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Nucleotide substitutions in a portion of the mitochondrial cytochrome *b* gene in water striders (Hemiptera: Gerridae)

By

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Summary : A portion of the mitochondrial cytochrome *b* gene was sequenced using 22 individuals from 14 species belonging to the subfamily Gerrinae and two outgroup taxa. As reported for mitochondrial genes in many other insects, a nucleotide content bias for A and T was observed in the sequences. Among taxa belonging to Gerrinae, a relatively high frequency of T-C transitions, as well as a high level of A-T transversions was observed. Comparisons of the nucleotide sequences between pairs of 22 samples revealed that nucleotide substitutions in this DNA fragment were biased by the amino acid composition of the cytochrome *b* enzyme. Pairwise comparisons also suggested that strong multiple substitutions have occurred in the first and third positions of codons between distantly related taxa. On the basis of these results, the utility of the nucleotide sequences of the cytochrome *b* gene for estimating the phylogenetic relationship among gerrinae taxa is discussed.

Key Words : nucleotide sequence, cytochrome *b* gene, mitochondrial DNA, Gerrinae, Gerridae.

Introduction

The heteropteran infraorder Gerromorpha includes approximately 1,500 species of semiaquatic bugs belonging to eight families[1]. Their taxonomy and classification have been studied intensively. Phylogenetic relationships among families and subfamilies are well established on the basis of morphology[2]. However, ambiguities still remain in relationships among taxa, especially at the species level.

Recently, in an attempt to obtain molecular markers to reconstruct the gerromorphan phylogeny, we analyzed nucleotide sequences in portions of the 28S ribosomal DNA and mitochondrial 16S ribosomal DNA (16S rDNA)[3]. In this analysis, we found that these sequences can be used to discriminate many genera and subgenera. However, phylogenetic trees based on these sequences were unable to solve the relationships among closely related taxa lower than the generic or subgeneric levels.

In the present study, we analyzed nucleotide sequences in a portion of the mitochondrial cytochrome *b* (cyt *b*) gene of water striders belonging to the subfamily Gerrinae. Nucleotide sequence of this gene has been frequently used to estimate evolutionary relationships among taxa within a number of vertebrate groups. Although several researchers have also analyzed the cyt *b* sequences of insect groups such as parasitic wasps and ants[4,5], the phylogenetic utility for many other insect groups is not clear. Thus in this study, we also analyzed the property of nucleotide substitutions in the DNA fragment of the gerrinae taxa and evaluated the usefulness of the DNA sequences for the gerrid phylogeny.

Materials and Methods

Materials used in this study are listed in Table 1. In addition to 22 individuals from 14 species belonging to the subfamily Gerrinae, two taxa belonging to another gerrid subfamily Halobatinae (*Metrocoris esakii*) and the gerroid family Hermatobatidae (*Hermatobates weddi*) were sequenced as outgroups. As a source of template for the PCR, total DNA was extracted from individual adult insects using a GenomicPrep™ Cells and Tissue DNA Isolation Kit according to the manufacturer's instructions (Amershan Pharmacia

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Biotech), with the exception that both RNase and the suggested incubation at 37℃ were omitted. Nucleic acids obtained were dissolved in 200 μl of sterile water.

The two primers TAGGATATGTTTTACCTTG AGGACA and CTCCTCCTAATTTATTAGGAATTG[6] were used to amplify a portion of the cyt *b* gene and to sequence the amplified product. These primers were designed based on a high degree of similarity in aligned corresponding sequences of the honey bee *Apis mellifera* (L06178), the migratory locust *Locusta migratoria* (X80245), the mosquito *Anopheles quadrimaculatus* (L04272), and the fruit fly *Drosophila yakuba* (X03204). Amplification was carried out in a 25 μl volume using a Taq DNA polymerase (Takara Shuzo). One μl DNA template, 2.5 μl 10×PCR buffer, 1.6 μl dNTPs (2.5 mM each), 0.5 μl (10 mM) of each primer, and 0.15 unit of polymerase were used in each reaction. Temperature cycling was carried out in a Perkin Elmer Cetus Thermal Cycler (model 480). After an initial heating step at 92℃, samples were incubated for 40 cycles at 92℃ for 1 min, 47℃ for 30 sec and 72℃ for 1 min. The amplified product was separated in a 1.0% low-melting-temperature agarose (Agarose-L; Nippon-Gene) gel (1×TBE) and the appropriate band (stained with ethidium bromide) was excised. The gel piece containing the DNA band was ground with pellet pestle (Eppendorf) in a tube with 0.2 m*l* TE (10 mM Tris-HCl, 1 mM EDTA). The DNA was then recovered by centrifugation, purified by ethanol-precipitation, and then used as a template for nucleotide sequencing. The DNA was labeled using a ThermoSequenase™ Dye Terminator Cycle Sequencing Pre-mix Kit v 2.0 (Amersham Pharmacia Biotech) and electrophoresed on an Applied Biosystems 373S DNA sequencer (Perkin Elmer Cetus). Sequences obtained in this study were submitted to DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers from AB 028274 to AB028296.

Table 1 Species, collection locality and date of insects sequenced in this study

Species	Collection localities	Date
Gerroidea		
Hermatobatidae		
Hermatobates weddi	Tokuno-shima Island, Kagoshima Pref., Japan	28. III. 1997
Gerridae		
Halobatinae		
Metrocoris esakii	Ishigaki-jima Island, Okinawa Pref., Japan	29. III. 1995
Gerrinae		
Tenagogonus sp.	Los Baños, Lagna, Philippines	14. III. 1995
Limnogonus hungerfordi	Ishigaki-jima Island, Okinawa Pref., Japan	29. III. 1995
Limnogonus fossarum skusei	Bali, Indonesia	22. III. 1995
Neogerris parvulus	Amami-oshima Island, Kagoshima Pref., Japan	25. V. 1995
Aquarius elongatus	Fujioka, Gunma Pref., Japan	29. IV. 1995
	Usue, Taiwan	21. IV. 1998
Aquarius paludum paludum	Akkeshi, Hokkaido Pref., Japan	21. VIII. 1995
Aquarius paludum amamiensis	Yonaguni-jima Island, Okinawa Pref., Japan	2. IV. 1997
Limnoporus genitalis	Akkeshi, Hokkaido Pref., Japan	21. VIII. 1995
Limnoporus esakii	Sakura, Chiba Pref., Japan	29. IV. 1995
Gerris (Gerris) babai	Akkeshi, Hokkaido Pref., Japan	21. VIII. 1995
Gerris (Gerris) latiabdominis	Akkeshi, Hokkaido Pref., Japan	21. VIII. 1995
	Omogo, Ehime Pref., Japan	7. VII. 1995
Gerris (Gerris) nepalensis	Sakura, Chiba Pref., Japan	29. IV. 1995
	Himeji, Hyogo Pref., Japan	6. X. 1997
Gerris (Macrogerris) yezoensis	Asahikawa, Hokkaido Pref., Japan	20. VI. 1998
	Katashina, Gunma Pref., Japan	15. VIII. 1994
Gerris (Macrogerris) insularis	Tsukuba, Ibaraki Pref., Japan	6. V. 1995
	Hachijo-jima Island, Tokyo Pref., Japan	21. V. 1997
Gerris (Macrogerris) gracilicornis	Akkeshi, Hokkaido Pref., Japan	21. VIII. 1995
	Yaku-shima Island, Kagoshima Pref., Japan	26. III. 1997

Table 2 Average contents (%) of nucleotides in a portion of the cyt *b* gene among taxa belonging to the subfamily Gerrinae

	A	T	C	G
overall	34.94	35.90	18.50	10.66
1st position	31.74	28.37	20.49	19.41
2nd position	25.11	42.83	22.06	10.00
3rd position	47.97	36.52	12.95	2.57

Nucleotide sequences were aligned using the computer program CLUSTAL W version 1.5[7] with default parameters (gap open penalty 10, gap extension penalty 0.1 in pairwise alignment and 0.05 in multiple alignments). The aligned sequences were used to perform phylogenetic analyses with the PHYLIP (Phylogeny Inference Package) version 3.5c[8]. The programs DNADIST, NEIGHBOR, and DNAPARS of the PHYLIP package were used to construct UPGMA, neighbor-joining, and maximum parsimony trees. Bootstrap values were calculated using SEQBOOT and CONSENSE as well as the programs mentioned above.

Results

Sequence alignment

In this study, we sequenced a portion of the cyt *b* gene using 22 individuals from 14 species belonging to the subfamily Gerrinae and two outgroup taxa, *M. esakii* and *H. weddi*. Because the nucleotide sequences at the 3′ and 5′ ends of the DNA fragment in several samples could not be determined unambiguously, we excluded these regions from the sequence alignment, and a 393 bp portion corresponding to the *D. yakuba* sequence[9] from bp 10,949 to bp 11,341 was used in the following analyses (Fig. 1).

Table 2 shows the average frequencies of different nucleotides in the cyt *b* fragment of gerrinae taxa. From this table, it is apparent that the nucleotide compositions were biased for A and T. This tendency became more obvious when only the third position of codons was considered. From aligned sequences of gerrinae taxa, 153 variable sites were detected; 129 of which were informative for parsimony analysis. Among the variable sites, 38 (29.0%), 4 (3.1%), and 111 sites (84.7%) were observed at the first, second, and third position of codons, respectively. Among 130 residues of amino acid sequences inferred by translation of the nucleotide sequences, variability was observed at 27 (20.8%) amino acid sites. Comparison of triplets among nucleotide sequences revealed that 29 variable nucleotide sites were responsible for changes in amino acid residues and that frequencies of replacement sites

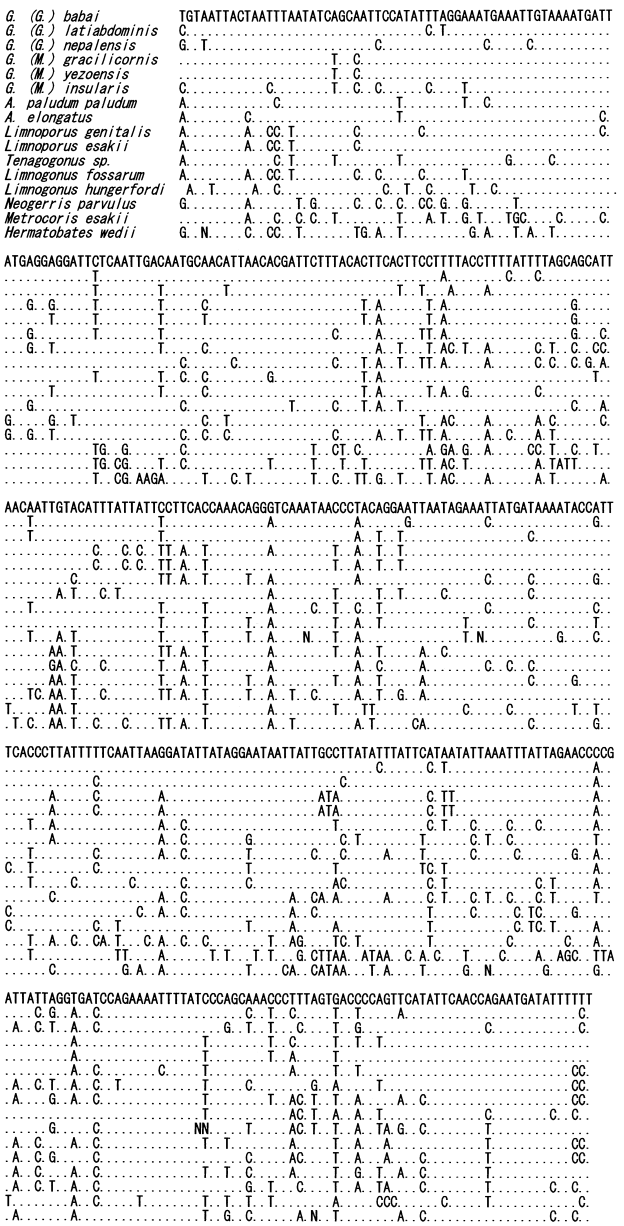

Fig. 1 Nucleotide sequences of the mitochondrial cyt *b* gene in species used in this study. Nucleotides identical to the reference, *Gerris babai,* are indicated by a dot.

were 55.3%, 100%, and 3.6% for the first, second, and third positions of codons, respectively.

Nucleotide substitutions

From comparisons of the sequences between pairs of 22 gerrinae taxa, 12,617 nucleotide substitutions were detected. Among them, 22.6%, 1.2%, and 76.2% were observed in the first, second, and third position of codons, respectively. As shown in Table 3, the frequency of different types of nucleotide substitutions was different among the codon positions. In the first position, T-C transitions showed the highest frequency. Comparisons of triplets including nucleotide substitu-

Table 3 Number of nucleotide substitutions detected among taxa belonging to the subfamily Gerrinae sequenced in this study

	T/C	A/G	A/T	A/C	G/T	G/C
overall	5518	1406	4141	1208	232	112
1st position	1779	374	493	171	35	2
2nd position	92	0	0	17	6	35
3rd position	3647	1032	3648	1020	191	75

tions among samples revealed that 96.7% (1,720 substitutions) of T-C transitions in this position were silent substitutions. Of these silent substitutions, 100% were due to base changes that occurred between triplets (CTN and TTR) within the degenerate codon family encoding leucine. When silent substitutions within this degenerate codon family, which accounted for 62.3% of all the nucleotide substitutions in this position, were excluded from pairwise comparison, the frequencies of T-C transitions and A-T transversions become 4.9% and 43.5%, respectively. In the second position, T-C transversions showed the highest frequency. However, all the nucleotide changes in this position were owing to replacement substitutions in only 4 nucleotide sites. A relatively high frequency of T-C transitions, as well as a high level of A-T transversions, was observed in the third position of codons. In this position, silent substitutions accounted for 97.5% (9,376 substitutions) of all the nucleotide changes.

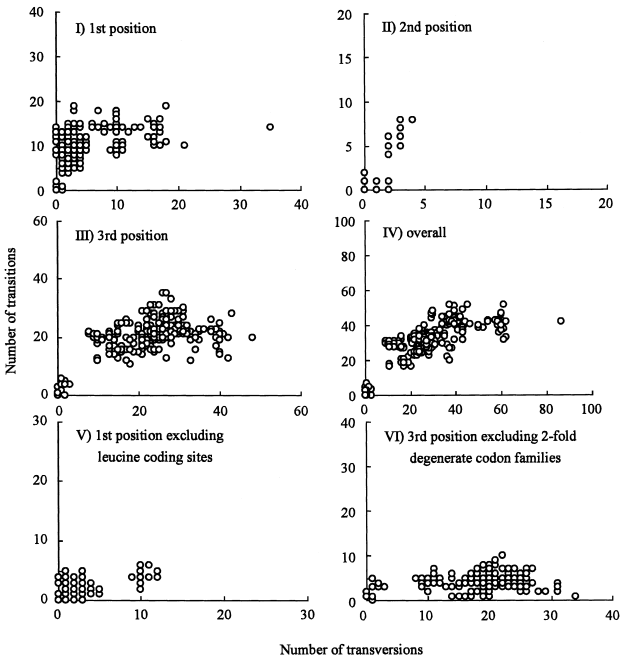

Fig. 2 Number of transitions plotted against number of transversions detected by pairwise comparison of the cyt *b* sequences between taxa.

When triplets including nucleotide changes were compared among taxa, it was revealed that 58.4% (7,371 substitutions) of all the substitutions in this position occurred within 2-fold degenerate codon families which allow only A-G or T-C substitutions at the third position. When substitutions in such codon families were excluded from pairwise comparisons, the frequency of A-T transversions increased to as high as 60.5%, and the frequency of T-C transitions decreased to 5.3%.

Fig. 2 shows the relationship between the numbers of transitions and transversions. In the second position of codons, the number of transitions increased linearly with an increase of transversional substitutions (Fig. 2–II). However, in the first position, the number increased very quickly and a plateau was reached at a low level of nucleotide divergence, *i.e.* around 5 transversions (Fig. 2–I). Because such a tendency became ambiguous when leucine coding sites were excluded (Fig. 2–V), saturation in transitions in this position is

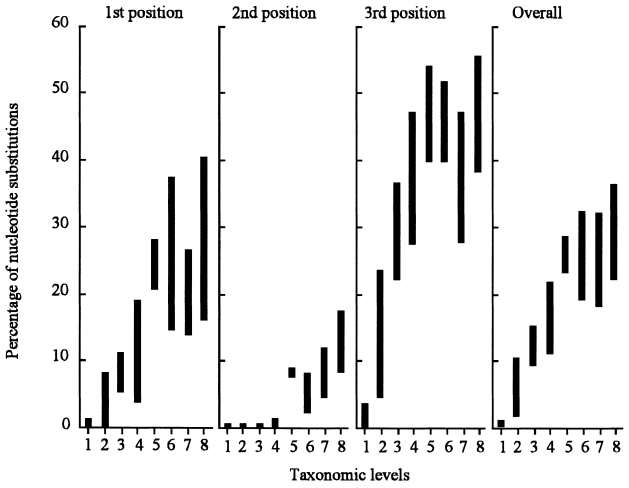

Fig. 3 Percentage of nucleotide substitutions in the cyt *b* sequences between pair of taxa at various taxonomic levels. Vertical lines show the range of nucleotide divergence between taxa on a given taxonomic level. Pairwise comparisons were made between taxa on eight different taxonomic levels: 1, between strains within a species; 2, between species within the same subgenus; 3, between subgenera; 4, between genera; 5, between subfamilies; 6, between families within the same suborder; 7, between suborders; 8, between orders. In addition to nucleotide sequences obtained in this study, previously reported corresponding sequences of the anthocorid bug *Orius sauteri* (AB020508), the pentatomid bug *Nezara viridula* (AB020514) (Muraji *et al.*, unpublished), the honey bee *Apis mellifera* (L06178)[10], the migratory locust *Locusta migratoria* (X80245)[11], and the fruit fly *Drosophila yakuba* (X03204)[9] were used to generate the figure.

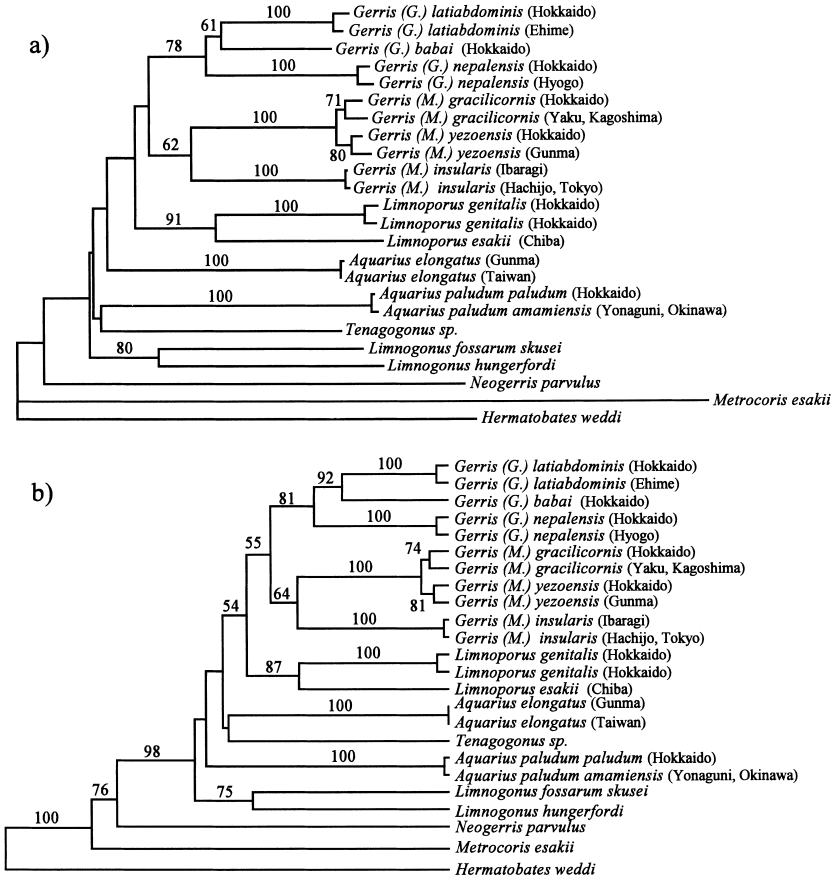

Fig. 4 Neighbor-joining tree generated based on maximum likelihood distances (a) and UPGMA dendrogram based on Jukes-Cantor distances (b). Bootstrap confidence limits are shown above the branches of clades supported in more than 50% of 500 replications.

considered to be due to nucleotide changes between triplets within the degenerate codon family encoding leucine. The third position also showed saturation in the number of transitions (Fig. 2–III). This tendency became more obvious when only the 4- and 6-fold degenerate codon families were analyzed (Fig. 2–VI). In this case, the number of transitions was strongly saturated at a very low level of nucleotide divergence, and the number did not exceed 10 transitions.

Nucleotide divergence

Fig. 3 shows the percentage of substituted nucleotides at different positions of codons. In the third position, the values increased quickly with an increase of taxonomic levels and saturated at the between-subgenus level. In this position, the values reached a plateau around 30 to 50% of the nucleotide substitutions. The first position also showed saturation in nucleotide substitutions. However, when compared with the third position, the values increased a little slower, and a plateau was reached at a lower level of nucleotide substitutions, *i.e.* around 20 to 40%. The values of the second position increased very slowly and did not show a plateau.

In this figure, homoplasies in nucleotide sequences were observed between distantly related taxa. For example, in the third position, the level 4 (between different subgenera) showed similar values to level 7 (between different families within the same order). Such a phenomenon was reflected in the fact that *Gerris* taxa showed a higher uncorrected pairwise nucleotide similarity with the pentatomid bug *Nezara viridula* (32.8–37.4%) than with a gerrid taxon *Neogerris parvulus* (40.5–45.8%).

Phylogenetic analysis

In order to generate phylogenetic trees, several methods were used in this study, *i.e.* UPGMA and neighbor-joining analyses based on the maximum likelihood, Jukes-Cantor and Kimura's 2-parameter distances and the maximum parsimony analysis. When the data set including all the codon positions were used, the neighbor-joining, UPGMA, and maximum parsimony analyses successfully discriminated all of the species used in this study (Fig. 4). Clades including individuals of respective species were supported by

high bootstrapping values. In the maximum likelihood analysis, the topology of the phylogenetic trees agreed generally with the morphological classification presented in Table 1 with respect to the relationships among taxa belonging to *Gerris* and *Limnoporus*. Bootstrapping analysis supported most of relationships among these taxa. The UPGMA and neighbor-joining analyses based on Jukes-Cantor and Kimura's 2-parameter distances generated virtually the same topologies as those generated by the maximum likelihood analysis (Fig. 4–a). However, relationships among taxa higher than the subgenus level became more ambiguous. Maximum parsimony analysis generated the 6 most parsimonious trees (570 steps). The topology of the consensus tree did not agree with the morphological classification (Table 1) with respect to the relationships among genera and subgenera.

When the data set excluding the third position of codons was analyzed, none of the phylogenetic methods could recognize monophyletic groupings for *Limnoporus, Aquarius, Gerris,* and *Gerris* subgenus *Gerris* and *Macrogerris*. In addition, the maximum likelihood, Jukes-Cantor and Kimura's 2-parameter methods could not discriminate *G.(M.) yezoensis* and *G.(M.) gracilicornis*. As expected from a small number of variable sites, phylogenetic relationships became ambiguous when the first position in leucine coding sites and the third position were excluded from the analysis.

Discussion

As observed in this study, a nucleotide composition bias for A and T have been observed in mitochondrial DNA genes in a number of insect species[12~16]. In relation to this phenomenon, several researchers reported a strong A-T transversion bias in insect mitochondrial genes[12~14]. On the other hand, A-T transversion bias was not obvious in the cyt *b* fragment sequenced in this study (Table 3). When compared with the 16S rDNA of gerrid insects (accession numbers: AB026585-AB026595, AB026597-AB026602)[3], the cyt *b* fragment showed a considerably higher frequency of T-C transitions (cyt *b*: 43.7%, 16S rDNA: 16.8%) and a lower frequency of A-T transversions (cyt *b*: 32.8%, 16S rDNA: 54.5%), even though both the cyt *b* and 16S rDNA showed similar nucleotide compositions.

In the case of invertebrate mitochondrial DNA, codons encoding leucine can take either T or C at the first position. Because, nucleotide changes in this position other than T-C transitions at leucine coding sites are responsible for changes in amino acid residues, it is likely that T-C transitions of this kind occur much more easily than other types of nucleotide substitutions. Actually, the result of pairwise comparisons indicated that T-C transitions were the most frequent in the 6 types of nucleotide substitution. The results also showed that the frequency of A-T transversions became higher when leucine coding sites were excluded. Therefore, the reduced frequency of A-T transversions in this position (Table 3) was not due to an unbiased mode of nucleotide substitutions but due to constraints by the amino acid composition of the cytochrome *b* enzyme.

A similar tendency was observed in the third position of codons, although nucleotide changes in this position are known to be less constrained by coding function than the other two positions. In the case of 2-fold degenerate codon families, silent substitutions can occur only between A and G or between T and C. On the other hand, the 4- and 6-fold degenerate codon families can take any nucleotides at the third position. Therefore, the mode of nucleotide substitutions are constrained differently among different degenerate codon families, and the frequencies of different types of substitutions in this position are expected to change depending on the amino acid composition of the enzyme. Such a situation was reflected in the results where A-T transversion bias became obvious, and the frequency increased as high as that reported for mitochondrial rDNA in which nucleotides are not constrained by coding function when only substitutions within the 4- and 6-fold degenerate codon families were considered. From these results, it is apparent that nucleotide substitutions are constrained by amino acid composition of the enzyme even at the third codon position.

It is well known that transitional substitutions occur much more easily than transversions and that the frequency of transitions is usually high between closely related taxa. On the other hand, transversional substitutions are accumulated between distantly related taxa through multiple substitutions that obscure transitions that have previously occurred[17]. The results of the present study confirm the general tendency that the frequency of transitions is initially high and the value decreases gradually with an increase of genetic divergence between taxa (Fig. 2–IV). Such a tendency was most obvious in the first position (Fig. 2–I) and the third position within the 4- and 6-fold degenerate codon families (Fig. 2–VI), in which the numbers of transitions were strongly saturated. Therefore, strong multiple substitutions are considered to have occurred in these positions.

When changes in amino acid residues are strongly constrained, the total number of nucleotide changes is

saturated, and multiple substitutions that occur under such conditions sometimes cause homoplasy in nucleotide sequences between distantly related taxa[17]. Such phenomena were evident in the cyt *b* sequences of taxa analyzed in this study (Fig. 3). The total number of nucleotide substitutions was strongly saturated in both the first and third position of codons. Apparent homoplasies were also observed between taxa higher than subfamily and genus in the first and third position, respectively. These results suggest that phylogenetic information contained in the cyt *b* sequences is strongly obscured by multiple substitutions especially among taxa higher than subgenus.

In many protein-coding genes, the third position of codons have been frequently excluded from phylogenetic analyses of distantly related taxa to exclude homoplasy due to intensive multiple substitutions and to alter phylogenetic information contained in nucleotide sequences. However, in the case of the cyt *b* fragment, such treatment needs attention to be paid to evolutional constraints acting on the first position. As mentioned before, 62.3% of nucleotide substitutions at the first position occurred within the degenerate codon family encoding leucine. Substitutions of this kind also accounted for as many as 59.2% of the nucleotide changes in the data set excluding the third position. Because strong multiple substitutions are considered to occur in the first position within leucine coding sites (Fig. 2), phylogenetic information contained in such a data set must be obscured by multiple substitutions. Therefore, exclusion of the third position is not expected to alter phylogenetic information much.

In order to generate phylogenetic trees, we applied several different methods to the data sets obtained in this study. Although many of the phylogenetic trees successfully discriminated species used in this study, the relationships among genera and higher taxa were not resolved unambiguously. As topologies for relatively higher taxa in these trees changed depending on methods and data sets used in the analysis, the cyt *b* sequences may be not very suitable for use as a molecular marker to estimate the phylogeny of a wide range of Gerrinae taxa. Among various factors that affect phylogenetic utility of DNA sequences, multiple substitutions obscure phylogenetically informative nucleotide changes[17] and cause homoplasy among distantly related taxa. Therefore, the utility of the cyt *b* sequences may be restricted to the phylogeny of closely related taxa in which nucleotide substitutions are not likely to be saturated.

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アメンボ類のミトコンドリア DNA cyt *b* 遺伝子における塩基置換の特性

村路雅彦*・立川周二**

（**平成** 13 **年** 3 **月** 20 **日受付/平成** 13 **年** 7 **月** 19 **日受理**）

要約 : Gerrinae **亜科に属する** 14 **種** 22 **個体のアメンボ類および** 2 **種のアウトグループ（サンゴアメンボおよびタイワンシマアメンボ）についてミトコンドリア** DNA **の** cyt *b* **遺伝子の部分的な塩基配列を調べた。その結果，他の多くの昆虫と同様に，これらの種の** cyt *b* **遺伝子の塩基組成は** A **と** T **に大きく偏っていることが明らかとなった。また，これらの種間における塩基置換は** T–C **トランジションおよび** A–T **トランスバージョンに偏っていた。さらに** 22 **塩基配列の比較の結果，それらの塩基置換は** cytochrome *b* **酵素のアミノ酸組成に支配されていること，各コドンの第** 1 **および第** 3 **塩基では激しい多重置換が生じていることなどが明らかとなった。これらの結果にもとづき，** Gerrinae **亜科昆虫の系統解析における** cyt *b* **遺伝子の塩基配列の有用性について検討した。**

キーワード : DNA **塩基配列，チトクローム** *b* **遺伝子，ミトコンドリア** DNA，**アメンボ上科**

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