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Molecular, Morphological, and Behavioral Analyses of Japanese *Gerris* (*Macrogerris*) Water Striders (Heteroptera; Gerridae): Evidence for a New Species

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Abstract. Nucleotide sequences of a 0.7 kb portion of the mitochondrial cytochrome *b* gene were analyzed using Japanese species of water striders belonging to the subgenus *Macrogerris*. Although the sequences were rather homogeneous among individuals within both *Gerris gracilicornis* and *G. yezoensis*, considerable variation was observed among insects showing conventionally recognized diagnostic characteristics of *G. insularis*. Results of molecular phylogenetic analyses clearly indicated that there are three distinct lineages, designated as types A, B, and C, among these latter insects. Comparisons of nucleotide sequences indicated that genetic distances among these lineages were much larger than those between *G. gracilicornis* and *G. yezoensis*. From PCR-RFLP analyses of insects collected from 35 localities, it was revealed that the geographic distributions of types B and C were divided between northeastern Honshu island and western Japan including Kyushu island and central to western Honshu island, whereas that of type A largely overlapped with those of the other two types. Although male adults of the three types were homogeneous in conventionally recognized diagnostic characteristics, apparent differences were observed in the shape of the head, pronotum, fore femur, and color pattern of the anterior pronotum between type A and the other two types. In addition, types B and C showed apparent dimorphism in hind wings, containing both long- and short-winged individuals, whereas type A showed only long and wide hind wings. Preliminary observations of mating behavior indicated that a precopulatory reproductive isolation is operative between type A and other two types. Based on these results, evolutionary relationships among the three lineages are discussed.

Key words: New species, mtDNA sequence, morphological traits, mating behavior, *Gerris* (*Macrogerris*).

Introduction

The water strider genus *Gerris* is distributed widely in all continents except South America, Australia, and the Antarctic. The taxonomy of this and several closely related genera have been studied intensively (Andersen, 1990; Andersen & Spence, 1992; Andersen, 1993), and species relationships appear to be well understood (Spence & Andersen, 1994). However, due to the regional nature of much of the early research, one group of these insects has scarcely been investigated. The *Gerris* subgenus *Macrogerris* currently includes 8 species of water striders distributed in eastern Asia (Andersen & Chen, 1993). Of these, three described species, *Gerris insularis*, *G. gracilicornis*, and *G. yezoensis*, are known from Japan. Most species of this group are difficult to identify by external appearance, and thus the geographical distribution of several species has not been studied sufficiently. Given this situation, Andersen (1993) predicted the existence of undescribed species in this insect group. In fact, Tachikawa (1999) found populations of an unknown water strider distributed in mountainous regions in Honshu island, Japan. Although the adult male of this insect shows the conventionally recognized diagnostic characteristics of *G. insularis*, difference was observed in the shape of the fore-legs (Tachikawa, personal communication). However, its phylogenetic and taxonomic status have not yet been studied.

Recently, a number of researchers have analyzed

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DNA sequences to examine phylogenetic relationships among water striders (Sperling *et al.*, 1997; Damgaard *et al.*, 2000; Muraji & Tachikawa, 2000). Mitochondrial DNA (mtDNA) sequence was demonstrated to be a useful marker for discriminating among closely related species. Such a method may also help to detect genetic variation, and to establish the classification of water striders belonging to *Macrogerris*. In this study, nucleotide sequences of a 0.7 kb portion of the mitochondrial cytochrome *b* gene (cyt *b*) were analyzed using male adults of the undescribed water strider discovered by Tachikawa (1999) as well as other *Macrogerris* species known in Japan. PCR-RFLP (restriction fragment length polymorphism in PCR amplified sequence) analysis was used to know the geographic distributions of mtDNA lineages detected in insects showing diagnostic characteristics of *G. insularis*. Preliminary analyses were also performed on morphological characters and copulatory compatibility. These results are combined to discuss evolutionary relationships among mtDNA lineages discovered in water striders exhibiting diagnostic characteristics of *G. insularis*.

Materials and Methods

Nucleotide sequencing and PCR-RFLP analysis

In 1998 and 1999, *Macrogerris* water striders were collected from 39 localities in Japan (Fig. 1). All of the three known Japanese species were included in the samples. They were stored in 99.5% ethanol. As the source of a template for the PCR, DNA was extracted from the hind legs of individual adults using a GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amershan Pharmacia Biotech). Two primers, CYTF1 (5′-CTTGATGAAATTTTGGATC-3′) and CYTR1 (5′-CTCCTCCTAATTTATTAGGAATTG-3′) (Muraji *et al.*, 2000), were used for amplification and sequencing of the mtDNA fragment. In addition, a primer, CYTR2 (5′-GCAGATATTAAGTTAGTAATTAC-3′), which anneals within the PCR products, was used for sequencing. These primers were designed based on the high degree of similarity of corresponding sequences in *Apis mellifera* (Accession number: L06178), *Locusta migratoria* (X80245), *Anopheles quadrimaculatus* (L04272), and *Drosophila yakuba* X03240). The amplification products correspond to bp 10605 to bp 11398 of the *D. yakuba* sequence. Amplification, purification, labeling, and sequencing of mtDNA fragments were carried out

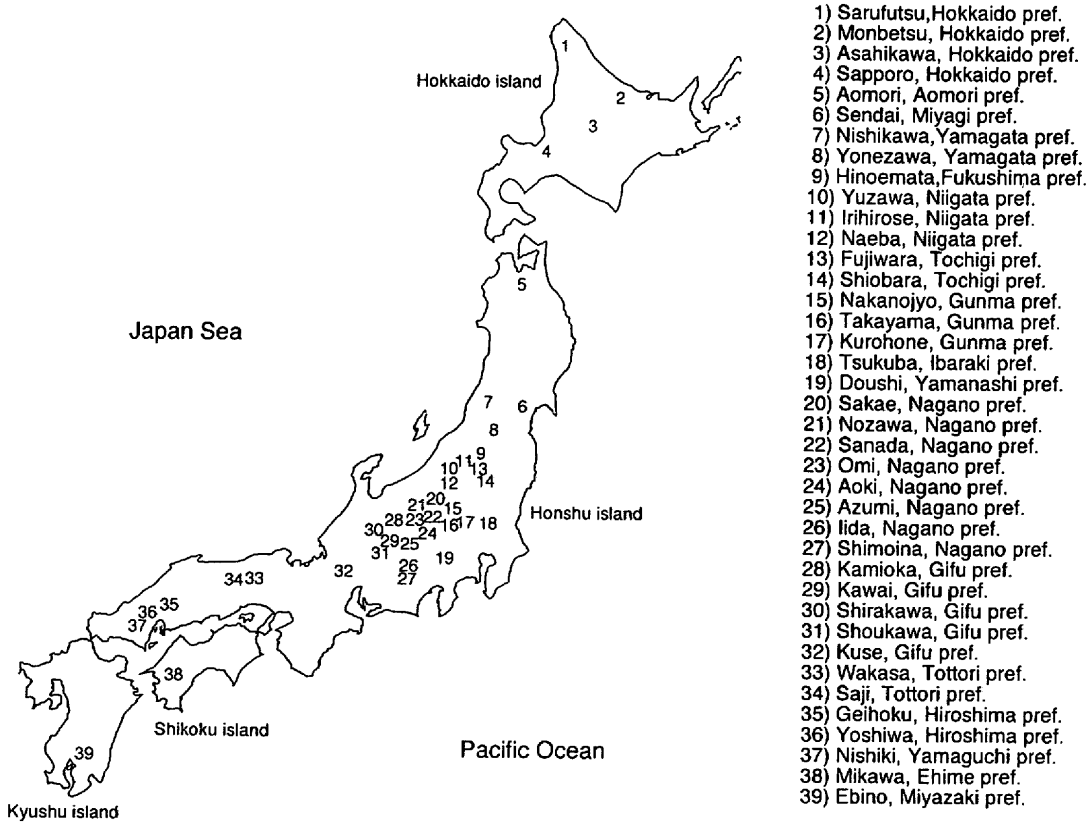

Fig. 1. Map showing localities from which *Macrogerris* water striders were collected.

according to the methods described in Muraji *et al.* (2000).

In addition to *Macrogerris* water striders, the sequence was analyzed using *Gerris* (*Gerris*) *latiabdominis* collected from three localities; Niseko, Hokkaido pref., Tsukuba, Ibaraki pref., and Kamiyaku, Kagoshima pref. These data were used as outgroups in the phylogenetic analyses. Some of nucleotide sequences obtained in this study were submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases (Accession number: AB051392–AB051395, AB059398, AB059399).

Nucleotide sequences were aligned using the program TreeAlign (Hein, 1990). Basic sequence statistical data were computed using MEGA, version 1.01 (Kumar *et al.*, 1993). This program was also used for neighbor-joining analyses based on the Jukes-Cantor, Tamura, Tamura-Nei, and Kimura 2-parameter distances. Maximum parsimony analyses were performed with PAUP* version 4.0 b4a (Swofford, 2000), using an heuristic search procedure with TBR swapping. The support for each node was assessed by bootstrapping, performing 1000 replications.

In order to determine the geographic distribution of mtDNA types, PCR-RFLP analyses were also performed using insects collected from 35 localities in Japan. To do this, a 498 bp long mtDNA fragment were amplified using a set of previously reported PCR primers, CYTR1 and CYTF2 (5′-TAGGATATGTTTTACCTTGAGGACA-3′) (Muraji *et al.*, 2000). The primer CYTF2 anneals nucleotide sites within the section of mtDNA used for the sequencing. The amplified fragment was treated with restriction enzyme *Mse*I (Gibco BRL) according to the manufacturer's instructions, electrophoresed in 3% MetaPhor™ agarose (FMC BioProducts) gel in 1 X TBE buffer at 120 volts (5.5 volts/cm) for 2 hours, then visualized by staining with ethidium bromide. This enzyme was used because aligned sequences showed it to be useful for discriminating among mtDNA lineages.

Morphological characteristics

Observation of external morphology was performed using male adults whose mtDNA type had been determined by DNA sequencing or PCR-RFLP analyses. In order to analyze morphological differences precisely, the lengths of 10 different traits were measured using 103 male adults collected from populations for which mtDNA types had been determined using representative individuals. The characters measured are summarized in Fig. 2.

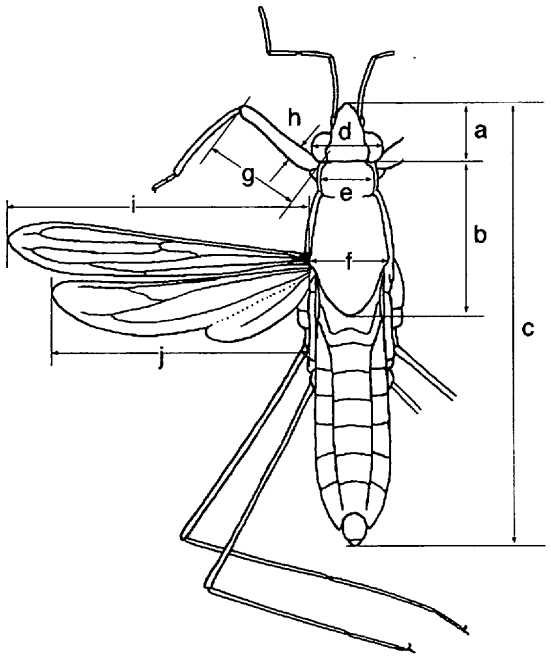

Fig. 2. Morphological characters measured in this study. a: head length, b: length of pronotum, c: body length, d: head width, e: width of anterior pronotum, f: maximum width of pronotum, h: maximum width of fore femur, g: length of fore femur, i: fore wing length, and j: hind wing length.

Observation of mating behavior

Twenty to thirty adults collected in June 1999 from each of three local populations (Shiobara, Tochigi pref., Tsukuba, Ibaraki pref., and Iida, Nagano pref.) were used for the observation. Mitochondrial DNA types of these populations were determined using several representative insects. They were reared in same-sex groups using plastic containers (diameter: 15.0 cm, depth: 9.2 cm) containing dechlorinated tap water (to 0.5 cm depth) and several pieces of styrene foam as resting sites. They were fed various kinds of small insects that were collected in the field and stored in a deepfreezer (−80°C). Food and fresh water were given daily. For observation of mating behavior, one female and two male adults were transferred into a clear plastic cup (diameter: 9.5 cm, depth: 4.5 cm) with a transparent lid, containing water and pieces of styrene foam. For analyses of mating compatibility, the frequency of behaviors in which males grabbed the female, mounted the back of the female, and inserted the genitalia into the female ovipositor were examined for nine different combinations of insects collected from the three localities. In addition, the duration of copulation, in which males remain in place on the back of a female inserting genitalia into the female ovipositor, was also examined. After 3 or 4 hour observation, females and males were separated and reared in

same-sex groups until the next observation. Each female was used for 3–8 times of successive observations. In each observation, they were paired with various males collected from different localities.

Results

mtDNA sequence

Nucleotide sequences of the mitochondrial cyt *b* gene were determined using 15 male adults collected from 15 different populations. Table 1 shows variable nucleotides detected in the 728 bp portion of the gene that correspond to bp 10608–11335 of the *D. yakuba* mtDNA sequence (X03240). These sequences showed no insertion/deletion mutations, and were aligned unambiguously by TreeAlign. Base substitutions were detected at 100 nucleotide sites. Of these, 14, 2, and 84 sites were at the first, second, and third position of codons, respectively. These sequences were homogeneous in terms of deduced amino acid sequences, and only five variable amino acid sites were detected. Nucleotide sequences of *Macrogerris* water striders showed an apparent A+T content bias, which is a characteristic of insect mitochondrial DNA (Simon *et al.*, 1994). The relative frequencies of different nucleotides were as follows: A: 34.1%, T: 37.8%, C: 14.7%, T: 13.4%. This tendency was most obvious at the third position of codons, where 88.6% of all nucleotides were A or T. Pairwise comparisons of the sequences revealed that base substitutions were biased for T–C transitions. The relative frequencies of different types were as follows: A–G: 16.1%, T–C: 54.4%, A–T: 25.5%, A–C: 3.4%, T–G: 0.6%, C–G: 0.0%. The T–C transition bias was observed at all codon positions.

Among sequences obtained in this study (Table 1), five major groups of haplotypes were detected. Two of them corresponded to *G. gracilicornis* and *G. yezoensis*, respectively. The other three groups were detected among insects showing conventionally recognized diagnostic characteristics of *G. insularis*. In this study, the three groups were designated as types A, B, and C. Table 2 shows the number of variable sites between pair of nucleotide and deduced amino acid sequences. This table indicates that genetic distances among the three groups (26–44 variable nucleotide sites) were larger than that between the different species *G. gracilicornis* and *G. yezoensis* (9–12 variable nucleotide sites).

In order to estimate relationships among the different groups of *Macrogerris* water striders, phylogenetic analyses were performed using aligned sequences. In these analyses, corresponding cyt *b* sequences of *G.* (*G.*) *latiabdominis* were included as outgroups. Figure 3 shows the molecular phylogenetic tree generated by the neighbor-joining method based on Jukes-Cantor distances. In this study, nucleotide composition and substitution biases were evident in the cyt *b* sequences. Thus, the neighbor-joining analyses were also performed based on Tamura, Tamura-Nei, and Kimura 2-parameter distances. However, all of these analyses produced the same results in terms of the topology of phylogenetic trees. In parsimony analyses, the unweighted parsimony method produced the most parsimonious 4 trees with a length of 184 steps and a consistency index of 0.799. Among these, differences were seen only in relationships among populations of *G. latiabdominis*. In order to minimize the effect of the nucleotide composition and substitution biases, various weighting schemes were applied to the aligned sequences. Initially, weighted parsimony analyses were performed by changing relative weights among different codon-positions. Subsequently, down-weighting the C–T transition (1/2, 1/4, 1/8, and 0/1 relative to other types of base substitution) was applied. However, these weighting schemes did not alter the topologies of phylogenetic trees from unweighted parsimony analyses. Topology of these trees agreed well with those of trees generated by the neighbor-joining analyses, except that the parsimony methods could not resolve relationships among types A, B, and C unambiguously.

In the molecular phylogenetic trees (Fig. 3), Japanese *Macrogerris* species were divided into five major groups. Among these, *G. gracilicornis* and *G. yezoensis* formed a monophyletic clade. The other three groups, types A, B, and C, formed a sister clade to *G. gracilicornis*+*G yezoensis*. Among those, water striders of types B and C formed a sister clade to type A insects.

Figure 4 shows the banding patterns of the cyt *b* fragment treated with the restriction enzyme *Mse*I. The picture indicates that the three mtDNA types can be easily identified by PCR-RFLP analysis. When this analysis was applied to 105 insects collected from 35 localities (i.e., 3 individuals/site) in Japan (Fig. 5), it was revealed that geographic distribution of type A sequences widely overlapped with the other two types, whereas those of type B and C were divided between northeastern Honshu island and western Japan, including Kyushu island and the central to western region of Honshu island, respectively.

Morphological characters

Among the three known Japanese species of the

Table 1. Variable nucleotide sites detected among 15 male adults of Japanese water strides belonging to the subgenus *Macrogerris*.

Species/collection sites	Nucleotide position 1111111111222222222222223333333333333444444444444444555555555555555556666666666666667777 1122235667890111567889123344555667899012334566778001223455889990011223334556678891112333446678990012 5914794062408147684065051501128126847381362469561581698719043584439281470284732843451149033657032510
Type A/Asahikawa	CCGTGCTCCTCTTCTCAAACTCAACCTCCAAAGTTTACAGAACCCCTCTGAATAACCTGCGCTTATTATCAAACCAGTTTCGCTTACCTCATCACATATA
Type A/Sakae	.....T.....C.....
Type A/Mikawa	.....T.....T.....G.....
Type B/Aomori	..AAAT..T.T.CT.T...T.T..T.....T.....TT....TTT.CT.AT..C..T..TAT...C..C ....T.....T...C....TT.TT.TCT..
Type B/Shiobara	..AAAT..T.T..T.T...T.T..T.....T.....TT.A..TTT.CT..T..C..T..TAT...C..C.....TG....T.....TT.TT.TCT..
Type B/Kurohone	..AAAT..T.T..T.T...T.T..T...T.T.....TTGA..TTT.CT..T..C..T..TAT...C.....T.....T.....TT.TT.TCT..
Type C/Doushi	A.A.AT..T.TC.T.T.....T.C.....T..A..TT..CT.ATGCCGTT.ATAT...C.....CTTTTC..T
Type C/Wakasa	A.A.AT..TCTC.T.T.G.....T.C.....T..A..TT..CT.ATGCCGTT.ATAT...C.....C.....C..CTTTTC.CT
Type C/Nishiki	A.A.AT..TCTC.T.T.....T.C.....T..AG.TT..CT.ATGCCGTT.ATAT...C.....CC.....C..CTTTTC.CT
G. gracilicornis/Sarufutsu	TTA...ATACT..T.TG.T.CTTTTT.T.G.TAAA.TT.A..TT.T.TA.G..C.TTC.T.ACCC.A..T.TTTT.ACC.TATA.TTT.T..T.TCCT..
G. gracilicornis/Sakae	TTA...ATACT..T.TG.T.CTTTTT.T...TAAACTT.A..TT.T.TA.G..C.TTC.T.ACCC.A..T.TTTT.ACC.TATA.TTT.T..U,UCCT..
G. gracilicornis/Yoshiwa	TTA...ATACT..T.TG.T.CTTTTT.T...TAAACTT.A..TT.T.TA.G..C.TTC.T.ACCC.A..T.TTTT.ACC.TATA.TTT.T..T.TCCT..
G. yezoensis/Sapporo	T.A...ATACT..TCTG.TTCTTTTT.T.G.TAAACTT.A..TT.T.TAAT..T.TTCAT.ACCC.AG.T.TTTT.ACC.TATA.TTT.T..T.TCC...
G. yezoensis/Nakanojyo	T.A...ATACT..TCTG.TTCTTTTT.T...TAAACTT.A..TT.T.TAAT..T.TTCAT.ACCC.AG.T.TTTT.ACC.TATA.TTT.T..T.TCC...
G. yezoensis/Nozawa	T.A...ATACT..TCTG.TTCTTTTT.T...TAAACTT.A..TT.T.TAAT..T.TTCAT.ACCC.AG.T.TTTT.ACC.TATA.TTT.T..T.TCC...

Nucleotides identical to the reference, type A insect collected in Asahikawa, are indicated by a dot.

Table 2. Number of variable sites between pair of nucleotide (below the diagonal) and deduced amino acid (above the diagonal) sequences.

		:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.	Type A/Asahikawa	:		0	0	1	1	2	1	1	1	3	3	3	3	3	3
2.	Type A/Sakae	:	2		0	1	1	2	1	1	1	3	3	3	3	3	3
3.	Type A/Mikawa	:	3	5		1	1	2	1	1	1	3	3	3	3	3	3
4.	Type B/Aomori	:	39	41	38		0	1	0	0	0	4	4	4	4	4	4
5.	Type B/Shiobara	:	38	40	37	5		1	0	0	0	4	4	4	4	4	4
6.	Type B/Kurohone	:	38	40	37	7	4		1	1	1	5	5	5	5	5	5
7.	Type C/Doushi	:	38	38	39	27	26	26		0	0	4	4	4	4	4	4
8.	Type C/Wakasa	:	42	42	43	31	30	30	4		0	4	4	4	4	4	4
9.	Type C/Nishiki	:	43	43	44	32	31	31	5	3		4	4	4	4	4	4
10.	*G. yezoensis*/Sapporo	:	68	70	67	61	60	60	63	65	64		0	0	0	0	0
11.	*G. yezoensis*/Nakanojyo	:	67	69	66	60	59	59	62	64	63	1		0	0	0	0
12.	*G. yezoensis*/Nozawa	:	67	69	66	60	59	59	62	64	63	1	0		0	0	0
13.	*G. gracilicornis*/Sarufutsu	:	63	65	62	60	57	57	64	66	65	11	12	12		0	0
14.	*G. gracilicornis*/Sakae	:	64	66	63	59	56	56	63	65	64	10	9	9	3		0
15.	*G. gracilicornis*/Yoshiwa	:	64	66	63	59	56	56	63	65	64	10	9	9	3	0	

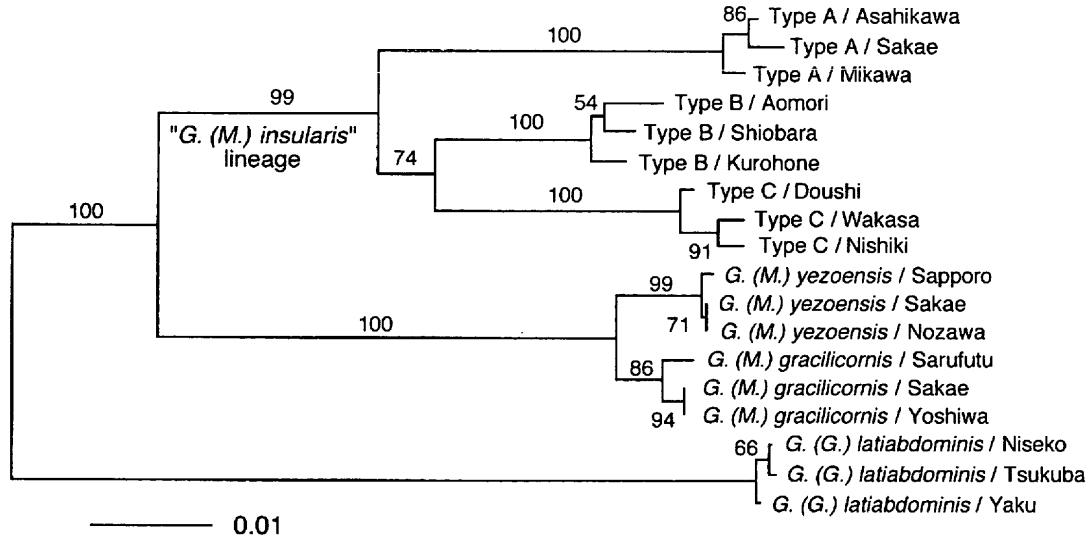

Fig. 3. Phylogenetic tree of Japanese *Macrogerris* water striders generated by the neighbor-joining method based on Jukes-Cantor distances. Three populations of *G.* (*Gerris*) *latiabdominis* were used as outgroups. Bootstrap confidence limits are shown above the branches (1,000 replications).

subgenus *Macrogerris*, *G. insularis* can be discriminated from other species based on spinules on the abdominal sternum 7, and the shape of the ventral sclerite of vesica and abdominal sternum 7 of male adults (Andersen, 1993). In this study, mtDNA types of 120 male adults showing diagnostic characteristics of *G. insularis* were determined by DNA sequencing or PCR-RFLP analyses. Although all these samples were homogeneous in terms of the diagnostic characters mentioned above, insects showing the type A sequence were clearly different from insects of the other two types in the shape of the fore femur (Fig. 6 c) and the color pattern of the pronotum (Fig. 6b). In addition, the shape and length of the hind wings were considerably different between type A and other types; type A insects showed only long and wide hind wings, whereas types B and C showed apparent dimorphism, containing both long- and short-winged individuals within a population (Fig. 6d).

In order to compare morphological characteristics more precisely, 10 morphological traits (Fig. 2) were measured in 102 male adults collected from 35 populations for which mtDNA types had been determined using representative insects. For each mtDNA type, 9–16 populations were used (1–6 individuals/population). Results are summarized in Table 3. Significant differences were detected in all of the 10 characters between type A and the other types. Figure 7 shows

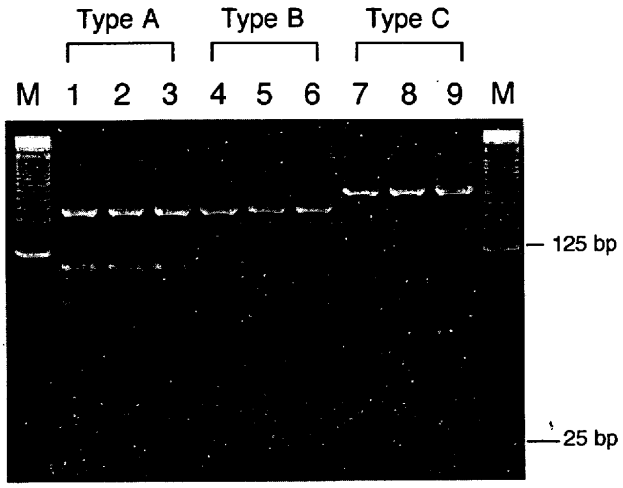

Fig. 4. Banding patterns of the PCR amplified fragment of the mitochondrial cyt *b* gene treated with the restriction enzyme *Mse*I. Lane 1: Asahikawa, Hokkaido pref., Lane 2: Sakae, Nagano pref., Lane 3: Mikawa, Ehime pref., Lane 4: Aomori, Aomori pref., Lane 5: Shiobara, Tochigi pref., Lane 6: Kurohone, Gunma pref., Lane 7: Doushi, Yamanashi pref., Lane 8: Wakasa, Tottori pref., Lane 9: Nishiki, Yamaguchi pref., Lane M: DNA size marker (25 bp ladder).

that type A and the other types can be clearly discriminated by the shape of the fore femur and the pronotum. Although significant differences were also observed between types B and C in many of the characters examined (Table 3), the length of these characters overlapped by a large amount between these two types (Fig. 7).

Mating behavior

The general sequence of mating behavior observed under laboratory conditions consisted of the following four phases. 1) Males approached a feeding or walking female without any apparent courtship behavior and grabbed a part of female body (mainly legs or abdomen) using the fore legs. 2) Males moved slowly along the female body using fore legs then mounted the back of the female. 3) Males extended the genitalia and inserted it into the female ovipositor, then remained in the same posture. 4) Males withdrew the genitalia from the female and remained in the same posture. During these phases, most females tried to reject copulation, and many of the males were thrown

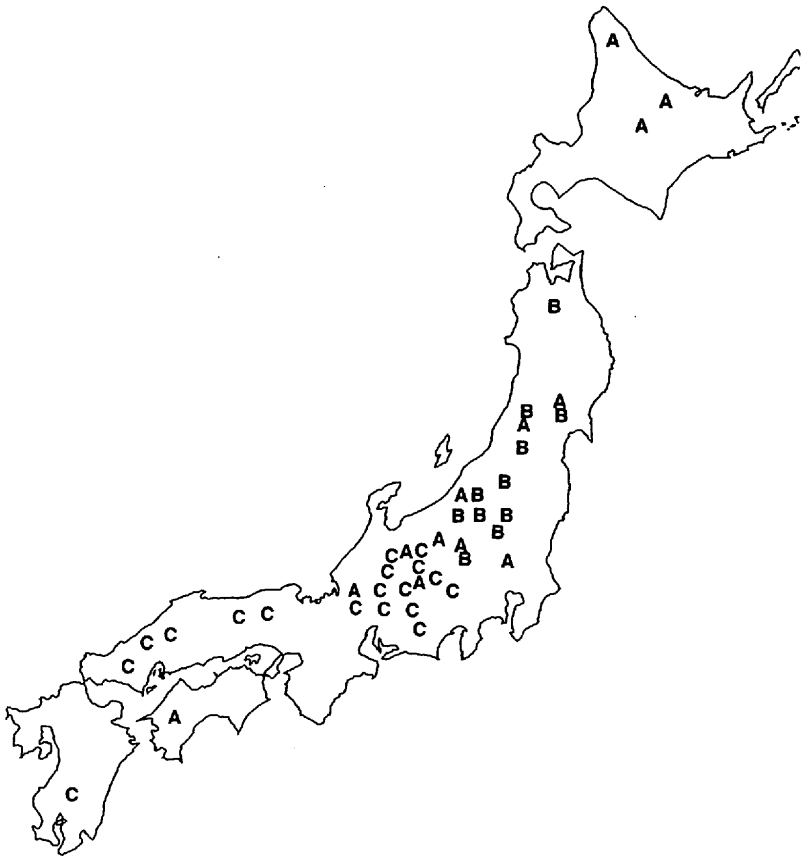

Fig. 5. Distribution of the three different mitochondrial lineages detected among water striders exhibiting conventionally recognized diagnostic characteristics of *G. insularis*. The mitochondrial haplotype of individual insects was determined by DNA sequencing or PCR-RFLP analysis.

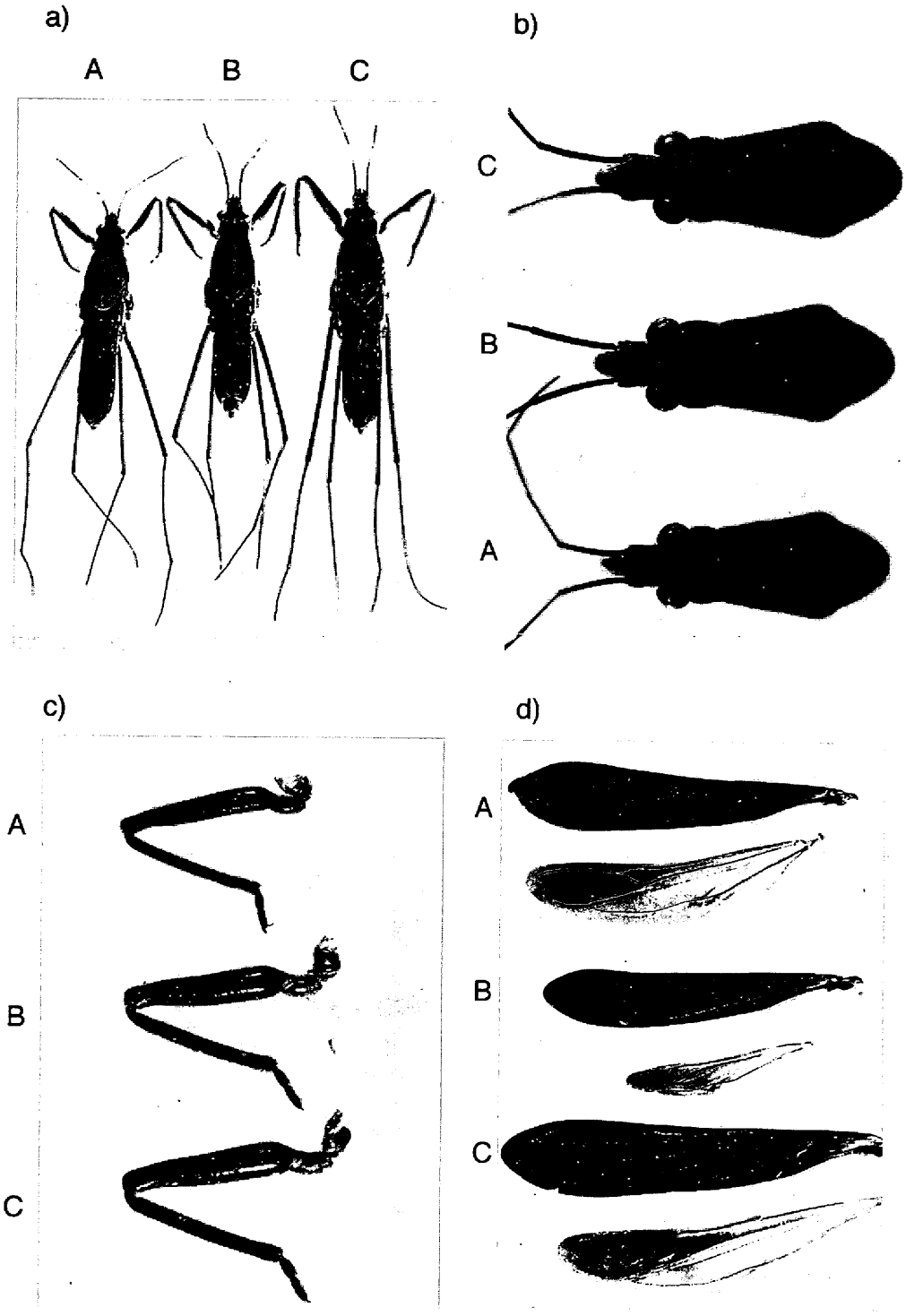

Fig. 6. Pictures showing differences in external appearance in the whole body (a), head and pronotum (b), fore leg (c), and fore and hind wings (d) among male adults of water striders exhibiting conventionally recognized diagnostic characteristics of *G. insularis*. A: type A (Sakae Nagano pref.), B: type B (Naeba, Niigata pref., short-winged individual), C: type C (Iida, Nagano pref., long-winged individual).

off the female in the course of mating behavior. Thus, the duration of phases 3 and 4 varies from several seconds to several hours.

In this study, the frequency of matings that reached several different phases was examined between female and male adults collected at several different localities. The results are summarized in Table 4. The three populations, Tsukuba, Shiobara, and Iida, included in this observation had mtDNA sequences of type A, B, and C, respectively (Fig. 5). This table indicates that

Table 3. Measurements of morphological characters of male adults collected from populations for which mtDNA type was determined by DNA sequencing or PCR-RELP analysis of representative insects.

Characters	Type A			Type B			Type C		
	min.–max.	mean±S.E.	n	min.–max.	mean±S.E.	n	min.–max.	mean±S.E.	n
a. Head length	1.11– 1.56	1.32a±0.016	33	1.19– 1.67	1.46b±0.020	35	1.31– 1.72	1.55c±0.017	34
b. Length of pronotum	3.47– 3.89	3.69a±0.019	33	3.68– 4.11	3.87b±0.022	35	3.89– 4.32	4.08c±0.022	34
c. Body length	10.04–11.19	10.51a±0.044	33	10.49–11.93	11.11b±0.061	35	10.82–13.38	11.70c±0.086	34
d. Head wigth	1.50– 1.67	1.58a±0.007	33	1.67– 1.81	1.75b±0.007	35	1.72– 1.89	1.79c±0.009	34
e. Width of anterior pronotum	1.28– 1.56	1.38a±0.010	33	1.56– 1.67	1.60b±0.007	35	1.56– 1.81	1.67c±0.010	34
f. Maximum width of pronotum	1.97– 2.17	2.07a±0.010	33	2.00– 2.28	2.15b±0.012	35	2.11– 2.50	2.28c±0.015	34
h. Maximum width of fore femur	0.41– 0.50	0.45a±0.003	33	0.54– 0.66	0.59b±0.005	35	0.53– 0.66	0.60b±0.005	34
g. Length of fore femur	2.63– 2.97	2.81a±0.014	33	2.79– 3.26	3.07b±0.020	35	2.87– 3.34	3.13b±0.019	34
i. Fore wing length (LW)	6.11– 7.05	6.70a±0.030	33	6.63– 8.00	7.22b±0.095	16	6.95– 8.21	7.55c±0.054	29
i. Fore wing length (SW)				5.53– 6.97	6.27a±0.080	18	6.16– 6.79	6.51a±0.134	5
j. Hind wing length (LW)	5.58– 6.21	5.90a±0.024	33	5.89– 7.11	6.33b±0.068	16	5.95– 7.05	6.51c±0.048	29
j. Hind wing length (SW)				3.79– 4.95	4.27a±0.066	18	4.42– 4.74	4.52b±0.058	5

LW and SW refer the results obtained from long- and short-winged males, respectively.
Means in a row with the same letter are not significantly different at 5% level in Kruskal-Wallis test or Mann-Whitney's U-test (fore and hind wings of short winged individuals).
Number of populations examined were; type A: 10, type B: 9, and type C: 14.

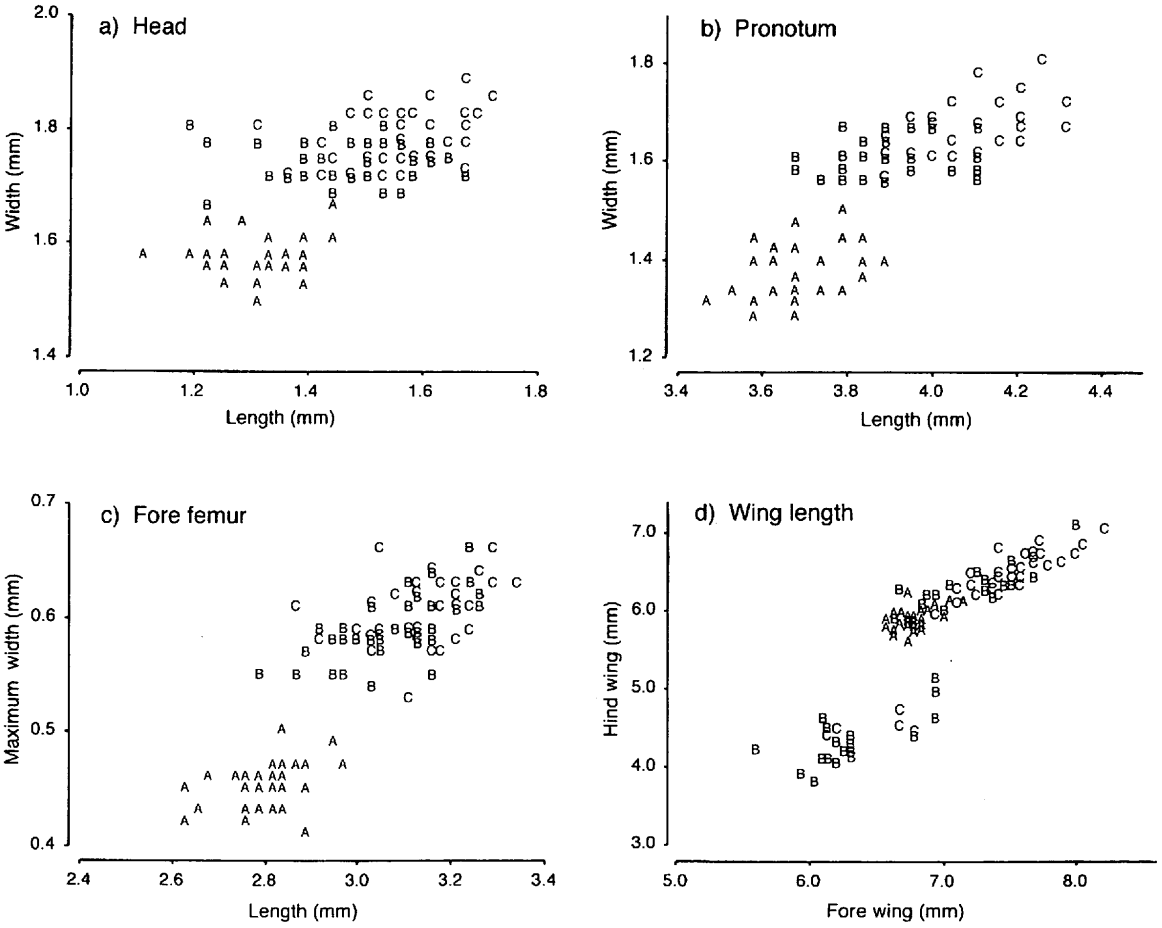

Fig. 7. Variations in the length of morphological characters measured for the head (a), pronotum (b), fore femur (c), and wings (d) of male adults collected from populations for which mitochondrial haplotypes were determined by DNA sequencing or PCR-RFLP analyses of representative insects.

Table 4. Frequency of mating behaviors that reached several different phases under laboratory conditions. Observations were made using insects originated from three local populations that are revealed to have mitochondrial cyt *b* sequences of type A, B, and C.

Female×Male	Mounting (%)	Genitalia insertion <1 hour (%)	Genitalia insertion >1 hour (%)	Total number of matings observed	Total number of pairs used
A×A	69.4	57.7	40.0	85	40
A×B	29.6	9.9	0.0	71	17
A×C	43.0	21.5	1.3	79	31
B×A	23.0	8.1	5.4	74	21
B×B	55.8	44.2	32.6	43	28
B×C	39.0	34.2	26.8	41	22
C×A	38.1	21.4	11.9	84	22
C×B	62.1	56.9	39.7	58	44
C×C	63.2	46.1	26.3	76	42

Populations used were; Tsukuba, Ibaraki prefecture (type A), Shiobara, Tochigi prefecture (type B), and Iida, Nagano prefecture (type C). The type of mitochondrial cyt *b* sequence was determined by the DNA sequencing or PCR-RFLP analysis of representative insects.

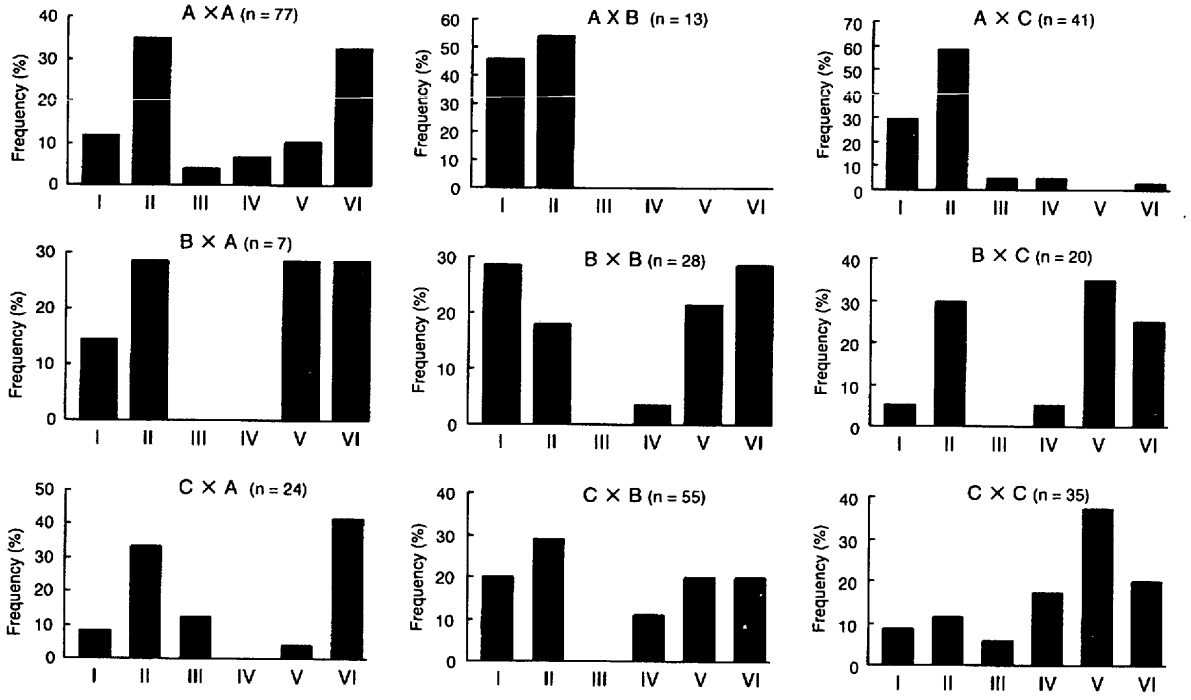

Fig. 8. Duration of copulations observed between insects collected from three different populations, Tsukuba, Shiobara and Iida, that were revealed to have mitochondrial cyt *b* sequences of type A, B, and C, respectively. Characters joined by an "×" sign indicate mtDNA types of insects (female×male). Roman numerals indicate the duration of copulation. I: <1 min. II: 1–10 min. III: 11–30 min. IV: 31–60 min. V: 1–2 hr. VI: ≥2 hr.

males of type A mounted and copulated with females of type A at considerably higher frequencies than with those of types B or C ($\chi^2=45.67$, 78.01, and 49.24 for mountings, genitalia insertions <1 hour, and genitalia insertions >1 hour, respectively. df=2, $P<0.001$). Similarly, males of type B and C copulated much more successfully with females of types B or C (Type B male: $\chi^2=17.31$ ($P<0.001$), 61.51 ($P<0.001$), and >232.36 ($P<0.001$) for mountings, genitalia insertions <1 hour, and >1 hour, respectively. df=2. Type C male: $\chi^2=9.29$ ($P<0.01$), 11.60 ($P<0.01$), and 149.59 ($P<0.001$). df=2). Of a total of 150 males of types B and C that attempted to mate with type A females, only one succeeded in inserting his genitalia into the female ovipositor for longer than 1 hour. All others were strongly resisted by females of type A lineage. With regard to the relationship between types B and C, no significant differences were

detected among frequencies of mating behaviors observed between and within these types ($\chi^2=7.46$, 5.43, and 3.10 for mountings, genitalia insertions <1 hour, and >1 hour, respectively. df$=3$. $P>0.05$).

Figure 8 presents a comparison of the duration of copulation (phase 3) among nine different combinations of insects. In this figure, many of the combinations showed a bimodal frequency distribution, consisting of two peaks at copulation times shorter than 10 min and longer than 1 hour. However, when males of type B and C were paired with females of type A (A×B and A×C), more than 90% of copulations ended within 10 min. Copulations of this kind became less frequent when they were paired with females of type B and C. On the other hand, similar patterns were found in the frequency distribution among combinations including type A males (A×A, B×A, and C×A).

Discussion

According to the biological species concept advocated by Mayr (1940), species are groups of actually or potentially interbreeding natural populations that are reproductively isolated from other groups. Under laboratory conditions, females of type A rejected almost all males of the other two types after a very short period of copulation (Table 4 and Fig. 8). Males of this type copulated with females of other types, but only at frequencies significantly lower than those within the same type. Tachikawa (personal communication) made observations of water striders of this group copulating in the field at several localities in Japan. He found that all type A insects copulated with insects of the same type even in habitats where type A insects coexist with insects of other types. Thus, some degree of precopulatory reproductive isolation appears to exist between type A and the other types. The results of morphological analyses also showed that type A insects were morphologically distinct among the three types (Fig. 7). Thus, this lineage seems to meet biological, morphological, and evolutionary criteria for species status (Wiley, 1978). These results strongly suggest that there are at least two distinct species among Japanese water striders exhibiting conventionally recognized diagnostic characteristics of *G. insularis*. The external appearance of type A insects seems to fit with the description of several morphological characteristics, such as color pattern of head, of *G. insularis* (Miyamoto, 1961).

With regard to the relationship between types B and C, no clear differences were detected in the shape of the several body parts measured in this study (Fig. 7). Copulation can occur freely between males and females of these different types under laboratory conditions (Table 4). However, in order to determine whether they meet the criterion for single species status, further studies are needed to examine mating behaviors of these types under natural conditions at the area where their geographic ranges border each other (Fig. 5), as well as to analyze postcopulatory reproductive isolation between the lineages.

For heteropteran insects including water striders, there are a number of reports on heterospecific mating (Spence, 1990; Zimmermann, 1993; Sperling *et al.* 1997). Spence (1990) reviewed these phenomena and suggested that pre-mating isolation in heteropteran species evolves when negative effects of hybridization, such as lower viability or fertility of hybrids, are expressed between populations that have differentiated in allopatry. Similarly, it may be possible to expect a case in which pre-mating isolation has not evolved between allopatric species that have not re-established contact after geographical separation. Such a situation seems to be the case for the Japanese and North American species of water strider, *Limnoporus esakii* and *L. canaliculatus*, which are distributed on different continents but can copulate under laboratory conditions (Dr. J. R. Spence, personal communication). In the present study, the evolution of pre-mating isolation coincides with geographic relationships among different lineages. Among these, pre-mating isolation is observed only between sympatric lineages (i.e., between types A and B and between types A and C). It was not detected between lineages having an parapatric relationship (i.e., between types B and C). Thus, types B and C might have been separated geogrphically in their evolutionary history, and therefore they have not developed a mate-recognition system.

Considering that mtDNA sequences of these types were divergent, they must have been separated for a long period. For the genus *Limnoporus*, which is a close relative of the genus *Gerris*, the fossil record indicated that intrageneric divergence occurred at least 50 million years ago (Andersen *et al.*, 1993). Sperling *et al.* (1997) examined the mitochondrial cytochrome oxidase subunit I (COI) gene sequence of this group, and indicated that the sequence is evolving more slowly than a commonly employed molecular clock, and that an uncorrected nucleotide divergence of 8.2–9.5% occurred over 50 million years. Recently, Muraji (unpublished) sequenced the mitochondrial cyt *b* gene of two *Limnoporus* species, *L. esakii* and *L. genitalis*, (AB051396 and AB051397). Comparison of

these and previously reported COI sequences of the same species (U83341 and U83339) revealed that the cyt *b* sequence is evolving at almost the same rate as the COI sequence. When such estimates are directly applied to the *Macrogerris*, a divergence time of 18.9–26.8 million years is estimated from an uncorrected nucleotide divergence of 3.6–4.4% detected between type B and C lineages. It is interesting to note that their divergence occurred around the early Miocine. Tectonic and paleomagnetic studies of Japan and the adjacent area indicates that the Japanese islands split from the Eurasian Continent and that the northeastern and southwestern portions of the islands rotated differently as the Japan Sea expanded during this period (Otofuji *et al.*, 1985; Tamaki, 1995). Thus, geographical isolation of these two lineages may have occurred with diastrophic and paleogeographic events during this period. Water striders of lineages B and C usually live in highlands at altitudes of higher than 1,000 meters. Although habitats of the type A lineage overlap with those of other lineages in vertical distribution (Tachikawa, 1999), this lineage mainly lives in mountainous areas lower than 500 meters above sea level. Thus, the movement of the earth crust may have influenced distribution areas of lineages B and C more strongly than that of type A lineage.

The tectonic zone separating northeastern and southwestern parts of the Japanese islands is known as the Fossa Magna, whose eastern and western borders are denoted by the Itoigawa-Shizuoka and Kashiwazaki-Chiba Tectonic Lines, respectively, which traverse the central part of Honshu island from the Japan Sea to the Pacific Ocean. Thus, geographic areas of the type B and C lineages seem to be separated by the Fossa Magna (Fig. 5). Similar phenomena have been reported for several insects, such as damselfly, ground beetle, and firefly, in which distribution areas of sibling species (Suzuki, 1984), and genetically and behaviorally distinct strains (Su *et al.*, 1998; Suzuki, 1997) are separated along the tectonic zone. In the case of the ground beetle, *Damaster bloptoides*, Su *et al.* (1998) estimated a divergence time of 16.8 ± 1.2 million years between phylogenetically distinct two lineages based on the mitochondrial NADH dehydrogenase subunit 5 gene sequences and a molecular clock calibrated using other carabid species. Their estimate is roughly similar to that between lineages B and C of *Macrogerris* water striders, although these estimates were based on nucleotide sequences and molecular clocks of different kinds. These results suggest a relationship between insect evolution and diastrophic events occurred in the Japanese islands during the early to middle Miocene.

Although mtDNA sequences of the three lineages were considerably divergent (Fig. 3), morphological differences were evident only between type A and the other two types (Fig. 7). Thus, between the type B and C lineages, morphological evolution seems to proceed more slowly than with respect to mtDNA sequences. Although morphological differences are usually apparent between distantly related species, these are not necessarily linked to evolution of mtDNA sequences. A possible explanation for the discordance is that the mtDNA of types B and C are evolving very quickly, perhaps due to natural selection acting on the mtDNA. However, considering that 89% of nucleotide substitutions among the three types occurred at the third position of codons and that these sequences were almost identical in terms of resulting amino acid residues, this explanation is unlikely. On the other hand, it may be possible that morphological characteristics are diverging very slowly between lineages B+C. Such a phenomenon can occur between a pair of allopatric species that have been under similar conditions of natural selection during their evolutionary history after geographical separation. As far as the author observed, there are no apparent differences in habitat preference, life cycles, and mating and foraging behaviors of these lineages between northeastern and southwestern parts of Honshu island (Muraji, unpublished). Thus, evolutionary factors may not have differentiated these lineages morphologically.

Another possible explanation is that the morphological characteristics have been homogenized by genetic introgression between types B and C. Under this hypothesis, mtDNA and nuclear DNA are considered to be exchanged asymmetrically between populations in the type B and C lineages. Because mtDNA is maternally inherited, this phenomenon can occur when viability or fertility of hybrids are different between reciprocal crosses. As was reported for hybridization within several insect groups (Hall & Muralidharan, 1989), such a phenomenon is also expected when hybridization is occurring between populations in which dispersal ability differs between sexes. Such an explanation may also be possible for the relationship between lineages B and C, which are distributed in contiguous geographic areas and have not developed a precopulatory reproductive isolation system. Thus, the difference between mtDNA sequence and morphological traits may reflect secondary contact of distribution areas after their mtDNA sequences differentiated in allopatry. In order to test this hypothesis, further analyses must be performed to

examine genetic population structures in terms of both nuclear and mtDNA sequences, and to determine the pattern of postcopulatory reproductive isolation between these types.

In this study, three distinct mitochondrial lineages were detected among water striders exhibiting conventionally recognized diagnostic characteristics of *G. insularis*. Morphological and behavioral analyses indicated that there are at least two species among them (i.e., type A and others). However, these analyses could not determine whether the remaining two lineages (types B and C) are different species. Considering the number of variable nucleotide sites, they were apparently divergent, and as clearly related as different species of *Macrogerris* (Table 2). In order to determine whether they are biological species, further analyses are needed to examine whether they exchange genetic materials. Nevertheless, as Spence & Andersen (1994) pointed out, the biological species concept might not be appropriate to determine the relationship between the two lineages clearly. Due to hybridization and reticulate evolution, biological species in several groups of water strider do not always coincide with morphologically and/or genetically recognized species. Thus, at present, it seems more important to accumulate genetic, ecological, and evolutionary data on these lineages than to determine hastily whether these lineages are different species.

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