
Original Article

New taxonomic status of *Tripteroides bambusa* (Yamada), 1917 from Japan, based on experimental crossing and COI sequence divergence

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Abstract: *Tripteroides bambusa bambusa* (Yamada) from the Palaearctic region of Japan, and *Tp. b. yaeyamensis* Tanaka, Mizusawa and Saugstad from Yaeyama Islands of the Ryukyu Archipelago, Japan were elevated to the rank of full species, *Tp. bambusa* and *Tp. yaeyamensis*, respectively. The specific status was based on the demonstration of the morphological distinction, the allopatric distribution, the sterile reciprocal cross, and the DNA sequence diversity of the cytochrome *c* oxidase subunit I (COI).

Key words: Reproductive isolation, COI, taxonomic status, *Tripteroides bambusa* subspecies, Ryukyu Archipelago, Japan

INTRODUCTION

Tripteroides bambusa (Yamada), 1917 was originally described based on specimens from Tokyo, Fukushima, Kyoto, Hiroshima, Nagasaki, Kumamoto, and Kagoshima in the Palaearctic region of Japan (Palaearctic Japan), and known to occur throughout Japan, i.e., Hokkaido to the Ryukyu Archipelago (Yaeyama and Tokara Islands), Taiwan, Korea and China (Yamada, 1917, 1927; Morishita and Okada, 1955; Lien, 1962, 1978; Knight and Stone, 1977; Miyagi et al., 1983; Tanaka, 2014). After that, Tanaka et al. (1979) have described as new subspecies, *Tp. b. bambusa* from Palaearctic Japan, and *Tp. b. yaeyamensis* from Ishigakijima and Iriomotejima of Yaeyama Islands in the Ryukyu Archipelago, respectively. *Tripteroides b. yaeyamensis* is not found from Amamioshima, Okinawajima and Miyakojima. The specimens from Nakanoshima of Tokara Islands located in the northern most of the Ryukyu Archipelago belonging to the Oriental Region, were tentatively treated as *Tp. b. yaeyamensis* by Miyagi et al. (1983) morphologically. The distributions of two subspecies of the *Tp. bambusa* in Japan are apparently allopatric and discontinuous. The newly described *Tp. b. yaeyamensis* from Yaeyama Islands is very similar to *Tp. b. bambusa* from Palaearctic

Japan including Tsushima and Yakushima, but can be easily distinguished from the latter by having light yellowish brown integument of scutum, while in the scutum of *Tp. b. bambusa* is chocolate brown (Tanaka et al., 1979). The constant and unique difference in the color of scutum between the two groups suggests the possibility that *Tp. b. yaeyamensis* is a distinct species. However, reproductive isolation and genetic difference between the two subspecies have not been examined.

Reproductive isolation and hybrid sterility are important in taxonomical and evolutionary studies among closely related species. Nucleic acid sequence diversity in the internal transcribed spacers (ITS1 and ITS2) of the ribosomal DNA (rDNA) gene array and the mitochondrial cytochrome *c* oxidase subunit I (COI) gene have been proven to be useful for studying phylogenetic relationships in closely related mosquito species/subspecies groups (Porter and Collins, 1991; Crabtree et al., 1995; Miller et al., 1996; Mukwaya et al., 2000; Toma et al., 2000; Cywinska et al., 2006; Kumar et al., 2007; Taira et al., 2012; Maekawa et al., 2016). To further investigate and clarify the taxonomic relationships of the subspecies of *Tp. bambusa* distributed in Japan and Taiwan, we carried out cross examinations and the DNA sequence analyses.

This paper presents the results of the experimental crosses and COI sequence divergence, and notes on

the new taxonomic status of the subspecies of *Tp. bambusa* from Japan and Taiwan.

MATERIALS AND METHODS

1. Cross examination

Four laboratory strains of the subspecies of *Tp. bambusa* collected from 3 geographical regions (Palearctic Japan, Ryukyu Archipelago, and Taiwan) were established in our insectary. Their origin and the collection year used in cross examinations are as follows: *Tripteroides b. bambusa* (Tbb), Nagasaki (NGA), Kyushu, Palearctic Japan, in 1978 and Taipei, Taiwan (TWN) in 1979; *Tp. b. yaeyamensis* (Tby), Iriomotejima (IRI) and Yonagunijima (YON) of Yaeyama Islands, Ryukyu Archipelago, Japan in 1978 (Fig. 1). The strains were maintained by connection cage devised by Toma and Miyagi (1978). The mosquitoes used in the experiments were reared according to standard procedures employed by Miyagi (1973). The mosquitoes used for colonization and experiments were kept in a insectary with temperature and relative humidity controlled at 26°C and 80±5%, respectively. A photoperiod of 16-hr light and 8-hr dark were provided and a crepuscular period was simulated by gradual dimming for 30-mins.

To obtain the adult mosquitoes for experiments, 200 larvae were reared in rectangular pans (41 cm in length, 29 cm in width and 5 cm in height) with 2,500 mL water and given powder mixture of fine-

grained mouse pellet and dried yeast (1:1). Each pan was covered with a plastic sheet to avoid contamination. Pupae were sorted by sex under stereomicroscope. The newly-emerged virgin females and males were kept separately until experiments. Reciprocal crosses between 2 strains from inter- or intra-subspecies were performed (see the combinations in Table 5). Fifty pairs of 1 day old females and males were introduced in a screen cages (20×20×30 cm) and reared 7 days which was previously estimated enough for mating of intra-strain (100% insemination rate for all 4 strains). Basically, experiments for controls followed the same procedures described above. To obtain eggs, females were provided with a blood meal from an anesthetized mouse. The generations of strains used for experiments were from P (parent) to F₂. In designating a cross, for example NGA×IRI, the female is always placed first.

1) Insemination and "Hide-and-see" test

In order to test the sexual ability of males, a single male of 7 days after emergence was put in a cage (20×20×30 cm) with 10 females of the same subspecies of the same age for 24 hr. All females were dissected to determine the presence of sperms.

The "hide-and-see" test (Hartberg and Craig, 1968) was applied to NGA and IRI strains. This experiment was designed to determine if each male of the *Tp. bambusa* subspecies could readily discriminate a single female of the same subspecies from same locality in the presence of an excess of females of another subspecies. The procedure followed Miyagi and Toma (1989). In the test, one IRI male was placed with one IRI female and with 19 NGA females; likewise, one NGA male was placed with one NGA female and 19 IRI females. After 24 hr, the presence of sperms was determined in the spermathecae of the female of the same strain as well as females of the other subspecies.

2) Fertility of eggs obtained in inter- and intra-subspecies crosses

In cross experiments, 50 females and 50 males of F₁ generation were put in one cage, and given blood meal for obtaining the eggs of next generation, F₂. Hatching rate, embryonated rate of unhatched eggs, and adult emergence and insemination rates were examined. Same examinations were done for cross experiments from F₃ to F₅. The rates of insemination were examined on 12–30 days and on 22 days after emergence for inter-subspecies and for intra-subspecies, respectively. In order to know the fertility of eggs and viability of larvae, the rates of hatching, unembryonated eggs, and adult emergence were examined for all experiments. All eggs obtained for each cross were basically utilized. However, for F₃ generations of NGA (Tbb) × TWN (Tbb), and YON (Tby) × TWN (Tbb), and for control experiments of YON (Tby), and TWN (Tbb), 300 eggs/cross experiment were utilized. Out of the eggs submerged for 8 days, 40 unhatched eggs

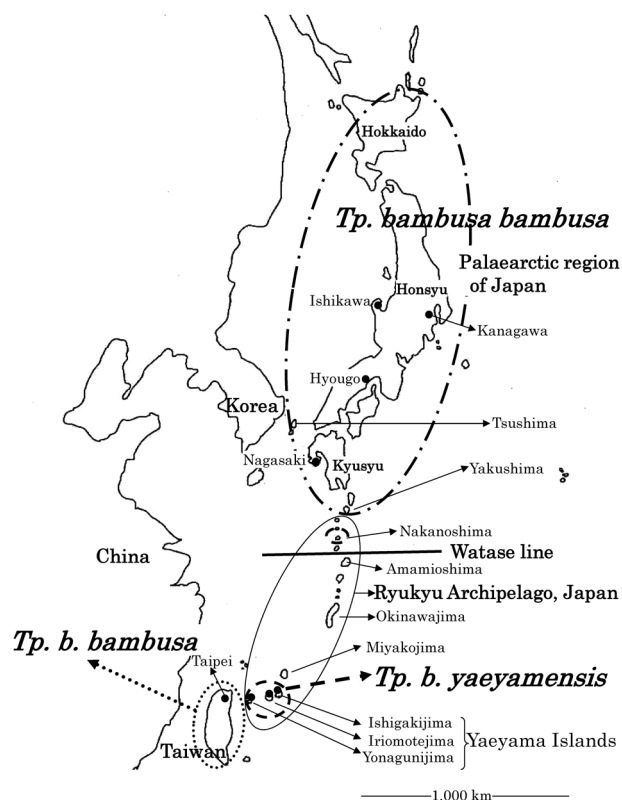


Fig. 1. Distribution of *Tripteroides bambusa* subspecies in Japan and Taiwan.

were dissected and examined microscopically for embryonation. The dissected eggs were judged as embryonated if development had passed a 40-hr stage when ocelli and hairs were visible, and as non-embryonated (sterile) if either at an earlier stage or no embryonation was observed (Miyagi and Toma, 1989). The fertile eggs indicate the total number of eggs hatched, and embryonated eggs in eggs unhatched. The percentage of fertile eggs was calculated as follows: $[(\text{No. of eggs hatched} + (\text{No. of eggs examined} - \text{No. of eggs hatched}) \times \% \text{ of embryonated eggs} / 100) / \text{No. of eggs examined}] \times 100$. The adult emergence rates were calculated based on the number of hatched larvae and the total number of the eggs examined for control and inter-subspecies crosses.

2. Analysis of DNA sequence

Mosquito specimens of *Tp. bambusa* from 8 locations of 3 geographical regions, Palaearctic Japan (Tbb), Honshu: Ishikawa, ISK and Kanagawa, KNG and Hyougo, HYG; Kyushu: Nagasaki, NGA,

the Ryukyu Archipelago, Japan (Tby), Yaeyama Islands: Ishigakijima, ISH; Iriomotejima, IRI and Yonagunijima, YON, and Taiwan, TWN (Tbb), from 2010 to 2011 (Fig. 1) were used for DNA examination. The larvae were collected in tree holes, cut bamboos, artificial containers and used tires, and the adults were collected by human bait collection in foothills and forests. Several larvae collected were reared to the adult stage for species identification.

Mosquito specimens in larval or adult stages were preserved in 99.5% ethanol and maintained at 4°C until used for DNA analysis. The specimens of the *Tp. bambusa* used for analyses of DNA sequences are listed in Table 1, including collection locality, habitat, their developmental life stages, code number for reference and GenBank accession number.

1) DNA extraction and amplification

DNA extraction and amplification from the mosquitoes followed Taira et al. (2012). One larvae or adult preserved in ethanol was homogenized in 300 µL of PBS using ceramic beads (1.5 mm diameter) and a

Table 1. Specimens of *Tripteroides bambusa* subspecies used in DNA study with collection locality, habit/habitat, development stage analyzed, code and GenBank accession number.

Serial no.	Geographic region Species	Collection locality*	Habit/habitat**	Development stage	Collection year	Code***	GenBank accession no.
Palaearctic region of Japan							
Honshu							
1	<i>Tp. b. bambusa</i>	ISK (36°32'N, 136°42'E)	C. bamboo	Larva	2010	394	LC441009
2	<i>Tp. b. bambusa</i>	KNG (35°22'N, 139°28'E)	Cont	Adult	2011	680	LC441010
3	<i>Tp. b. bambusa</i>	HYG (34°43'N, 135°10'E)	T. hole	Adult	2010	221	LC441011
4	<i>Tp. b. bambusa</i>	HYG (34°43'N, 135°10'E)	T. hole	Larva	2010	342	LC441012
5	<i>Tp. b. bambusa</i>	HYG (34°43'N, 135°10'E)	Cont	Larva	2010	383	LC441013
Kyushu							
6	<i>Tp. b. bambusa</i>	NGA (33°20'N, 129°47'E)	T. hole	Larva	2010	244	LC441008
Ryukyu Archi., Japan							
Yaeyama Islands							
7	<i>Tp. b. yaeyamensis</i>	ISH (24°27'N, 124°11'E)	Cont	Larva	2011	894	LC441023
8	<i>Tp. b. yaeyamensis</i>	ISH (24°27'N, 124°11'E)	Cont	Adult	2011	895	LC441024
9	<i>Tp. b. yaeyamensis</i>	IRI (24°19'N, 123°54'E)	Cont	Larva	2010	402	LC441016
10	<i>Tp. b. yaeyamensis</i>	IRI (24°19'N, 123°54'E)	Cont	Larva	2011	458	LC441017
11	<i>Tp. b. yaeyamensis</i>	IRI (24°20'N, 123°54'E)	Cont	Larva	2011	477	LC441018
12	<i>Tp. b. yaeyamensis</i>	IRI (24°20'N, 123°54'E)	Cont	Adult	2011	622	LC441019
13	<i>Tp. b. yaeyamensis</i>	YON (24°27'N, 122°58'E)	HB	Adult	2011	1012-1	LC441025
14	<i>Tp. b. yaeyamensis</i>	YON (24°27'N, 122°58'E)	HB	Adult	2011	1012-2	LC441026
15	<i>Tp. b. yaeyamensis</i>	YON (24°27'N, 122°58'E)	Cont	Larva	2011	1052	LC441027
Taiwan							
16	<i>Tp. b. bambusa</i>	TWN (23°43'N, 120°47'E)	U. tire	Adult	2011	522	LC441014
17	<i>Tp. b. bambusa</i>	TWN (23°43'N, 120°47'E)	U. tire	Larva	2011	524	LC441015
18	<i>Tp. b. bambusa</i>	TWN (23°43'N, 120°47'E)	U. tire	Adult	2011	681-1	LC441020
19	<i>Tp. b. bambusa</i>	TWN (23°43'N, 120°47'E)	U. tire	Adult	2011	681-2	LC441021
20	<i>Tp. b. bambusa</i>	TWN (23°43'N, 120°47'E)	U. tire	Adult	2011	681-3	LC441022
Outgroup							
21	<i>To. yanbarensis</i>	IRI (24°21'N, 123°45'E)	B. hole	Adult	2010	264	LC441029
22	<i>Ml. genurostris</i>	TOK (27°47'N, 128°56'E)	L. axil	Adult	2010	297	LC441030
23	<i>Tx. m. yaeyamae</i>	ISH (24°27'N, 124°11'E)	Cont	Larva	2011	893	LC441028

* ISK, Ishikawa; KNG, Kanagawa; HYG, Hyougo; NGA, Nagasaki; TOK, Tokunoshima; ISH, Ishigakijima; IRI, Iriomotejima; YON, Yonagunijima; TWN, Taiwan. ** HB, human bait; Cont, artificial container; U. tire, used tire; B. hole, bamboo hole; C. bamboo, cut bamboo; L. axil, leaf axil; T. hole, tree hole. *** Code indicated for reference.

FastPrep FP120 homogenizer (Q-Biogene, Carlsbad, CA, USA) for 30 sec at speed level five. DNA was extracted from homogenized specimen using QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) and was eluted into 100 μ L of nuclease-free water. The COI barcoding region was amplified using primer sets LCO1490 and HCO2198 (Folmer et al., 1994). The PCR temperature cycle, which was carried out in C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), consisted of an initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 10 sec, annealing at 45°C for 30 sec, extension at 72°C for 60 sec and a final extension at 72°C for 10 min.

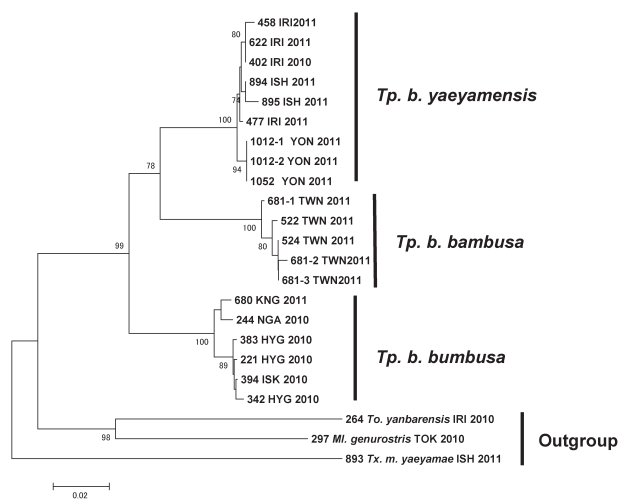


Fig. 2. Neighbor-joining analysis of the Kimura 2-parameter (K2P) sequences from *Tripteroides bambusa* subspecies in Japan and Taiwan, and outgroup. The specimens are indicated by code-location-year collected. ISK, Ishikawa; KNG, Kanagawa; HYG, Hyogo; NGA, Nagasaki; TOK, Tokunoshima; ISH, Ishigakijima; IRI, Iriomotejima; YON, Yonagunijima; TWN, Taiwan.

2) DNA sequence analysis

The amplification products were purified using the QIAquick PCR purification kit (Qiagen, Tokyo, Japan). Direct sequencing was performed with the same PCR primers using the ABI PRISM BigDye Terminator version 3.1 systems in ABIPRISM 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA). All the DNA sequence analyses were conducted using MEGA version 5.0 software (Tamura et al., 2011). DNA sequences obtained were aligned using CLUSTAL W (Thompson et al., 1994). Nucleotide sequence divergences were calculated using the Kimura two-parameter (K2P) distance model (Kimura, 1980). A neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) with 1,000 bootstrap replicates was constructed to provide a graphic representation of the subspecies divergence, with bootstrap values of more than 70% indicated at the branch. *Topomyia yanbarensis* Miyagi, *Malaya genurostris* Leicester and *Toxorhynchites manicatus yaeyamae* Bohart from the Ryukyu Archipelago were used as outgroups (Table 1). All specimens shown in Fig. 2 are labeled by a combination of individual ID number, collection locality and year collected.

RESULTS

1. Insemination frequencies and “Hide-and-see” test

In NGA and IRI strains, each male of 7 days after emergence could inseminate 2 to 3 and 3 to 4 females of the same strain during a 24-hr period, respectively (Table 2).

In one of the five replicates with NGA male, the male inseminated the homotypic female, although that male also inseminated two heterotypic females. In the other four replicates with NGA males, insemination was observed in 1–3 heterotypic females but not in the homotypic female. In all of the 5 replicates with IRI

Table 2. Frequencies of the number of females inseminated by a single male of the same subspecies in 24-hr period.

1 male × 10 females	No. of tests	No. of females inseminated among 10 females				
		1	2	3	4	5–10
NGA	3	0	2	1	0	0
IRI	4	0	0	3	1	0

Abbreviation of strains: NGA, Nagasaki; IRI, Iriomotejima. Single male was put in a cage with 10 females.

Table 3. Results of “Hide-and-see” test with one male mosquito caged with one homotypic female and 19 heterotypic females.

Combination	No. of replicates	Homotypic female	No. of cases				
			No. of heterotypic females inseminated				
			0	1	2	3	
NGA male and female, and IRI females	5	Inseminated	1	0	0	1	0
		Uninseminated	4	0	1	2	1
IRI male and female, and NGA females	5	Inseminated	0	0	0	0	0
		Uninseminated	5	0	1	1	3

Abbreviation of strains refer to Table 2.

male, insemination was observed in 1–3 heterotypic females but not in the homotypic female (Table 3).

2. Fertility of eggs obtained in inter- and intra-subspecies crosses

The average insemination rate after a 22-day mating period was 96–100% in the control crosses of NGA, IRI, YON and TWN. The percentages of hatching and fertile eggs were 76.1–89.1% and 78.4–95.7%, respectively (Table 4). The adult emergence rates for larvae hatched, and eggs used were 79.1–85.5% and 60.6–71.0%, respectively.

In the inter-subspecies cross, NGA×IRI, the insemination rate was 100%, although there were no fertile eggs. In the reciprocal cross, the insemination

rate was 100% with 1.9% hatching and 1.9% fertile eggs in the 1st generation. The emergence rates for larvae hatched and eggs examined were 22.2% and 0.4%, respectively (Table 5).

In NGA×YON, hatching and fertile rates were 0.2% and 2.6%, respectively. The emergence rate was 0.1% for eggs of the F₁ generation. In YON×NGA, the insemination rate was 100% with 1.6% hatching and 28.0% fertile eggs. The emergence rate was 0.4% for eggs of F₁ (Table 5).

The insemination rates by crosses, NGA×TWN, were 73–97.5% in P–F₂ generations. The hatching and fertile rates were 24.1–40.2% and 46.2–63.6% in F₁ and F₂, respectively. In TWN×NGA, the hatching rate was 1.0%, and fertile rate was 26.3% in F₁. The emergence

Table 4. Egg fertility and adult emergence of each subspecies of *Tripteroides bambusa* in control experiments.

Cross 50 females×50 males (subsp.)	No. of replicates	Ave. % Insemination of females ¹⁾	Total no. of eggs examined	Ave. % of eggs			Ave. % of adults for 1st larvae ⁴⁾	Ave. % of adults for eggs ⁵⁾
				Hatched	Embryonated ²⁾	Fertile ³⁾		
NGA×NGA (Tbb)	5	100	2,321	76.1	9.9	78.4	85.5	63.4
IRI×IRI (Tby)	5	96.0	3,274	78.5	13.7	80.9	79.3	62.3
YON×YON (Tby)	3	100	900	89.1	59.5	95.7	79.7	71.0
TWN×TWN (Tbb)	6	100	1,800	76.6	13.8	79.9	79.1	60.6

Abbreviation of strains: YON, Yonagunijima; TWN, Taiwan. NGA and IRI refer to Table 2. ¹⁾ Insemination rate was examined by 10 females after a 22 days after emergence. ²⁾ Percentage of embryonated eggs out of 40 unhatched eggs dissected. ³⁾ Percentage of fertile eggs = [(No. of eggs hatched + (No. of eggs examined - No. of eggs hatched) × % of embryonated eggs/100] / No. of eggs examined] × 100. ⁴⁾ Percentage of adults emerged from 1st instar larvae. ⁵⁾ Percentage of adults emerged from eggs.

Table 5. Egg fertility and adult emergence of inter- or intra-subspecies of *Tripteroides bambusa* in cross experiments.

Cross 50 females×50 males	Generation	No. of replicates	Ave. % Insemination of females ¹⁾	Total no. of eggs examined	Ave. % of eggs			Ave. % of adults for 1st larvae ⁴⁾	Ave. % of adults for eggs ⁵⁾
					Hatched	Embryonated ²⁾	Fertile ³⁾		
NGA (Tbb)×IRI (Tby)	P	1	100						
	F ₁	1	—	1,891	0	0.0	0	0	
IRI (Tby)×NGA (Tbb)	P	1	100						
	F ₁	1	—	965	1.9	0.0	1.9	22.2	0.4
NGA (Tbb)×YON (Tby)	P	3	100						
	F ₁	3	—	5,192	0.2	2.4	2.6	22.2	0.1
YON (Tby)×NGA (Tbb)	P	2	100						
	F ₁	2	—	647	1.6	26.8	28.0	14.3	0.4
NGA (Tbb)×TWN (Tbb)	P	4	97.5						
	F ₁	4	80	5,963	24.1	29.2	46.4	67.3	16.4
	F ₂	3	73	1,222	40.2	42.8	63.6	75.8	30.0
	F ₃	3	—	900	39.6	11.0	46.2	73.6	28.7
TWN (Tbb)×NGA (Tbb)	P	4	100						
	F ₁	4	—	7,471	1.0	25.5	26.3	60.9	0.5
YON (Tby)×TWN (Tbb)	P	4	100						
	F ₁	4	97.5	959	78.2	53.6	92.5	97.5	76.2
	F ₂	4	68.3	1,081	45.5	27.0	61.1	74.5	33.6
	F ₃	4	50	1,200	54.2	3.1	55.5	54.4	27.3
	F ₄	2	58.3	598	22.9	6.6	28.1	65.5	15.0
TWN (Tbb)×YON (Tby)	F ₅	1	—	334	0	1.0	1.0	0	0
	P	5	100						
	F ₁	5	62.9	6,351	63.3	22.9	71.2	70.6	44.3
	F ₂	5	—	1,183	0.8	0.9	1.7	35.0	0.5

Abbreviation of strains refer to Tables 2 and 4. ¹⁾ Insemination rate was examined by 10–12 females after a 12–30 days after emergence. ^{2), 3), 4), 5)} refer to Table 4.

rate for eggs of F_1 was 0.5%.

The insemination rates by crosses, YON×TWN, were 100% in P and 97.5% in F_1 , respectively. And the rates were 50–68.3% in F_2 – F_4 generations. The hatching and fertile rates were also high, 78.2% and 92.5% in F_1 , but became low, 22.9–54.2% and 28.1–61.1% in F_2 – F_4 . These rates in F_5 became to 0 and 1.0%, respectively. In the reciprocal crosses, TWN×YON, the insemination rates were 100% in P and 62.9% in F_1 . The adult emergence rates for larvae hatched and eggs were 35.0% and 0.5% in F_2 , respectively (Table 5).

Genetic distance analysis

A 658 bp fragment of the COI gene was sequenced for 20 specimens belonging to *Tp. bambusa* collected from 8 localities of 3 geographical regions (Table 1). Kimura two-parameter distances in each subspecies from 3 geographic regions were ranged from 0.3–1.9% in ISK, KNG and HYG of Honshu, and NGA of Kyushu, Palaeartic Japan, 0–1.1% in ISH, IRI and YON of Yaeyama Islands, the Ryukyus, and 0–0.9% in TWN, respectively (Table 6). The variation in each region was less than 2%. The divergences of the COI DNA sequences between intra- or inter-subspecies of *Tp. bambusa*, Palaeartic Japan (Tbb)-Yaeyama Islands (Tby), Palaeartic Japan (Tbb)-TWN (Tbb), and Yaeyama Islands (Tby)-TWN (Tbb), were 7.6–8.8%, 8.8–10.9%, and 6.5–8.1%, respectively.

Phylogenetic analysis

A NJ phylogenetic tree based on 658 bp nucleotide sequences of 20 specimens was constructed with the outgroup *Topomyia yanbarensis* Miyagi, *Malaya genurostris* Leicester, and *Toxorhynchites manicatus yaeyamae* Bohart (Fig. 2). From the phylogenetic

tree, 2 subspecies from 3 geographical regions were separated into 3 distinct clusters, corresponding to the specimens from Yaeyama Islands, Taiwan, and Palaeartic Japan with high bootstrap value (100%) at terminal branches. The COI sequences of specimens from Palaeartic Japan (ISK, KNG, HYG, and NGA) formed a single clade, whereas the sequences of those from Yaeyama Islands and Taiwan formed two related sister clades, which together formed a larger group.

DISCUSSION

Experimental crosses and DNA sequence analyses of 658 bp fragment of the COI gene were carried out in order to know the relationship of the subspecies of *Tp. bambusa* from the Palaeartic region of Japan, Yaeyama Islands and Taiwan. In the hide-and-see tests, no evidence for the male's preference of the homotypic females was obtained. It is apparent that mating and insemination were done randomly and the male had no strong preference for female of same subspecies. In crosses among the strains from different geographic regions, the Palaeartic region of Japan, Yaeyama Islands and Taiwan, as well as crosses between strain from the same geographic region, mating occurred easily and insemination rates were high, and no definite barriers were observed in the insemination in all crosses. However, in crosses between strains from the Palaeartic region of Japan and Yaeyama Islands, the eggs of the F_1 hybrids were sterilized or only a small number of larvae was hatched. In the crosses using males of Taiwan and females of Yaeyama Islands, the F_5 generation was obtained, although inviable eggs were increased, and

Table 6. Percent of pairwise divergence between specimens of *Tripteroides bambusa* subspecies from 8 locations of 3 geographical regions using the K2P model.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 <i>Tp. b. bambusa</i> ISK 2010 (394)																				
2 <i>Tp. b. bambusa</i> KNG 2011 (680)	1.9																			
3 <i>Tp. b. bambusa</i> HYG 2010 (221)	0.3	1.6																		
4 <i>Tp. b. bambusa</i> HYG 2010 (342)	0.3	1.9	0.3																	
5 <i>Tp. b. bambusa</i> HYG 2010 (383)	0.3	1.9	0.3	0.3																
6 <i>Tp. b. bambusa</i> NGA 2010 (244)	1.1	0.8	1.1	1.1	1.1															
7 <i>Tp. b. yaeyamensis</i> ISH 2011 (894)	7.8	8.0	8.2	8.2	7.8	7.8														
8 <i>Tp. b. yaeyamensis</i> ISH 2011 (895)	8.4	8.6	8.8	8.8	8.4	8.4	0.5													
9 <i>Tp. b. yaeyamensis</i> IRI 2010 (402)	7.8	7.6	7.8	8.2	7.8	7.8	0.3	0.8												
10 <i>Tp. b. yaeyamensis</i> IRI 2011 (458)	7.8	8.0	7.8	8.2	7.8	7.8	0.6	1.1	0.3											
11 <i>Tp. b. yaeyamensis</i> IRI 2011 (477)	7.8	7.6	7.8	8.2	7.8	7.8	0.3	0.8	0.3	0.6										
12 <i>Tp. b. yaeyamensis</i> IRI 2011 (622)	7.8	7.6	7.8	8.2	7.8	7.8	0.3	0.8	0.0	0.3	0.3									
13 <i>Tp. b. yaeyamensis</i> YON 2011 (1012-1)	8.2	7.6	7.8	8.2	7.8	7.8	0.6	1.1	0.6	0.9	0.6	0.6								
14 <i>Tp. b. yaeyamensis</i> YON 2011 (1012-2)	8.2	7.6	7.8	8.2	7.8	7.8	0.6	1.1	0.6	0.9	0.6	0.6	0.0							
15 <i>Tp. b. yaeyamensis</i> YON 2011 (1052)	8.2	7.6	7.8	8.2	7.8	7.8	0.6	1.1	0.6	0.9	0.6	0.6	0.0	0.0						
16 <i>Tp. b. bambusa</i> TWN 2011 (522)	9.8	9.4	9.8	10.2	10.2	10.0	7.5	7.7	7.1	7.5	7.1	7.1	7.5	7.5	7.5					
17 <i>Tp. b. bambusa</i> TWN 2011 (524)	10.0	10.0	10.0	10.5	10.5	10.2	7.3	7.5	7.3	7.7	6.9	7.3	6.9	6.9	6.9	0.5				
18 <i>Tp. b. bambusa</i> TWN 2011 (681-1)	9.6	8.8	9.6	10.0	10.0	9.4	6.9	7.1	6.5	6.9	6.5	6.5	6.9	6.9	6.9	0.5	0.9			
19 <i>Tp. b. bambusa</i> TWN 2011 (681-2)	10.5	10.0	10.5	10.9	10.9	10.7	7.7	7.9	7.7	8.1	7.3	7.7	7.3	7.3	7.3	0.5	0.3	0.9		
20 <i>Tp. b. bambusa</i> TWN 2011 (681-3)	10.0	10.0	10.0	10.5	10.5	10.2	7.3	7.5	7.3	7.7	6.9	7.3	6.9	6.9	6.9	0.5	0.0	0.9	0.3	

Abbreviation of collection locality and specimen codes shown in parentheses refer to Table 1.

hybrids in F_6 were not obtained. Post-mating isolation was seen in the allopatric subspecies from three areas of Palaearctic region of Japan, Yaeyama Islands and Taiwan.

Reproductive isolation without pre-mating isolation is also seen in the two sympatric species of the subgenus *Stegomyia* of *Aedes* in the Ryukyu Archipelago. The cross between females of *Ae. albopictus* (Skuse) and males of *Ae. flavopictus downsi* Bohart and Ingram, which are the member of the *Ae. albopictus* subgroup, produced a small number of fertile hybrids (Toma and Miyagi, 1989). The morphological form of male IX tergum resembled that of *Ae. f. downsi* except the absence marginal serration. In the field, the form was not observed. These hybrids were bred through the F_2 generation and appeared to be normal in every way. However the eggs from the reciprocal cross were completely sterile. In the cross, post-mating isolation mechanism was operative among these 2 species.

In molecular biological study of mosquitoes, it is reported recently that the mean intraspecific variations were less than 2% divergence (Kumar et al., 2007; Taira et al., 2012; Khrabrova et al., 2015). The mean intraspecific variations of 35 species including the medically important vector species of *Anopheles*, *Aedes* and *Culex* showed a less than 2% ranged with 0–1.5% divergences, but higher divergences of more than 2% were detected in *Aedes aureostriatus okinawanus* Bohart (2.2%) and *Culex hayashii ryukyuanus* Tanaka et al. (3.3%) in the Ryukyu Archipelago, Japan (Taira et al., 2012). *Aedes a. okinawanus* breeding in tree holes and *Cx. h. ryukyuanus* breeding in streams of the forest showed differences in divergence between specimens from different islands. In the Palaearctic region of Japan, 44 species also showed an average sequence divergence at less than 2%, except for *Anopheles lindesayi japonicus* breeding in streams of forest (Maekawa et al., 2016).

In this study, the divergences between two subspecies from Palaearctic region of Japan and Yaeyama Islands, and Yaeyama Islands and Taiwan were 7.6–8.8% and 6.5–8.1%, respectively. The pairwise divergence between *Tp. b. bambusa* from Palaearctic region of Japan and Taiwan were 8.8–10.9%, which are more than 2% intraspecific variation. The divergence of the specimens with in each geographical region was less than 2%. Taira et al. (2012) reported that the mean nucleotide divergences for interspecific variations were 9.9 (4.1–12.5) % for *Anopheles*, 12.4 (3.0–16.0) % for *Aedes*, and 9.7 (3.8–13.5) % for *Culex* in breeding in the Ryukyu Archipelago. In our study, the grouping of the taxa on the Neighbor-joining phylogenetic tree corresponded to not subspecies designation, but geographic regions, i.e., Palaearctic region of Japan, the Yaeyama Islands of the Ryukyu Archipelago and Taiwan. Specimens from the same geographic

region were grouped into a same clade irrespective of collection localities. From the results of cross tests and DNA analyses for COI region, the Yaeyama Islands population should be treated as full species, *Tp. yaeyamensis*, rather than subspecies.

Full descriptions for specimens of *Tp. yaeyamensis* from Yaeyama Islands were given by Tanaka et al. (1979). This species, morphologically, is closely similar to those of the Palaearctic region of Japan in almost all respects, including the male terminalia. Specific characters for separating these species may be found at only adult stage: Scutum, scutellum and postnotum are all light yellowish brown in specimens from Yaeyama Islands, while all chocolate brown in those of the Palaearctic region of Japan. It is interesting to note that no *Tripteroides* species has been recorded from comparatively large islands of the Ryukyu Archipelago, Amamiyoshima, Tokunoshima, Okinawajima and Miyakojima in spite of our extensive faunal studies (Toma and Miyagi, 1986). We want to make the treatment of species for specimens from Tokara Islands pending until getting additional specimens. The distribution of *Tp. bambusa* and *Tp. yaeyamensis* may be due to the long geographic insulation of the archipelagoes, together with adaptation of the species to uneven climatic conditions at the boundary between the Palaearctic and Oriental Regions. A theory supported by previous analyses of phylogeographical patterns of amphibian and reptile lineages in the archipelago that indicated distribution was strongly influenced by isolation in the geographical history of the archipelago (Ota, 1998; Kuramoto et al., 2011; Kizaki, 1980). We suspect that the Taiwan population belongs to a species which is different from *Tp. bambusa* and *Tp. yaeyamensis*. However, here we still treat as *Tp. bambusa* until the morphology and DNA of individuals from Taiwan and China are examined in detail.

The taxonomic treatments for *Tp. bambusa* and *Tp. yaeyamensis* are as follows:

***Tripteroides (Tripteroides) bambusa* (Yamada), 1917, new status**

Rachionotomyia bambusa Yamada, 1917: 61 (♂, ♀, P, L, E). Type locality: Tokyo, Higashiyama Spa (Fukushima Pref.), Kyoto and Hiroshima, Honshu; Omura (Nagasaki Pref.), Kumamoto and Kagoshima, Kyushu, Japan.

Tripteroides bambusa (Yamada): Edwards, 1932: 78. LaCasse and Yamaguti, 1950: 47 (♂, ♀, L).

Tripteroides b. bambusa (Yamada): Tanaka, Mizusawa and Saugstad, 1979: 478 (♀, ♂, L). Tanaka, 2014: 199.

Distribution: Palaearctic region of Japan (Hokkaido, Honshu, Shikoku, Kyushu, Tsushima and Yakushima), Korea, China and Taiwan (?).

Bionomics: The larvae of this species are commonly

occur in tree holes and bamboo stumps, occasionally in artificial containers in more or less natural environments (Tanaka et al., 1979). The females sometimes come to bite human in forest. It commonly bites chicken and mice in laboratory. The females drop eggs onto the surface of water while hovering above it, sometimes while resting on the wall of the container (Miyagi, 1972, 1973).

***Tripteroides (Tripteroides) yaeyamensis* Tanaka, Mizusawa and Saugstad, 1979, new status**

Rachionotomyia bambusa Yamada, 1927: 569 (in part: Ryukyu Archipelago).

Tripteroides b. yaeyamensis Tanaka, Mizusawa and Saugstad, 1979: 481 (♂, ♀). Holotype: male (#22393, K-0637-13) collected Mt. Banna, Ishigakijima, Ryukyu Archipelago. Toma and Miyagi, 1989: 45 (♂, ♀, L).

Distribution: Ryukyu Archipelago (?Nakanoshima of Tokara Islands, Ishigakijima, Iriomotejima and Yonagunijima).

Bionomics. The immature stages of *Tp. yaeyamensis* have been collected primarily in bamboo stumps, but have also been found in tree holes and in artificial containers at forest of mountainous areas. They have been frequently found in association with *Ae. a. taiwanus* Lien, *Ae. riversi* Bohart and Ingram, *Orthopodomyia anoptheroides* (Giles), *Cx. bicornatus* (Theobald) in forest and at the base of mountains (Toma and Miyagi, 1986). The females have been fed on man occasionally during the day-time in forest (Toma and Miyagi, 1986). Miyagi and Toma (2017) reported that the females feeding on turtle. Birds and the wild pigs also may be bitten in nature.

Tripteroides yaeyamensis as well as *Tp. bambusa* falls into the group B (*nitidoventor* group) of subgenus *Tripteroides* according to Edwards (1932). *Tripteroides bambusa*, and *Tp. yaeyamensis* are closely related to *Tp. cheni* Lien (Lien, 1968) from Lanyu Island, Taiwan, but differ from it by the following key:

1. Integument of thorax relatively light yellowish brown. Only one spermatheca present *cheni*
- Integument of thorax relatively brown to dark brown. Three spermatheca present 2
2. Anterior, posterior and lateral margins of scutum and paratergite, and postnotum light yellowish brown *yaeyamensis*
- Anterior, posterior and lateral margins of scutum and paratergite, and postnotum chocolate brown *bambusa*

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