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Current taxonomic status of *Eurema mandarina* (Pieridae: Lepidoptera) in Korea

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Abstract

We investigated the taxonomic status of the Korean populations of *Eurema hecabe* (Linnaeus, 1758) based on morphology and nuclear triose phosphate isomerase (*Tpi*). Up to now, the Korean population of *E. hecabe* was classified into *E. mandarina* (de l'Orza, 1869) based on morphological characteristics. A previous study raised the possibility of the Jeju-do population to be *E. hecabe* based on the color of forewing's fringe. However, morphological examination showed that the Korean population found in southern areas (Gyeonsang and Jeolla provinces), including Jeju-do is *E. mandarina*. In particular, the external morphology of autumn seasonal type of the Korean population was almost same as the Japanese mainland population, which is known as *E. mandarina*. Furthermore, sequence analysis of *Tpi* gene from nine individuals of *E. mandarina* collected in South Korean localities including Jeju-do clearly showed that all Korean specimens truly belong to *E. mandarina*.

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Introduction

The pierid butterfly genus *Eurema* Hübner, 1819 is widely known over the world and comprised of over 70 species. In the Korean peninsula, two species of the genus are known: *Eurema mandarina* de l'Orza, 1869 and *Eurema laeta* Boisduval, 1836. The former has long been named as *E. hecabe* Linnaeus, 1758, which is widely distributed from southern and middle Africa, southeast Asia, and northeast Asia to Pacific islands (Yata, 1989; Shirôzu, 2006). *E. hecabe,* however, was divided into two distinct groups, subtropical (B type) and temperate (Y type) groups, which differ in several morphological and ecological traits, along with different allelic frequency of allozymes (Kato, 1999; Kato and Honda, 1992; Nomura and Kato, 1993; Kato, 2000a, b, c; Kobayashi *et al.*, 2001). These two groups are geographically separated based on the location of Okinawa islands, Japan: one in Okinawa and neighboring islands (subtropical group); and the other in the northern part in Japan above Okinawa (temperate group). Morphologically, the subtropical group has brownish wing fringe in the forewings, whereas the temperate group has yellowish wing fringe in the forewings (Kato, 1999, 2000a). Based on several morphological and ecological traits, along with

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coloration pattern of wing fringe, the two types were treated as distinct species: Y type as *E. mandarina*, whereas B type as *E. hecabe* (Kato and Yata, 2005; Shirôuzu, 2006).

Taxonomically, nevertheless, *E. hecabe* is one of the most burdensome species, revealing seasonal and geographic variations in wing color pattern, so that numerous subspecies has been suggested (Yata, 1995). Furthermore, *E. hecabe* and *E. mandarina* are sympatric in some distributional range, such as Hong Kong, China, and Okinawa in Japan (Narita *et al*., 2006, 2007). In the laboratory conditions *E. hecabe* and *E. mandarina* are two biological species having premating isolation mechanism, but infrequent copulation has also been reported (Kato 2000b; Kobayashi *et al*., 2001). In nature, thus, it must be rare, but the possibility of hybridization over a long evolutionary time has been raised (Narita *et al*., 2006). Indeed, *E. hecabe* infected with the *Wolbachia* has been suggested to be introduced occasionally from *E. hecabe* (B type) to *E. mandarina* populations (Y type) through migration and subsequent interspecific hybridization (Narita *et al*., 2006, 2007). Consequently, mitochondrial DNA (mtDNA) sequences including DNA barcoding region, which typically is helpful for resolving taxonomic ambiguity (Hajibabaei *et al*., 2006) maybe uninformative in the *E. mandarina* populations, which are infected with the cytoplasmic incompatibility (CI)-causing *Wolbachia* (O'Neill *et al*., 1997; Werren, 1997).

E. hecabe collected in subtropical region and *E. mandarina* collected in temperate region in Japan formed independent group in the phylogenetic analysis using mitochondrial ND5 and *16S rRNA* sequences, accordingly to infection by *Wolbachia* rather than their species identity (Narita *et al*., 2006). On the other hand, autosomal nuclear elongation factor 1 alpha (*EF1- α*) gene provided two groups, consistent to their species identity (Narita *et al*., 2006). Later, Z chromosome-linked nuclear triose phosphate isomerase (*Tpi*) gene also separated two groups on the basis of species identity from the samples collected in Okinawa prefecture, Japan (Miyata *et al*., 2020) and was used for the development of molecular diagnostic method that distinguish the two species (Miyata *et al*, 2020).

In South Korea, *E. mandarina* is found in the southern islands including Jeju-do island, and middle part of mainland (Chungcheongnam-do and Gyeongsangbuk-do provinces) and coastal areas (Gyeonggi bay in the west and Gangneung in the east) (Kim, 2015). In South Korea, the species is designated as one of the indicators of climate change based on the availability of food plant and potential northward movement (Ministry of Environment, 2010; Kim *et al*., 2018). Meantime, the Korean populations were classified into *E. mandarina*, because they showed the similar geographic distribution and seasonal form with those living in the same latitude of Japan (Kim, 2015). Narita *et al*. (2007) reported that the *E. mandarina* is the endemic species in Japan and classified the Jeju-do population into *E. hecabe*, which has B-type coloration pattern of wing fringe. At that time, Narita *et al*. (2007) found that Tsushima island population also has Y-type coloration pattern of wing fringe. Considering the geographic closeness of Tsushima island to the Korean peninsula classification of the Jeju-do population into *E. hecabe* is further ambiguous.

The aim of this paper was to determine the taxonomic status of the Korean populations of *E. mandarina*. For the purpose of study, we examined 90 specimens collected in 11 South Korean localities including those from Jeju-do island. Among them nine individuals collected in six localities including two from Jeju-do Island were sequenced for *Tpi* gene. For the genetic comparison *Tpi* sequences of both *E. mandarina* and *E. hecabe* were downloaded from GenBank (Narita *et al*., 2006; Miyata *et al*., 2017, 2020).

Materials and methods

Sampling and morphological examination

Ninety specimens of *Eurema mandarina* were collected from 11 Korean localities from 2009 to 2020 using a butterfly net and used for morphological examination. For the male and female genitalia examination, the abdomen was boiled in 10% KOH solution for approximately 20 min, removed scales and tissues, stained with Chlorazol black, and mounted on slides in Euparal solution.

DNA sequencing

For DNA sequencing analysis nine specimens collected in six Korean localities including two from Jeju-do Island were used (Table 1). The genomic DNA was extracted from one or two legs using the DNA Purification kit according to the manufacturer's instructions (Bioneer, Daejeon, Korea). For amplifying the 501 - 512-bp of the nuclear *Tpi* gene primers were adapted from Jiggins *et al.* (2001): 5′-GGTCACTCTGAAAGGAGAACCA CTTT-3′ for forward direction and 5′-CACAACATTTGCCC AGTTGTTGCCAA-3′ for reverse direction. The polymerase chain reaction (PCR) was conducted using the AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) under the following

Table 1. Lists of *Eurema mandarina* and *E. hecabe* sequenced in this study and downloaded from public data.

No.	Collecting locality (*n*)	Collected date	Animal no.	Infection status*	*Tpi* haplotype	GenBank no.
E. mandarina						
1	Namhae-gun, Korea (1)	2020-09	CNU14436	NA	EUTPI01	MZ671037
2	Hamyang-gun, Korea (1)	2019-09	CNU14431	NA	EUTPI02	MZ671038
3	Hapcheon-gun, Korea (2)	2019-08	CNU14430	NA	EUTPI03	MZ671039
		2019-08	CNU14437	NA	EUTPI04	MZ671040
4	Muan-gun, Korea (1)	2018-11	CNU14433	NA	EUTPI05	MZ671041
5	Sinan-gun, Korea (2)	2020-07	CNU14434	NA	EUTPI06	MZ671042
		2020-09	CNU14435	NA	EUTPI07	MZ671043
6	Seoguipo, Jeju-do, Is, Korea (2)	2019-08	CNU10632	NA	EUTPI07	MZ671044
		2019-08	CNU10655	NA	EUTPI08	MZ671045
7	Sendai, Miyagi, Japan (1)	NA	NA	–	EUTPI06	AB231163
8	Nigata, Nigata, Japan (1)	NA	NA	+	EUTPI09	AB231171
9	Hitachi, Ibaraki, Japan (1)	NA	NA	+	EUTPI10	AB231169
		NA	NA		EUTPI11	AB231170
10	Honjo, Saitama, Japan (2)	NA	NA	+	EUTPI12	AB231167
		NA	NA	–	EUTPI13	AB231168
11	Matsudo, Chiba, Japan (2)	NA	NA	+	EUTPI14	AB231164
		NA	NA	–	EUTPI15	AB231165
12	Takao, Tokyo, Japan (1)	NA	NA	–	EUTPI16	AB231166
13	Kametomi, Yamanashi, Japan (1)	NA	NA	+	EUTPI17	AB231172
14	Omi, ShigaOmi, Shiga, Japan (1)	NA	NA	+	EUTPI18	AB231174
		NA	NA		EUTPI19	AB231175
15	Nara, Nara, Japan (1)	NA	NA	+	EUTPI20	AB231178
16	Ibaragi, Osaka, Japan (1)	NA	NA	+	EUTPI20	AB231173
17	Koya, Wakayama, Japan (1)	NA	NA	+	EUTPI21	AB231176
		NA	NA		EUTPI06	AB231177
18	Okayama, Okayama, Japan (1)	NA	NA	+	EUTPI22	AB231179
		NA	NA		EUTPI23	AB231180
19	Matsue, Shimane, Japan (1)	NA	NA	+	EUTPI24	AB231181
		NA	NA		EUTPI25	AB231182
20	Kagawa, Kagawa, Japan (1)	NA	NA	+	EUTPI26	AB231183
21	Tsushima Is., Nagasaki, Japan (3)	NA	NA	–	EUTPI27	AB231188
		NA	NA	–	EUTPI28	AB231189
		NA	NA	–	EUTPI29	AB231190
22	Fukuoka, Fukuoka, Japan (1)	NA	NA	+	EUTPI30	AB231184
		NA	NA		EUTPI31	AB231185
23	Kumamoto, Kumamoto, Japan (2)	NA	NA	+	EUTPI32	AB231186
		NA	NA	+	EUTPI33	AB231187

Table 1. Lists of *Eurema mandarina* and *E. hecabe* sequenced in this study and downloaded from public data.(Continued)

24	Kagoshima, Kagoshima, Japan (1)	NA	NA	+	EUTPI34	AB231191
25	Tanegashima Is., Kagoshima, Japan (31)	NA	NA	+	EUTPI35	AB231192
		2015-05	2015-F-01	+	EUTPI35	LC210521
		2015-05	2015-F-02	+	EUTPI36	LC210522
		2015-05	2015-F-03	+	EUTPI37	LC210523
		2015-05	2015-F-04	+	EUTPI38	LC210524
		2015-05	2015-F-05	+	EUTPI35	LC210525
		2015-05	2015-F-06	+	EUTPI39	LC210526
		2015-05	2015-F-07	+	EUTPI36	LC210527
		2015-05	2015-F-08	+	EUTPI40	LC210528
		2015-05	2015-F-09	+	EUTPI34	LC210529
		2015-05	2015-F-10	+	EUTPI41	LC210530
		2015-05	2015-F-11	+	EUTPI42	LC210531
		2015-05	2015-F-12	+	EUTPI43	LC210532
		2015-05	2015-F-13	+	EUTPI44	LC210533
		2015-05	2015-F-14	+	EUTPI36	LC210534
		2015-05	2015-F-15	+	EUTPI06	LC210535
		2015-05	2015-F-16	+	EUTPI45	LC210536
		2015-05	2015-F-17	+	EUTPI29	LC210537
		2015-05	2015-F-20	+	EUTPI46	LC210538
		2015-05	2015-F-21	+	EUTPI37	LC210539
		2015-05	2015-F-22	+	EUTPI47	LC210540
		2015-05	2015-F-23	+	EUTPI48	LC210541
		2015-05	2015-F-25	+	EUTPI49	LC210542
		2015-05	2015-F-27	+	EUTPI50	LC210543
		2015-05	2015-F-28	+	EUTPI35	LC210544
		2015-05	2015-F-29	+	EUTPI41	LC210545
		2015-05	2015-F-30	+	EUTPI51	LC210546
		2015-05	2015-F-31	+	EUTPI52	LC210547
		2015-05	2015-F-33	+	EUTPI38	LC210548
		2015-05	2015-F-34	+	EUTPI53	LC210549
		2015-05	2015-F-35	+	EUTPI54	LC210550
26	Yakushima Is., Kagoshima, Japan (1)	NA	NA	+	EUTPI55	AB231193
27	Okinawa Is., Okinawa, Japan (2)	NA	NA	+	EUTPI56	AB231194
		NA	NA	+	EUTPI57	AB231195
		NA	NA		EUTPI58	AB231196
E. hecabe						
28	Okinawa Is., Okinawa, Japan (3)	NA	NA		EUTPI59	AB231197
		NA	NA	+	EUTPI60	AB231198
		NA	NA	+	EUTPI61	AB231199
29	Kume Is., Okinawa, Japan (2)	NA	NA	+	EUTPI61	AB231200
		NA	NA		EUTPI57	AB231201
		NA	NA	+	EUTPI62	AB231202
30	Shigaki Is., Okinawa, Japan (1)	NA	NA	+	EUTPI63	AB231205
31	Yonaguni Is., Okinawa, Japan (2)	NA	NA	+	EUTPI64	AB231203
		NA	NA	+	EUTPI65	AB231204
32	Hateruma Is., Okinawa, Japan (2)	NA	NA	+	EUTPI66	AB231206
		NA	NA		EUTPI67	AB231207
		NA	NA	+	EUTPI68	AB231208
33	Iriomote Is., Okinawa, Japan (1)	NA	NA	+	EUTPI69	AB231209
		NA	NA		EUTPI70	AB231210

34	Ishigaki Is., Okinawa, Japan (61)	2015-09	2015-Eh-1-C	+	EUTPI71	LC468370
		2015-09	2015-Eh-2-C	+	EUTPI72	LC468363
		2015-09	2015-Eh-3-C	+	EUTPI73	LC468371
		2015-09	2015-Eh-6-C	+	EUTPI62	LC468372
		2016-04	2016-Eh-1-C	+	EUTPI73	LC468373
		2016-04	2016-Eh-3-C	+	EUTPI57	LC468358
		2016-04	2016-Eh-29-C	+	EUTPI71	LC468374
		2016-04	2016-Eh-49-C	+	EUTPI72	LC468364
		2016-04	2016-Eh-53-C	+	EUTPI74	LC468375
		2016-04	2016-Eh-58-C	+	EUTPI75	LC468376
		2016-04	2016-Eh-92-C	+	EUTPI76	LC468415
		2016-04	2016-Eh-95-C	+	EUTPI73	LC468377
		2016-04	2016-Eh-101-C	+	EUTPI73	LC468378
		2016-04	2016-Eh-122-CF	+	EUTPI73	LC468379
		2016-04	2016-Eh-132-C	+	EUTPI73	LC468380
		2016-04	2016-Eh-133-C	+	EUTPI77	LC468381
		2016-04	2016-Eh-134-C	+	EUTPI78	LC468417
		2016-04	2016-Eh-135-C	+	EUTPI71	LC468382
		2016-04	2016-Eh-136-C	+	EUTPI71	LC468383
		2016-04	2016-Eh-142-CF	+	EUTPI73	LC468384
		2016-04	2016-Eh-163-C	+	EUTPI71	LC468385
		2016-04	2016-Eh-165-C	+	EUTPI77	LC468386
		2016-04	2016-Eh-171-C	+	EUTPI78	LC468416
		2016-04	2016-Eh-172-C	+	EUTPI77	LC468387
		2016-04	2016-Eh-173-C	+	EUTPI57	LC468359
		2016-04	2016-Eh-174-C	+	EUTPI79	LC468388
		2016-04	2016-Eh-175-C	+	EUTPI80	LC468389
		2016-04	2016-Eh-176-C	+	EUTPI57	LC468360
		2016-04	2016-Eh-177-C	+	EUTPI81	LC468390
		2016-04	2016-Eh-178-C	+	EUTPI71	LC468391
		2016-04	2016-Eh-179-C	+	EUTPI82	LC468392
		2016-04	2016-Eh-180-C	+	EUTPI73	LC468393
		2017-04	2017-Eh-9-CF	+	EUTPI73	LC468394
		2017-04	2017-Eh-13-CF	+	EUTPI83	LC468418
		2017-04	2017-Eh-14-CF	+	EUTPI71	LC468395
		2017-04	2017-Eh-29-CF	+	EUTPI73	LC468396
		2017-04	2017-Eh-34-CF	+	EUTPI84	LC468397
		2017-04	2017-Eh-36-C	+	EUTPI73	LC468398
		2017-04	2017-Eh-54-CF	+	EUTPI73	LC468399
		2017-04	2017-Eh-81-CF	+	EUTPI73	LC468400
		2017-04	2017-Eh-84-CF	+	EUTPI85	LC468401
		2017-04	2017-Eh-85-CF	+	EUTPI73	LC468402
		2017-04	2017-Eh-89-CF	+	EUTPI71	LC468403
		2017-04	2017-Eh-96-CF	+	EUTPI71	LC468404
		2017-04	2017-Eh-97-CF	+	EUTPI86	LC468365
		2017-04	2017-Eh-98-CF	+	EUTPI72	LC468366
		2017-04	2017-Eh-105-CF	+	EUTPI73	LC468408
		2017-04	2017-Eh-114-CF	+	EUTPI86	LC468367
		2017-04	2017-Eh-126-CF	+	EUTPI57	LC468361
		2017-04	2017-Eh-136-CF	+	EUTPI87	LC468405
		2017-04	2017-Eh-182-CF	+	EUTPI57	LC468362
		2017-04	2017-Eh-193-CF	+	EUTPI71	LC468406
		2017-04	2017-Eh-196-CF	+	EUTPI73	LC468407
		2017-04	2017-Eh-208-CF	+	EUTPI72	LC468368
		2017-04	2017-Eh-225-CF	+	EUTPI71	LC468409
		2017-04	2017-Eh-226-CF	+	EUTPI73	LC468410
		2017-04	2017-Eh-228-CF	+	EUTPI71	LC468411
		2017-04	2017-Eh-229-CF	+	EUTPI77	LC468412
		2017-04	2017-Eh-230-CF	+	EUTPI88	LC468369
		2017-04	2017-Eh-248-CF	+	EUTPI73	LC468413
		2017-04	2017-Eh-251-CF	+	EUTPI89	LC468414

**Wolbachia* infection status: +, infected; –, uninfected; NA, Not available.

conditions: initial denaturation at 94°C for 7 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, with a subsequent final 7-min extension at 72°C. Electrophoresis was carried out to confirm successful DNA amplification using 0.5× TAE buffer on a 0.5% agarose gel. The PCR products were purified with a PCR purification Kit (Bioneer, Daejeon, Korea) and cloned using T-Blunt™ PCR Cloning kit (SolGent, Daejeon, Korea) and HITTM DH5α High 108 competent cells (Real Biotech Co., Banqiao City, Taiwan). The resultant plasmid DNA was isolated using a Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea). DNA sequencing was conducted using the ABI PRISM® BigDye® Terminator ver. 3.1 Cycle Sequencing kit with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All products were sequenced from both strands.

Sequence analysis

The sequences of both strands from each individual were aligned using SeqMan (DNASTAR, Madison, WI, USA) to generate qualified individual sequences. Each individual sequence was compared to those available in public sequence databases, such as GenBank, through a Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify the accuracy of the sequences.

Through BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) we obtained 139 *Tpi* sequences of *E. mandarina* and *E. hecabe* originated from Japan (Narita *et al*., 2006; Miyata *et al*., 2017, 2020). These were originated from 129 individuals in 28 locations in Japan, which represent each 48 haplotypes of *E. mandarina* and 33 haplotypes of *E. hecabe*.

Analyses of sequence divergence and phylogenetic relationships

The degree of divergence among and between *E. hecabe* and *E. mandarina* was obtained from the unrooted pairwise distances using PAUP* v4.01b10 (Swofford, 2002). For phylogenetic analysis, maximum-likelihood (ML) method was applied using RAxML-HPC2 ver. 8.0.24 (Stamatakis, 2014), which was incorporated into the CIPRES Portal ver. 3.1 (Miller *et al*., 2010). Substitution model (GTR+Gamma) was determined using IQ-TREE ver. 1.5.3 (Nguyen *et al*., 2015). Confidence values were determined with 1,000 bootstrap (BS) iterations. Phylogenetic trees were visualized using FigTree version 1.42 (http://tree.bio.ed.ac.uk/software/figtree/). *E. blanda* (GenBank acc. no. AB231211; Narita *et al*., 2006) was as an outgroup.

Results and discussion

Characteristics of the Korean *Eurema mandarina*

Our morphological examination of the Korean populations that are distributed in southern areas (Gyeonsang and Jeolla provinces), including Jeju-do are classified into *E. mandarina* (Fig. 1). Previously, Narita *et al*. (2007) reported that the *E. mandarina* is an endemic species in Japan and classified the Jeju-do population into *E. hecabe*, which has B-type coloration pattern of wing fringe. However, the external morphology of Korean population and Japanese mainland population are indistinguishable, and, especially, the low-temperature type is almost same as the Japanese mainland population, which is currently classified into *E. mandarina* (Fig. 1A).

The morphological differences between *E. mandarina* and *E. hecabe* are as follows (Shirôzu, 2006): forewing fringe yellow in *E. mandarina*, but mixed color of yellow and dark brown in *E. hecabe*; the black band on outer margin of forewing smaller and absent at low temperature in *mandarina*, but large and present at low temperature in *E. hecabe*; and underside of forewing with stronger reddish-brown dots in *E. hecabe*. Additionally, three different types in forewing outer margin depending on

Fig. 1. Fringe of the forewing of *Eurema mandarina* occurring in Korea. (A) low-temperature brood; (B) high temperature brood; and (C) intermediate temperature brood.

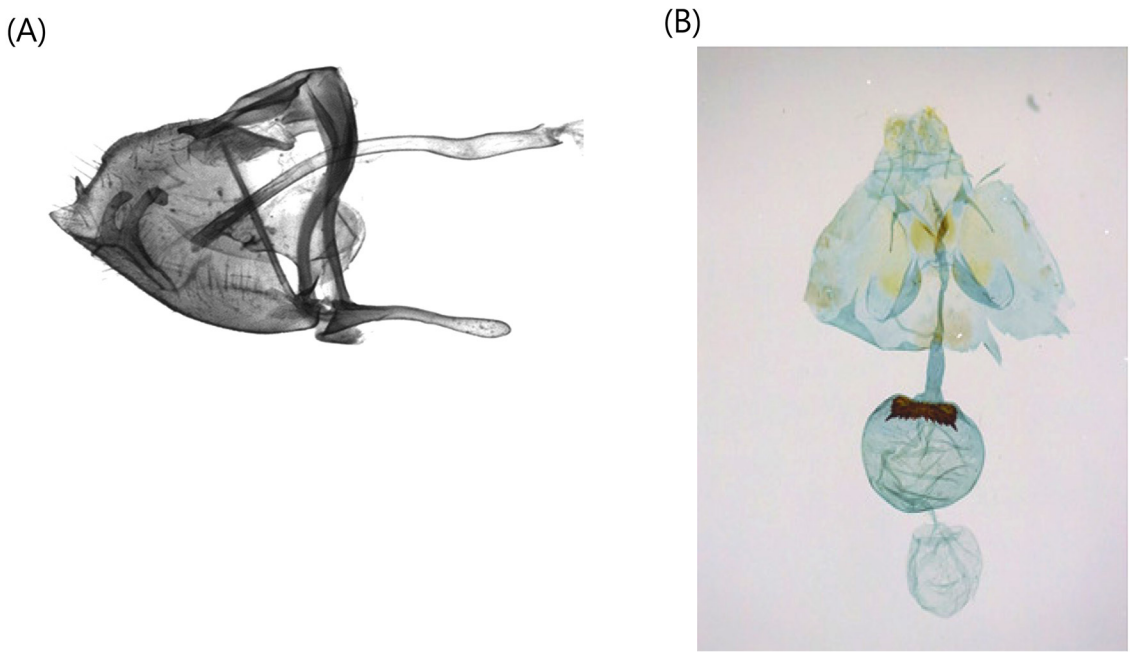

Fig. 2. Male (A) and female (B) genitalia of *Eurema mandarina* occurring in Korea.

the seasonal temperature (Fig. 1A, 1B, 1C) are observed in *E. mandarina*, while the only high temperature form (Fig. 1B) is observed in *E. hecabe*.

Male genitalia (Fig. 2A). Tegumen broad, triangular in dorsal aspect, somewhat concaved dorsomedially; saccus moderately long; uncus usually short, with apex strongly bifurcate. Valva with a process near the middle of the ventral margin of costa–ampulla region somewhat broad and flapped, a process beyond the middle of dorsal margin of costa–ampulla region long and slender, an apical process of valva broad and triangular with pointed apex, a process or processes at the basal portion or middle of harpe represented by two processes similar to each other. Phallus is moderately long, slender and arched dorsally.

Female genitalia (Fig. 2B). Ovipositor simple; posterior apophyses almost double size of anterior apophyses; ostium bursae V-shaped, sclerotized, narrow. Ductus bursae long, slender; corpus bursae ovate with large bar-shaped signum; appendix bursae ovate.

Specimens examined. 5♂3♀, Seoguipo, Is. Jejudo 15.VII.2019; 1♂, Seoguipo, Is. Jejudo 21.VIII.2019; 1♂, Seoguipo, Is. Jejudo 28.VIII.2019; 15♂2♀, Gochang, Jeonbuk Prov. 5.X.2013; 12♂2♀, Mt. Duryunsan, Jeonnam Prov. 21.X.2009; 3♂1♀, Muan, Jeonnam Prov. 21.VII.2019; 1♀, Muan, Jeonnam Prov. 5.XI.2018; 1♂, Sinan, Jeonnam Prov. 18.VII.2020; 1♂, Sinan, Jeonnam Prov. 29.IX.2020; 20♂12♀, Is. Geojedo, Gyeongnam Prov. 27.VII.2018; 3♂3♀, Uljin, Gyeongnam Prov. 13.IX.2016. 1♂, Namhae, Gyeongnam Prov. 18.IX.2020; 1♀, Hamyang, Gyeongnam Prov. 8.IX.2019; 1♂, Hapcheon, Gyeongnam Prov. 16.VIII.2019; 1♂, Hapcheon, Gyeongnam Prov. 20.VIII.2019.

Molecular results

From the *Tpi* sequences of nine individuals collected in six Korean localities eight haplotypes were obtained. Each one individual collected in Sinan-gun (locality 5) and Jeju-do Island (locality 6) shared EUPTPI07 (Table 1). EUPTPI06 detected in Sinan-gun (locality 7) as a single individual also was detected in Sendai, Miyagi, Japan as a single individual. The genetic divergence of the haplotypes detected in South Korea ranged from 1.275 (seven) to 8.743% (48 bp). When current eight haplotypes were combined with the 81 GenBank-registered haplotypes, representing each 48 haplotypes of *E. mandarina* and 33 haplotypes of *E. hecabe* originating from Japan, all eight haplotypes originating from South Korea were closer to those from *E. mandarina*, indicating that the nine individuals collected in current study can casually be assigned as *E. mandarina* in terms of *Tpi* sequences. The sequence divergence of *E. mandarina* including those from South Korea ranged from 0.182 (one) to 14.208% (78 bp), and that of *E. hecabe* ranged from 0.182 (one) to 6.922% (38 bp), showing a relatively larger sequence

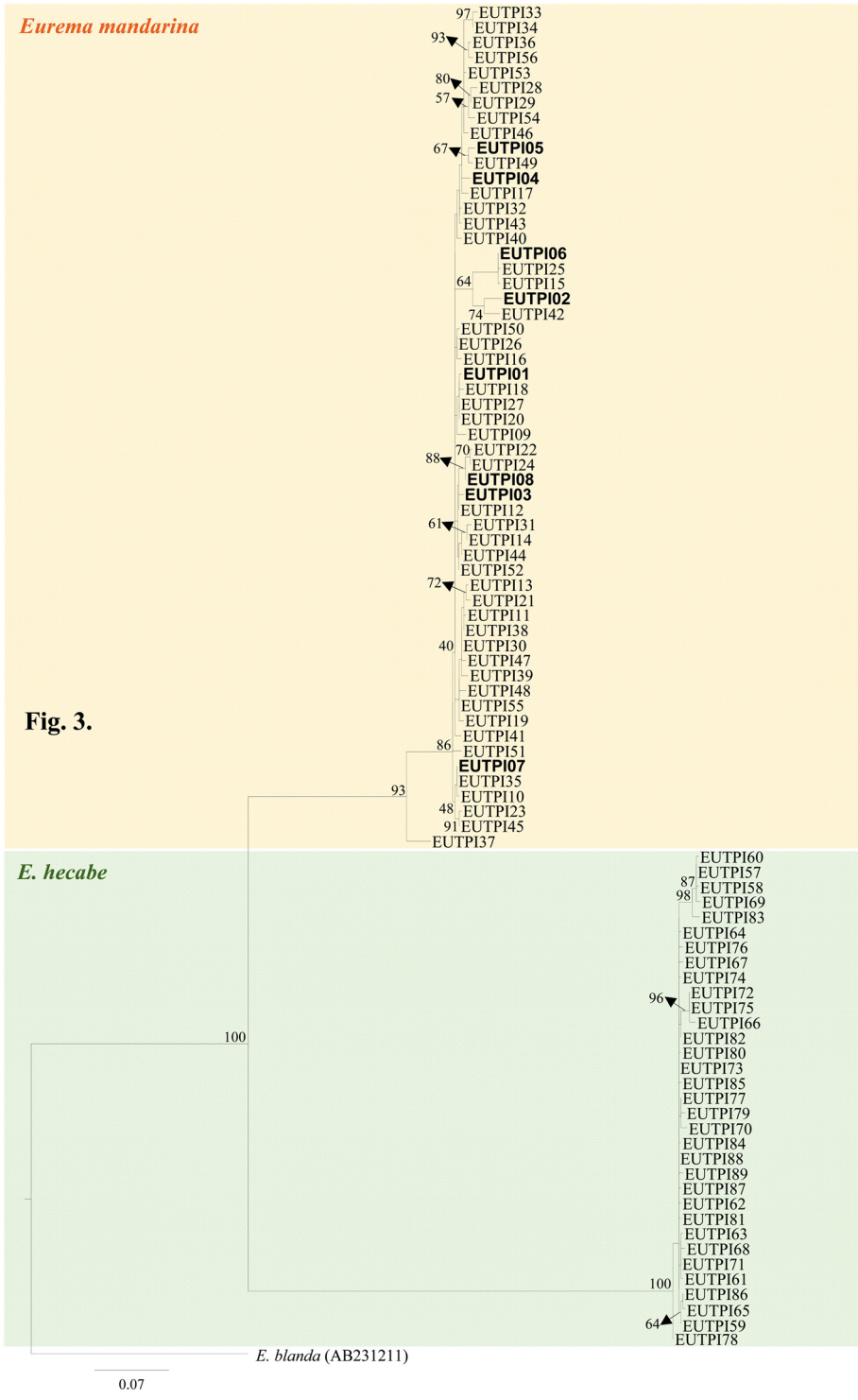

Fig. 3. Phylogeny of *Eurema mandarina* and *E. hecabe haplotypes* using the maximum-likelihood method. The numbers at each node specify bootstrap supports. The scale bar indicates the number of substitutions per site. Bold-faced haplotypes are those acquired from South Korea in current study. *E. blanda* (GenBank acc. no. AB231211) was utilized as an outgroup (Narita *et al*., 2006).

divergence in each species. Including the eight haplotypes detected in South Korea showed a substantial sequence divergence between the two species, ranging from 34.608 (190 bp) to 42.44% (233 bp).

Phylogenetic analysis of the two species with *Tpi* haplotypes including those obtained from current study formed each an

independent group, supported each with the higher (BS = 93% for *E. mandarina*) or the highest nodal supports (BS = 100% for *E. hecabe*) (Fig. 3). The eight haplotypes obtained in current study all well grouped together with the those of *E. mandarina*, indicating that all Korean specimens truly belong to *E. mandarina*.

Summarized, under the circumstances that typical mitochondrial COI gene, which is used as DNA barcode to aid species identification is not informative for *Eurema* species due to widespread *Wolbachia* infection of both *E. hecabe* and *E. mandarina* in majority of distributional range, additional study to identify the species status of Korean population has been necessitated. After we examined the morphology and sequenced *Tpi* gene of *E. mandarina* in South Korea, we found that the Korean populations that are distributed in southern areas can be classified into *E. mandarina*.

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