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Genetic identity of Japanese *Sympetrum frequens* and Korean *S. depressiusculum* inferred from mitochondrial 16S rRNA sequences (Odonata: Libellulidae)

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Abstract

The Japanese endemic *Sympetrum frequens* is considered as the insular vicariant of *S. depressiusculum*, widely distributed in the Eurasian Continent. In Korea, morphologically intermediate specimens have been collected, mixed with typical *S. depressiusculum*. The taxonomical status of these two species is thus questionable. To clarify their status, sequencing of mitochondrial 16S ribosomal RNA (rRNA) was performed on 77 specimens of *Sympetrum* species collected from Korea and Japan. The pairwise differences between 378 nucleotides of *S. frequens* and *S. depressiusculum*, including the intermediate type, revealed only minor differences (< 0.5%, average 0.48%). The neighbor-joining phylogenetic tree indicated that all *S. frequens* and *S. depressiusculum* form one clade, suggesting that they pertain to a single species. The tree also suggests that the *S. frequens* population from Hokkaido is different from all other populations.

Introduction

Sympetrum frequens (Selys) is a common anisopteran of the islands of Japan, where it is regarded as an endemic (Hamada & Inoue 1985; Sugimura et al. 1999). *S. depressiusculum* (Selys) is widely distributed on the Eurasian continent including the Korean Peninsula. *S. depressiusculum* occasionally emerges in Japan (Naraoka 1993, 1996), although it is usually considered not to be resident there. It is collected every year from the Tsushima Islands halfway between the Korean Peninsula and Kyushu (M. Aiura pers. comm.).

Asahina (1984) hypothesized that *S. frequens* is the insular vicariant of *S. depressiusculum*. During the early Quaternary, when the Japanese Archipelago was

connected to the continent, a common ancestor might have extended its range there. After the late Quaternary, when the Japanese Islands detached from the continent, *S. frequens* became established when a particular race of *S. depressiusculum* evolved on the Japanese islands, becoming larger in the warmer climate and acquiring a strong ability to migrate to the highlands in summer.

Evidence for the taxonomic status of *S. frequens* remains equivocal. For example, the morphology of the male vesica spermalis of *S. frequens* is very similar to that of *S. depressiusculum* (Asahina 1961). *S. frequens* is usually distinguished from *S. depressiusculum* with the aid of its colour pattern: a broad basifrontal dark stripe without deep invagination on the sides and a broad pterothoracic dark stripe (Asahina 1984). Transitional forms are often found, however, especially in the Korean Peninsula (Asahina 1958, 1961, 1984, 1990) and in Primorskii krai in Russia (K. Inoue pers. comm.). Therefore Asahina (1961) suggested that Japanese *S. frequens* should be included in the continental *S. depressiusculum*. Later, Kiauta & Kiauta (1984) revealed that chromosome numbers and karyotypic morphology of both species were essentially similar. This taxonomic problem has not been exhaustively addressed so far.

Several studies have used mitochondrial (mt) DNA sequence data to establish phylogenetic relationships among odonate species (e.g. Chippindale et al. 1999; Hirose 2000; Artiss et al. 2001; Turgeon & McPeek 2002). In the present study, we analyze mtDNA sequence data from the 16S rRNA region in specimens of *S. depressiusculum*, *S. frequens* and intermediate forms.

Material and methods

Odonata

Details of the collection sites of the *Sympetrum* specimens used in this study are given in Table 1 and Figure 1. A total of 77 *S. depressiusculum* (= Sdp) and *S. frequens* (= Sf) were obtained from Korea and Japan during 1999 - 2001. The Sdp specimens with a thick black stripe on the thorax being morpholologically similar to that of *S. frequens* were categorized as the intermediate type (= Sdp*). Two additional species, *S. darwinianum* (Selys) (= Sdw) and *S. striolatum imitoides* Bartenev (= Ss) were used for the DNA analysis as reference samples for the out-group. Each locality of the collection site was marked on the map (Fig. 1), and each specimen was numbered with a three-figure according to its locality and sample numbers, e.g. Sdp101 showed 01 of sample number and was collected from site no. 1, Chungchongnam-Do. All specimens were analyzed for morphological characteristics and stored at -80°C until used for extraction of total DNA.

DNA extraction and PCR amplification

We amplified a region of 16S rRNA because it is more preserved than the protein coding regions of mtDNA and is therefore suited to studying evolutionary relationships (Nei 1990). Total genomic DNA was extracted from individual Odonata

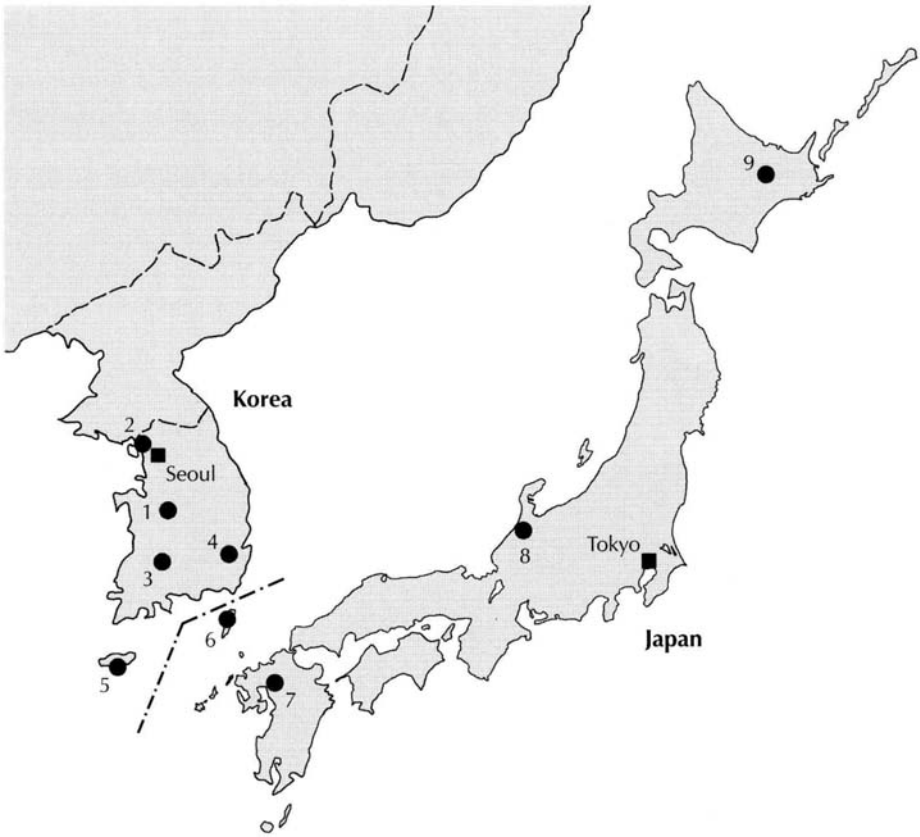

Figure 1: Map showing the localities where the specimens analyzed were collected from the Korean Peninsula (site numbers 1-5) and from the Japanese Islands (site numbers 6-9) — (1) Chungchongnam-Do; (2) Kyonggi-Do; (3) Chollabuk-Do; (4) Kyongsanbuk-Do; (5) Cheju-Do); (6) Nagasaki-Prefecture; (7) Saga-Pref.; (8) Ishikawa-Pref.; (9) Hokkaido. See Table 1 for site details.

using an IsoQuick DNA extraction Kit (Tanehashi, Tokyo, Japan). The mitochondrial 16S rRNA region was amplified in a final volume of 20 µl containing AccuPower PCR premix (Bioneer, Chungbuk, Korea) and primers used for PCR (Model 9600, PE Applied Biosystems, USA). The PCR cycling conditions were as follows: 30 s 95°C, 30 s 55°C and 1 min 72°C for 35 cycles followed by a 4 min final extension at 72°C. Primers used were designated based on the alignments of the mitochondrial 16S rRNA from *S. corruptum* (Hagen) (GenBank accession numbers AF037192-037193 and AF037171-037184); 5'-CGC TGT TAT CCC TAA GGT-3' and 5'-GTA TAA GGT CTG ACC TGC C-3', as a forward and reverse primer, respectively.

DNA sequencing

The PCR products, visualized by ethidium bromide staining, were isolated by excising individual bands from a 2% LMP agarose gel (SeaPlaque GTG agarose, USA) and then purified using GFX PCR DNA and a Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Each purified double-stranded PCR product was directly cycle-sequenced from both ends using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit v1.1 (PE Applied Biosystems) and the same primers used for PCR using the thermal profile 10 s 96°C, 5 s 55°C and 4 min 60°C for 25 cycles (Model 9600, PE Applied Biosystems), and ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Table 1. Collection sites of *Sympetrum* specimens in Korea and Japan. — Sdp: *S. depressiusculum*; Sdp*: *S. frequens*-like *S. depressiusculum*; Sf: *S. frequens*; Sdw: *S. darwinianum*; Ss: *S. striolatum imitoides*.

Country	Map no.	Region	Site	Date	Latitude	Taxa
Korea	1	Chungchongnam-Do	Cheong-yang, Chilgapsan	viii 1999	36°52′N	Sdp, Sdp*, Sdw
			Puyo	x 2000	36°15′N	Sdp, Sdp*
	2	Kyonggi-Do	Kapyong	vii 2000	38°00′N	Sdp, Sdp*
			Yong-in, Sougjun	ix 2000	37°01′N	Sdp, Sdp*
			Kanghwado	ix 2000	37°35′N	Sdp
	3	Chollabuk-Do	Namwon	vii 2000	35°25′N	Sdp, Sdp*
	4	Kyongsangbuk-Do	Kyongju	ix 2000	35°05′N	Sdp, Sdp*
	5	Cheju-Do	Seogwipo	ix 2000	33°15′N	Ss
Japan	6	Nagasaki-Pref.	Kami-tsushima	ix 2001	34°35′N	Sdp, Sf
	7	Saga-Pref.	Kyuragi-machi	ix 2001	33°20′N	Sf
	8	Ishikawa-Pref.	Nonoichi-machi	x 2000	36°30′N	Sf
	9	Hokkaido	Bihoro-machi, Fukuzumi	x 2000	43°45′N	Sf

Table 2. Percentage of nucleotid variation between pairs of *Sympetrum specimens*. The pairwise distances of the mitochondrial 16S rRNA were analyzed. For abbreviations, see Table 1.

	Sdp	Sdp*	Sf	Sdw	Ss
Sdp	0.44	-	-	-	-
Sdp*	0.44	0.48	-	-	-
Sf	0.48	0.50	0.50	-	-
Sdw	0.89	1.48	1.17	0.82	-
Ss	1.07	1.90	1.41	3.36	0.27

Phylogenetic analysis of mitochondrial 16S rRNA

Alignment analyses were performed using the program GENETYX-WIN ver. 5 (Software Development Co., Tokyo, Japan). Phylogenetic analysis was performed using distance and parsimony methods in MEGA (ver. 2.1; Kumar et al. 2001). Alignment gaps were treated as missing data. For the distance method, the neighbor-joining (NJ) approach was applied using Kimura's 2-parameter model. For the maximum parsimony (MP) method, the heuristic search was used. The topologies of the inferred trees were assessed by 1,000 bootstrap resamplings for each method.

Results

Similar sized PCR products were amplified at the 16S rRNA region from all *Sympetrum* specimens. The partial 16S rRNA sequences from all Sdp, Sdp* and Sf specimens were 378 base pairs long with no major variations in alignment and were highly A+T rich (GC content: 26.86 - 27.67%) while the 16S rRNA sequences of Sdw and Ss specimens had slightly higher G+C contents (29.03 - 29.57%).

Alignments of 378 base pairs in length showed few insertions and deletions, but generally little variation in *Sympetrum* species. The levels of nucleotide variation detected between pairs of specimens are presented in Table 2. Percentage nucleotide differences within and also between groups were very low, < 0.5% (average 0.48%). No differences were found between intra- and interspecific variations.

Two trees using the NJ and parsimony methods inferred from the partial mtDNA 16S rRNA sequences are shown in Figures 2 and 3. In the NJ tree (Fig. 2), none of the Sdp and Sf populations, including Sdp*, separated into distinct clusters, while the outgroup species, Sdw and Ss formed a distinct clade with bootstrap confidences greater than 95%. Bootstrap values are not shown at the nodes of Figure 2 because, except for a few individuals, all values were less than 50%. Despite this, when focusing only on Sf populations, the NJ tree separated into two groups. The lower small cluster, group II, consisting of Sdp103 down to Sf801, contained all the Japanese Sf populations except that from Hokkaido (Sf901-906).

All the Hokkaido Sf specimens were distributed on the larger cluster, group I, along with specimens of Sdp from all regions of Korea and two Sf specimens from Japan.

A similar trend of the clustering arose in the MP tree (Fig. 3). All the populations were nearly equal distributed on this tree, and the out-group species, Sdw and Ss, were in the different cluster, showing greater than 95% bootstrap confidences. The MP tree separated into two groups as in NJ tree. All Japanese Sf specimens were included in group II, but no Hokkaido specimen (Sf901-906).

Discussion

In the present study based on the mitochondrial 16S rRNA sequences, hardly any nucleotide differences were found among the three groups of putative *Sympetrum* species, Sf, Sdp and Sdp*. This result suggests that *S. frequens* and *S. depressiusculum* should be assigned to a single species, with the senior name *depressiusculum* taking precedence. The population of *S. frequens*-like *S. depressiusculum*, Sdp*, is clearly included in the above-mentioned single species. This is a new argument in an old discussion about the taxonomic status of *S. frequens*. Asahina (1961) indicated that the morphology of the male vesica spermalis of *S. frequens* is the same as that of continental *S. depressiusculum*. Furthermore, Asahina (1990) found out that a *S. depressiusculum* population from the northern highland of the Korean Peninsula, Nomagodae, had a hindwing length ranging 23-29 cm, and did not show any differences from *S. depressiusculum* from Europe to Manchuria. The hindwing length of *S. depressiusculum* gradually increases (29-34 cm) southward toward the southern Korean Peninsula. The population has a black stripe on the thorax as broad as that of *S. frequens* of Japan. This suggests that the body size of *S. depressiusculum* – expressed as the hindwing length – is gradually increasing towards the south to nearly the size of *S. frequens*. Asahina (1990) argued that Japanese *S. frequens* should therefore be included in the continental *S. depressiusculum*. Our results support this opinion.

In our study, the phylogenetic trees were constructed using two different methods, the distance method, NJ, and the parsimony method. Both trees indicate the same result, that *S. depressiusculum* and *S. frequens* should be regarded as a single species. However, this species could be split into at least two minor groups. Almost no specimens in group I of the NJ tree (Fig. 2) are included in the lower small cluster, group II of Figure 3, consisting of Sdp607 down to Sf803, except three specimens, Sdp110*, Sdp604 and Sf802. No Hokkaido Sf specimen (Sf901-906) is found in group II, but all are included in group I.

Both trees indicate that the Hokkaido population is closer to some Korean populations than the other Japanese populations. This is probably caused (1) by differences of the source (the ancestor) population, due to different expansion routes from the continent into the Japanese Archipelago during the Quaternary era and/or (2) by the difference of selection pressure among at least three localities – Korean Peninsula, Hokkaido and Japanese islands except for Hokkaido – taken into account in our study. Unfortunately, no specimens from Russia were analyzed so

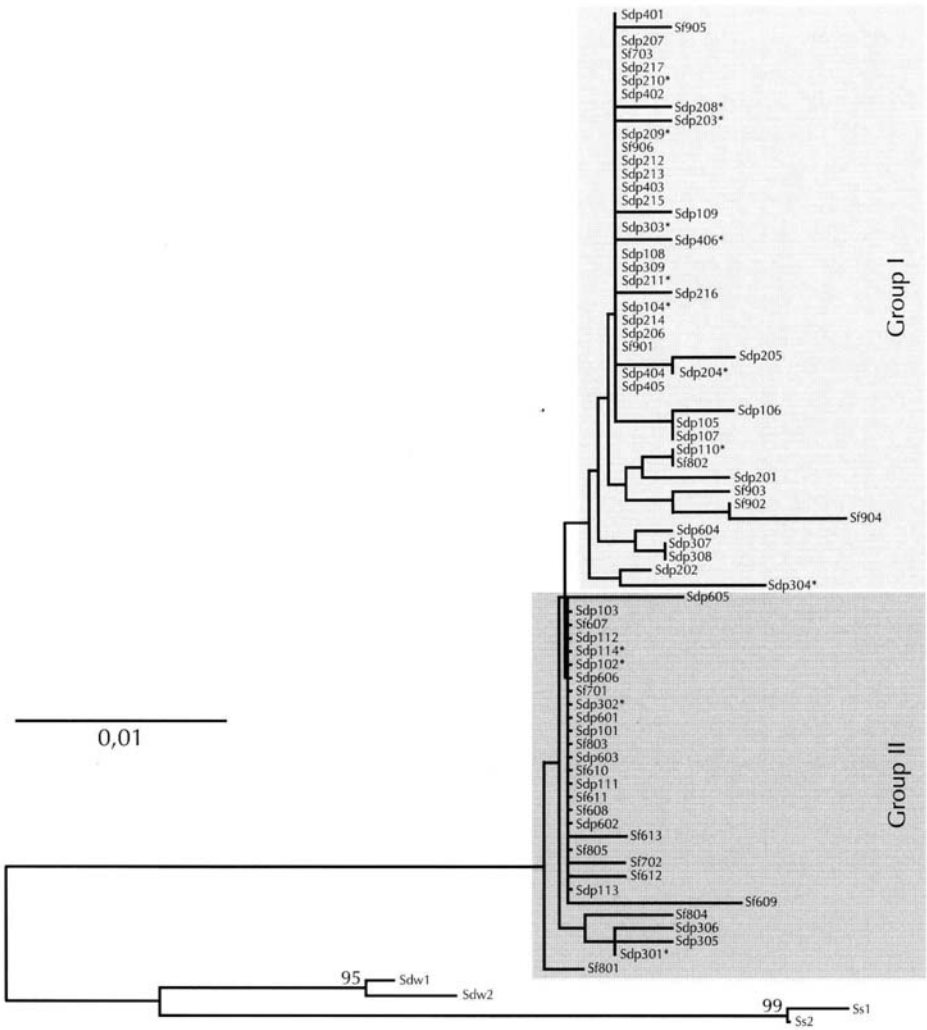

Figure 2: Neighbor-joining (NJ) tree of *Sympetrum* species based on partial mitochondrial 16S rRNA gene nucleotide sequences. A distance matrix was calculated using the Kimura's 2-parameter evolutionary model and the tree was constructed using the neighbor-joining approach in the MEGA ver. 2.1. Scale bar indicates the proportion of sites changing along each branch. Numbers on internodes indicate percentage of 1,000 bootstrap replicates.

far, so we cannot discuss possibility (1) in more detail. Possibility (2) attracts our interest because, in Hokkaido, *S. frequens* does not always perform the long distant migration for upland aestivation as that is typical of populations from other localities in Japan (Uéda 1988). We will discuss this problem in another paper. In other focus, according to the low levels of the bootstrap confidences, it could be inferred that the genetic divergence is now in progress. The speed of genetic divergence in mtDNA is five to ten times greater than that in coding regions of nuclear DNA (Brown et al. 1979; Nei 1985). Therefore, mtDNA gene sequences typically show more differences among the populations in terms of genetic divergence than nuclear sequences do.

One limitation of this study was that we estimated genetic differentiations among the three *Sympetrum* populations using the mtDNA 16S rRNA region only. Since, unlike mtDNA, the nuclear genome is biparentally inherited, nuclear sequences may be used to detect heterozygotes for an investigation of the gene flow between populations or hybridization between species. Furthermore, while most regions of the nuclear genome are conserved relative to the mitochondrial genome, recent work by Samraoui et al. (2003) used variation at the ITS1 intergenic spacer region, but not at ITS2 or by morphology, to separate *Lestes virens* (Charpentier) into two taxa. By contrast, Samraoui et al. (2002) found no significant nucleotide differences in the 18S rDNA or the ITS1 and 2 intergenic spacers among morphologically distinct members of the recently diverging *Enallagma cyathigerum* (Charpentier). Selecting the appropriately variable genetic marker therefore is not always straightforward. We believe that the mitochondrial 16S rRNA sequences are more useful to estimate the intraspecific variation between closely related sibling species than nuclear genomes. Among all gene regions of the mtDNA, the rRNA region is more highly conserved than the other protein coding region of mtDNA so that it is recognized as the most appropriate genetic marker to study and the major lines of descent during interspecific evolutionary research (Nei 1985). The establishment of adequate phylogenetic systematic is anticipated from mutation comparisons of the base sequence in this region. Thus, we used the mitochondrial 16S rRNA sequences as the first step of this study, however, a combined analysis that includes more variable regions of the mitochondrion, such as cytochrome oxidase I, will likely shed further light on the phylogenetic relationships of Asian *Sympetrum* species (Artiss et al. 2001).

Only when phylogenetic trees inferred from different markers, preferably from both mitochondrial and nuclear genomes show the same topologies can we have a high degree of confidence that we have uncovered an accurate phylogeny of the organisms. For the *Sympetrum* species, only a few partial sequence data have recently been reported from the 18S and 28S rRNA genes of *S. vulgatum* (Linnaeus), *S. sanguineum* (O.F. Müller) and *S. danae* (Sulzer) by Hovmoller et al. (2002). We obtained clear results indicating the genetic differentiation between three populations of *S. frequens* and *S. depressiusculum* from the mtDNA region. However, more sequence data from nuclear regions may be provided to confirm this notion, and in particular, data from nominal *S. depressiusculum* populations in Eurasia, especially from Russia, would be very useful as a next step.

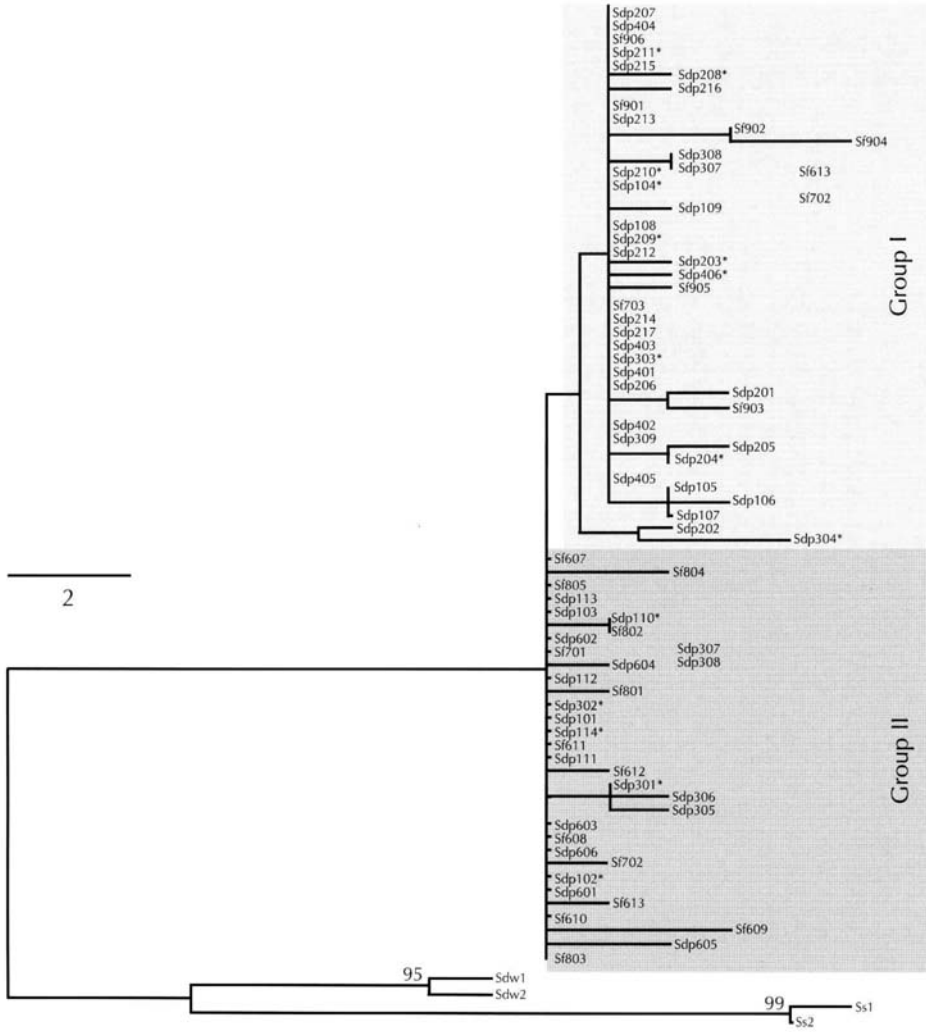

Figure 3: Maximum parsimony (MP) tree of *Sympetrum* species based on partial mitochondrial 16S rRNA gene nucleotide sequences. The tree was constructed using heuristic search of parsimony method in the MEGA ver. 2.1. Scale bar indicates the number of changes inferred as having occurred along each branch. Numbers on internodes indicate percentage of 1,000 bootstrap replicates.

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