Biogeographical Distribution and Phylogenetic Analysis of *Simulium* (*Wallacellum*) (Diptera: Simuliidae) Based on the Mitochondrial Sequences

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Abstract

The blackfly subgenus *Wallacellum* of genus *Simulium* (Diptera: Simuliidae) is a small subgenus, represented by 17 species, and distributed insularly in Southeast Asia. Phylognetic relationships among *Wallacellum* were surveyed using mitochondrial sequences of cytochrome *c* oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) regions. Combined with morphological data, *Wallacellum* was divided into three groups (A, B, C). The group A has ancestral characters and is distributed widely, whereas the group B has derivative characters and is distributed only in the central Philippines, suggesting that the group A is basal and might have been distributed widely in the early period, and then species radiation of the group B occurred in the center of the distribution of this subgenus. The interspecific and intraspecific divergence values were not overlapped both for the COI and 16S rRNA regions, indicating that both regions were effective to identify the species of *Wallacellum*.
Key words: blackfly, distribution, phylogeny, *Simulium*, *Wallacellum*

Introduction

The subgenus *Wallacellum* Takaoka is one of the subgenera of the genus *Simulium* Latreille s. l. (Diptera: Simuliidae) and is known to have insular distribution (TAKAOKA 1983, 2003). It is consisted of 14 species recorded from the Philippines (TAKAOKA 1983, 2006, 2009), two species from Indonesia (TAKAOKA 2003) and one species both from Yonakuni Island, the Ryukyu Islands, Japan (TAKAOKA 1972) and Lanyu Island, Taiwan (CHUNG 1986). According to TAKAOKA (1983), this subgenus is characterized

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by combination of following characters: in the adult, the enlarged calcipala and the hind tibia with a narrow ridge on the anterointernal surface along basal 1/2 or 1/3, the haired pleural membrane and katepisternum; in the pupa, the abdominal segments 6-9 devoid of dorsal spine-combs, abdominal segments 6 and 7 each having an inner hook and lacking an outer hook ventrally on each side.

The subgeneric status of *Asiosimulium* Takaoka and Choochote, *Daviesellum* Takaoka and *Wallacellum*, all recently-established small subgenera of the genus *Simulium*, was confirmed by phylogenetic analysis based on mitochondrial sequences (Otsuka *et al*. 2007). In the phylogenetic analysis, *Wallacellum* was placed basally with a sister group of the counter clade including other eight subgenera of genus *Simulium* in the Oriental Region. The subgenus *Wallacellum* is the only subgenus having insular distribution in the Oriental Region. In the Pacific, two subgenera, *Hebridosimulium* Grenier and Rageau and *Inseliellum* Rubtsov, also have insular distribution. *Hebridosimulium* is endemic in Vanuatu, Fiji and Society islands. *Inseliellum* is distributed in Micronesia (Guam and Chuuk islands in Federated States of Micronesia) and disparately in the southern central Pacific (Marquesas islands, Society islands and Cook islands). Takaoka (2012) also investigated the phylogenetic relationships of 10 subgenera distributing in Southeast Asia and the Pacific using morphological data. In the cladogram, *Wallacellum* has a sister taxon relationship with *Hebridosimulium*. The clade of *Wallacellum and Hebridosimulium* was a sister relation with other subgenera and clades. The analysis both from mitochondrial sequences and morphological data suggested that *Wallacellum* might have been separated from other subgenera of the Oriental Region in the early period. In this paper, we surveyed phylogenetic relationships of 15 of the 17 species of *Wallacellum* based on mitochondrial sequences, and explored the process of distribution of this insular subgenus with morphological data.

Materials and Methods

Sample collection and DNA extraction

Pupae and larvae of each blackfly species examined in this study were collected at localities shown in Table 1. The pupae were individually reared in tubes until adult emergence. Identification was done according to the original descriptions (Takaoka 1972, 1983, 2003, 2006, 2009). DNA was extracted from a single larva, pupa or adult using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Extracted DNA was dissolved in 200μl AE provided in the kit.

PCR amplification and sequencing

The cytochrome *c* oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) regions were amplified by polymerase chain reaction (PCR) using the following primers: LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3' and

Table1. Species of *Wallacellum* and accession numbers and localities of the samples uesd in this study.

species	Accession no.		locality of sample
	CO1	16S	
Simulium alfurense	-	-	
S. amplum	LC034954	LC034985	Calamba, Luzon, Philippines
S. cabrerai	-	AB093128*	Banaue, Luzon, Philippines
S. carinatum	LC034955	AB093129*	Los Baños, Luzon, Philippines
S. celebesense	-	AB334095*	Tomohon, Sulawesi, Indonesia
	-	LC034986	Kendari, Sulawesi, Indonesia
S. claveriaense	LC034956	AB334096*	Claveria, Luzon, Philippines
	LC034957	LC034987	Claveria, Luzon, Philippines
	LC034958	LC034988	Claveria, Luzon, Philippines
S. makilingense	-	-	
S. marilogense	LC034959	LC034989	Davao, Mindanao, Philippines
	LC034960	LC034990	Davao, Mindanao, Philippines
	LC034961	LC034991	Davao, Mindanao, Philippines
S. molawinense	LC034962	LC034992	Calamba, Luzon, Philippines
S. ogonukii	LC034963	LC034993	Davao, Mindanao, Philippines
S. recurvum	-	AB334097*	Banaue, Luzon, Philippines
S. resimum	LC034964	LC034994	Katanglad, Mindanao, Philippines
	LC034965	LC034995	Katanglad, Mindanao, Philippines
S. spinosibranchium	LC034966	AB334098*	Banaue, Luzon, Philippines
	LC034967	LC034996	Banaue, Luzon, Philippines
S. suyoense	LC034968	AB334099*	Luzon, Philippines
	LC034969	LC034997	Luzon, Philippines
	LC034970	LC034998	Luzon, Philippines
	LC034971	LC034999	Cagayan de Oro, Mindanao, Philippines
	LC034972	LC035000	Cagayan de Oro, Mindanao, Philippines
S. tenederoi	LC034973	LC035001	Samar, Philippines
	LC034974	LC035002	Samar, Philippines
S. tuyense	LC034975	AB334100*	Luzon, Philippines
	LC034976	LC035003	Luzon, Philippines
	LC034977	LC035004	Mindoro, Philippines
	LC034978	LC035005	Mindoro, Philippines
	LC034979	LC035006	Mindoro, Philippines
	LC034980	LC035007	Cagayan de Oro, Mindanao, Philippines
	LC034981	LC035008	Cagayan de Oro, Mindanao, Philippines
S. yonakuniense	LC034982	AB334101*	Yonakuni, Japan
*Prosimulium kiotoense***	LC034983	LC035009	Kumamoto, Japan
P. yezoense **	LC034984	LC035010	Hokkaido, Japan

* Sequences were determined in Otsuka *et al* . 2003, 2007.

** *Prosimulium kiotoense* and *P. yezoense* were used as outgroup in phylogenetic analysis.

HCO2198, 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' for COI (FOLMER *et al*. 1994); and primer A, 5'-CGCCTGTTTATCAAAAACAT-3' and primer B, 5'-CTCCGGTTTGAACTCAGATC-3' for 16S rRNA region (XIONG and KOCHER 1991). PCR was carried out using 20 μL volumes containing 0.5 units of *Ex Taq* (TaKaRa), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 1 μL of the extracted DNA. The amplified products were electrophorised through a 1% agarose gel. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the PCR primers. Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit and run on a 3130 Genetic Analyzer (Applied Biosystems). The sequence data of this paper

have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers LC034954-LC035010 (Table 1).

Phylogenetic analysis

Sequences of COI and 16S rRNA of *Wallacellum* were aligned using the CLUSTAL W multiple alignment program (THOMPSON *et al*. 1994) with deposited sequences. *Prosimulium kiotoense* and *P. yezoense* were used as outgroups. Gap sites were excluded from the following analysis. The Kimura two-parameter model was employed to calculate genetic divergence (KIMURA 1980). Using the divergence values, construction of neighbor-joining (NJ) trees (SAITOU and NEI 1987) and the bootstrap test with 1,000 replications were performed with the MEGA version 6.0 program (TAMURA *et al*. 2013). Bayesian analysis was conducted with MrBayes 3.2 (RONQUIST *et al*. 2012) by using two replicates of 1 million generations with the nucleotide evolutionary model. The best-fit model was chosen for each gene separately using the Akaike Information Criterion in MrModeltest version 2.3 (NYLANDER 2004). The general time reversible with gamma distribution shape parameter and invariable sites (GTR+G+I) was selected for both regions. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burn-in.

Results and Discussion

Sequence diversity

The sequences of 29 samples for COI and 24 samples of *Wallacellum* for 16S rRNA region were determined (Table 1). Combined with the published sequences (OTSUKA *et al*. 2003, 2007) and those of the outgroup species (*P. kiotoense and P. yezoense*), the sequences were aligned and compared. Means of interspecific divergence in *Wallacellum* were 15.79% (range 3.94-20.28%) for COI and 3.80% (range 0.78-6.07%) for 16S rRNA region (Table 2). Low levels of interspecific divergence were observed between *S. tuyense* and *S. yonakuniense* (3.94-4.59% for COI and 0.98-1.38% for 16S rRNA) and between *S. marilogense* and *S. ogonukii* (6.08% for COI and 0.78% for 16S rRNA). *Simulium tuyense* and *S. yonakuniense* were morphologically similar especially in the adult and pupal stages, having slight differences in the number of rows and hooks of the larval posterior circle (TAKAOKA 1983). Means of intraspecific

Table 2. Interspecific and intraspecific divergences of *Wallacellum* , and divergences between the groups.

gene	interspecfic divegence		intraspecfic divergence		mean (%) between the groups
	mean (%)	range (%)	mean (%)	range (%)	
COI	15.79	3.94 - 20.28	0.75	0 - 2.49	A - B 17.51 A - C 14.64 B - C 16.30
16S rRNA	3.80	0.78 - 6.07	0.09	0 - 0.39	A - B 4.89 A - C 3.83 B - C 4.55

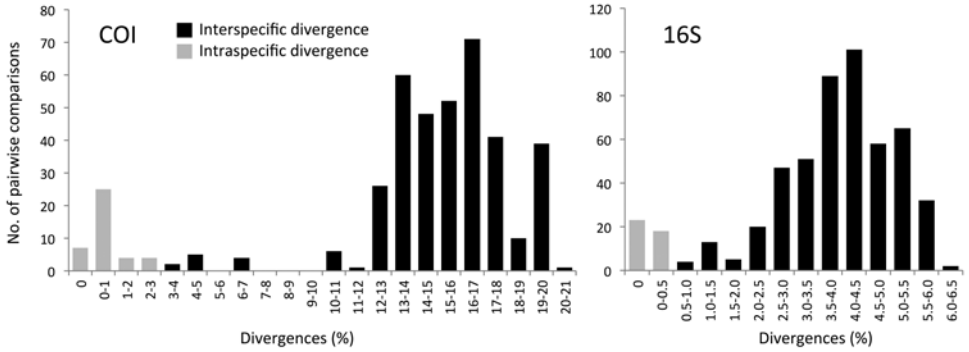

Fig. 1. Histograms showing interspecific (black) and intraspecific (gray) genetic divergences for COI and 16S rRNA regions.

divergence were 0.75% (range 0-2.49%) for COI and 0.09% (range 0-0.39%) for 16S rRNA (Table 2). For both genes, interspecific and intraspecific divergence values were not overlapped (Fig. 1). In COI, since most of the interspecific divergences were larger than 10%, the peak of intraspecific divergence is clearly separated from that of intraspecific divergence. The COI region was used for the identification of animal species known as DNA barcoding (Hebert *et al*. 2003). DNA barcoding has showed to be effective in various taxa of animal including blackfly (Rivera and Currie 2009, Pramual *et al*. 2010, Hernández-Triana *et al*. 2012, Pramual and Adler 2014). In the previous works of Thai blackflies using DNA barcoding, interspecific and intraspecific divergence values overlapped in some taxa (Pramual *et al*. 2010, Pramual and Adler 2014), resulting difficulties of species identification. Our results showed that DNA barcoding is effective for *Wallacellum*. Moreover, 16S rRNA region was also proved to be useful for the identification of species of *Wallacellum*.

Phylogenetic analysis

Phylogentic analysis was conducted for COI and 16S rRNA regions by two methods (NJ and bayes). In Figs. 2 & 3, bayesian trees were shown for COI and 16S rRNA regions, respectively, since bayesian analysis revealed the relationships of species of *Wallacellum* with higher confident values than NJ, although the bootstrap values of NJ were shown in the nodes of the trees. In the analysis, *S. celebesense*, *S. marilogense*, *S. ogonukii*, *S. tuyense* and *S. yonakuniense* had a clade with high confidences without NJ of 16S rRNA region. These species are assigned to the group A. Furthermore, *S. marilogense* and *S. ogonukii* were clearly separated from the other species of the group A. Although *S. marilogense* and *S. ogonukii* were endemic only in Mindanao, the other species of the group A have different distribution. *Simulium tuyense* is known to be distributed in many islands of the Philippines (Luzon, Mindoro, Samar, Palawan and Mindanao). Despite *S. yonakuniense* in Yonakuni island, Japan and Lanyu island, Taiwan and *S. celbesense* in Sulawesi and Biak, Indonesia were geographically separated, the two species were phylogenetically related. In the trees,

Simulium amplum, S. cabrerai, S. carinatum, S. molawinense, S. recurvum , S. resimum and *S. spinosibranchium* also had a clade with high confidences, all of which are assigned to the group B. Although *S. resimum* is endemic only in Mindanao, most

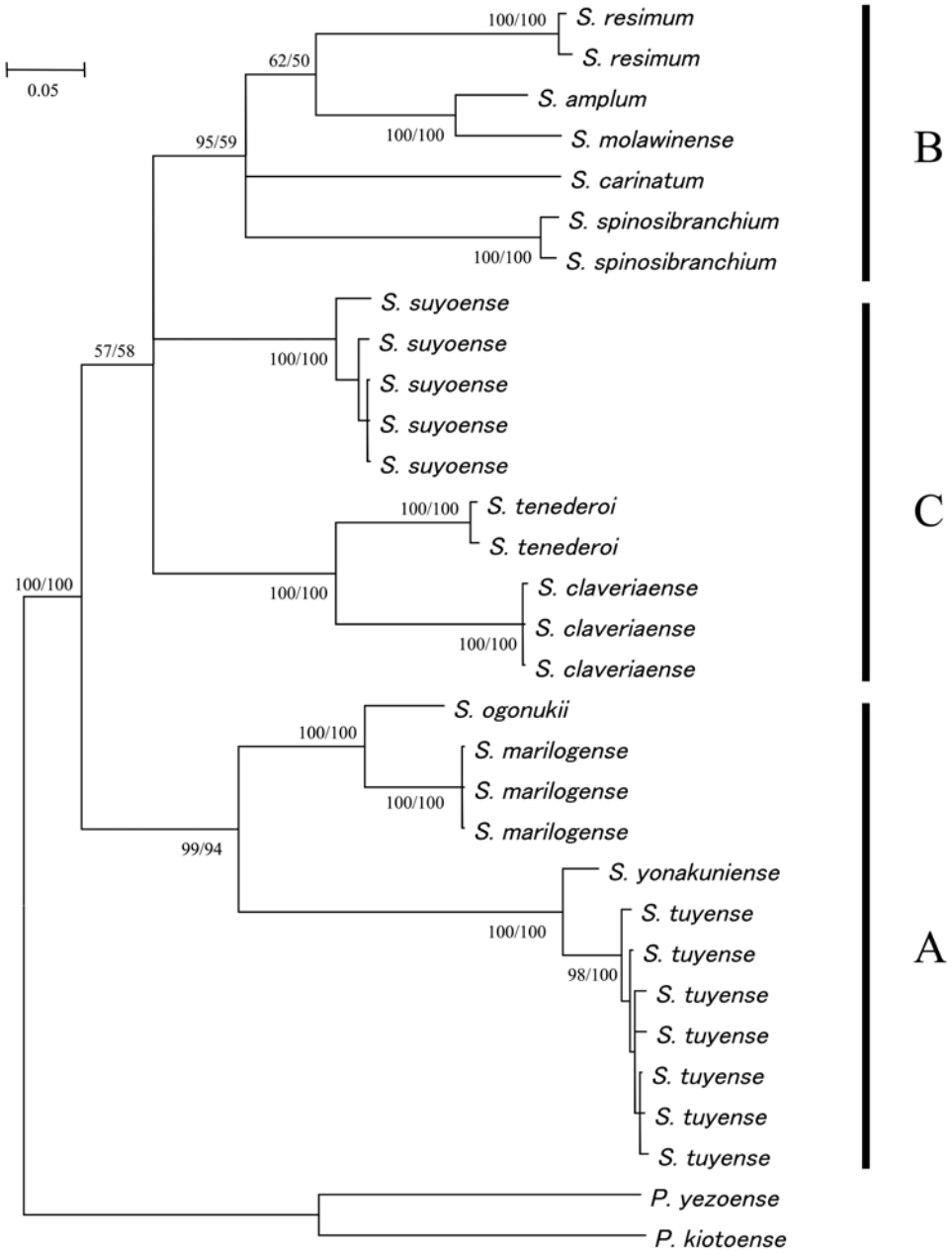

Fig. 2. A bayesian tree of the species of *Wallacellum* based on the sequences of COI. Numbers at tree nodes are bayesian posterior probabilities (%) and bootstrap values (%) of NJ analysis, with dashes indicating a lack of support for the analysis. Branch lengths are proportional to genetic distance (scale bar).

species of the group B are mainly endemic in Luzon. *Simulium carinatum* and *S. recurvum* are also known in Negros, and were slightly related in Bayesian analysis of 16S rRNA. Three other species of *Wallacellum, S. claveriaense, S. suyoense* and *S.*

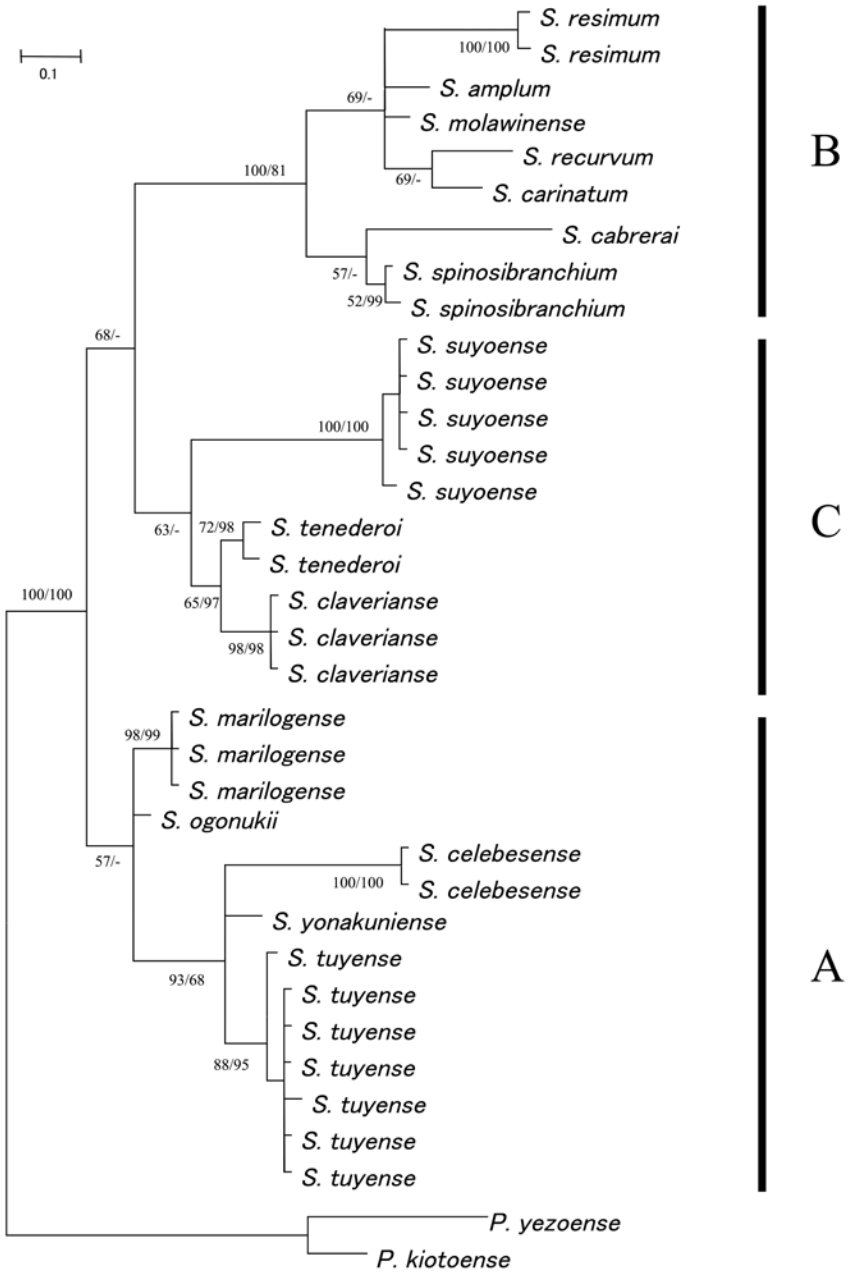

Fig. 3. A bayesian tree of the species of *Wallacellum* based on the sequences of 16S rRNA region. Numbers at tree nodes are bayesian posterior probabilities (%) and bootstrap values (%) of NJ analysis, with dashes indicating a lack of support for the analysis. Branch lengths are proportional to genetic distance (scale bar).

tenederoi, all assigned to the group C, were a monophyletic group only in bayesian analysis of 16S rRNA region. Means of divergence between the three groups were compared (Table 2). Means of divergence between the groups A and B were highest both for COI (17.51%) and 16S rRNA regions (4.89%), whereas those of between the groups A and C were lowest both for COI (14.64%) and 16S rRNA regions (3.83%).

Morphological character and distribution

The three groups also can be separated by morphological characters. The pupae of the groups A and C bear long and slender gill filaments. Moreover, the cuticles of the filament are thick, with numerous transvers ridges. On the other hand, pupal gill filaments of the group B are of short, inflated tubular form, lacking transvers ridges. In the female genitalia, paraprocts of the group B are large, strongly extending ventroanteriorly, and thrusting the posterior margin of the ovipositor valves inward, whereas those of the group A are not so large. The genitalia of female adult of group C are intermediate between the groups A and B (Takaoka 1972, 1983, 2003, 2006, 2009). In this work, *S. alfurense* and *S. makilingense* were not analyzed with mitochondrial sequences. From the original descriptions, *S. alfurense* and *S. makilingense* are likely to belong to the groups A and C, respectively (Takaoka 1983, 2003). The distribution of all the 17 species of *Wallacellum* is listed in Table 3, and the distribution of the three groups is shown in Fig. 4. The group A is widely distributed in the Philippines, and extends northward to Taiwan and Japan and southward to Indonesia. On the other hand, the groups B and C are endemic in the central parts of the Philippines, mainly in Luzon. Certain morphological characters are presiomorphic in the group A and apomorphic in

Table 3. Distribution of *Wallacellum.*

group	species	Country and island											
		J	T	the Philippines						Indonesia			
		Y	La	Lu	Mr	Sa	P	N	Mn	Su	F	B	Se
A	*Simulium alfurense*											○	○
	S. celebesense									○	○*		
	S. marilogense								○				
	S. ogonukii								○				
	S. tuyense			○	○	○	○		○**				
	S. yonakuniense	○	○										
B	*S. amplum*			○									
	S. cabrerai			○									
	S. carinatum			○				○					
	S. molawinense			○									
	S. recurvum			○				○					
	S. resimum								○				
	S. spinosibranchium			○									
C	*S. claveriaense*			○									
	S. makilingense			○									
	S. suyoense			○	○				○**				
	S. tenederoi					○							

J = Japan, T = Taiwan, Y = Yonakuni, La = Lanyu, Lu = Luzon, Mr = Mindoro, Sa = Samar, P = Palawan, N = Negros, Mn = Mindanao, Su = Sulawesi, F = Flores, B = Biak, Se = Seram.

* A larva of *Wallacellum* collected from Flores is similar to that of *S. celebesnse* (Takaoka 2003).

** Unpublished data (Takaoka H.).

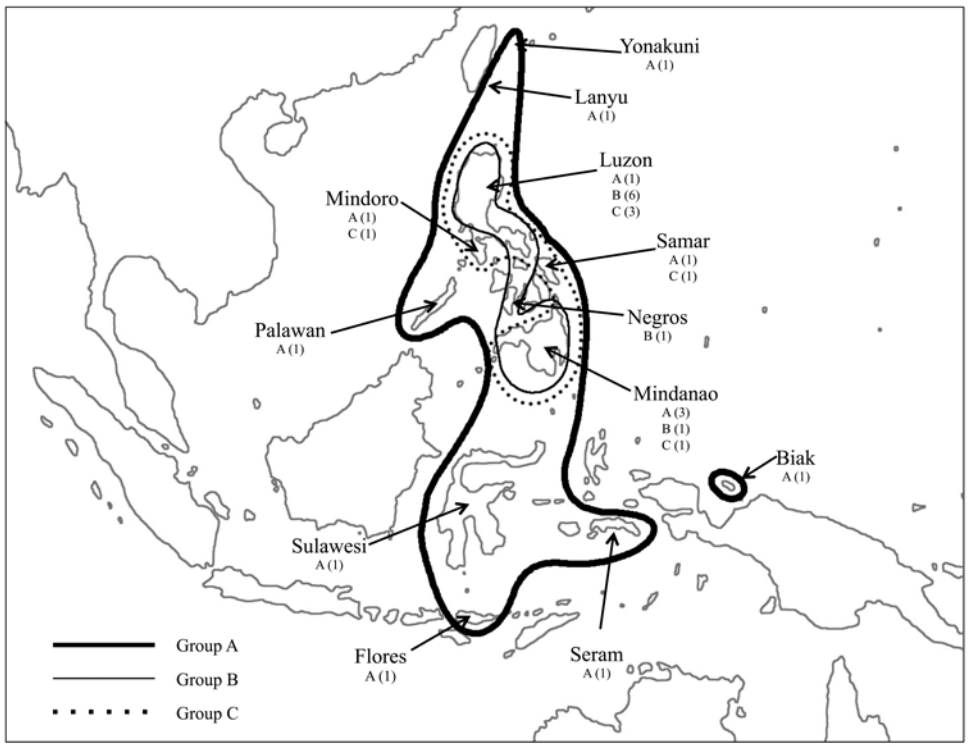

Fig. 4. Distribution of *Wallacellum* in Southeast Asia; group A (bold line), group B (thin line), group C (dot line). The numbers of species of each group in the island are shown under the name of the island.

the group B (TAKAOKA 1983). Combined these results and information, it is suggested that the group A is the basal group, having been distributed widely in the early period, and then species radiation of the group B occurred in the center of the distribution of this subgenus.

Geographical history of the subgenus *Inseliellum*, which also has insular distribution in the Pacific, was surveyed with phylogenies and information on island ages of hot-spot archipelagoes (CRAIG *et al*. 2001). In *Inseliellum*, basal species and clades are widely distributed in separated old islands. Moreover, *S. malardei* and *S. lotti*, which are basal among the species of *Inseliellum* in the Society Islands, are widely spread in the islands. In contrast, derived species are limited to younger islands, where species radiation occurred. The insular subgenera *Wallacellum* and *Inseliellum* have similarities in their distribution; 1) basal species or clade are widely distributed, 2) in some certain islands, species radiation occurred. In Sulawesi, Indonesia, the *Simulium variegatum* species-group of subgenus *Simulium* Latreille s.str. of the genus *Simulium* is represented by 11 species (TAKAOKA 2003), most of which have various shapes of inflated pupal gill filaments, like those of the group B of *Wallacellum*. This variation in the pupal gill filaments of the *S. variegatum* species-group might be due to species radiation. Although the geographical history of *Inseliellium* was considered

with evolution of running water habitats (Craig *et al*. 2001), mechanisms of radiation of blackfly species in islands still remain unknown.

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References

Chung, C. L. 1986. A New Record of *Simulium* (*Wallacellum*) *yonakuniense* Lanyu Is., Taitung Country, Taiwan (Diptera: Simuliidae). Journal of Taiwan Museum, 39:1-10.

Craig, D. A., Currie, D. C. and Joy, D. A. 2001. Geographical History of the Central-Western Pacific Black Fly Subgenus *Inseliellum* (Diptera: Simuliidae: *Simulium*) Based on a Reconstructed Phylogeny of the Species, Hot-Spot Archipelagoes and Hydrological Considerations. Journal of Biogeography, 28: 1101-1127.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. 1994. DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates. Molecular Marine Biology and Biotechnology, 3: 294-299.

Hebert, P. D., Cywinska, A. and Ball, S. L. 2003. Biological Identifications through DNA Barcodes. Proceedings of the Royal Society of London B: Biological Sciences, 270: 313-321.

Hernández-Triana, L. M., Crainey, J. L., Hall, A., Fatih, F., Mackenzie-Dodds, J., Shelley, A. J., Zhou, X., Post, R. J., Gregory, T. R. and Hebert, P. D. N. 2012. DNA Barcodes Reveal Cryptic Genetic Diversity within the Blackfly Subgenus *Trichodagmia* Enderlein (Diptera: Simuliidae: *Simulium*) and Related Taxa in the New World. Zootaxa, 3514: 43-69.

Kimura, M. 1980. Simple Method for Estimating Evolutionary Rates of Base Substitution Through Comparative Studies of Nucleotide Sequences. Journal of Molecular Evolution, 16: 111-120.

Nylander, J. A. A. 2004. Mr Modeltest v2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University.

Otsuka, Y., Takaoka, H., Aoki, C. and Choochote, W. 2003. Phylogenetic Analysis of the Subgenus *Himalayum* within the Genus *Simulium* s. l. (Diptera: Simuliidae)

Using Mitochondrial 16S rRNA Gene Sequences. Medical Entomology Zoology, 54: 113-120.

Otsuka, Y., Aoki, C., Choochote, W., de la Llagas, L. and Takaoka, H. 2007. Phylogenetic Analysis of Three Subgenera: *Asiosimulium*, *Daviesellum* and *Wallacellum*, of the Genus *Simulium* s. l. Endemic in the Oriental Region. Medical Entomology Zoology, 58: 329-233.

Pramual, P. and Adler, P. H. 2014. DNA Barcoding of Tropical Black Flies (Diptera: Simuliidae) of Thailand. Molecular Ecology Resources, 14: 262-271.

Pramual, P., Wongpakam, K. and Adler, P. H. 2010. Cryptic Biodiversity and Phylogenetic Relationships Revealed by DNA Barcoding of Oriental Black Flies in the Subgenus *Gomphostilbia* (Diptera: Simuliidae). Genome, 54: 1-9.

Rivera, J. and Currie, D. C. 2009. Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). Molecular Ecology Resources, 9: 224-236.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. 2012. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology, 61: 539-542.

Saitou, N. and Nei, M. 1987. The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. Molecular Biology and Evolution, 4: 406-425.

Takaoka, H. 1972. A New Species of Simuliidae from Yonakuni Island, Ryukyu Islands, Japan (Diptera: Simuliidae). Journal of Medical Entomology, 9: 521-523.

Takaoka, H. 1983. The Blackfiles (Diptera: Simuliidae) of the Philippines. Xii+199 pp., Japan Society for the Promotion of Science, Tokyo.

Takaoka, H. 2003. The Black Flies (Diptera: Simuliidae) of Sulawesi, Maluku and Irian Jaya. XXii+581 pp., Kyushu University Press, Fukuoka.

Takaoka, H. 2006. Three New Species of *Simulium* (*Wallacellum*) from Luzon Island, Philippines (Diptera: Simuliidae). Medical Entomology and Zoology, 57: 327-346.

Takaoka, H. 2009. Three New Species of *Simulium* (*Wallacellum*) (Diptera: Simuliidae) from the Philippines. Medical Entomology and Zoology, 60: 39-63.

Takaoka, H. 2012. Morphotaxonomic Revision of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) in the Oriental Region. Zootaxa, 3577: 1-42.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Positions-Specific Gap Penalties and Weight Matrix Choice. Nucleic Acids Research, 22: 4673-4680.

Xiong, B. and Kocher, T. D. 1991. Phylogeny of Sibling Species of *Simulium venustrum* and *S. verecundum* (Diptera: Simuliidae) Based on Sequences of the

Mitochondrial 16S rRNA Gene. Molecular Phylogenetics and Evolution, 2: 293-303.