, and another species pair, *N.* aff. *palmata* and *N. palmata*. Another large clade with moderate support consists of 11 species. *Neocaridina* sp. 1, *N.* sp. 2, *N.* sp. 3 (see Remarks under *N. ikiensis*), *N.* aff. *fukiensis* and *N. ikiensis* are more closely related, and form two groups, one of which includes *N.* sp. 1, *N.*

Table 2. Other *COI* haplotypes of *Neocaridina* species from GenBank used in figure 3. Accession numbers with identical haplotypes are parenthesized

Species	Original name (if different)	Locality	Accession no.	Reference
N. davidi		Taiwan: Sinshe, Taichung; Dounan, Yunlin	AB300183 (AB300184)	Shih and Cai 2007
		Taiwan: Kinmen	AB300187	
		Hawaii: Oahu	AB300185; AB300186	
N. davidi	"*N. denticulata*"	Canada: Big Al's Fish Store	MG319788	unpublished
	"*N. denticulata*"	Hungary	MH780819 (MH780820, MH780821)	unpublished
	"*N. denticulata*"	Israel	MN336479	Levitt-Barmats et al. 2019
			MN336445 (MN336446, MN336449, MN336450, MN336451, MN336452, MN336453, MN336461, MN336473, MN336482, MN336483); MN336447 (MN336448, MN336454, MN336455, MN336456, MN336457, MN336459, MN336460, MN336462, MN336463, MN336465, MN336475, MN336480, MN336481, MN336484)	
N. denticulata		Japan: Biwa Lake	AB300191	Shih and Cai 2007
	"*N. davidi* Type II"	Japan: Okinawa: Nanjo: Kakinohana spring	LC659919	Nagai and Imai 2021
	"*N. denticulata*"	Israel: HaHula	MN336458	Levitt-Barmats et al. 2019
N. fonticulata		Taiwan: Kenting, Pingtung	LC427866	Shih et al. 2019
N. hofendopoda		China: Hubei: Yichang: Sanxia	MN701609 (MN701610)	Chen et al. 2020
N. ketagalan		Taiwan: Sijhih, New Taipei City	AB300180 (AB300181); AB300182	Shih and Cai 2007
N. koreana	"*N. heteropoda koreana*"	Korea: Gyeongsangnam: Geojedo	MK907783	Park et al. 2019
N. palmata		China: Meizhou, Guangdong; Jiangle, Fujian	LC324769	Shih et al. 2017
		China: Leye, Guangxi	LC324770	
N. saccam		Taiwan: Longci, Tainan; Houjha, Tainan	AB300177 (AB300179)	Shih and Cai 2007
		Taiwan: Longci, Tainan	AB300178	
N. spinosa		China: Tong-an, Fujian	AB300188	
N. aff. *fukiensis*	"*N. denticulata*"	Japan: Oita: Machida R.	LC612372	Fuke et al. 2021
		Japan: Oita: Miya R.	LC612374 (LC612376, LC612378); LC612379	
N. aff. *palmata*	"*N. koreana*"	aquarium in South Korea	LC324768	Shih et al. 2017
	"*N.* sp. aff. *davidi*"	Japan: Hokkaido: Sapporo	LC664097 (LC664098, LC664099)	Kakui and Komai 2022
		Japan: Chiba	LC664096	
N. sp. 1	"*N. denticulata*"	Japan: Kumatoto: Kuro R.	LC612358 (LC612359, LC612360, LC612361, LC612362, LC612363); LC612364; LC612357	Fuke et al. 2021
		Japan: Nagasaki: Ishiki R.	LC612349; LC612352; LC612356	
N. sp. 2	"*N. denticulata*"	Japan: Nagasaki: Goto Islands: Fukue-jima	LC612339 (LC612340, LC612342, LC612343, LC612344, LC612345, LC612346, LC612348); LC612341 (LC612347)	
N. sp. 3	"*N. denticulata*"	Japan: Oita: Machida R.	LC612365; LC612368; LC612366; LC612371; LC612370	
N. sp. 4	"*N.* sp."	Taiwan: Hualien	MG734216; MG734217; MG734218; MG734219; MG734220; MG734221	Han et al. 2019

sp. 2 and *N.* aff. *fukiensis*, and the other consists of *N. ikiensis* and *N.* sp. 3. The remaining six species do not exhibit clear groupings, except for *N.* aff. *denticulata*, which is closely related to *N. koreana*.

The sequences of the species introduced to Hawaii, Canada, Hungary and Israel cluster within the same clade as *N. davidi*. Another species found in common between Okinawa and Israel appears to belong to *N. denticulata* (but see Remarks under *N. denticulata*). Some specimens collected from Japan share identical haplotypes with those from China and Vietnam, including *N. davidi* (NCHUZOOL 14941

Table 3. Geographic positions of the collection sites in the main islands of Japan, with the species of *Neocaridina* collected in this study

No. on Fig. 1	Prefecture	City	Latitude	Longitude	Species collected			
					N. davidi	*N. denticulata*	*N. koreana*	*N. ikiensis*
1	Gifu	Mizunami City	35°22'03.9"N	137°14'07.6"E		*		
2	Hyogo	Himeji City	34°56'31.9"N	134°38'19.0"E	*			
3	Hyogo	Awaji City (Awaji Island)	34°26'09.8"N	134°53'22.8"E	*	*		
4	Okayama	Akaiwa City	34°53'48"N	134°01'05"E		*		
5	Okayama	Okayama City	34°45'47"N	133°55'05"E				
6	Okayama	Ihara City	34°33'57.4"N	133°28'50.4"E		*		
7	Hiroshima	Fukuyama City	34°36'43"N	133°15'33"E		*		
8	Shimane	Izumo City	35°23'33.3"N	132°43'37.1"E	*			
9	Shimane	Izumo City	35°22'42.9"N	132°50'35.4"E		*		
10	Hiroshima	Miyoshi City	34°46'51"N	132°54'39"E		*	*	
11	Hiroshima	Higashihiroshima City	34°23'14"N	132°43'50"E		*		
12	Hiroshima	Hiroshima City	34°29'11"N	132°31'51"E	*	*		
13	Hiroshima	Hiroshima City	34°22'53"N	132°38'17"E		*		
14	Yamaguchi	Ube City	N34°01'08"	131°14'46"E	*	*		
15	Nagasaki	Sasebo City	33°09'45.5"N	129°50'32.9"E				
16	Nagasaki	Iki City (Iki Island)	33°47'26.7"N	129°42'49.4"E				*
17	Nagasaki	Tsushima City	34°14'1.9"N	129°17'45.7"E				
18	Nagasaki	Tsushima City	34°15'32.4"N	129°18' 47.5"E				

No. on Fig. 1	Prefecture	City	Latitude	Longitude	Species collected			
					N. palmata	*N.* aff. *denticulata*	*N.* aff. *fukiensis*	*N.* aff. *palmata*
1	Gifu	Mizunami City	35°22'03.9"N	137°14'07.6"E		*		
2	Hyogo	Himeji City	34°56'31.9"N	134°38'19.0"E	*	*		*
3	Hyogo	Awaji City (Awaji Island)	34°26'09.8"N	134°53'22.8"E				
4	Okayama	Akaiwa City	34°53'48"N	134°01'05"E				
5	Okayama	Okayama City	34°45'47"N	133°55'05"E		*		
6	Okayama	Ihara City	34°33'57.4"N	133°28'50.4"E				
7	Hiroshima	Fukuyama City	34°36'43"N	133°15'33"E			*	
8	Shimane	Izumo City	35°23'33.3"N	132°43'37.1"E				*
9	Shimane	Izumo City	35°22'42.9"N	132°50'35.4"E		*		
10	Hiroshima	Miyoshi City	34°46'51"N	132°54'39"E				*
11	Hiroshima	Higashihiroshima City	34°23'14"N	132°43'50"E				
12	Hiroshima	Hiroshima City	34°29'11"N	132°31'51"E				
13	Hiroshima	Hiroshima City	34°22'53"N	132°38'17"E				
14	Yamaguchi	Ube City	34°01'08"N	131°14'46"E				
15	Nagasaki	Sasebo City	33°09'45.5"N	129°50'32.9"E		*		
16	Nagasaki	Iki City (Iki Island)	33°47'26.7"N	129°42'49.4"E				
17	Nagasaki	Tsushima City	34°14'1.9"N	129°17'45.7"E		*		
18	Nagasaki	Tsushima City	34°15'32.4"N	129°18' 47.5"E		*		

from Hyogo, Japan; NCHUZOOL 13347 from Fujian, China), *N. palmata* (NCHUZOOL 14940 from Hyogo, Japan; NCHUZOOL 14975 from Guangxi, China; NCHUZOOL 15174, 15175, 15176 from northern Vietnam) and *N.* aff. *palmata* (NCHUZOOL 14940, 14941 from Hyogo, Japan; NCHUZOOL 15170 from Yunnan, China) (Fig. 3).

Morphological identification

Based on the taxonomic literature for *Neocaridina* from mainland China, Japan and Taiwan (see "MATERIALS AND METHODS"), five of the eight clades from Japan are identified with precise published names (Fig. 3), including the native *N. denticulata* and *N. ikiensis*, as well as the introduced *N. davidi*, *N. palmata*, and *N. koreana*. The remaining three species are temporarily referred to as *N.* aff. *fukiensis*, *N.* aff.

palmata, and *N.* aff. *denticulata*. The identity of these three species will be further compared morphologically, in conjunction with known species in the region, and the results will be published separately.

TAXONOMY

Family Atyidae De Haan, 1849
Neocaridina Kubo, 1938
Neocaridina denticulata (De Haan, 1844)
(Figs. 4, 5)

Specimens examined: **Japan**: 1 male, cl 6.5 mm, 1 male, cl 5.2 mm, 6 males, cl 4.2–4.7 mm, ZRC 2023.0211, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015; 1 female, cl 6.2 mm,
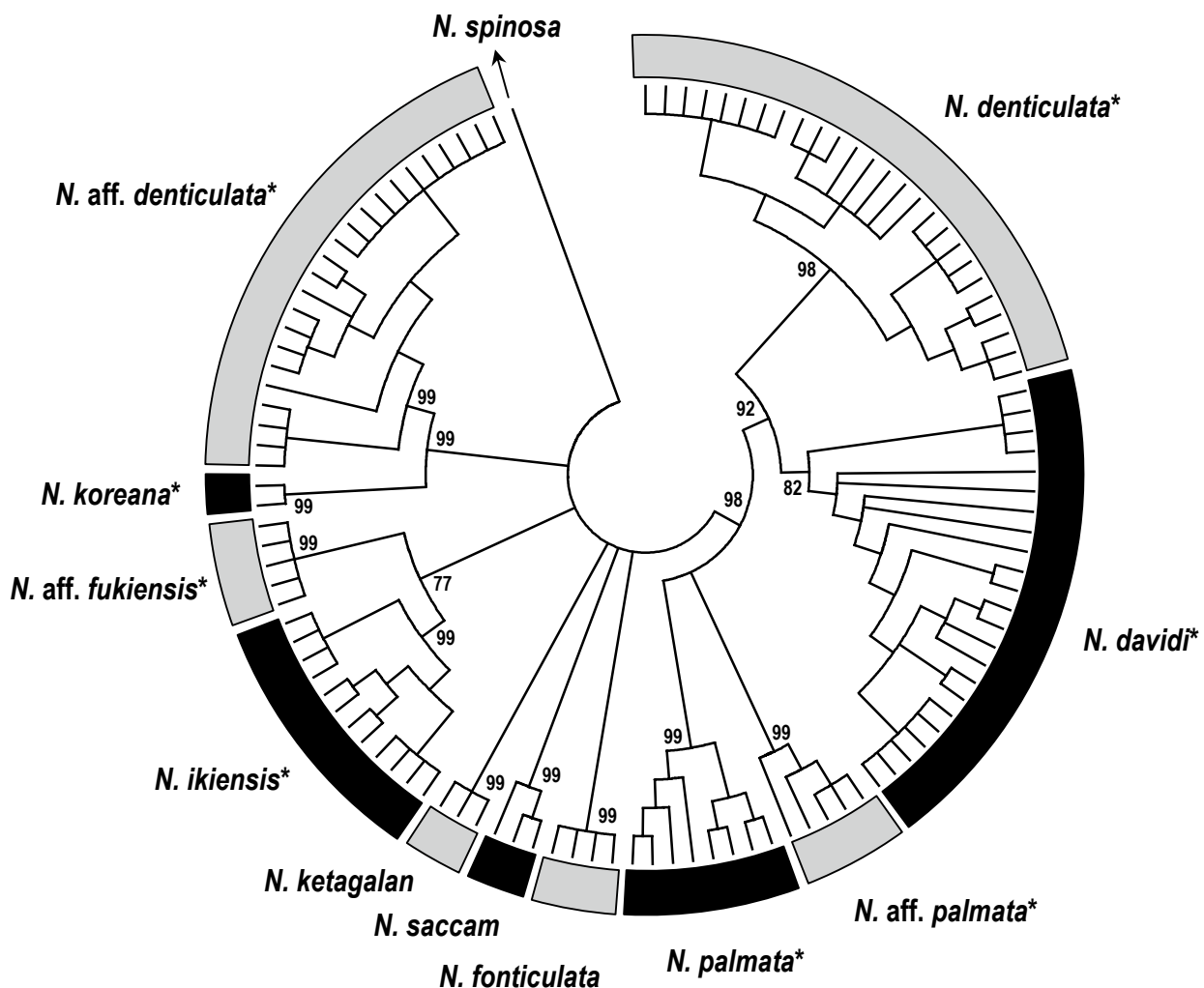

Fig. 2. A neighbor-joining *COI* tree, based on 53 haplotypes (115 specimens) of *Neocaridina* species from the main islands of Japan and the related taxa from East Asia. Only bootstrap values > 50% are shown on the nodes. *, species collected from Japan in this study.

Table 4. Matrix of percentage pairwise nucleotide divergences with K2P distance and mean number of differences based on 658 bp of *COI* within and between species of *Neocaridina* used in this study. In the interspecific (right) part, lower-left values are K2P distances and upper-right ones are bp differences. Range of values are given in parentheses

	Intraspecific		Interspecific				
	Nucleotide divergence	Mean nucleotide difference	*N. davidi*	*N. denticulata*	*N. ikiensis*	*N.* aff. *fukiensis*	*N. koreana*
N. davidi	0.89 (0–2.17)	5.8 (0–14)		18.42 (11–25)	44.68 (39–50)	51.87 (50–54)	46.96 (45–49)
N. denticulata	1.17 (0–2.01)	7.59 (0–13)	2.88 (1.7–3.93)		45.2 (41–51)	54.77 (52–59)	52.85 (50–56)
N. ikiensis	1.22 (0–2.49)	7.86 (0–16)	7.21 (6.25–8.13)	7.31 (6.58–8.31)		36.92 (35–40)	41.92 (41–43)
N. aff. *fukiensis*	0	0	8.48 (8.14–8.85)	9 (8.52–9.76)	5.91 (5.59–6.43)		47 (47–47)
N. koreana	0	0	7.6 (7.26–7.95)	8.64 (8.12–9.21)	6.74 (6.59–6.92)	7.63 (7.63–7.63)	
N. aff. *denticulata*	0.77 (0–2.01)	5.02 (0–13)	9.32 (8.62–10.08)	9.71 (8.8–10.27)	7.17 (6.39–7.77)	8.64 (8.12–9.01)	3.97 (3.78–4.43)
N. palmata	0.41 (0–0.61)	2.68 (0–4)	4.77 (4.24–5.56)	5.46 (4.9–5.89)	8.39 (7.56–8.95)	9.47 (9.31–9.49)	9.12 (8.79–9.5)
N. aff. *palmata*	0.7 (0.15–1.23)	4.6 (1–8)	4.51 (3.77–5.58)	4.99 (4.26–5.59)	8.63 (8.12–9.18)	9.88 (9.72–10.1)	8.84 (8.63–9.15)
N. fonticulata	0	0	5.69 (5.4–6.24)	6.14 (5.57–6.59)	6.76 (6.43–6.94)	8.36 (8.36–8.36)	6.08 (6.08–6.08)
N. saccam	0.31 (0–0.46)	2 (0–3)	7.13 (6.74–8.14)	8.02 (7.43–8.68)	7.85 (7.11–8.33)	8.88 (8.52–9.06)	6.53 (6.42–6.59)
N. ketagalan	0.1 (0–0.15)	0.67 (0–1)	7.2 (6.57–7.78)	8.06 (7.44–8.5)	6.58 (6.08–7.11)	7.7 (7.64–7.81)	6.32 (6.26–6.43)
N. spinosa	—	—	14.41 (13.94–14.7)	15.06 (14.51–15.3)	15.56 (14.73–16.12)	16.27 (16.27–16.27)	15.68 (15.68–15.68)

	Interspecific						
	N. aff. *denticulata*	*N. palmata*	*N.* aff. *palmata*	*N. fonticulata*	*N. saccam*	*N. ketagalan*	*N. spinosa*
N. davidi	56.78 (53–61)	30.21 (27–35)	28.53 (24–35)	35.74 (34–39)	44.17 (42–50)	44.59 (41–48)	84.52 (82–86)
N. denticulata	58.98 (54–62)	34.39 (31–37)	31.4 (27–35)	38.38 (35–41)	49.33 (46–53)	49.56 (46–52)	87.85 (85–89)
N. ikiensis	44.5 (40–48)	51.83 (47–55)	52.88 (50–56)	41.92 (40–43)	48.25 (44–51)	40.92 (38–44)	90.17 (86–93)
N. aff. *fukiensis*	52.87 (50–55)	57.88 (57–58)	59.8 (59–61)	51 (51–51)	54 (52–55)	47.33 (47–48)	94 (94–94)
N. koreana	25.22 (24–28)	55.88 (54–58)	54.2 (53–56)	38 (38–38)	40.67 (40–41)	39.33 (39–40)	91 (91–91)
N. aff. *denticulata*		65.04 (61–68)	63.6 (60–67)	47 (44–48)	50.86 (47–52)	50.12 (47–52)	97.22 (95–101)
N. palmata	10.76 (10.04–11.3)		36.28 (34–40)	46.63 (45–47)	52.46 (51–53)	48.21 (47–49)	91.25 (90–92)
N. aff. *palmata*	10.51 (9.84–11.13)	5.77 (5.39–6.4)		44.4 (43–46)	51.93 (50–54)	47.33 (46–49)	89.4 (89–90)
N. fonticulata	7.61 (7.1–7.77)	7.5 (7.22–7.56)	7.14 (6.9–7.41)		34.33 (34–35)	34.67 (34–35)	83 (83–83)
N. saccam	8.29 (7.61–8.49)	8.52 (8.27–8.62)	8.45 (8.11–8.81)	5.49 (5.43–5.6)		38 (37–39)	83 (82–85)
N. ketagalan	8.17 (7.61–8.51)	7.79 (7.58–7.93)	7.66 (7.43–7.95)	5.53 (5.42–5.58)	6.08 (5.91–6.25)		85.67 (85–86)
N. spinosa	16.94 (16.49–17.72)	15.75 (15.5–15.9)	15.35 (15.26–15.46)	14.13 (14.13–14.13)	14.15 (13.95–14.54)	14.67 (14.54–14.73)	
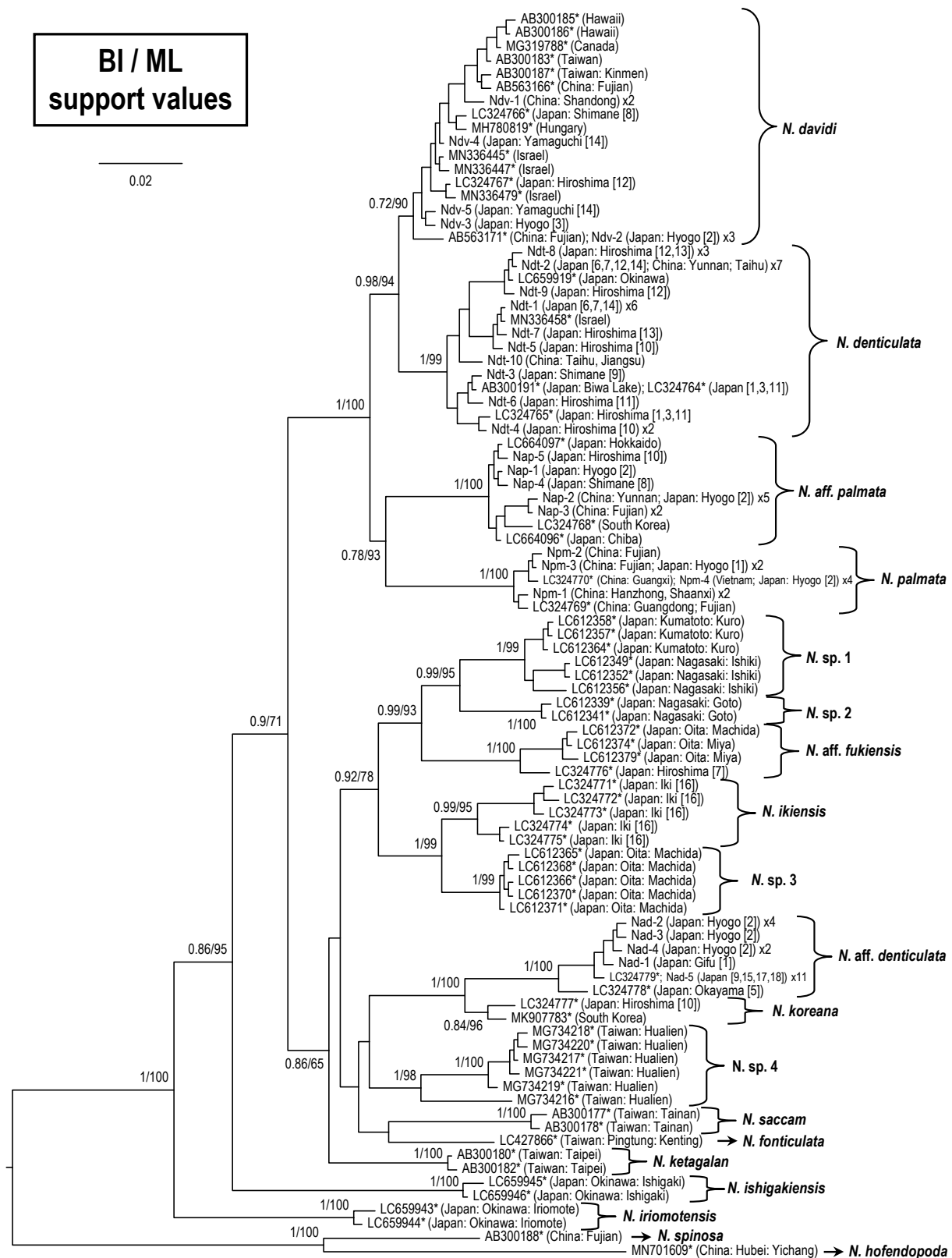
Fig. 3. Bayesian inference (BI) tree for species of the genus *Neocaridina* from the East Asia Arc and the related taxa from East Asia, based on the cytochrome *c* oxidase subunit I (*COI*) gene. Probability values at the nodes represent support values for BI and maximum likelihood (ML). Only support values > 50% are shown on the nodes. For haplotype names, see table 1. *, sequences downloaded from GenBank.

1 female, cl 5.9 mm, 11 females, cl 4.1–7.5 mm, NCHUZOOL 14965, 1 male, cl 4.2 mm, 9 males, cl 4.5–6.0 mm, NCHUZOOL 14964, Basen-gawa R., Go-no-gawa system, Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016; 1 female, cl 3.5 mm, NCHUZOOL 14944, Kurose R., Kurose River system, Higashihiroshima City, Hiroshima Prefecture, N34°23'14", E132°43'50", coll. H. Yoshigou, 27 Sep. 2015; 1 male, cl 3.3 mm, NCHUZOOL 14942, Shizuki R., Shizuki River system, Awaji City, Awaji Island, Hyogo Prefecture, N34°26'09.8", E134°53'22.8", coll. Y. Nakahara, 17 Sep. 2015; 1 female, cl 3.8 mm, 1 male, cl 3.7 mm, NCHUZOOL 14968, Iwatuki-gawa R., Takahashigawa system, Odagawa, Ihara City, Okayama Prefecture, N34°33'57.4", E133°28'50.4", coll. H. Yoshigou, 10 Oct. 2016; 1 female, cl 4.5 mm, 1 male, cl 4.1 mm, NCHUZOOL 14960, Misasa R., Ota-gawa system, Hiroshima City, Hiroshima Prefecture, N34°29'11", E132°31'51", coll. H. Yoshigou, 11 Feb. 2016; 2 males, cl 5.8, 5.9 mm, ZRC 2024.0055, Misasa R., Ota-gawa system, Hiroshima City, Hiroshima Prefecture, N34°29'11", E132°31'51", coll. H. Yoshigou, 11 Feb. 2016; 1 male, cl 3.6 mm, NCHUZOOL 14946, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015; 1 male, cl 4.7 mm, NCHUZOOL 14958, Hazamagawa R., Shonaigawa system, Mizunami City, Gifu Prefecture, N35°22'03.9", E137°14'07.6", coll. Y. Nakahara, 21 Oct. 2015; 1 female, cl 4.7 mm, NCHUZOOL 14966, Ato-gawa R., Seno-gawa system branch of Kumano-gawa R., Hiroshima City, Hiroshima Prefecture, N34°22'53", E132°38'17", coll. H. Yoshigou, 5 Mar. 2015; 1 male, cl 4.3 mm, ZRC 2023.0212, Sunagawa R., Asahigawa system, Akaiwa City, Okayama Prefecture, N34°53'48", E134°01'05", coll. H. Yoshigou, 23 Nov. 2015. **Mainland China**: 1 female cl 8.7 mm, NCHUZOOL 15168, Pingyuan, Yunnan, coll. H.-T. Shih, 4 Nov. 2002; NCHUZOOL, Taihu (Lake), Changzhou, Jiangsu, coll. W.-R. Yun, 30 Jun. 2018; 5 males, cl 3.2–3.4 mm, 6 females, cl 3.9–4.5 mm, ZRC 2023.0213, Taihu (Lake), Changzhou, Jiangsu, coll. W.-R. Yun, 30 Jun. 2018.

Native distribution: Western part of the main islands of Japan (Toyota et al. 2014; Mitsugi et al. 2017; Mitsugi and Suzuki 2018).

Remarks: *Neocaridina denticulata*, the type species of this genus, was considered the only native species of the genus in the main islands of Japan (Cai 1996; Liang 2004) until the publication of the name *N. ikiensis* for specimens from Iki Island, off the northern coast of Kyushu (Shih et al. 2017). Key characters of *N. denticulata* include its relatively long rostrum, which generally reaches beyond the end of the antennular peduncle; the third and fifth pereiopods do not display sexual dimorphism; the endopods of the male first pleopods are distinctly broad, about 1.2 times as long as wide, and the appendix interna of the male second pleopods is short, not reaching the end of the endopod (Cai 1996; Liang 2004). Our specimens agree well with these characters (Figs. 4, 5). Based on our study supported by molecular evidence, the species is confirmed to be distributed in the main islands of Japan, as well as the Jiangsu (Taihu Lake and Nanjing City) and Yunnan Provinces of China (Fig. 3). Due to the limited samples across China used for genetic comparison, we are unable to comment if these populations in China are native.

When describing two new *Neocaridina* species from Taiwan, Shih and Cai (2007) presented genetic relationships by examination of *COI* sequences of their new species with a few known species from Taiwan, China and Japan. In their study, *Neocaridina denticulata* was represented by specimens from Lake Biwa (AB300191). Nishino (2017) and Fuke et al. (2021) questioned the validity of the species name used for the genetic material, as *N. denticulata* was presumed to be extinct from Lake Biwa before the incidental introduction of *N. davidi* in the 2000s, and *N. davidi* has since spread widely in and beyond the lake (Nishino and Niwa 2004). Based on molecular and morphological evidence, however, Onuki and Fuke (2022) recently confirmed that both species are found in Lake Biwa. With more samples available, our current study shows that the Lake Biwa specimen (AB300191) is genetically closely clustered with many *N. denticulata* specimens from western Japan and China (Jiangsu and Yunnan) (Fig. 3). The attribution of the name *N. denticulata* to this specimen by Shih and Cai (2007) was therefore correct.

Nagai and Imai (2021) reported a species of *Neocaridina* from Kakinohana spring, Nanjo City, Okinawa Island, the Ryukyus. They tentatively assigned their specimens to "*N. davidi* Type II (*N. davidi koreana*?)" based on *COI* sequences. These sequences shared only one haplotype and genetically clustered together with our *N. denticulata* specimens collected from Shiga (Lake Biwa), Gifu, Hyogo, Okayama, Shimane, Hiroshima, and Yamaguchi (Fig. 3), as well as one of Fuke et al. (2021)'s Oita specimens (LC612373) (Nagai and Imai 2021: fig. 3). This result suggests that the Okinawa specimens should be assigned to *N. denticulata*, and this species also occurs in Oita. The Chinese material (from Henan Province) named as *N. davidi koreana* (MW069609–MW069622) in Zhou et al. (2021) should also be assigned to *N. denticulata*.

Levitt-Barmats et al. (2019) reported the occurrence of "*N. denticulata*" in Israel. According to
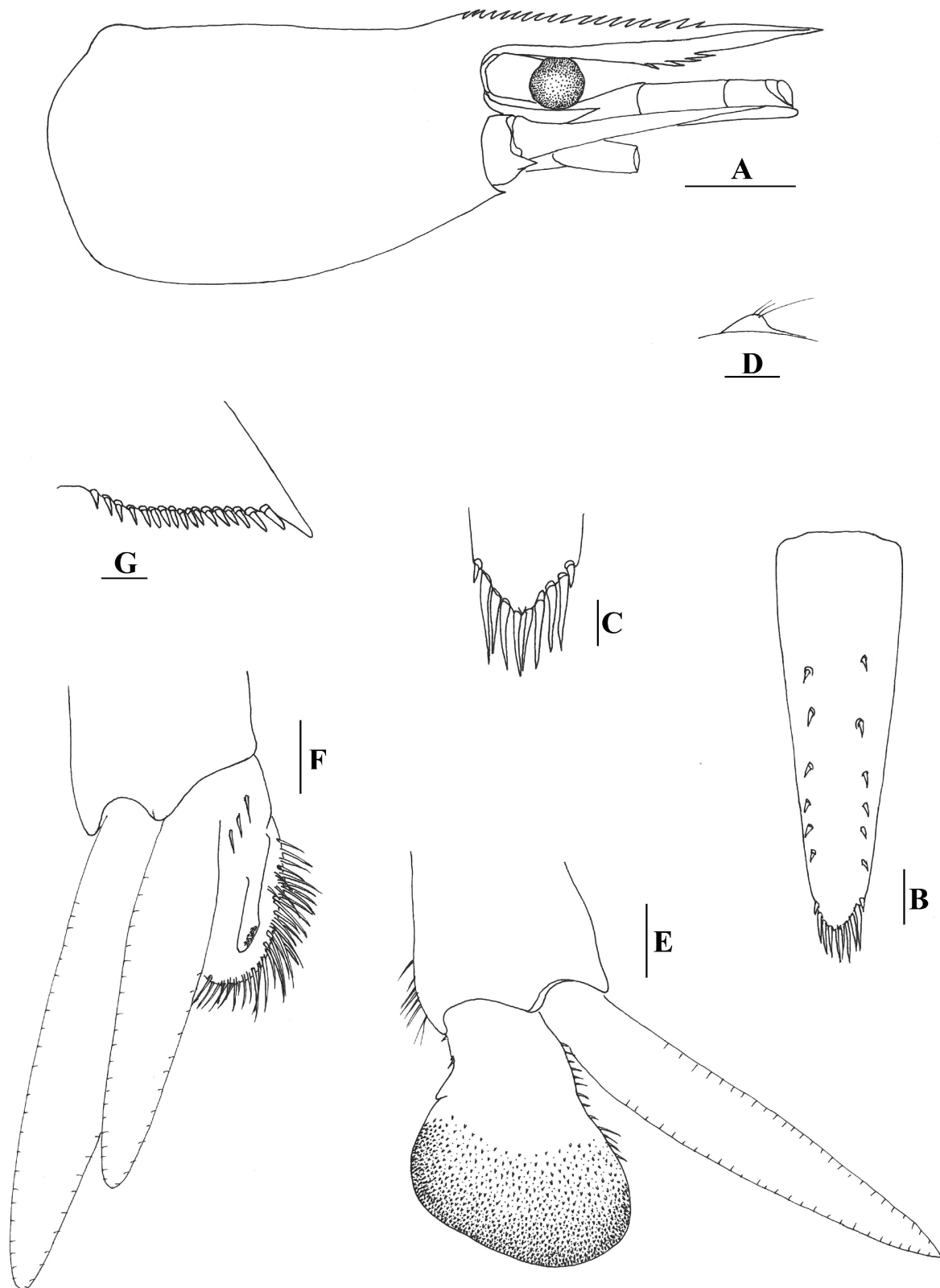
Fig. 4. *Neocaridina denticulata* (De Haan, 1844). (A) cephalothorax and cephalic appendages, lateral view; (B) telson; (C) distal portion of telson; (D) preanal carina; (E) male 1st pleopod; (F) male 2nd pleopod; (G) uropodal diaeresis. Scale bars: A = 2 mm; B, D, E, F = 0.5 mm; C, G = 0.2 mm (A, D–G, male, cl 6.5 mm, B, C, male, cl 5.2 mm, ZRC 2023.0211, Kaya R., Ashida river system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015).
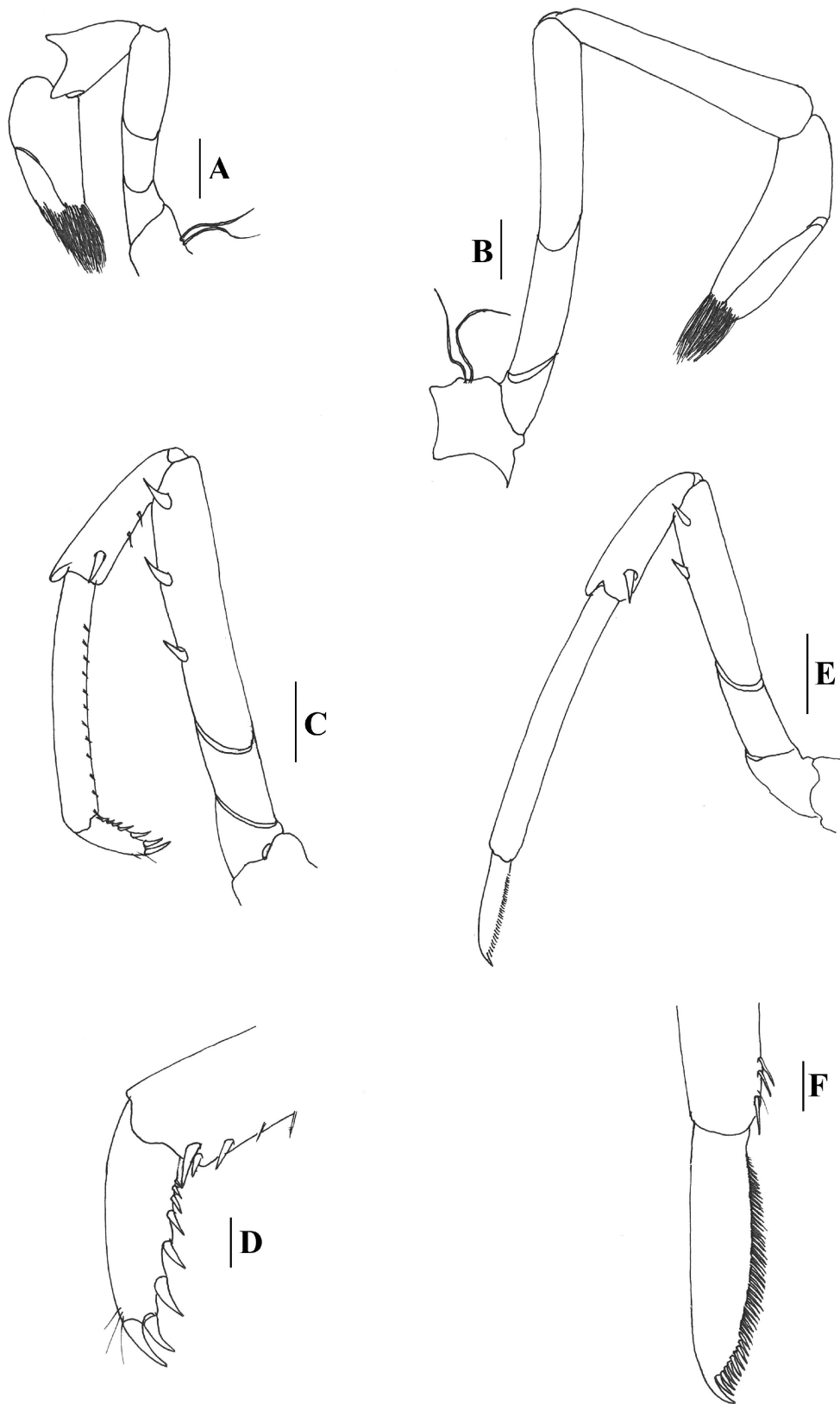
Fig. 5. *Neocaridina denticulata* (De Haan, 1844). (A) 1st pereiopod; (B) 2nd pereiopod; (C) 3rd pereiopod; (D) the same, dactylus; (E) 5th pereiopod; (F) the same, dactylus. Scale bars: A, B, C, E = 0.5 mm; D, F = 0.2 mm (male, cl 6.5 mm, ZRC 2023.0211, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015).

the authors, the Israeli specimens are most probably *N. denticulata*, as they showed no sexual dimorphism in the third pereiopods. However, their *COI* sequences of 15 specimens (MN336485–MN336499) from Japan (Okayama), cluster into two distinct clades, with three specimens belonging to *N. denticulata* and 12 belonging to *N.* aff. *denticulata* (results not shown). In our study, these two species have also been identified from Okayama (Table 3). By including the longer Israeli *COI* sequences of *Neocaridina* (Table 2) into our phylogenetic tree (Fig. 3), all samples clustered well within the clade of *N. davidi*, except for one (MN336458) from HaHula Nature Reserve, which belonged to *N. denticulata*. While this specimen may represent the first recorded introduction of *N. denticulata* outside its natural range (Japan and possibly China), we cannot exclude the possibility of mislabelling.

Neocaridina davidi (Bouvier, 1904)
(Figs. 6, 7)

Specimens examined: **Japan**: 6 males, cl 3.9–5.6 mm, ZRC 2023.0214, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015; 1 female, cl 4.0 mm, 1 female, cl 4.4 mm, NCHUZOOL 14952, Koto R., Tanai, Ube City, Yamaguchi Prefecture, N34°01'08", E131°14'46", coll. H. Yoshigou, 23 Jan. 2016. **Mainland China**: 2 males, cl 4.7–5.3 mm, 1 female, cl 4.8 mm, 1 damaged, cl 4.7 mm, NCHUZOOL 13342, Putian, Fujian, coll. H.-T. Shih, 7 Jul. 2004. **Taiwan**: 1 ovig. female, cl 5.4 mm, NCHUZOOL 13108, Sinshe, Taichung, coll. H.-T. Shih, 17 Apr. 2003; 1 female, cl 5.3 mm, NCHUZOOL 13109, Dounan, Yunlin, coll. H.-T. Shih, 28 Jul. 2006; 1 ovig. female, NCHUZOOL 13323, Guangfu, Hualien, coll. 7 Mar. 2009; 1 female, cl 3.3 mm, 1 damaged, cl 4.2 mm, NCHUZOOL 15178, Zuoying, Kaohsiung, coll. Oct. 2006; 2 damaged, NCHUZOOL 15177, Siaoliouciou, Pingtung, coll. 13 Nov. 2010.

Native distribution: Mainland China and Taiwan (Cai 1996; Liang 2004).

*Remarks*: Cai (1996) transferred *C. davidi* to the genus *Neocaridina* and treated it as a subspecies of *N. denticulata*. In the same publication, Cai (1996) redescribed *Neocaridina denticulata sinensis* based on specimens from Taihu, Jiangsu Province, China, the type locality, and highlighted that the characters separating *N. d. sinensis* from *N. d. denticulata* are the sexual dimorphism of the third pereiopods; the distinctly shorter appendix interna of the male second pleopods, and the relatively shorter rostrum. Liang (2002) described a new species, *N. heteropoda*, from

Zhejiang Province, China and referred some of Cai's (1996) *N. d. sinensis* to his new species. Liang (2002 2004) also considered that the characters separating *N. d. denticulata* (De Haan) and *N. d. sinensis* (Kemp) are not reliable, and treated *N. d. sinensis* from mainland China and Taiwan as a synonym of *N. d. denticulata*, referring the form introduced to Hawaii as *N. heteropoda heteropoda*. In response to this taxonomic action, Shih and Cai (2007) commented, "…The validity of *N. heteropoda* Liang, 2002 is also doubtful as Liang (2004: 91) listed *Caridina davidi* Bouvier, 1904 as a synonym of *N. heteropoda*, which in turn, should make *H.* [sic. *N.*] *heteropoda* a junior synonym, if Liang's (2004) conclusion is correct." Klotz et al. (2013) examined the co-type material of *Caridina davidi* Bouvier 1904 and tentatively assumed that *N. heteropteran* [sic. *heteropoda*] *heteroptera* [sic. *heteropoda*] Liang, 2002 and *N. denticulata sinensis* (Kemp, 1913 [sic. 1918]) are synonyms of *N. davidi*. Based on our current examination, *N. davidi* is closely related to *N. denticulata*, sharing a similar pear-shaped endopod of the male first pleopods, but it can be distinguished by the sexual dimorphism in the third pereiopods and the distinctly shorter appendix interna of the male second pleopods (Figs. 6, 7). The species is widely distributed in mainland China and Taiwan and has been introduced to Hawaii (Englund and Cai 1999), the main islands of Japan (see below), Germany (Klotz et al. 2013), Poland (Jabłońska et al. 2018), Hungary (Weiperth et al. 2019), Israel (Levitt-Barmats et al. 2019), and Canada (Toronto, based on GenBank sequence) (cf. Fig. 3).

Nishino and Niwa (2004) first reported the occurrence of *N. davidi* (under the name *Neocaridina denticulata sinensis*) in Japan (Lake Biwa). Although *N. denticulata* was firstly recorded from Lake Biwa in 1915 (Kemp 1918), this indigenous shrimp had never been reported since then. In 2001, many individuals of another congeneric shrimp *Neocaridina* sp. were found in the lake (Nishino and Niwa 2004). *Neocaridina davidi* (as *N. denticulata sinensis*), presumed to be introduced from China or Korea as live bait for game fishing, has rapidly spread throughout Japan (Nishino 2017). Onuki and Fuke (2022) recently rediscovered the native species *N. denticulata* in Lake Biwa but found that in 11 of the 19 surveyed sites, the introduced species was dominant. Their results suggest that the native species has largely been replaced by the invasive species, and the native populations around Lake Biwa are in a critically threatened state. Fujita et al. (2011) also reported the occurrence of *N. davidi* (as *N. d. sinensis*) at Gono R. (Shimane) and Kako R. (Kakogawa, Hyogo). Fujita et al. (2011) found that *N. denticulata* has high genetic diversity in the sequences of ND2-tRNATrp + ND5, with four distinct clades
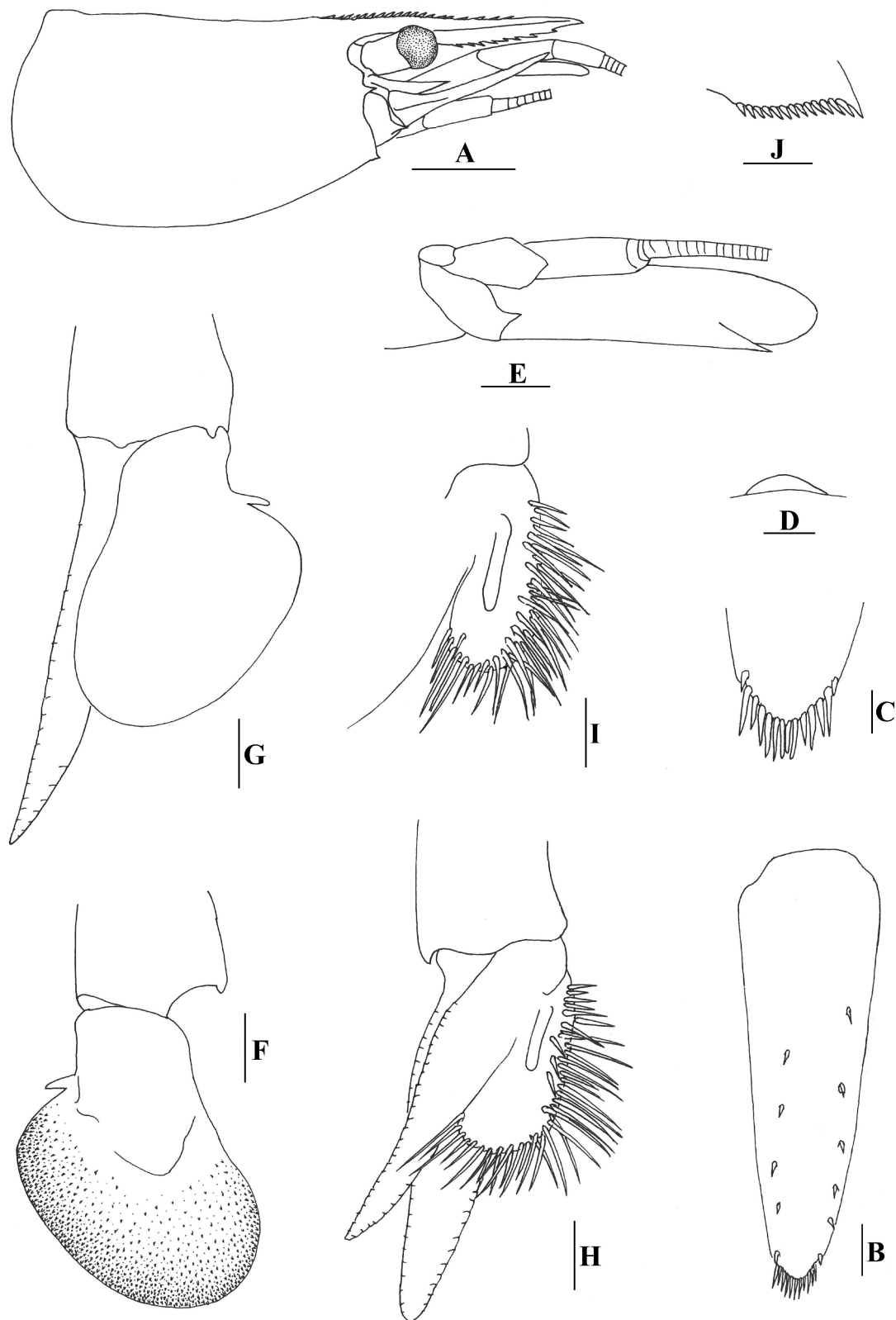
Fig. 6. *Neocaridina davidi* (Bouvier, 1904). (A) cephalothorax and cephalic appendages, lateral view; (B) telson; (C) distal portion of telson; (D) preanal carina; (E) scaphocerite; (F, G) male 1st pleopod; (H, I) male 2nd pleopod; (J) uropodal diaeresis. Scale bars: A = 2 mm; B, D, F–J = 0.5 mm; C = 0.2 mm; E = 1 mm (A–H, J, male, cl 5.6 mm; I, male, cl 5.3 mm, ZRC 2023.0214, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015).
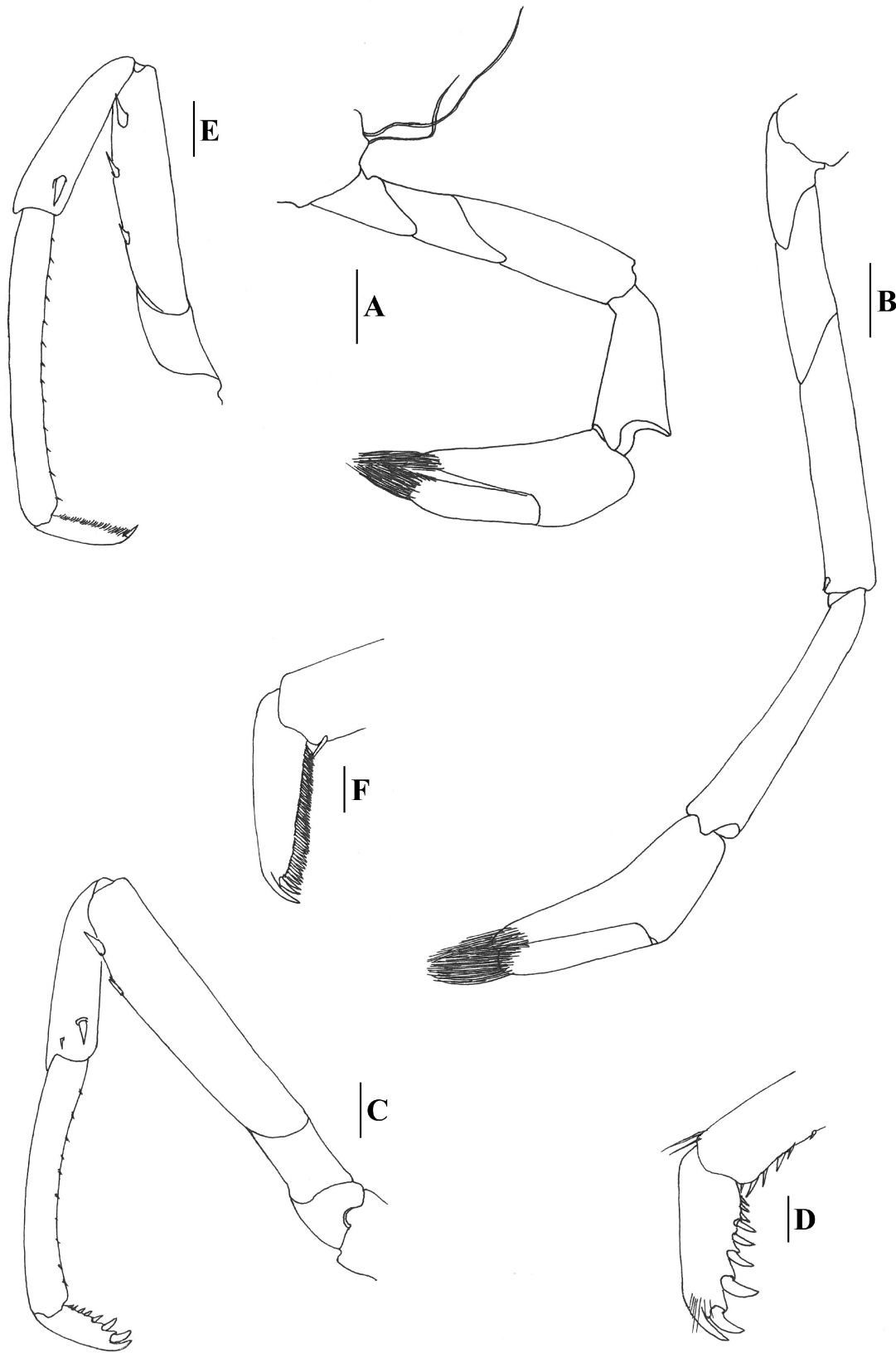
Fig. 7. *Neocaridina davidi* (Bouvier, 1904). (A) 1st pereiopod; (B) 2nd pereiopod; (C) 3rd pereiopod; (D) the same, dactylus; (E) 5th pereiopod; (F) the same, dactylus. Scale bars: A, B, C, E = 0.5 mm; D, F = 0.2 mm (male, cl 5.6 mm, ZRC 2023.0214, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015).

that roughly correspond to local populations of *N. denticulata*, but one clade was confirmed as *N. davidi*, and it is sympatric with *N. denticulata* in three rivers in western Japan. *Neocaridina davidi* has also been found in eastern Japan (Chiba) where no populations of *N. denticulata* were found (Toyota et al. 2014; Mitsugi et al. 2017; Mitsugi and Suzuki 2018).

Mitsugi et al. (2017) reported the occurrence of *N. davidi* at Tomoe R., in Boso Peninsula, Chiba, eastern Japan. They found that their specimens are conspecific genetically with samples collected from Gono R. in Shimane Prefecture, Kako R. in Hyogo Prefecture, and Saba R. in Yamaguchi Prefecture. Mitsugi and Suzuki (2018) studied the life history of that population. Onuki (2021) investigated the distribution of *N. davidi* in relation to environmental parameters at Mama-shita Springs Park at Kunitachi, Tokyo.

Neocaridina palmata (Shen, 1948)
(Figs. 8, 9)

Specimens examined: **Japan**: 1 male, cl 4.6 mm, 1 male, cl 4.5 mm, NCHUZOOL 14940, 7 males, cl 3.6–5.5 mm, ZRC 2023.0215, 1 male, cl 4.2 mm, 1 male, cl 5.0 mm, ZRC 2023.0216, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015.

Native distribution: Mainland China and northern Vietnam (Cai 1996; Liang 2004).

Remarks: Shen (1948) described *Caridina palmata* based on specimens from "Sha-Ping-Pa", Chungking (= Chongqing), southwestern China, with no mention of specific comparison with other congeners. It had been totally ignored in the Chinese fauna until Dai et al. (1993) redescribed and illustrated it in detail and transferred it to *Neocaridina*. Morphologically, *N. palmata* is similar to *N. denticulata* and *N. davidi*. However, it can be distinguished from *N. denticulata* and *N. davidi* by its distinct palm-shaped endopod of the male first pleopods (vs. pear-shaped in the latter two species); and the much longer and stouter appendix interna of the male second pleopods, as well as the stouter spinules surrounding the appendix masculina (Figs. 8, 9). It can be further distinguished from *N. denticulata* by the sexual dimorphism in the third pereiopods, and from *N. davidi* by the distinctly longer appendix interna of the male second pleopods.

Two specimens (1 male, cl 4.2 mm; 1 male, cl 5.0 mm; ZRC 2023.0216) from Himeji City, Hyogo are morphologically typical of *N. palmata*, but the *COI* data show them to be clustered within *N. davidi* (Fig. 3). We suspect that interspecific hybridization might have occurred between the two species living in the same waterbody (see "DISCUSSION"). In their natural range

in China, the two species have not been found from the same collection sites (Cai 1996; Liang 2004), while in Japan they were found to be sympatric in the Sugo River.

Neocaridina palmata is mainly distributed in central and southern China, with the northern limit in the Hubei and Anhui provinces and a western limit in the Yunnan and Sichuan provinces, and its range extends to southern China (Liang 2004) and northern Vietnam (Li and Liang 2004). The sequences of specimens from northern Vietnam have been included in the phylogenetic tree (Fig. 3). The occurrence of *N. palmata* at Sugo River, Himeji City, Hyogo Prefecture, represents the first confirmed record of the species (Figs. 2, 3) being introduced outside its natural range in China and Vietnam.

According to Cai (1996) and Liang (2004), *Neocaridina palmata* is a hardy, well-adapted species that can be found in various freshwater habitats, *e.g.*, small streams, large rivers, and open water like ponds, reservoirs, and lakes in temperate and subtropical areas, and always occurs in abundance. The species may pose threats to native Japanese atyid species. Close monitoring of the species is highly recommended for follow-up action.
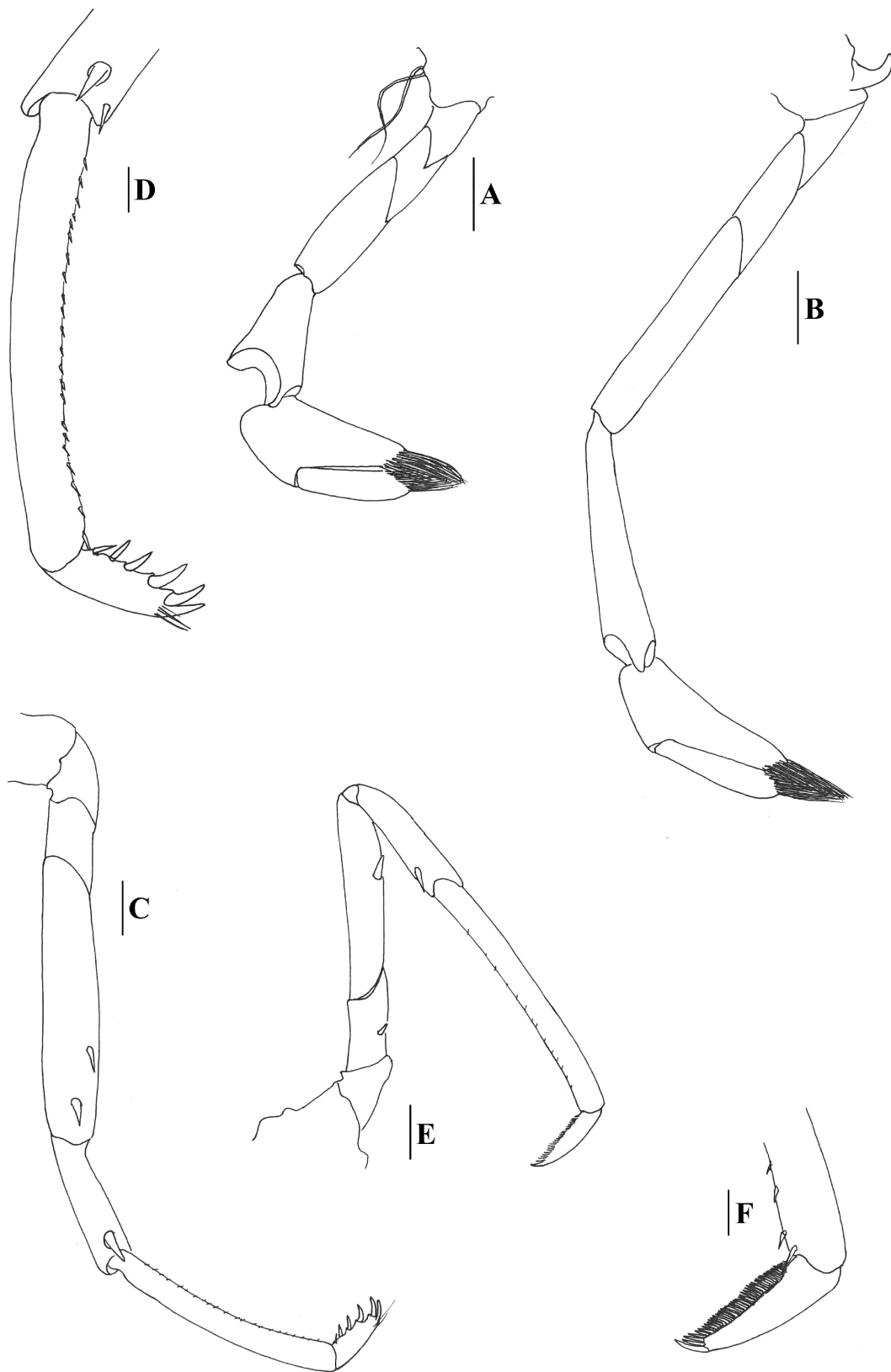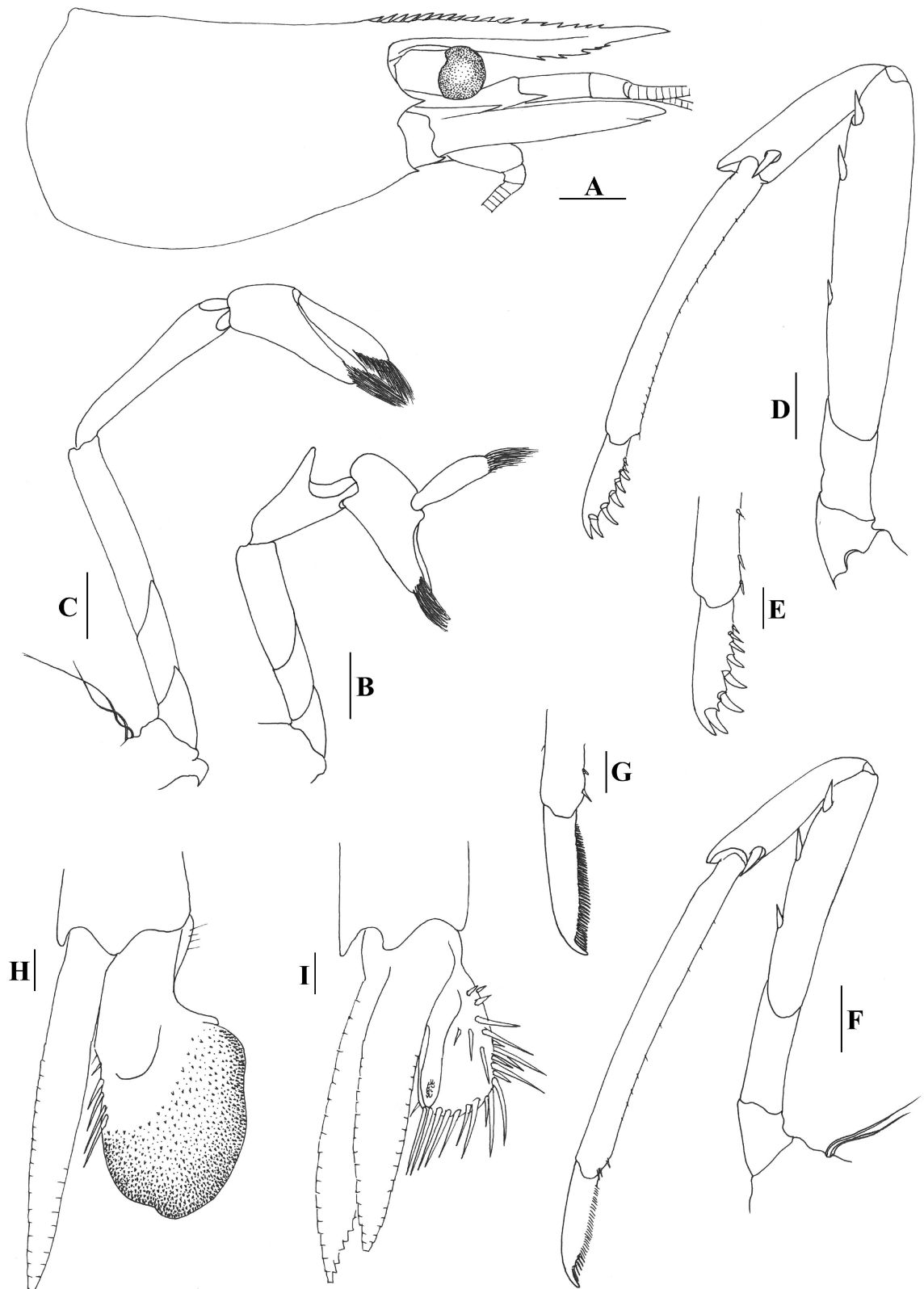
Neocaridina aff. *palmata*
(Fig. 10)

Specimens examined: **Japan**: 1 male, cl 4.2 mm, NCHUZOOL 14940, 1 female, cl 5.5 mm, 1 ovig. female, cl 5.1 mm, non-eyed eggs 1.0 × 0.7 mm, NCHUZOOL 14941, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015; 1 female, cl 4.1 mm, NCHUZOOL 14954, Takahamagawa R., Takahamagawa system, Izumo City, Shimane Prefecture, N35°23'33.3", E132°43'37.1", coll. Y. Nakahara, 10 Mar. 2015; 1 female, cl 6.1 mm, NCHUZOOL 14965; Basen-gawa R., Go-no-gawa system, Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016. 1 female, cl 4.7 mm, ZRC 2023.0217; Basen-gawa R., Go-no-gawa system, Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016. **Korea**: 1 female, cl 5.0 mm, NCHUZOOL 14972, aquarium, coll. Jul. 2005. **Mainland China**: 1 female, cl 3.9 mm, NCHUZOOL 15169, Jiangle, Fujian, coll. H.-T. Shih, 5 Jul. 2004; 1 female, cl 4.3 mm, NCHUZOOL 15172, Nanping, Fujian, coll. H.-T. Shih, 5 Jul. 2004; 1 male, cl 4.1 mm, NCHUZOOL 15170, Yunnan, coll. H.-T. Shih, 5 Nov. 2002; 1 ovig. female, cl 6.2 mm, non-eyed eggs 0.9 × 0.7 mm, ZRC 2023.0218, Shilin, Yunnan, coll. Y. Cai, 11 Apr. 2005; 1 female, cl 6.0 mm, NCHUZOOL
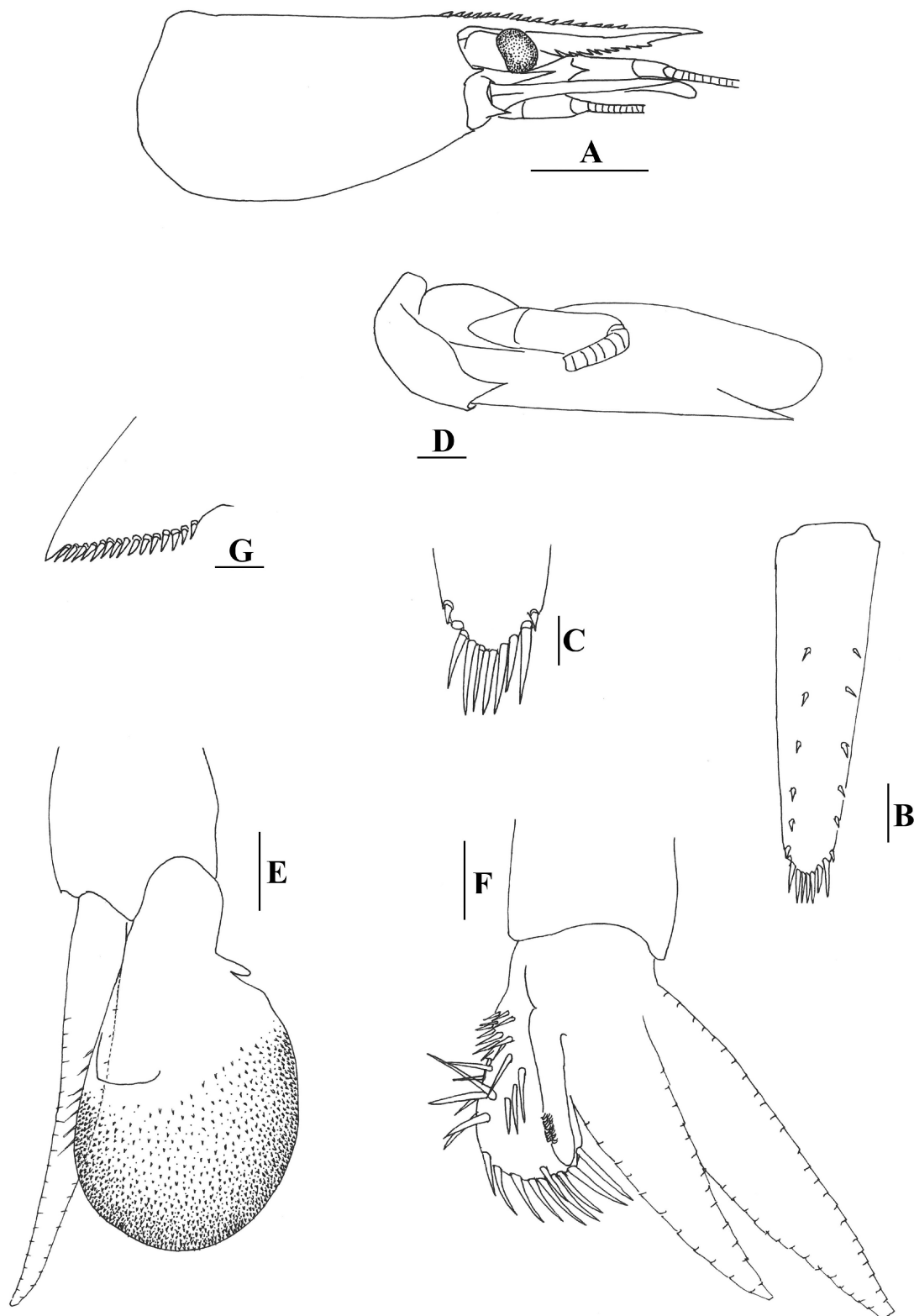
Fig. 8. *Neocaridina palmata* (Shen, 1948). (A) cephalothorax and cephalic appendages, lateral view; (B) telson; (C) distal portion of telson; (D) scaphocerite; (E) male 1st pleopod; (F) male 2nd pleopod; (G) uropodal diaeresis. Scale bars: A = 2 mm; B, D, E, F = 0.5 mm; C, G = 0.2 mm (A, D–G, male, cl 4.6 mm, NCHUZOOL 14940, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015; B, C, male, cl 4.3 mm, ZRC 2023.0215, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015).

Fig. 9. *Neocaridina palmata* (Shen, 1948). (A) 1st pereiopod; (B) 2nd pereiopod; (C) 3rd pereiopod; (D) the same, dactylus; (E) 5th pereiopod; (F) the same, dactylus. Scale bars: A, B, C, E = 0.5 mm; D, F = 0.2 mm (male, cl 4.6 mm NCHUZOOL 14940, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015).

Fig. 10. *Neocaridina* aff. *palmata.* (A) cephalothorax and cephalic appendages, lateral view; (B) 1st pereiopod; (C) 2nd pereiopod; (D) 3rd pereiopod; (E) the same, dactylus; (F) 5th pereiopod; (G) the same, dactylus; (H) male 1st pleopod; (I) male 2nd pleopod. Scale bars: A–D, F = 0.5 mm; E, G, H, I = 0.2 mm (male, cl 4.2 mm, NCHUZOOL 14940, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture, N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015).

15171, Kunming, Yunnan, coll. H.-T. Shih, 5 Nov. 2002.

Native distribution: Korea and mainland China.

Remarks: This species is morphologically very close to *N. palmata*, and currently we can only separate them based on subtle differences displayed by the available specimens (Fig. 10), *e.g.*, the position of the appendix interna located at 0.35 times the length of the endopod (vs. 0.30 in *N. palmata*); the telson terminating in a prominent projection (vs. hardly discernible in *N. palmata*) and a broader scaphocerite (2.8 times as long as wide vs. 3.4 in *N. palmata*) (cf. Figs. 8, 9).

Shih et al. (2017) assigned a specimen that they obtained from South Korea (aquarium dealer) to "*N. koreana*". The specimen (NCHUZOOL 14972) was re-examined morphologically in the current study, but we are unable to confirm its identity morphologically as it is a female specimen. However, *COI* sequence data (Figs. 2, 3) firmly indicated that it should be re-assigned to *N.* aff. *palmata* instead.

Kakui and Komai (2022) reported the first occurrence of the freshwater ectoparasitic platyhelminth *Scutariella japonica* from Yasuharu River, Sapporo, Hokkaido, Japan and discussed the identity of the host shrimps, a species of *Neocaridina*. Phylogenetic analyses using *COI* (Kakui and Komai 2022: fig. 5) showed that the host species is clustered with "*N. koreana*" identified by Shih et al. (2017) (see above), sister to *N. palmata*, and also close to two clades, "*N. davidi*" (types I and II, after Nagai and Imai 2021). The authors thus tentatively referred their shrimps to "*N.* sp. aff. *davidi*". The Hokkaido *COI* sequences (LC664097, LC664098, LC664099) were re-analyzed in our study and the results show that genetically they are conspecific with our material of "*N.* aff. *palmata*" (Fig. 3). The specimens from the same clade in Kakui and Komai (2022: fig. 5) would also be assigned to the same species, including specimens from Hyogo (AB524970), Shimane (AB524966, AB524968), and Chiba (LC664096), and Chinese material from Henan Province (MW069628, MW069631, MW069644, MW069650, MW069652, MW069653, MW069657, MW069661, MW069670).

Notably, based on one of the specimens, the drawings of the endopod of the male first pleopods, the appendix masculina of the male second pleopods, and the form of the dactylus and propodus of the male third pereiopods provided by Kakui and Komai (2022: fig. 3E, F, H) clearly exhibit morphological characteristics consistent with *N. davidi*, despite the authors initially believing this specimen to be a young male. It is worth noting that our current study observed both *N.* aff. *palmata* and *N. davidi* living sympatrically in the streams at Hyogo, Himeji City and Shimane, Izumo City. The conflict between genetic and morphological findings suggests the possibility of interspecific hybridization (see "DISCUSSION"). Further investigation with both morphology and DNA testing would facilitate a taxonomic decision.

The species is found in Fujian (Jiangle and Nanping) and Yunnan (Shilin and Kunming) in southern China, as well as the Korean Peninsula, so we presume that China and Korea are likely its native range. We have recorded its presence in several Japanese localities, including Hyogo, Shimane, Hiroshima, Chiba, and Hokkaido (Fig. 3). These localities are considered to represent one or more introductions, especially since the sites (Sites 2, 8 and 10 in Fig. 1) are also associated with other introduced species (Table 3).

Neocaridina ikiensis Shih, Cai, Niwa & Nakahara, 2017

Specimens examined: See Shih et al. (2017).

Native distribution: This species is only distributed in Iki Island, Nagasaki Prefecture, western Japan (Shih et al. 2017).

Remarks: This species was the second native species of *Neocaridina* discovered in the main islands of Japan; and its distribution is restricted to Iki Island, an offshore island in northern Kyushu (Shih et al. 2017).

Fuke et al. (2021) questioned the validity of *N. ikiensis*, commenting that Shih et al. (2017) may not have adequately considered the genetic and morphological variations between their new species and the populations of *N. denticulata* in nearby regions, including what they referred to as "*N. denticulata*" from Fukue-jima Island, Goto, Nagasaki; Ishiki River, Kawatana, Nagasaki; Kuro River, Aso, Kumamota; and Machida R., Kokoneo, Oita. Their *COI* tree showed that *N. ikiensis* clustered together with several populations of their "*N. denticulata*". By re-analyzing their sequences using longer lengths (Table 3), along with ours, the results (Fig. 3) indicate that *N. ikiensis* forms a sister clade with the Machida population. The samples collected from Kuro River and Ishiki River form one clade, which is sister to a clade composed of samples from the Goto Islands. The supported values of these two clades suggest that each clade may represent a separate species not previously recognized, here assigned as *N.* sp. 1 and *N.* sp. 2, from the Kuro/Ishiki River and from the Goto Islands, respectively. These two clades are distinctly differentiated from the taxa *N. denticulata* and *N.* aff. *denticulata* that our study recognizes based on both genetic and morphological data (cf. Fig. 3).

The group formed by *N.* sp. 1, *N.* sp. 2, and *N.* aff. *fukiensis* is sister to another group comprising *N. ikiensis* and the previously mentioned Machida population (Fig.

3). *Neocaridina* aff. *fukiensis* is morphologically very distinct from *N. denticulata* (see Remarks under *N.* aff. *fukiensis*), and the Machida specimens may represent another separate species, named here as *N.* sp. 3, that is genetically similar to *N. ikiensis.* Since Fuke et al. (2021) did not provide morphological characters for the species they identified, we cannot compare these three potential unknown species to any of the known ones.

Neocaridina aff. *fukiensis*
(Fig. 11)

Specimens examined: **Japan**: 1 male, cl 6.6 mm, 1 male, cl 5.7 mm, 1 male, cl 6.0 mm, 10 males, cl 3.7–5.8 mm, NCHUZOOL 14946, 1 female, cl 7.7 mm, 1 female, cl 4.5 mm, NCHUZOOL 14947, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015; 1 male, cl 4.5 mm, 1 male, cl 4.2 mm, 14 males, cl 3.8–5.7 mm, ZRC 2023.0219, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 1 Dec. 2016.

Remarks: This species morphologically resembles *Neocaridina fukiensis* (Liang & Yan, 1978) including the form of its rostrum, which is elongated, reaching beyond the end of the antennular peduncle and with a slight upward curve distally; the telson terminates in a prominent projection; no sexual dimorphism in the third pereiopods; and the form of the appendix interna and the appendix masculina of the male second pleopods, with the appendix interna projecting out distinctly from the appendix masculina (Fig. 11). However, the endopod of the male first pleopods does not possess a distinct projection at its base (vs. with a distinct projection in *N. fukiensis*) and the fewer spines on the flexor margin of dactylus of the third pereiopods (6–7 vs. 9–10 in *N. fukiensis*) can be used to separate the two species easily.

Fuke et al. (2021) assigned specimens collected from Miya River, Yufuin, Oita to "*Neocaridina* sp." based on both morphological and molecular analyses. Upon re-analysis of their *COI* sequence data (LC612372, LC612374, LC612376, LC612378, LC612379) in conjunction with our own data, the findings revealed that the Miya material clusters in a clade with our Fukuyama specimens, suggesting they are conspecific (Fig. 3). Despite these findings, a pending detailed morphological examination is necessary to confirm the identity of this clade, as it may represent an undescribed native species endemic to Japan. Additional geographic data are requisite to accurately establish its distributional range.

Neocaridina koreana Kubo, 1938
(Fig. 12)

Specimens examined: **Japan**: 1 male, cl 4.1 mm, 3 males, cl 4.1–5.3 mm, NCHUZOOL 14964, 1 female, cl 4.1 mm, NCHUZOOL 14965, Go-no-gawa River system, Basen-gawa R., Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016; 2 males, cl 5.0–6.5 mm, ZRC 2023.0220, Basen-gawa R., Go-no-gawa system, Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016.

Native distribution: Korea and probably China (Cai 1996; Liang 2004).

Remarks: The original description of *Neocaridina denticulata koreana* Kubo, 1938 mentioned that "first cheliped rather short, with proportions against finger: palm 0.9, carpus 1.2, merus 1.4…" and "Second cheliped much longer than first, with proportions against finger: palm 0.9, merus 2.0." "Endopodite of first pleopod pear-shaped, length about 1.7 times as long as breadth in male…" (Kubo 1938: 81). Our specimens agree well with this description (Fig. 12), but with a slightly more elongated endopod of the male first pleopods (1.85 times as long as wide) in adult males, which could be within the range of individual variation if a larger sample size were available.

Liang (2004) redescribed the species as having characteristic sexual dimorphism of the third pereiopods and transferred the taxon to the subspecies status under *Neocaridina heteropoda* (= *N. davidi*), based on specimens from Zhejiang Province (Jinhua, Quxian). However, this reassignment could not be confirmed, as Kubo's (1938) original description did not mention this character, and no topo-type specimens are available for comparison. Notably, our specimens do not exhibit any sexual dimorphism in the third pereiopods (Fig. 12).

Park et al. (2019) reported the complete mitochondrial genome of *Neocaridina koreana* (as *Neocaridina heteropoda koreana*) based on specimens collected from Geojedo, which is close to the type locality Huzan (Busan). Comparison of the available sequences shows that our specimens are conspecific with those from Geojedo (Fig. 3). The Japanese record in our study represents the first confirmed record of the species to be introduced outside its natural range, Korea, and probably China (Cai 1996; Liang 2004).

Neocaridina aff. *denticulata*
(Fig. 13)

Specimens examined: **Japan**: 3 males, cl 3.5–4.6 mm, ZRC 2023.0221, Sugo R., Yumesaki River system, Himeji City, Hyogo Prefecture,
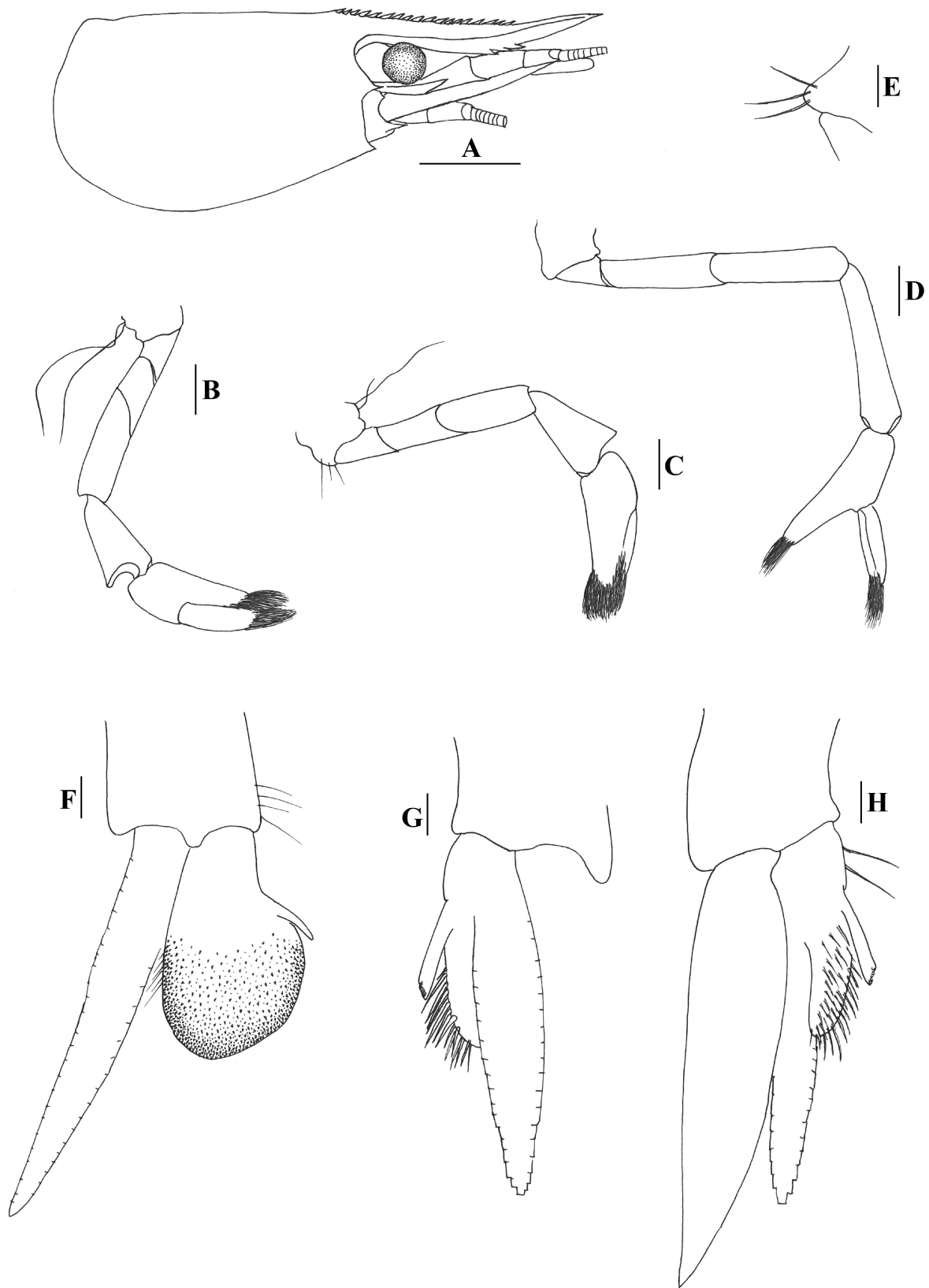
Fig. 11. *Neocaridina* aff. *fukiensis.* (A) cephalothorax and cephalic appendages, lateral view; (B, C) 1st pereiopod; (D) 2nd pereiopod; (E) same, basis; (F) male 1st pleopod; (G, H) male 2nd pleopod. Scale bars: A–D = 0.5 mm; E–H = 0.2 mm (male, cl 5.7 mm, NCHUZOOL 14946, Kaya R., Ashida River system, Fukuyama City, Hiroshima Prefecture, N34°36'43", E133°15'33", coll. H. Yoshigou, 17 Oct. 2015).
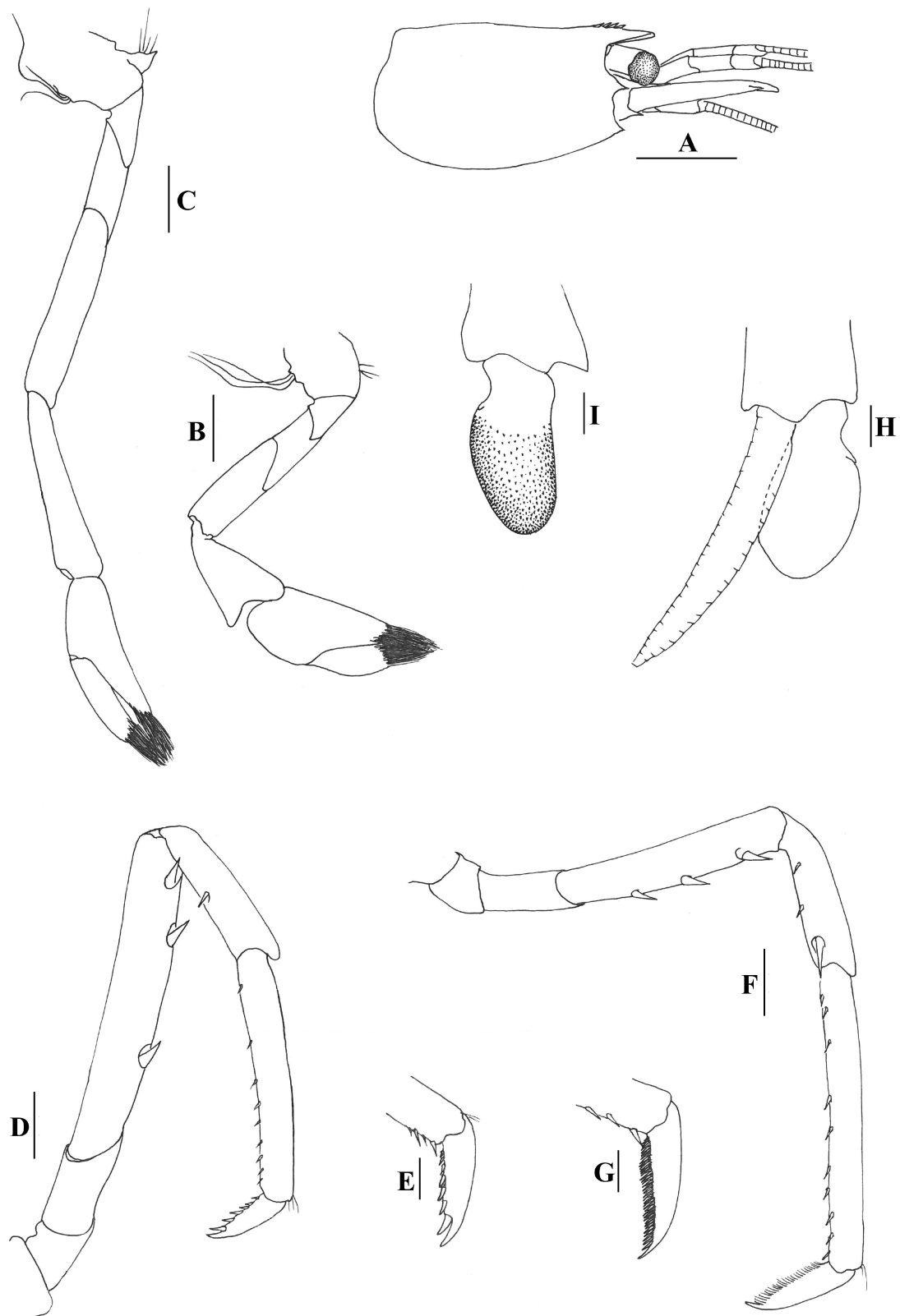
Fig. 12. *Neocaridina koreana* Kubo, 1938. (A) cephalothorax and cephalic appendages, lateral view; (B) 1st pereiopod; (C) 2nd pereiopod; (D) 3rd pereiopod; (E) the same, dactylus; (F) 5th pereiopod; (G) the same, dactylus; (H, I) male 1st pleopod. Scale bars: A = 2 mm; B–D, F = 0.5 mm; E, G–I = 0.2 mm (male, cl 4.1 mm, NCHUZOOL 14964, Basen-gawa R., Go-no-gawa system, Miyoshi City, Hiroshima Prefecture, N34°46'51", E132°54'39", coll. H. Yoshigou, 28 Feb. 2016).

N34°56'31.9", E134°38'19.0", coll. N. Niwa, 14 Aug. 2015; 8 males, cl 4.6–5.5 mm, 15 females, cl 5.5–5.9 mm, ZRC 2023.0222, Oura upstream way (on Oura Dam), Izuharamachi-koura, Tsushima City, Nagasaki Prefecture, N34°14'1.9", E129°17'45.7", coll. H. Yoshigou, 15 Dec. 2016; 7 males, cl 5.2–6.0 mm, 10 females, cl 4.5–7.4 mm, ZRC 2023.0223, Takahama R., Mitsushimamachi-kechikou, Tsushima-shi, Tsushima City, Nagasaki Prefecture, N34°15'32.4", E129°18'47.5", coll. H. Yoshigou, 16 Dec. 2016.

Remarks: *Neocaridina* aff. *denticulata* is similar to *N. denticulata* in the general appearance of the rostrum, the absence of sexual dimorphism in the third pereiopods, and the appendix masculina of the male second pleopods. However, it can be distinguished by its relatively shorter rostrum which reaches to or near to the end of the antennular peduncle (vs. mostly reaching beyond the antennular peduncle in *N. denticulata*); the exopod of the male first pleopods is 1.5 times as long as wide (vs. 1.2 times in *N. denticulata*); and the carpus of the first pereiopods is stouter (Fig. 13) (vs. slender in *N. denticulata*).

Its distribution is confined to western Japan, encompassing western Honshu and Kyushu. The phylogenetic relationship with other species (Fig. 3) suggests that it is most probably a cryptic native species, which may have long been mistaken for *N. denticulata*.

DISCUSSION

In Japan, several introduced species of *Neocaridina*, including *N. davidi*, were found in western Japan in the early 2000s (Niwa et al. 2005; Niwa 2010; Fujita et al. 2011; Toyota et al. 2014). These species have recently spread to eastern Japan (Nishida 2016; Katayama et al. 2017; Mitsugi et al. 2017). Nishino (2009) reported that among the *Neocaridina* species in Japan tested with mtDNA sequencing of *COI* and 16S, there are two major haplotype clades, one composed of the haplotypes detected only from Japan, while the other is composed of those observed from both Japan and China, including haplotypes that are identical or show only a few nucleotide differences between the two countries. They considered the former to be indigenous to Japan (*N. denticulata denticulata*) and the latter to be exotic (*i.e.*, *N. davidi*, see Nishino 2020). "In four localities of Japan, the two clades were observed sympatrically, implying the genetic pollution through hybridization or introgression between indigenous and exotic populations" (Nishino 2009).

Several studies have successfully used DNA barcoding to distinguish closely related species of freshwater shrimps (*e.g.*, Klotz and von Rintelen 2014;

Shih et al. 2017 2019; Chen et al. 2020; Xu et al. 2020; Feng et al. 2021; Zhou et al. 2021). Genetic distances of *COI* among related species can serve as important references for molecular thresholds in certain groups of crustacean taxa (Lefébure et al. 2006; Costa et al. 2007; Chu et al. 2015). Most of the minimum interspecific *COI* distances (either uncorrected *p*-distances or K2P) of atyid shrimps are larger than 3.5%, *e.g.*, 3.5% of K2P between *Caridina serrata* and *C. tetrazona* (Chen et al. 2020); 8.7% of *p*-distance between *C. pacho* and *C. pseudoserrata* (Do et al. 2020) and 5.57% of K2P between *N. fonticulata* and *N. davidi* (Shih et al. 2019). In our study, eight species of *Neocaridina* from Kyushu and southern Honshu of Japan were detected using the *COI* marker, with the K2P distances ≥ 3.77% among most species (Table 4), which is larger than the 3.5% threshold found in most studies and could treated as separate species. However, the distance between *N. davidi* and *N. denticulata* is small (1.7%; Table 4) and similar to values found in previous studies (*e.g.*, Shih et al. 2017 2019; Han et al. 2019) (Table 4) although they are morphologically very distinct. Additionally, mitochondrial *COI* barcoding may encounter problems in species delimitation and identification, including asymmetrical introgression (genes introgressed to another species) and incomplete lineage sorting (where some mitochondrial and/or nuclear genes of descendants inherited from a common ancestor do not sort neatly). Furthermore, the coalescence times in mitochondrial markers are 3–4 times faster than those in nuclear markers. Therefore, using additional evidence from nuclear markers is recommended to strengthen the morphology-based taxonomy (Chu et al. 2015; Eberle et al. 2019 2020; Ahrens et al. 2021).

Among the introduced species of *Neocaridina* in Japan, three species (*N. davidi*, *N. koreana* and *N.* aff. *palmata*) are difficult to identify. This is mainly due to the lack of distinct and consistent morphological characters, and the unavailability of *COI* sequences for most Chinese species. Based on Japanese literature (Niwa 2010; Toyota et al. 2014; Hasegawa et al. 2015; Mitsugi et al. 2017), these alien species are believed to have been introduced from China and Korea, either for the aquarium trade or as live fish bait.

The extent of environmental impact from some invasive freshwater decapod crustaceans has been well documented. Examples include, the Chinese mitten crab (*Eriocheir sinensis*; Dittel and Epifanio 2009), the red swamp crayfish (*Procambarus clarkii*; Gherardi 2006; Gherardi and Acquistapace 2007), the giant river prawn (*Macrobrachium rosenbergii*; Iketani et al. 2016), and land-locked freshwater crabs (*Sayamia germaini*; Shih et al. 2011). Regarding atyid shrimps, Weber and Traunspurger (2016) reported that introduced *N. davidi*
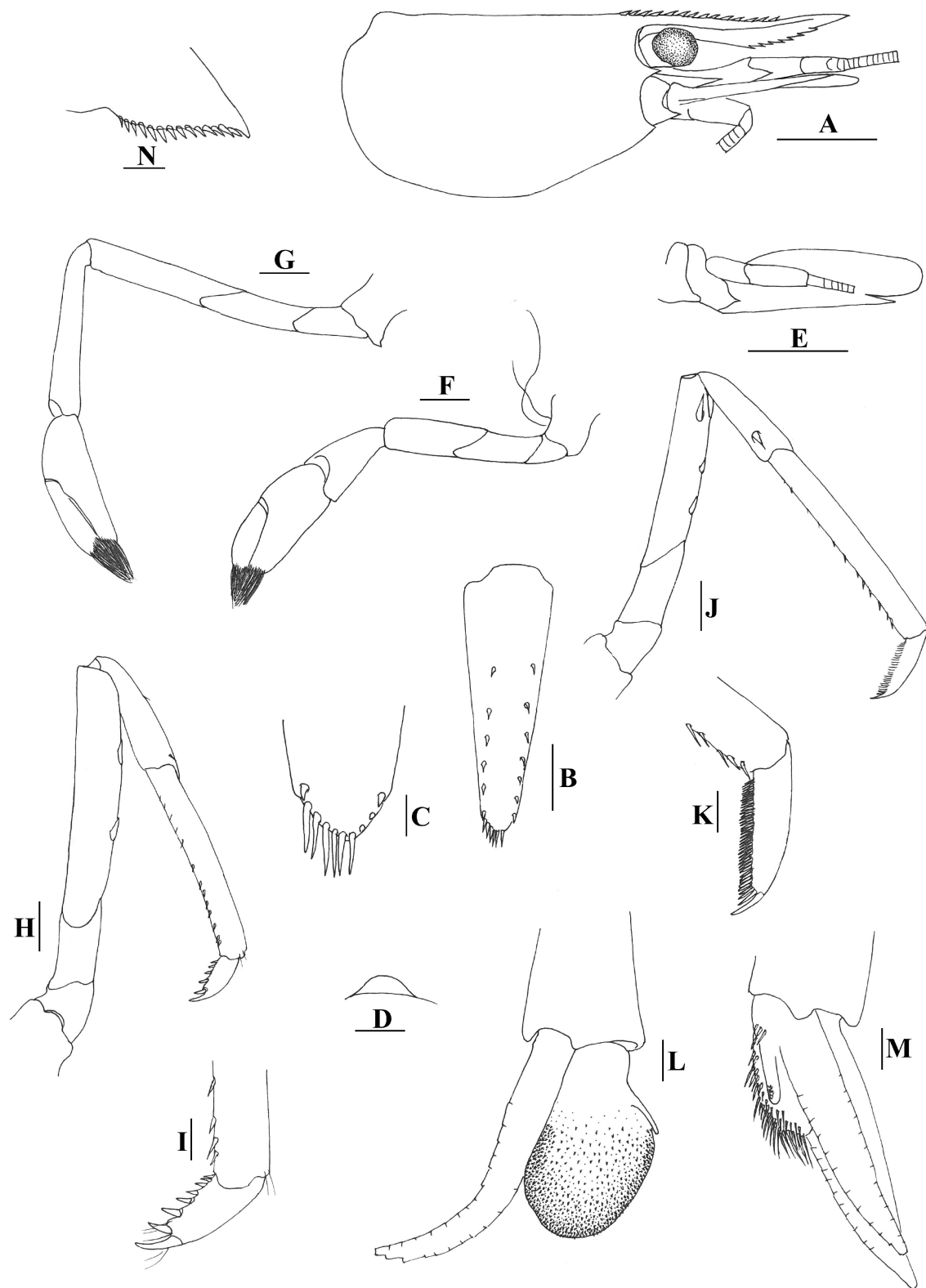
Fig. 13. *Neocaridina* aff. *denticulata* Kubo, 1938. (A) cephalothorax and cephalic appendages, lateral view; (B) telson; (C) distal portion of telson; (D) preanal carina; (E) scaphocerite; (F) 1st pereiopod; (G) 2nd pereiopod; (H) 3rd pereiopod; (I) the same, dactylus; (J) 5th pereiopod; (K) the same, dactylus; (L) male 1st pleopod; (M) male 2nd pleopod; (N) uropodal diaeresis. Scale bars: A, E = 2 mm; B, D, F–H, J = 0.5 mm; C, I, K, L, M, N = 0.2 mm (male, cl 5.5 mm, ZRC 2023.0222, Oura upstream way (on Oura Dam), Izuharamachi-koura, Tsushima City, Nagasaki Prefecture, N34°14'1.9", E129°17'45.7", coll. H. Yoshigou, 15 Dec. 2016).

has impacted the stream ecology of Germany, especially through its predatory behavior on the meiofaunal assemblages of streams and, in turn, the food web of freshwater ecosystems. The introduction and expansion of the introduced *Neocaridina* spp. in Japan could lead to interbreeding with, and the competitive exclusion of, native shrimps (Niwa 2010; Nishida 2016; Katayama et al. 2017). Based on the collections from each site (Table 3), some localities have 2–4 species living sympatrically, and might even have excluded the endemic *N. denticulata* (*e.g.*, at sites 2, 5, 8, 15; see Fig. 1) which implies the negative impact of these alien species. However, the ecological impacts of *N. davidi* and the other alien species that were detected in this study are still unknown and need further evaluation for the purposes of biodiversity conservation and management.

While we are of the view that the postulated hybridization or introgression between the sympatric *N. denticulata* and *N. davidi* still requires genetic evidence for confirmation, the current study observed that hybridization may have occurred between the sympatric introduced species *N. davidi* and *N. palmata* (Sugo River, Himeji City) as well as *N. davidi* and *N.* aff. *palmata* (Sugo River, Himeji City; Hanegawa River, Izumo City), as there are discrepancies between the morphological evidence and the *COI* sequences. Hybridization of different species of atyids has previously been suggested (*e.g.*, von Rintelen and Cai 2009; Mitsugi and Suzuki 2018; Onuki and Fuke 2022), and breeding experiments are needed to demonstrate the possibility of hybridization in these atyid shrimps.

CONCLUSIONS

In our study, eight species of *Neocaridina* from Kyushu and southern Honshu of Japan were identified by molecular and morphological evidence. There are two native species, *N. denticulata* and *N. ikiensis*; four introduced species, viz. *N. davidi*, *N. koreana*, *N. palmata*, *N.* aff. *palmata*; and two cryptic and probably native species, viz. *N.* aff. *denticulata* and *N.* aff. *fukiensis*. Phylogenetic analyses, including sequences from other Japanese studies, suggest that at least three additional species may be present among the Japanese fauna of freshwater shrimps.

Authors' contributions: HTS conceived this study, performed the molecular genetic analysis, and drafted the manuscript. YC performed the morphological description and discussion and drafted the manuscript. NN, HY and YN collected and processed the samples and drafted the manuscript. HTS, YC, HY and YN read and approved the final manuscript.

Competing interests: HTS, YC, NN, HY and YN declare that they have no conflicts of interest.

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

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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