

Comparison of Mitochondrial DNA Sequences among Japanese Cicadas, with Special Reference to Three Tibicenine Species

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Abstract. Nucleotide sequences of cytochrome oxidase I gene were compared among Japanese Cicadidae species. Phylogenetic analysis based on these sequences indicated that *Cryptotympana facialis* (Walker) and *C. atrata* Fabricius were closely related, regardless of their difference in each song. Considerable resemblance was also found between these species and *Tibicen japonicus* (Kato). Two Cicadinini species, *Terpnosia vacua* (Olivier) and *Tanna japonensis* (Distant) formed a group. These results were in accordance with morphology-based classification.

Key words: Cicada, Tibicenini, DNA sequence.

More recently, occurrence of *Cryptotympana atrata* Fabricius, a cicada species ranging from Korea, China, Formosa to the northern part of Southeast Asia, but unknown from Japan, was found in Kanazawa City (Tokumoto et al., 2001). Adult insect of this species is morphologically similar to *C. facialis* (Walker), but song of *C. atrata* resembles that of *Tibicen japonicus* (Kato), not of *C. facialis*. In order to elucidate the relationship among these three species, and to provide for future elucidation of the origin of *C. atrata* in Kanazawa, analysis was made on nucleotide sequences of the gene for mitochondrial cytochrome oxidase subunit I (COI). For comparison, 7 other cicada species, together with another hemipteran insect *Halyomorpha picus* Fabricius (Pentatomidae) were also subjected to nucleotide sequencing.

Materials and Methods

Specimens analyzed

Except for *Cryptotympana facialis* caught by H.F. at Yamatokohriyama, Nara, 10 hemipteran species (imagos) were captured by A.T.: *Platypleura kaempferi* and *Tanna japonensis* from Shicho-mati, Komatsu, *Cryptotympana atrata* from Hatta, Kanazawa, *Tibicen japonicus* from Kigoyama, Kanazawa, *Graptopsaltria nigrofuscata*,

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Oncotempana maculaticollis, *Meimuna opalifera* and *Haryomorpha picus* from Ishibiki, Kanazawa, *Terpnosia vacua* from Utatsuyama, Kanazawa, and *Cicadetta radiator* from Togi, Ishikawa Pref.

Extraction of DNA

Flight muscle was removed from each adult insect, minced, and DNA was extracted using DNeasy tissue Kit (QIAGEN): About 50 mg of the muscle was treated with proteinase K in a detergent solution, then with RNase A, and purified by silica gel chromatography. Concentration of each DNA was determined from UV absorbance, with Gene Quan II (Pharmacia Biotech).

Amplification and purification of COI region

A 554 base pairs region of mitochondrial cytochrome oxidase subunit I gene was amplified, with forward (CARCAYYTRTTYTGATTYTTYG) and reverse (RAARTGYTG NGGRAARAADGT) primers, using *Taq* DNA polymerase (QIAGEN). Polymerase chain reaction (PCR) was performed under the condition of denaturation (at 94°C for 1 min), annealing (45°C, 30 min), and extension (72°C, 1 min), with Programtemp Control Ssystem PC-707 (Astec). For check of chain length and purity, each PCR product was subjected, together with size markers, to 2% agarose gel electrophoresis at 100 V for 45 min, using a Mupid 21 apparatus (Cosmo BIO). The PCR products were purified using QIA quick PCR Purification Kit, according to supplier's recommendation.

Sequencing and data analysis

DNA sequencing was carried out by dideoxy method, using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, template DNA, and the above mentioned primers. The reaction conditions were as follows : denaturation (at 96°C for 10 sec), annealing (45 °C, 5 sec), and extension (60°C, 4 min). After 25 cycles reaction, the products were purified by ethanol precipitation, denatured at 95°C, and then loaded onto a DNA sequencer (ABI PRISM 310 Genetic Analyzer). The nucleotide sequence data were analyzed using Sequencing Analysis 3.3, Auto Assembler 2.1 and GENETIC-MAC ver. 10.1 softwares.

Results and Discussion

The nucleotide sequences (total 554 residues) of COI gene of 10 cicada species were compared in Fig. 1, with that of *Halyomorpha picus* (belonging to the same order

Hemiptera, but different family Pentatomidae). From these data, each amino acid sequence was deduced and analyzed as well.

In Fig. 2, numbers of substituted nucleotides among the 11 hemipteran species were compared. When compared with *Halyomorpha picus*, the cicada species (Hemiptera, Cicadidae) varied 18.8 - 21.5%, in the nucleotide sequences, and 14.1 - 17.4% in the amino acid sequences (Table 1). Between *Cicadetta radiator* (Subfamily Tibicininae) and other 9 cicadas (Subfamily Cicadinae), substitution was 14.6 - 17.9% in nucleotides, and 10.3 - 13.6% in amino acids. Among these hemipteran insects, transversion type was somewhat more frequent than transition.

Based on the nucleotide sequences, phylogenetic relationship among the Cicadidae species was analyzed by neighbor joining (NJ) method (Fig. 3), using *Haliomorpha picus* (A) or *Cicadetta radiator* (B), as the out group. Although song of *Cryptotympana atrata* closely resembles that of *Tibicen japonicus*, the former is morphologically similar to *C. tympana*. Besides morphological similarity in the imagos, exuviae of *C. atrata* and *C. facialis* resemble each other, having a prominent processs between midleg and hindleg. These two species, varying in 41 nucleotide residues and 3 amino acid residues, fell into the same group, in the phylogenetic tree as well. A phylogenetic tree based on the amino acid sequences also showed a close relationship between *C. atrata* and *C. tympana* (bootstrap value 98%). The two *Cryptotympana* species and *Tibicen japonicus* belong to a same Tribe Tibicenini, and occupy considerably close positions in the tree. Moreover, *Tanna japonensis* and *Terpnosia vacua*, members of Cicadinini, resemble each other phylogenetically. Regardless of difference in Tribe (Dundubini and Tacuarini), *Meimuna opalifera* and *Graptopsaltria nigrofusca* showed a relatively close relationship.

These results presented above roughly coincide with traditional classification based on morphology (e.g. Ishihara, 1961), and demonstrate validity of phylogenetic analysis based on DNA sequence. For elucidation of intrageneric relationship, e.g. in Tibicenini, such species as *Tibicen bihamatus*, *T. flammatus* and *T. kyushuensis* should be analyzed in the future. In order to clarify intergeneric relationship among Japanese cicadas, further studies are required using more species specimens, as well as genes other than COI.

References

- Ishihara, T. (1961). *Hemiptera: Cicadidae*. Insecta Japonica. Series 1, Part 2. Hokuryukan, Tokyo, (In Japanese with English summary.)
- Tokumoto, H. Ohgushi, R., Matsui, M., & Tomisawa, A. (2001). The first record of *Cryptotympana atrata* from Japan (Hatta, Kanazawa City). Tokkuribachi, (68), 1-3, (In Japanese.)

Table.1. Number and proportion (%) of substituted nucleotides and amino acids as compared with *Halyomorpha picus* (I) or *Cicadetta radiator* (II).

Species	Nucleotides			Amino acids
	Total	Transition	Transversion	
<i>Platypleura kaempferi</i>	109 (19.7)	47 (43.1)	62 (15.8)	29 (15.8)
<i>Tibicen japonicus</i>	115 (20.8)	57 (49.6)	58 (50.4)	30 (16.3)
<i>Cryptotympana atrata</i>	115 (20.8)	47 (40.6)	68 (59.1)	26 (14.1)
<i>Cryptotympana facialis</i>	111 (20.0)	42 (37.8)	69 (62.2)	28 (15.2)
<i>Graptosaltria nigrofuscata</i>	112 (20.2)	41 (36.6)	71 (63.4)	30 (16.3)
I <i>Terpnosia vacua</i>	104~107 (18.8~19.3)	37~40 (35.6~37.4)	67 (64.4~62.6)	29 (15.8)
<i>Tanna japonensis</i>	104 (19.5)	34 (32.7)	70 (67.3)	27 (14.7)
<i>Oncotympana maculaticollis</i>	108 (19.5)	38 (35.2)	70 (64.8)	29 (15.8)
<i>Meimuna opalifera</i>	119 (21.5)	45 (37.8)	74 (62.2)	28 (15.2)
<i>Cicadetta radiator</i>	116 (20.9)	50 (43.1)	66 (56.9)	32 (17.4)
<i>Platypleura kaempferi</i>	90 (16.7)	36 (40.0)	54 (60.0)	19 (10.3)
<i>Tibicen japonicus</i>	99 (17.9)	51 (51.5)	48 (48.5)	22 (12.0)
<i>Graptosaltria nigrofuscata</i>	96 (17.3)	43 (44.8)	53 (55.2)	24 (13.0)
II <i>Terpnosia vacua</i>	81~84 (14.6~15.1)	30~33 (37.0~39.3)	51 (63.0~60.7)	19 (10.3)
<i>Tanna japonensis</i>	92 (16.6)	38 (41.3)	54 (58.7)	23 (12.5)
<i>Oncotympana maculaticollis</i>	95 (17.1)	41 (43.2)	54 (56.8)	25 (13.6)
<i>Meimuna opalifera</i>	99 (17.9)	41 (41.4)	58 (58.6)	23 (12.5)

Fig.1. Nucleotide sequences of 10 cicada species as compared with *Halyomorpha picus*. In cicadas, residues varied from those of *H. picus* were shown selectively.

	0	40	80
Hp	*GG CAC CCC GAA GTT TAT ATT TTA ATT CTA CCA GGA TTT GGC TTA ATT TCC CAT ATT ATT AGA CAA GAA AGA GGA AAA AAT		
1	*.T..T..T... ..G...T...G... ..A... ..A... ..CT... ..T... ..T.		
2	*.T..T..A... ..G..C.T... ..T... ..A... ..CT... ..G... ..G...		
3	*.A..T..T..G... ..T... ..T... ..A..C... ..CT... ..G... ..T.		
4	*..T..T... ..G... ..T..G... ..A... ..CT... ..T...		
5	*.T..T..T... ..A... ..A.T..C..T... ..A... ..T..C... ..CT... ..T.		
6	*.T..T..T... ..A... ..C.T...T... ..T... ..T... ..CT... ..T.		
7	*..T... ..A... ..C.T...C..T... ..A... ..T... ..CT... ..G... ..GT.		
8	*.A..T... ..G... ..T..C... ..A... ..A... ..C..G... ..T.		
9	*.T..T..T... ..T... ..T..G... ..A... ..T..C... ..CT... ..T..G..T.		
10	*.T..T..T... ..T... ..T... ..C..T... ..CT... ..G..G... ..GG..T.		

	120	160
Hp	GAA ACA TTT GGT AAT ATT GGA ATA ATT TAC GCT ATA CTA TCA ATT GGA ATT TTA GGA TTC ATT GTA TGA GCA CAT CAT ATA	
1	...T... ..A.TC..T.A..G..G... ..T..A... ..A... ..C... ..T.G... ..G... ..C... ..G	
2	...T.G... ..C.TCA.C.A... ..T..A... ..A... ..G.G... ..C.T... ..T.G... ..G... ..C..G	
3	...T... ..G.TCA.T.A... ..T... ..G.A... ..C.T... ..T.G... ..C...	
4	..G.T... ..G.TCA.T.A... ..T... ..A... ..C.T... ..T.G... ..G..G... ..C...	
5	...T.T... ..TCA.T.A... ..T..A... ..A... ..T... ..C.T... ..T.G... ..T... ..C..G	
6	...T.T... ..A.TCA.T.A... ..T..A... ..A... ..C.T..T..T.G... ..T... ..t...	
7	...T... ..A.TCA.C.G... ..T..A... ..A... ..T... ..T... ..T.G... ..T...	
8	...T.T... ..C.TCA.T.A... ..T..C..G.T... ..T... ..T.G... ..G	
9	...T... ..TCA.T.A..T... ..T... ..A... ..T... ..C.T... ..T.G...	
10	...T... ..A.TCA.T.A... ..G..C..T... ..A... ..T... ..T.G...	

	200	240
Hp	TTT ACA GTA GGC ATG GAT GTT GAT ACC CGA GCC TAC TTC ACA TCA GCC ACA ATA ATC ATT GCA GTA CCA ACA GGT ATT AAA	
1T..T..A..A... ..A... ..T..T..T..T..T... ..T... ..G..G... ..T... ..G	
2G..T... ..A..C... ..T... ..T..T..T..T..T... ..T... ..T... ..T...	
3G..C..G..A... ..T... ..T..T..T..T..T..T... ..T... ..T... ..A... ..G	
4T..A..A... ..T... ..T..T..T..T..T..T... ..T... ..T... ..T... ..A..C...	
5T.A.T..T... ..G... ..T..T..T..T... ..T... ..T..T... ..T... ..T..T... ..A...	
6A..A... ..A... ..A..T..T..T..T..T... ..T..T... ..T... ..T... ..T...	
7G... ..A..T..T..T..T..T... ..T..C... ..T... ..T..T..C... ..A...	
8	..C..T.A.T..A..A... ..A..T..T..T..T..T... ..T..T... ..T... ..T..T..G... ..A...	
9A.T..A..A... ..G... ..A..T..A..T... ..T... ..T..T... ..T... ..T..T..T... ..A...	
10T... ..A..A... ..A..C..A..T..A..T..T..T... ..T..T... ..T... ..T... ..T...	

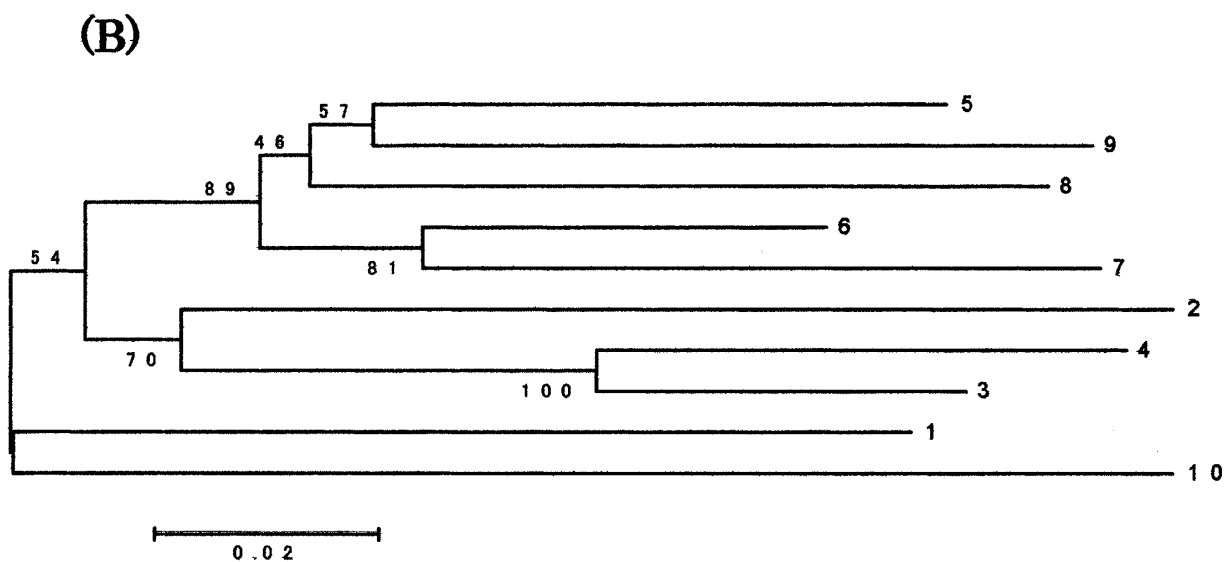
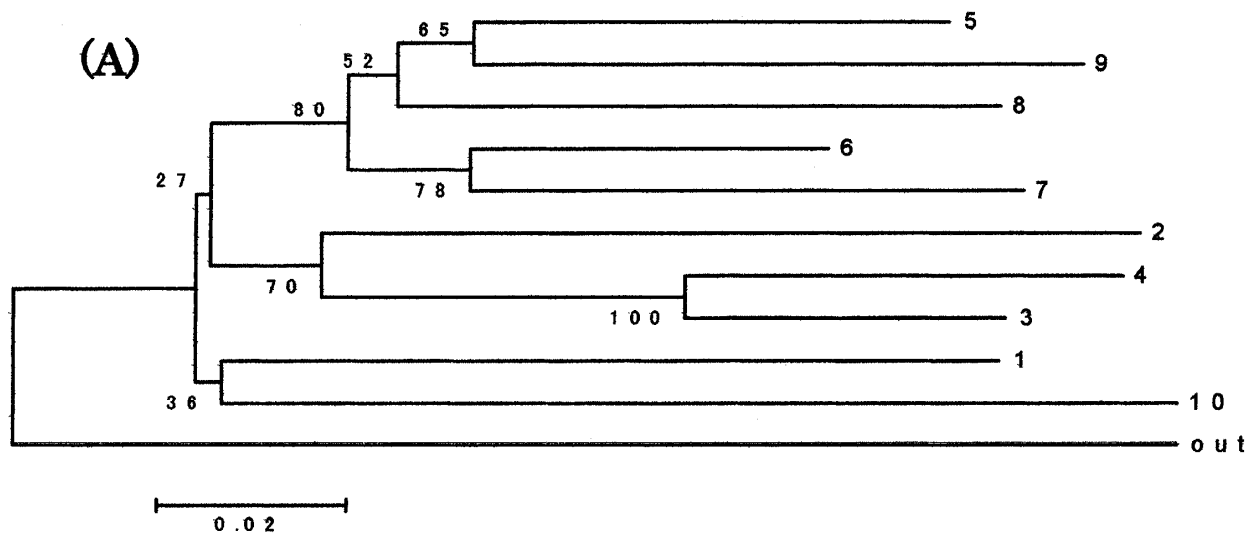
Fig.1. continued

											280										320									
Hp	ATT	TTC	AGA	TGA	CTA	GCC	ACA	TTA	CAT	GGT	GTA	AAA	ATA	AAT	TAT	TCA	CCC	GCT	ATA	ATA	TGA	GCA	TTA	GGA	TTT	GTA	TTT			
1	G.G	..T	T..	..T	A..	..A	AC.	...	C..	..TT	TGT	T..	..T	T..C	..G	...			
2	G..	..T	..T	..G	T..	..T	..T	...	A..	..A	ACT	G.G	AT.	AAT	T.A	T..	..T	C..	..G	..T	C..T	...			
3	G.C	..T	..T	...	T.G	..A	..T	..G	A..	...	AG.A	ATA	AGT	T.T	T..	..T	T..	...	T.TTT	...			
4	G.A	..T	..T	...	T..	..A	..T	...	A.C	...	AG.A	TA	AGT	T.T	T..	..T	T..	...	T.TTT	...			
5	G.A	..T	T..	..G	A..	...	TC.	..TGG	..T	..T	T.T	T..	..T	T..T			
6	G..	..T	..T	...	T.r	..T	A..	...	TC.	..TTA	AT.	...	T.T	T..	..T	T..			
7T	..T	...	T..	..A	A..	..G	TC.	TTT	..T	..A	AT.	A..	T.T	T..	..TT			
8	G.A	..TG	..A	A..	...	TC.	..TA	AT.	..T	T.A	T..	..T	T.TG			
9	G..	..T	T.G	..T	...	C.T	A..	...	TC.	TTTA	AT.	...	T.T	T..	..C	T..T	C.T			
10	G..	..T	..T	...	T..	..A	..T	...	AGA	..A	A.GT	...	ATC	A..	T.T	T.A	GCT	T..TG	...			

											360										400									
Hp	TTA	TTC	ACT	ATT	GGA	GGA	TTA	ACA	GGA	GTA	ATT	CTA	GCC	AAC	TCA	TCT	ATT	GAT	ATC	ATT	TTA	CAT	GAT	ACA	TAT	TAT	GTA			
1T	..AG	..CC	..T	..GTATGT			
2	..G	..TAG	..T	...	T..	..T	..TATC			
3	..G	..TG	..GTG	..T	..T	..T	..AT	G.GTTT			
4ATT	..CT	..T	..C	..GT	G.ATTT			
5T	..ATT	...	T.GTAT	...	C.TTT			
6T	..AT	..TT	...	T.r	..T	..TAT	G..	C.Tyy	...			
7T	..AG	..CT	...	T.GTTT	..CT	..C			
8T	..ATT	...	T..	..T	..TT	...	C.TTT	...			
9T	..ATT	...	T.G	..T	..T	..GT	...	C.CC	..TTT			
10T	..G	C..	..T	...	T..T	..T	..AT	G.A	..G			

											440										480									
Hp	GTA	GCC	CAT	TTC	CAT	TAT	GTA	CTT	TCA	ATA	GGT	GCA	GTA	TTT	GCT	ATT	ATA	GGA	AGA	TTT	ATT	CAA	TGA	TTT	CCC	TTA	TTC			
1	..T	..TTT	T.A	..TG	..T	T.G	..TTA	..A	..G	..A	...			
2ATG	T.AGGA	T..CA	T.T	..G	..T	...			
3TT	T.AATA	T.G	..TTA	..AT	...			
4T	..C	..TAATA	T.G	..T	...	G.C	..TA	T.A			
5	..T	..ATG	T.AGA	C.G	..TTA	T.A	..G	..A	...			
6	..T	..TT	T.AA	..T	T..CTA	T.A	C.T	..A	...			
7	..T	..TTT	T.AA	..T	T..TTA	T.A	C.T	..A	...			
8	..T	..ATT	T.A	T..TTA	T.AA	...			
9	..T	..ATT	T.AA	..TA	T.G	..TTA	..T	C.G	A.A	...			
10	..T	..ATT	T.ATCTC	..AT			

Fig.3. Phylogenetic tree based on mitochondrial CO I sequences (neighbor joining method).
AS the out group, *Halyomorpha picus* (A), or *Cicadetta radiator* (B) was used.



OUT *Halyomorpha picus* , 1 *Platypleura kaempferi* , 2 *Tibicen japonicus* ,
 3 *Cryptotympana atrata* , 4 *Cryptotympana facialis* , 5 *Graptopsaltria nigrofuscata* ,
 6 *Terpnosia vacua* , 7 *Tanna japonensis* , 8 *Oncotympana maculaticollis* ,
 9 *Meimuna opalifera* , 10 *Cicadetta radiator* .

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