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 μ*l* reaction mixture using 2 μ*l* of the DNA solution. The primers (primer A, 5'-CGCCTGTTTATCAAAAACA-T-3'; primer B, 5'-CTCCGGTTTGAACTCAGATC-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 $MgCl_2$, 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℃ for 1 min, annealing at 50℃ for 2 min, extension at 72℃ for 2 min and a final extension at 72℃ 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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Table 1 Materials used in this study

Genus	Subgenus	Species-group	Species	Locality	GenBank Acc.
Simulium	*Nevermannia*	*feuerborni*	*S. feuerborni* Edwards	Java, Indonesia	AB056728
				Peninsular Malaysia	AB056729
			S. mie Ogata & Sasa	Kanagawa, Japan	AB056730
				Nagano, Japan	AB056731
				Yakushima, Japan	AB056732
			S. saitoi Takaoka	Kanagawa, Japan	AB056733
			S. sasai Rubtsov	Hakone, Japan	AB056734
		ruficorne	*S. aureohirtum* Brunetti	Indonesia	AB056735
				Peninsular Malaysia	AB056736
			S. ornatipes Skuse	Irian Jaya, Indonesia	AB056737
		vernum	*S. bonninense* Shiraki	Ogasawara, Japan	AB056738
			S. subcostatum Takahasi	Kanagawa, Japan	AB056739
				Oita, Japan	∗
			S. uchidai Takahasi	Oita, Japan	AB056740
				Yakushima, Japan	†
		ungrouped	*S. konoi* Takahasi	Tochigi, Japan	AB056741
	Gomphostilbia		*S. palauense* Stone	Palau	AB056742
			S. whartoni Takaoka & Davies	Peninsular Malaysia	AB056743
	Morops		*S. farciminis* Smart & Clifford	Irian Jaya, Indonesia	AB056744
	Simulium s.str.		*S. aokii* Takahasi	Oita, Japan	AB056745
			S. quinquestriatum Shiraki	Oita, Japan	AB056746
Prosimulium			*P. kiotoense* Shiraki	Oita, Japan	AB056747

∗ Sequence of *S. subcostatum* from Oita, Japan was identical to that from Kanagawa, Japan
† Sequence of *S. uchidai* from Yakushima, Japan was identical to that from Oita, Japan

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 and 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-