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DNA barcording and molecular phylogenetic analyses of *Siphlonurus* mayfly species (Ephemeroptera: Siphlonuridae) in Japan

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Abstract
Mayflies of the genus *Siphlonurus* (Ephemeroptera, Siphlonuridae) are distributed throughout the Northern Hemisphere. However, information of species distribution and the presence of DNA sequences in reference databases for species identification of *Siphlonurus* are limited in Japan. To enhance species identification and gather DNA information within this genus, DNA sequences from the mitochondrial cytochrome c oxidase subunit I (COI), nuclear DNA of histone H3, and the 18S rRNA regions were analyzed. The study focused on three species (*S. binotatus*, *S. sanukensis*, and *S. yoshinoensis*), with eighteen specimens collected from eastern Japan. The phylogenetic tree of a phylogeny using all three genes (COI + histone H3 + 18SrRNA) strongly supported monophyly at species level. For *S. yoshinoensis*, we found that they have high genetic difference within the species for COI and histone H3 regions.

Keywords: Mitochondrial DNA COI region, nuclear DNA histone H3, nuclear DNA 18S rRNA, species identification, freshwaters

Introduction

Biological monitoring of insects is important for conserving biodiversity and protecting ecosystems (Gerlach *et al*., 2013) [5]. However, a lack of experts with the taxonomic skills to classify various insect species is problematic because taxonomic information is an important foundation to understand and evaluate species diversity. Therefore, the development of genetic-based methods of species identification, such as DNA barcoding, has become a prominent sequencing technology that uses information. Some DNA data is available at Barcode of life (BOLD) data systems or National Centre for Biotechnology Information (GenBank etc.), facilitating researchers. These data are useful not only species identification, but also phylogenetic analysis.

Aquatic insects have high value as good indicators of water quality. So identification skill of aquatic insects is useful and needed for evaluating for environmental condition such as to understand the water quality. Recently, DNA-based identification including DNA barcoding of aquatic insects has advanced (Zhou *et al*., 2010; Gattolliat *et al*., 2015; Saito *et al*., 2016; Selvakumar *et al*., 2016; Wakimura *et al*., 2016; Kranzfelder *et al*., 2017; Molina *et al*., 2017; Stauffer-Olsen *et al*., 2017; Jo and Tojo, 2019) [24, 4, 16, 17, 23, 10, 12, 18, 8] and these studies contributed for species identification and to know phylogenetic relationship among aquatic insects.

Mayflies of the genus *Siphlonurus* Eaton, 1868 [2] (Ephemeroptera, Siphlonuridae) are distributed throughout the Northern Hemisphere. Larvae are good swimmers and they have characteristic large leaf-like tracheal gills (Ishiwata *et al*., 2018) [7]. Four *Siphlonurus* species are known to distribute in Japan: *Siphlonurus binotatus* Eaton, 1892 [1]; *Siphlonurus sanukensis* Takahashi, 1929 [19]; *Siphlonurus yoshinoensis* Gose, 1979 [6]; *Siphlonurus zhelochovtsevi*, Tshernova, 1952 [22]. However, *S. zhelochovtsevi* has only been recorded in larval stage in Hokkaido and detailed information was unrecorded. Morphological identification of species level for three species except S. *zhelochovtsevi* in larvae stage is only possible at: an individual in the state just before hatching, with the male's genitalia visible from the outside, or an individual in which the body color of the adult stage is transparent (Ishiwata *et al*., 2018) [7]. From these reasons, detailed species distributional information and DNA sequence data of *Siphlonurus* in Japan were difficult to gather. For example, much DNA information of Japanese mayfly species was obtained by Wakimura *et al*. (2016) [23]. However, for *Siphlonurus* species, they registered only one species (*S. sanukensis*, three individuals) and others were registered as *Siphlonurus* sp. (five individuals).

In this study, we collected adults and larvae of three *Siphlonurus* species (*S. binotatus*, *S. sanukensis*, and *S. yoshinoensis*) in Japan to accumulate DNA information used for species identification and phylogenetic analysis.

Materials and methods

Sampling

A total of 18 specimens of *Siphlonurus* species from six locations mainly in Hokkaido and north-east Japan (Table 1) were collected (nine specimens of *S. binotatus*, two specimens of *S. sanukensis*, and seven specimens of *S. yoshinoensis*). Larvae were collected using hand nets and emerged in the laboratory under plastic container filled with water at room temperature (ca. 25°C). Some adults were collected using sweep nets and some were collected using light traps. All specimens were fixed in 99.9% ethanol. Our species identifications were conducted based on the

morphological key characteristic sets by Ishiwata *et al*. (2018) [7].

Table 1: Sample information and accession number of sequences

Species	Stage	Location	ID	CO1	18SrRNA	Histone h3
Siphlonurus binotatus	Adult	Tamura, Fukushima, JPN	156	LC579582	LC579596	LC579612
Siphlonurus binotatus	Adult	Tamura, Fukushima, JPN	157	LC579583	LC579597	LC579613
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	162	LC579587	LC579601	LC579617
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	163	LC579588	LC579602	LC579618
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	164	-	LC579603	LC579619
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	165	-	LC579604	LC579620
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	166	-	LC579605	LC579621
Siphlonurus binotatus	Adult	Namie, Fukushima, JPN	167	LC579589	-	LC579622
Siphlonurus binotatus	Adult	Inawashiro, Fukushima, JPN	153	LC579579	LC579593	LC579609
Siphlonurus yoshinoensis	Adult	Eniwa, Hokkaido, JPN	95	LC579575	LC579590	LC579606
Siphlonurus yoshinoensis	Adult	Eniwa, Hokkaido, JPN	96	LC579576	LC579591	LC579607
Siphlonurus yoshinoensis	Adult	Horokanai, Hokkaido, JPN	97	LC579577	-	-
Siphlonurus yoshinoensis	Adult	Horokanai, Hokkaido, JPN	98	LC579578	LC579592	LC579608
Siphlonurus yoshinoensis	Adult	Oshu, Iwate, JPN	154	LC579580	LC579594	LC579610
Siphlonurus yoshinoensis	Adult	Oshu, Iwate, JPN	155	LC579581	LC579595	LC579611
Siphlonurus yoshinoensis	Adult	Namie, Fukushima, JPN	158	LC579584	LC579598	LC579614
Siphlonurus sanukensis	Adult	Namie, Fukushima, JPN	159	LC579585	LC579599	LC579615
Siphlonurus sanukensis	Adult	Namie, Fukushima, JPN	160	LC579586	LC579600	LC579616

DNA extraction, amplification, sequencing and estimating divergence times

Total genomic DNA was extracted from ethanol-preserved tissue (pectoral muscle or legs) of specimens using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden), according to the manufacturer's instructions. Total DNA was used to amplify DNA fragments by polymerase chain reaction (PCR) with sets of primer (Table 2). PCR performed using the enzyme, rTaq (TOYOBO, Osaka) and KOD FX NEO (TOYOBO, Osaka) for three regions; mitochondrial cytochrome c oxidase subunit I (hereafter, mtDNA COI), and nuclear DNA (hereafter, nuDNA) of histone H3 and 18S rRNA. PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon genetics, Tokyo). Purified DNA fragments were sequenced by consignment (Eurofins, Tokyo) as follow; DNA fragments were directly sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, MA) on an automated DNA Sequencer (3730xl DNA Analyzer, Thermo Fisher Scientific, MA). We added the sequences of *Siphlonurus* species from GenBank for phylogenetic analysis (Figures 1-3). For outgroup, we selected Odonata, *Anax parthenope* [mtDNA COI (MN701549, MN701501), nuDNA histone H3 (MT010074) and 18S rRNA (MK774267)].

Table 2: DNA primers used for PCR and sequencing analysis refer to Wakimura *et al*., (2016) [22]

Region	Primer name	Sequene (5'-3')	Annealing temperature
CO1	LCO1490	atatccttgggcatgatggtgac	45℃
	HCO2198	taaacttcagggtgaccaaaaaatca	
18SrRNA	KOBO18SF1	tggcgtatattaaagttgttgcggt	55℃
	KOBO18SR1	agtttcagctttgcaaccatacttc	
Histone h3	HexAF	atggctcgtaccaagcagacggc	55℃
	HexAR	atatccttgggcatgatggtgac	

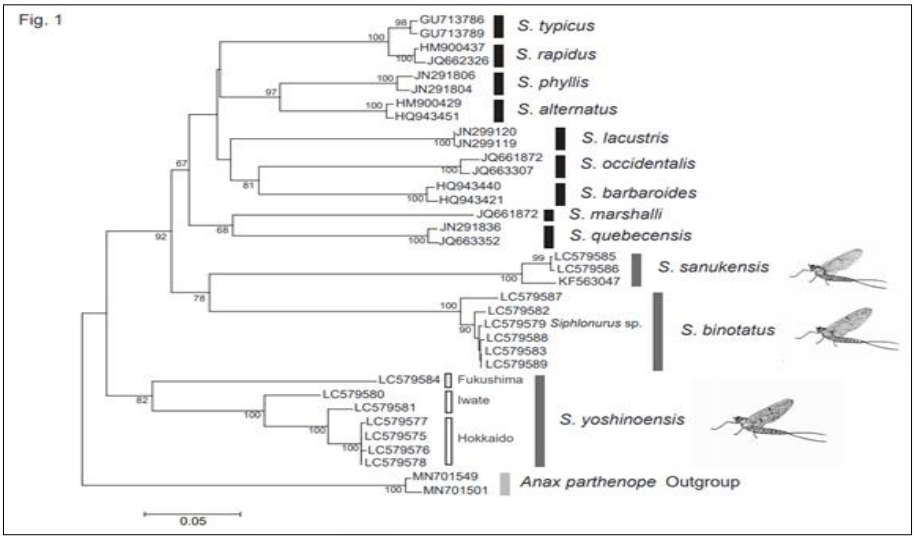

Fig 1: Maximum likelihood (ML) phylogram of 12 *Siphlonurus* species based on the sequence data of the mtDNA COI region (549 bp). The OTUs indicate the detected GenBank accession numbers. The accession numbers of analysis in this study referred to in Table 1. The numbers around major nodes indicate ML bootstrap supports (>60).

All of DNA sequences data have been registered to DDBJ, and the accession numbers are given in Table 1. Sequence alignment and editing were performed for each gene separately using CLC Main Workbench software (CLC bio, Aarhus). All sequence data were aligned using Clustal W (Thompson *et al*., 1994) [20] instrumented with MEGA 7 (Kumar *et al*., 2016) [11].

Phylogenetic analyses were performed by the maximum likelihood method (ML; Felsenstein, 1981) [3] using MEGA 7 with 1,000 bootstrap replications. Prior to the ML phylogenetic estimations, best-fit ML models were chosen based on Schwarz's Bayesian Information (BIC) as follows using MEGA 7: General Time Reversible + G + I for the mtDNA COI region, Tamura-Nei + G for histone H3 region, Kimura 2-parameter for 18S rRNA region, and Tamura 3-parameter model region for combined their regions (COI + histone H3 + 18S rRNA). Mean of genetic distance between eleven species and intraspecies of *S. yoshinoensis* for mtDNA COI region was estimated by Kimura 2 Parameter using MEGA 7.

Results

About the phylogenetic tree of the mtDNA COI region (549 bp) of *Siphlonurus* species (Figure 1), the 11 species monophyly at the species level was strongly supported (Figure 1). *Siphlonurus marshalli* Traver, 1934 [21] was analyzed only one sample in this study, therefore, it's monophyly could not be tested (Figure 1). The ML bootstrap support levels of each species were above 80 % (Figure 1). *S. sanukensis* was basal to all other species (Figure 1). In addition, *S. binotatus* and *S. yoshinoensis* were also basal clades (Figure 1). The range of genetic distance between *Siphlonurus* species is 0.023-0.234 between species (Table 3). The phylogenetic tree of nuDNA histone H3 region strongly supported three species monophyly at the species level for *S. yoshinoensis* and *S. sanukiensis*, but it was not for S. *binotatus* (Figure 2). In addution, the phylogenetic tree of nuDNA 18S rRNA region was not supported species monophyly except for *S. binotatus* group (Figure 3). Furthermore, we also constructed a phylogeny using all three genes, then, monophyly at the species level was strongly supported (Figure 4). As the DNA sequence data registered in NCBI as *Siphlonurus* spp. collected from Japan (KP970718 - KP970721 for COI, KP970742 - KP970746 for histone H3; KP970792 - KP970794 for 18rRNA from Wakimura *et al.*, 2016) [23] made monophyly clades with our samples (Figure 1, 2). Moreover, these clades were strongly supported at the species level (Figure 1, 2). However, monophyly at the species level wasn't strongly supported for two DNA sequence data for nuDNA 18S rRNA region (Figure 3).

Table 3: Average of genetic distance for mtDNA COI region (549 bp) between Siphlonurus species

Species	*S. typicus*	*S. rapidus*	*S. sanukiensis*	*S. quebecensis*	*S. phyllis*	*S. pccodentalis*	*S. maesahallis*	*S. lcustris*	*S. barbaroides*	*S. altermatus*	*S. binotatus*	*S. yoshinoensis*
S. typicus	-	0.006	0.021	0.019	0.016	0.018	0.018	0.019	0.018	0.015	0.020	0.019
S. rapidus	0.023	-	0.021	0.019	0.016	0.018	0.019	0.018	0.017	0.016	0.019	0.019
S. sanukiensis	0.214	0.206	-	0.023	0.023	0.022	0.022	0.021	0.022	0.021	0.021	0.021
S. quebecensis	0.161	0.164	0.23	-	0.017	0.020	0.018	0.018	0.018	0.017	0.021	0.019
S. phyllis	0.126	0.129	0.221	0.154	-	0.018	0.019	0.015	0.016	0.012	0.017	0.017
S. pccodentalis	0.149	0.161	0.229	0.178	0.156	-	0.020	0.020	0.017	0.018	0.022	0.020
S. maesahallis	0.165	0.175	0.230	0.157	0.181	0.191	-	0.020	0.019	0.019	0.021	0.018
S. lcustris	0.149	0.144	0.216	0.163	0.122	0.168	0.181	-	0.017	0.016	0.021	0.020
S. barbaroides	0.143	0.138	0.220	0.165	0.123	0.128	0.173	0.134	-	0.015	0.020	0.019
S. altermatus	0.119	0.128	0.207	0.144	0.074	0.151	0.176	0.144	0.128	-	0.018	0.018
S. binotatus	0.170	0.170	0.196	0.202	0.145	0.203	0.200	0.190	0.186	0.152	-	0.020
S. yoshinoensis	0.201	0.204	0.224	0.207	0.168	0.197	0.190	0.200	0.187	0.174	0.208	-

Average of genetic distance using Kimura-2-parameter nucleotide divergences (below diagonal) and estimation of standard erroes (above doagonal).

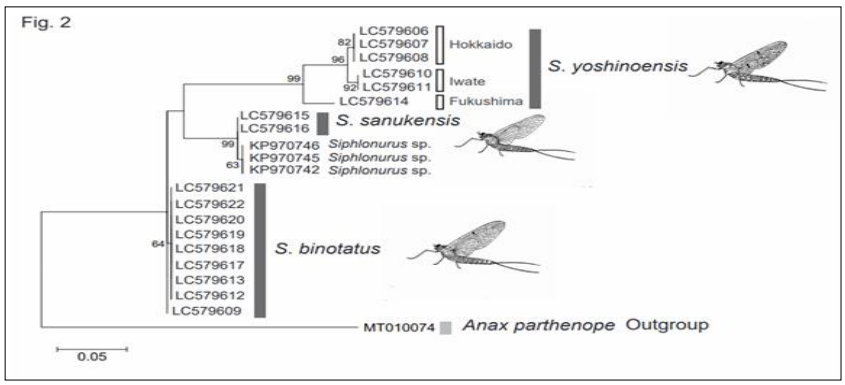

Fig 2: Maximum likelihood (ML) phylogram of of the 3 *Siphlonurus* species based on the sequence data of the nuDNA of histone H3 region (327 bp). The OTUs indicate the detected GenBank accession numbers. The accession numbers of analysis in this study referred to in Table 1. The numbers around major nodes indicate ML bootstrap supports (>60).

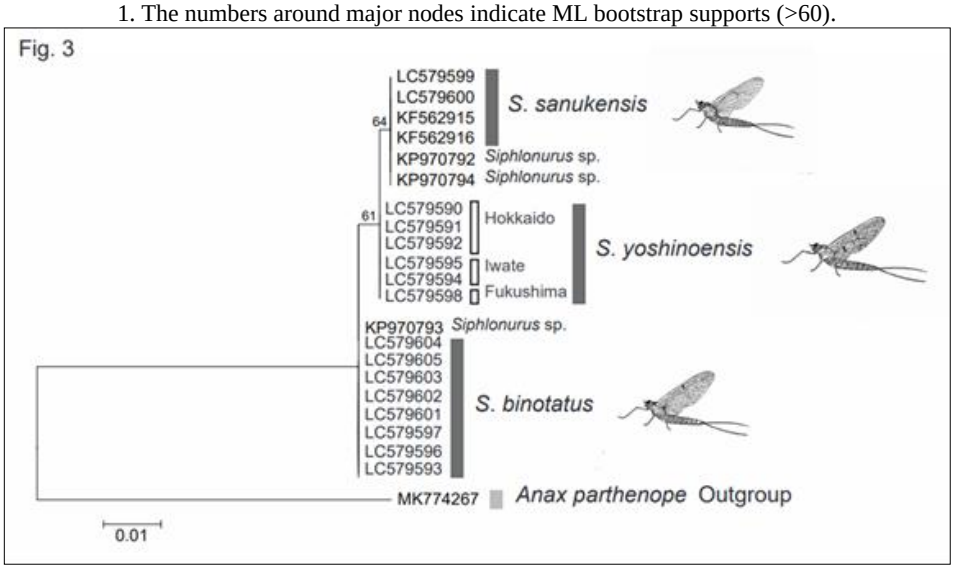

Fig 3: Maximum likelihood (ML) phylogram of of the 3 *Siphlonurus* species based on the sequence data of the 18S rRNA region (533 bp). The OTUs indicate the detected GenBank accession numbers. The accession numbers of analysis in this study referred to in Table 1. The numbers around major nodes indicate ML bootstrap supports (>60).

In addition, we detected genetic difference between the *S. yoshinoensis* collected from Namie town in Fukushima with other *S. yoshinoensis* samples in the phylogenetic tree of mtDNA COI region. Genetic distance between the *S. yoshinoensis* collected from Namie town Fukuhima and specimens collected in Iwate and Hokkaido was 0.150 ± 0.018. Its genetic distance is similar interspecific level. It also indicated the genetic difference of *S. yoshinoensis* at results of phylogenetic tree of nuDNA histone H3 region (Figure 2). However, phylogenetic tree results of nuDNA 18S rRNA did not indicate this trend (Figure 3).

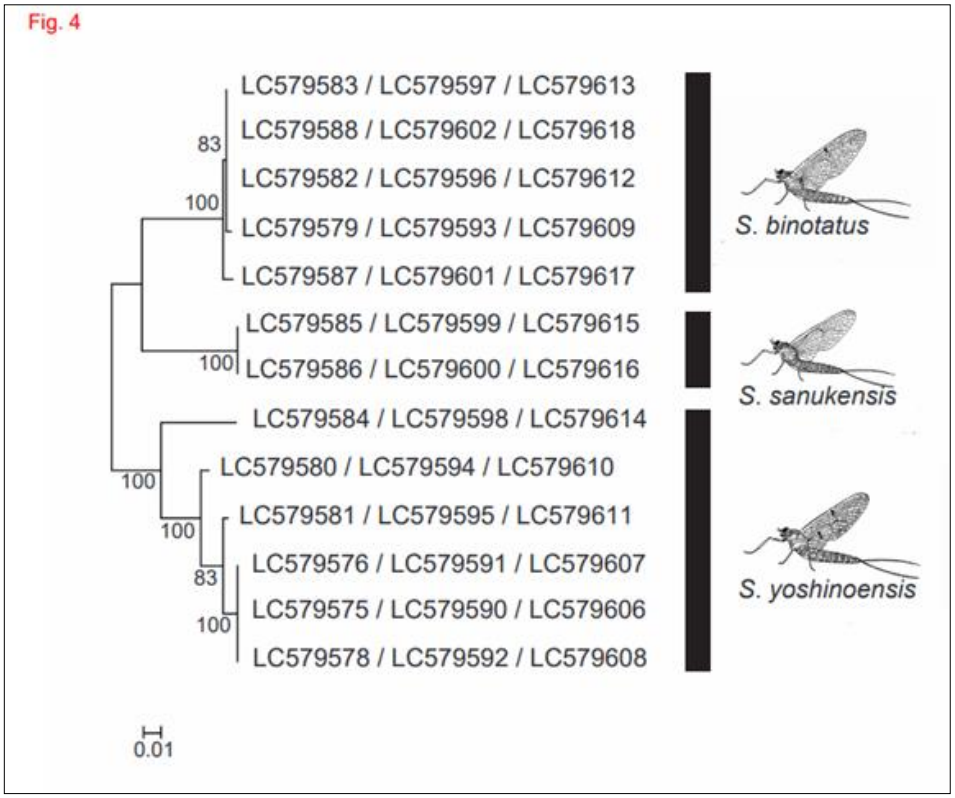

Fig 4: Maximum likelihood (ML) phylogram of of the 3 *Siphlonurus* species based on the sequence data of combined the mtDNA COI (549 bp) + histone H3 (327 bp) + 18S rRNA (533 bp) region (1409 bp). The OTUs indicate the detected GenBank accession numbers. The accession numbers of analysis in this study referred to in Table 1. The numbers around major nodes indicate ML bootstrap supports (>60).

Discussion

Our results showed that species monophyly for 11 species of *Siphlonurus* strongly supported by the bootstrap for mtDNA

COI region levels (Figure 1). In addition, three species are distributed in Japan (i.e., *S. sanukensis*, *S. binotatus*, and *S. yoshinoensis*) were basal clades. Otherwise, other nine species which were used in our phylogenetic analysis for mtDNA COI region (Figure 1, Supplemental material Table S1) were derivative clade and these species are distributed mainly in North America or Europe.

Table S1: Sample information (species name, GenBank ID, country, and reference) utilized in this study in GenBank

Scientific name	GenBank ID (Accession number)			Country	Reference
	COI	H3	18s		
Siphlonurus lacustris	JN299119			Norway Norway	Kjaerstad *et al*., 2012 [9]. A review of the Ephemeroptera of Finnmark DNA barcodes identify Holarctic relations
	JN299120				
Siphlonurus barbaroides	HQ943421			Canada	Unpublished (GenBank only)
	HQ943440				
Siphlonurus phyllis	JN291804			Canada	Unpublished (GenBank only)
	JN291806				
Siphlonurus quebecensis	JQ663352			Canada	Unpublished (GenBank only)
	JN291836				
Siphlonurus occidentalis	JQ661872			U.S.A.	Unpublished (GenBank only)
	JQ663307				
Siphlonurus rapidus	JQ662326			Canada	Unpublished (GenBank only)
	HM900437			U.S.A.	
Siphlonurus typicus	GU713786			U.S.A.	Unpublished (GenBank only)
	GU713789				
Siphlonurus alternatus	HM900429			Canada	Unpublished (GenBank only)
	HQ943451				
Siphlonurus sanukensis	KF563047			Japan	Wakimura *et al*., 2016 [23]. A reference collection of Japanese aquatic macroinvertebrates
Siphlonurus sp.		KP970746	KP970792	Japan	Wakimura *et al*., 2016 [23]. A reference collection of Japanese aquatic macroinvertebrates
		KP970745	KP970793		
		KP970742	KP970794		
			KF562915		
			KF562916		
Anax parthenope	MN701549			Malta	Rewicz *et al*., 2020 [13]. First records raise questions: DNA barcoding of Odonata in the middle of the Mediterranean
	MN701501			Poland	
	MK774267			Japan	Unpublished (GenBank only)

Focusing on three *Siphonurus* species, *S. binotatus*, *S. sanukensis*, and *S. yoshinoensis*, their species monophyly was strongly supported (Figure 1, 4). From our analysis, the unknown species samples from Japan that registered in NCBI were identified at the species level. As the DNA sequence data registered in NCBI as *Siphlonurus* spp. collected from Japan (KP970718–KP970721 for COI, KP970742–KP970746 for histone H3; KP970792–KP970794 for 18rRNA from Wakimura *et al*., 2016) [23], monophyly at the species level was supported in our analyzed in each species samples for mtDNA COI and nuDNA histone H3 regions (Figures 1-2) So, these unknown species's samples could be identified as species level. Otherwise, DNA sequence data for nuDNA 18S rRNA region is not useful species identification, however, it may be useful in phylogenetic analysis of higher taxonomic level (e.g., Wakimura *et al*., 2016) [23].

Finally, we found that *S. yoshinoensis* has high intraspecific genetic difference (Figures 1-2). Previous phylogenetical and phylogeographical studies revealed that some aquatic insects had high intraspecific genetic difference in Japan (e.g., *Isonychia japonica* Saito and Tojo, 2016a; *Drunella* spp., Jo and Tojo, 2019) [14, 8]. In case of mayfly *Isonychia japonica* (Saito *et al*., 2016; Saito and Tojo, 2016a) [16, 13] and *Stenopsyche marmorata* (Saito and Tojo, 2016b) [15], they have some genetic difference within species and which are caused by the influence of the geographical historical events (e.g., formation of Japanese Archipelago and formation of mountain) and habitat selection (e.g., distribution up-stream or down-stream). The origin of the high intraspecific genetic difference within *S. yoshinoensis* is unclear, however it may be possibly related with geographical history or ecological factors. If we try the additional analysis using *S. yoshinoensis* collected from a wider range, it may reveal why the genetic difference within *S. yoshinoensis* has occurred. Furthermore, it was difficult to detect morphological difference within this species because we didn't have adult male samples. As a future subject, we need to confirm the morphology within *S. yoshinoensis* using an adequate sample of adult males.

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Data availability statement

The data that support the findings of this study are openly available in NCBI and DDBJ at nucleotide database at (https://www.ncbi.nlm.nih.gov, https://www.ddbj.nig.ac.jp/index.html) with the accession numbers (from LC579575 to LC579622) which permits

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