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Citation
日本熱帯医学会雑誌, vol.29(2), pp.261-266; 2001

Issue Date
2001-06-15

URL
http://hdl.handle.net/10069/22435

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PHYLOGENETIC ANALYSES OF A BLACKFLY SUBGENUS SIMULIUM (NEVERMANNIA) BASED ON MITOCHONDRIAL 16S RIBOSOMAL RNA GENE SEQUENCES

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Received April 2, 2001/Accepted May 20, 2001

Abstract: Nucleotide sequences of a subregion of the mitochondrial 16S ribosomal RNA gene of 10 species of a blackfly subgenus Simulium (Nevermannia), which include four species of feuerborni species-group, two species of ruficorne species-group, three species of vernum species-group and an ungrouped species (S. konoi), were determined. Phylogenetic analyses of the sequences of the Nevermannia species and other species of related subgenera of Simulium s.l. showed that the feuerborni and vernum species-groups were closely related, but the ruficorne species-group and S. konoi were not. Variations between the ruficorne species-group and other Nevermannia species were larger than those between Nevermannia species (excluding the ruficorne species-group) and other subgenera species. These molecular data suggest that revision of the definition of the subgenus Nevermannia is needed.

Key words: Black fly, Simulium, Nevermannia, Phylogeny, Mitochondrial rRNA

INTRODUCTION

A blackfly subgenus Simulium (Nevermannia) is distributed worldwide. In Asia, there are 3 species-groups (i.e. feuerborni, ruficorne and vernum species-group), and some ungrouped species (Crosskey and Howard, 1997). To investigate the relationship within subgenus Nevermannia species and between subgenus Nevermannia and other subgenera, we analyzed sequence variations in a subregion of the mitochondrial 16S ribosomal RNA (rRNA) gene of 10 species of subgenus Nevermannia and related species.

MATERIALS AND METHODS

Materials used in this study and their origin are listed in Table 1. Total DNA was extracted from single larva using the crude STE boiling method (O’Neill et al., 1992). Polymerase chain reactions (PCR) were performed in a 50 µl reaction mixture using 2 µl of the DNA solution. The primers (primer A, 5’-CGCCTGTTTA TCAAAAACAT-3’; primer B, 5’-CTCCGGTTTGAACTCAGACTC-3’) were used to amplify the mitochondrial 16S rRNA region as described by Xiong and Kocher (1991). The reaction mixture contained 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 250 µM dNTPs, 2.5 units of Tag DNA polymerase and 50 pM of each of the primers. The thermal cycling conditions were 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 2 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products were purified by using the QIAquick PCR purification kit (Qiagen), and cloned into pGEM-T Easy vector (Promega). At least 4 independent clones from each blackfly sample were sequenced to identify polymerase error using the fmol DNA sequencing system (Promega). Sequences were deposited in DDBJ/EMBL/GenBank databases under accession numbers AB056728-AB056747.

The sequences were aligned by using the program CLUSTAL W ver. 1.7 (Thompson et al., 1994). Sites containing alignment gaps were removed in the following analyses. The number of nucleotide substitution per site was estimated between each pair of the sequences, using Jukes-Cantor methods (Jukes and Cantor, 1969). Construction and bootstrap probability estimation of the neighbor-joining tree (Satiou and Nei, 1987) were performed by PHYLIP 3.57c (Felsenstein, 1995).

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RESULTS

We determined the mitochondrial 16S rRNA region of 10 *Nevermannia* species including three species-groups (*feuerborni*, *ruficorne*, *vernum*) and an ungrouped species (*S. konoi* Takahasi), five species of other subgenera and *Prosimulium kiotoense* Shiraki, and aligned (Fig. 1). All of the *Nevermannia* species had 516 bases in this region. As for the five species (*S. feuerborni* Edwards, *S. mie* Ogata & Sasa, *S. aureohirtum* Brunetti, *S. subcostatum* Takahasi, *S. uchidai* Takahasi), we determined the sequences of two or three samples from different localities. *S. subcostatum* and *S. uchidai* did not have any intraspecific variations, but *S. feuerborni*, *S. mie* and *S. aureohirtum* had. These intraspecific variations were not larger than the interspecific variations.

To study relationships between *Nevermannia* species and between *Nevermannia* and other subgenera, a neighbor-joining tree was constructed based on the estimated *d* values (the number of the nucleotide substitutions per site) between each pair of the samples (Fig. 2). *P. kiotoense* was used as an outgroup. The three species-groups of *Nevermannia* were separated into different clusters with high bootstrap probabilities. The *feuerborni* and *vernum* species-groups were clustered, but the *ruficorne* species-group was placed in a distinct cluster. One of the objectives of this study was to determine the relationship of the ungrouped species, *S. konoi*, to the known species-groups. But *S. konoi* was not related to any species-groups of *Nevermannia* in the tree.

Table 2 summarizes the average *d* values among species-groups of *Nevermannia* and other subgenera. The average *d* values between the *ruficorne* species-group and the other species-groups of *Nevermannia* were higher than those between the species-groups (without the *ruficorne* species-group of *Nevermannia*) and other subgenera, and were approximately the same level as those between the *ruficorne* species-group and other subgenera.

**DISCUSSION**

Our phylogenetic analyses of subgenus *Nevermannia* based on the mitochondrial 16S rRNA gene sequences showed that the *feuerborni* and *vernum* species-groups were closely related, but the *ruficorne* species-group and the ungrouped species, *S. konoi*, were not. The *ruficorne* species-group was largely divided from other *Nevermannia* species and other subgenus species. The *ruficorne* species-

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**Table 1  Materials used in this study**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Subgenus</th>
<th>Species-group</th>
<th>Species</th>
<th>Locality</th>
<th>GenBank Acc.</th>
</tr>
</thead>
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<td><em>Simulium</em></td>
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<td></td>
<td><em>P. kiotoense</em> Shiraki</td>
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</tr>
</tbody>
</table>

* Sequence of *S. subcostatum* from Oita, Japan was identical to that from Kanagawa, Japan

 rentals, Japan was identical to that from Oita, Japan
Figure 1-1 DNA alignment of mitochondrial 16S rRNA region for the 16 species. A period indicates the site identical to *S. feuerborni* (Indonesia); a dash indicates a gap site.
Figure 1-2
group and the ungrouped species, *S. konoi*, have morphological characters which depart from the other species of *Nevermannia*. One of such characters in the *ruficorne* species-group is the male genitalia with ventral plate with a distinct median keel (Crosskey, 1969). On the other hand, *S. konoi* has a female adult cibarium with a distinctive armature consisting of several oblique rows of denticles on each side (Bentinck, 1955); its larval antennae have a few unpigmented annulations on the second segment (unpublished data). These molecular and morphological data taken into consideration, revision of the definition of the subgenus *Nevermannia* may be needed.

**ACKNOWLEDGMENTS**

This research was supported by the Grant-in-Aid for Scientific Research (C) of the Ministry of Education, Science, Culture & Sports, Japan (No. 11670246 to HT & YO).

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