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Original article

Classification and Distribution of Chironomidae (Diptera) using DNA Barcoding at Urban Streams in Gwangju, South Korea

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Abstract Chironomid communities are indicators of water pollution because of their ability to thrive under freshwater conditions. However, it is difficult to distinguish between chironomid larvae based on morphology. DNA barcoding, based on nucleotide sequences of marker genes, can be used to identify chironomid larvae. Samples of chironomid larvae were collected from Gwangju Stream and Pungyeongjeong Stream, tributaries of the Yeongsan River in South Korea. We identified 3 subfamilies, 13 genera, 16 species, and 1 cryptic species. There were 7 genera and 10 species from the subfamily Chironominae, 5 genera and 5 species from subfamily Orthocladiinae, 1 genus and 1 species from subfamily Tanipodinae, and the cryptic chironomid species of the family Chironomidae. There were 21 individuals from, 7 species and 1 cryptic species from the Gwangju Stream and 24 individuals, belonging to 10 species from the Pungyeongjeong Stream. The only species detected in both streams was *Cricotopus bicinctus*. The relationship between water quality and the species detected was difficult to explain, but the number of species showed a tendency to increase at sites where water quality was poor. Additional investigations and studies are needed to understand the relationship between water quality and the chironomid species occurring in these two streams.

Key words: benthic macroinvertebrate index, Chironomidae, *Cricotopus bicinctus*, DNA barcoding, ecosystem health

INTRODUCTION

Chironomid, or non-biting midges, comprise more than 10,000 species worldwide (Ekrem and Willassen, 2004). Although several genera belonging to the family Chironomidae have a global distribution, others are endemic to tropical areas in Africa and South America (Ekrem and Willassen, 2004). The identification of chironomid species is mainly based on the morphological characteristics of larvae and imagoes (Makarevich *et al*., 2000). Members of the family Chironomidae are frequently used by freshwater biologists to assess and monitor environmental conditions (Verneaux and Verneaux, 2002; Aagaard *et al*., 2004). Chironomid larvae are important indicator organisms for water pollution because of their ability to thrive under a wide variety of freshwater conditions (Wright, 1984; Armitage, 1995; Lindergaard, 1995; Aagaard *et al*., 2004), including exposure to heavy metals, organic pesticides, and other xenobiotics (Di Veroli *et al*., 2014; Matt *et al*., 2017). However, it is generally difficult to distinguish between the larvae and females of closely related species based on morphology, and species identification frequently depends on the association of these

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life stages with identified pupal exuviae of adult males, which tend to possess more species-specific characteristics (Ekrem *et al*., 2007).

DNA barcoding is a molecular method based on nucleotide sequences of marker genes and is among the most informative techniques in phylogenetic analysis (Matsuhashi *et al*., 1999; Makarevich *et al*., 2000). In order to use DNA-based techniques, an appropriate molecular marker is critical (Kuncham *et al*., 2016). Mitochondrial DNA (mtDNA) is used in most molecular studies, and often only one or several genes can act as effective markers. The most commonly used gene is the cytochrome c oxidase I gene (*COI*). This gene has been widely used in evolutionary studies and, population genetics, as well as in species identification due to its relatively high degree of variation (Carew *et al*., 2003; Sharley *et al*., 2004). Use of *COI* has two advantages. First, the universal primers for this gene are robust, enabling recovery of its 5′ end from representatives of most, if not all, animal phyla (Folmer *et al*., 1994; Zhang and Hewitt, 1997). Second, *COI* appears to possess a greater range of phylogenetic signal than any other mitochondrial gene (Hebert *et al*., 2003).

River and stream ecosystem health assessment started as a Korean national project in 2016 (MOE, 2016). Algae, fish, benthic macroinvertebrates, and others are used in biotic water quality assessment. For the purposes of this ecosystem health assessment, the family chironomidae is divided into two types, based on the color of the larval body (Red or white).

Both Gwangju Stream and Pungyeongjeong Stream are approximately 20-km-long tributaries of the Yeongsan River. They are typical urban streams with anthropogenic disturbances such as rapid urbanization since the 1970's water pollution, purification projects, and public park projects. The two streams have had insufficient maintenance flow and several kinds of water sources have been used to supplement the maintenance flow. There were three maintenance flows discharged into the Gwangju Stream: water processed from a sewage treatment plant (STP), the lake water of the Juam Reservoir, and the water of the Yeongsan River. Meanwhile only the water from the Yeongsan River had been discharged into the Pungyeongjeong Stream.

Studies on the benthic macroinvertebrates, especially Chironomidae, of small-scale urban streams such as Gwangju Stream and Pungyeongjeong Stream have been almost nonexistent. The aims of this study were to identify species of chironomid larvae using the DNA barcoding method and to determine the characteristics of the distribution and abundance of chironomid larvae along the two urban streams. We also investigated the applicability of members of the family Chironomidae, as identified here, as a water quality indicator.

MATERIALS AND METHODS

1. Study area and sampling

Chironomid larvae were collected in 2016 from 10 sites in two urban streams: four sites (St.1-1 to St.1-4) in the Gwangju Stream and six sites (St.2-1 to St.2-6) in the Pungyeongjeong Stream; each site was sampled once. Sampling was performed at St.1-1 on January 12, at St.1-2 to St.1-4 on July 13∼15, and at St.2-1 to St.2-6 on July 29. Benthic macroinvertebrates were also collected and identified along with the chironomid larvae. Stream ecosystem health was assessed using the Benthic Macroinvertebrate Index (BMI) of Korea, incorporating the species and individuals of benthic macroinvertebrates (MOE, 2016). Chemical water quality of each sampling site was analyzed. The measured parameters included biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solid (SS), total nitrogen (TN), and total phosphorus (TP) (MOE, 2015).

St.1-1 was a branch of the Gwangju Stream and was not affected by any maintenance flow additions. St.1-2 to St.1-4 were affected by the STP discharge water and the water of the Yeongsan River. St.1-2 was also affected by the lake water of the Juam Reservoir during summer. St.2-1 to St.2-6 were affected by the water of the Yeongsan River. St.2-1 was located in an agricultural area, St.2-2 and St.2-3 were located in industrial areas, and St.2-4 to St.2-6 were located in residential areas.

Chironomid larvae were collected using a Surber net (50 × 50 cm, mesh 1.0 mm). The larvae were washed through a 1-mm sieve, preserved in water for 5 days to excrete gut contents and fecal materials for preventing contamination, and stored in 80% ethanol (Pfenninger *et al*., 2007). The head capsule and the tail, with anal setae, were stored in 80% ethanol for future morphological examination (Yoon *et al*., 2011). The larval thorax and abdomen were used for DNA sequencing.

2. DNA extraction, PCR amplification

Genomic DNA was extracted larvae using a DNeasy Blood and Tissue Kit (Qiagen, Germany) following the

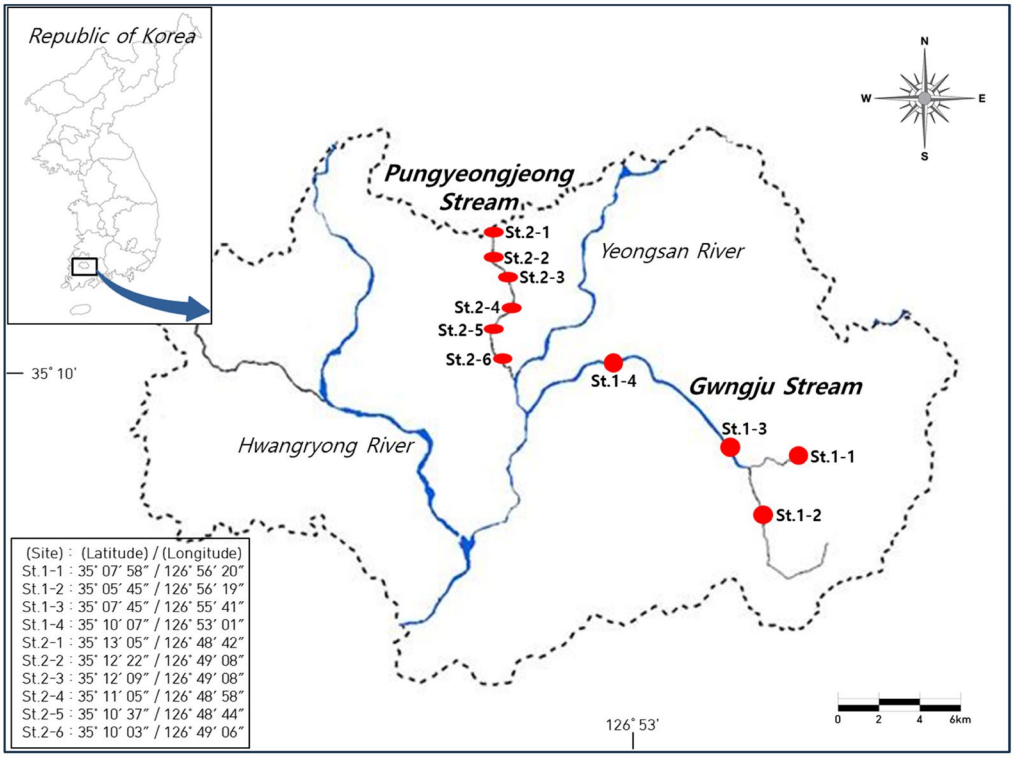

Fig. 1. Map of sampling sites. There were 4 sites (St.1-1 to St.1-4) in the Gwangju Stream and 6 sites (St.2-1 to St.2-6) in the Pungyeongjeong Stream.

manufacturer's instructions. A 658 bp fragment of the mitochondrial *COI* gene was amplified using the universal primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer *et al*., 1994). The first PCR was performed with 2 μL of DNA extract and 18 μL distilled water treated with diethyl pyrocarbonate (DEPC; Gendpot, USA) in the first PCR premix tube (Intron, Korea). PCR conditions were as follows: initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec, and final elongation at 72°C for 5 min, using a Geneamp 9700 biosystem (ABI, USA). PCR products were confirmed by electrophoresis on a 1.5% agarose gel with ethidium bromide.

3. Sequence alignment and phylogenetic analysis

The amplified PCR products were sent to Cosmogenetech (Daejeon, Korea) for sequencing using the ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City CA). The nucleotide sequence downloaded from the National Center for Biotechnology Information database, and phylogenetic analyses were conducted via the MEGA6 program. Genetic distances were calculated using the Kimura-2-parameter (K2P) distance model (Kimura, 1980). Intraspecific and interspecific sequence divergences based on K2P distances were calculated for all species, and mean intraspecific and interspecific K2P divergences were calculated from pairwise comparisons within each species. Neighbor-joining (NJ) and maximum likelihood (ML) analyses were performed using MEGA v6.0 with 1,000 bootstrap replicates.

RESULTS

In total, 46 individuals were sequenced from 40 specimens from the Gwangju Stream and 30 specimens from the Pungyeongjeong Stream (Table 1). We identified 3 subfamilies, 13 genera, 16 species, and 1 cryptic species. These were classified as 7 genera and 10 species of the subfamily Chironominae, 5 genera and 5 species of the subfamily Orthocladiinae, 1 genus and 1 species of the subfamily Tanipo-

Table 1. Summary of the dataset: collection location, number of sequences, divergences, and GenBank accession numbers.

Subfamily	Genus	Species	Collection location		No. of sequences		Divergence (%)		GenBank accession number (COI)	Identity (%)
			Gwangju Stream	Pungyeongjeong Stream	Total	Sample ID	Intraspecific	Interspecific		
Chironominae	*Chironomus*	*Chironomus flaviplumus*	St.1-2, St.1-4		13	GJ-11, 12, 13, 14, 15, 20, 29, 30, 31, 33	16.4	35.3	JF412075	99
		Chironomus kiiensis		St.2-4	1	PY-16		31.4	JF412086	99
	Dicrotendipes	*Dicrotendipes nervosus*		St.2-1, St.2-2, St.2-5	5	PY-1, 2, 8, 22, 24	12.0	32.9	JF412128	97
	Harnishia	*Harnishia* sp.		St.2-3	1	PY-15		29.2	KP902778	98
	Microtendipes	*Microtendipes pedellus*	St.1-3		1	GJ-23		29.4	KR748799	92
	Polydedilum	*Polydedilum nubifer*		St.2-4, St.2-5	2	PY-17, 23	3.8	32.8	JF412147	99
		Polypedilum cultellatum		St.2-4	2	PY-19, 20	4.5	30.9	JF412156	99
		Polypedilum japonicum		St.2-1	1	PY-3		28.9	KU497071	99
	Rheotanytarsus	*Rheotanytarsus* sp.		St.2-6	1	PY-30		31.2	KR653512	89
	Tanytarsus	*Tanytarsus formosanus*		St.2-3, St.2-5, St.2-6	3	PY-14, 25, 26	8.2	31.5	KU720133	99
Orthocladiinae	*Cricotopus*	*Cricotopus bicinctus*	St.1-1	St.2-1, St.2-2, St.2-6	9	GJ-1, 3, 9 PY-4, 7, 9, 10, 27, 29	13.7	32.1	KP902804	99
	Orthocladius	*Orthocladius rivulorum*		St.2-2, St.2-3	2	PY-6, 13	2.4	34.4	KR749115	88
	Paracricotopus	*Paracricotopus* sp.	St.1-2		1	GJ-18		46.7	HQ105232	91
	Paratrichocladius	*Paratrichocladius rufiventris*	St.1-2		1	GJ-16		86.8	JN887090	99
	Tvetenia	*Tvetenia paucunca*	St.1-4		1	GJ-32		57.4	KR291093	88
Tanipodinae	*Conchapelopia*	*Conchapelopia* sp.	St.1-1		1	GJ-2		31.4	KR427331	93
–	–	*Chironomidae* sp.	St.1-4		1	GJ-35		90.9	KM927497	89

Table 2. Environmental variables and Benthic Macroinvertebrate Index (BMI) assessment.

Site	BOD (mg L^{-1})	COD (mg L^{-1})	SS (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})	BMI
St.1-1	3.3	6.4	2.0	4.255	0.178	B
St.1-2	1.0	4.0	6.0	1.447	0.040	A
St.1-3	1.9	10.4	5.5	3.283	0.048	B
St.1-4	2.0	5.2	8.5	2.491	0.067	C
St.2-1	1.2	6.0	7.0	1.604	0.097	C
St.2-2	1.7	6.4	13.5	1.275	0.113	C
St.2-3	1.9	6.6	12.5	1.388	0.158	C
St.2-4	2.1	7.0	8.5	1.456	0.162	C
St.2-5	2.6	6.6	11.5	1.472	0.125	E
St.2-6	2.1	7.8	7.0	1.569	0.135	C

BOD: Biological Oxygen Demand; COD: Chemical Oxygen Demand; SS: Suspended Solid; TN: Total Nitrogen; TP: Total Phosphorus

dinae, and the cryptic species of the Chironomidae family. There were 21 individuals from 7 species and 1 cryptic species from the Gwangju Stream and 24 individuals from 10 species from the Pungyeongjeong Stream. The only species detected in both streams was *Cricotopus bicinctus*. The dominant and subdominant species were *Chironomus flaviplumus* (13 individuals) and *Cricotopus bicinctus* (3 individuals) in the Gwangju Stream, and *Cricotopus bicinctus* (6 individuals) and *Dicrotendipes nervosus* (5 individuals) in the Pungyeongjeong Stream.

Interspecific divergences of sequenced samples were higher than intraspecific divergences. Mean intraspecific divergences of species with more than two individuals were 2.4~16.4%, and that of *Cricotopus bicinctus* was 13.7%. Mean interspecific divergences were 28.9~90.9%. The species with the minimum intraspecific divergence was *Orthocladius rivulorum*, and that with maximum interspecific divergence was *Chironomus flaviplumus*.

The result of the neighbor-joining (NJ) analysis calculated by genetic distances was similar to that of the maximum likelihood (ML) analysis calculated by base locations of genetic characters (Fig. 2). Both NJ and ML trees showed clear groupings at the species level. In the NJ tree, subfamily Chironominae, including *Chironomus flaviplumus*, *Chironomus kiiensis*, *Dicrotendipes nervosus*, *Harnishia* sp., *Microtendipes pedellus*, *Polydedilum nubifer*, *Polypedilum cultellatum*, *Polypedilum japonicum*, *Rheotanytarsus* sp., and *Tanytarsus formosanus* produced monophyletic groups. Subfamily Orthocladiinae including *Cricotopus bicinctus*, *Paratrichocladius rufiventris* and subfamily Tanipodinae, including *Conchapelopia* sp. were also monophyletic groups. Some specimens were not shown in both trees because their homologies were below 90%. PY-6 and PY-13 was similar to *Orthocladius rivulorum* (homology 88%), GJ-18 were *Paracricotopus* sp. (homology 88%), GJ-32 was similar to *Tvetenia paucunca* (homology 88%), and GJ-35 was similar to *Chironomidae* sp. (homology 89%) (Table 1, Fig. 1).

The environmental variables at the sampling sites in the Pungyeongjeong Stream (St.2-1 to St.2-6) did not show large differences, but those in the Gwangju Stream (St.1-1 to St.1-4) did show some differences (Table 2). The mean concentration of BOD was 2.1 mg L^{-1} in the Gwangju Stream and 1.9 mg L^{-1} in the Pungyeongjeong Stream. Notably, the concentrations of BOD, TN, and TP were the highest at St.1-1 because of the inflow of domestic sewage. The concentrations of SS and TP in the Pungyeongjeong Stream were higher than those in Gwangju Stream. The mean concentration of SS was 5.5 mg L^{-1} in the Gwangju Stream and 10.0 mg L^{-1} in the Pungyeongjeong Stream. The mean concentration of TP was 0.083 mg L^{-1} in the Gwangju Stream and 0.132 mg L^{-1} in the Pungyeongjeong Stream.

The biotic water quality, as assessed using the BMI, was class A (very good) at St.1-2, class B (good) at St.1-1 and St.1-3 in the Gwangju Stream, and class C (neutral) at most sites of the Pungyeongjeong Stream. In particular, the BMI of St.2-5 was class E (very bad) because the bed structure of St.2-5 was mostly composed of sand and the majority of the benthic macroinvertebrates collected were chironomid larvae.

DISCUSSION

Chironomid larvae are indicator organisms used in the assessment of water quality and toxicity in rivers and lakes (Kim *et al*., 2001; Kim *et al*., 2009). However, community

Fig. 2. (a) Neighbor-joining (NJ) tree and (b) Maximum likelihood (ML) tree of K2P distances based on mtDNA *COI* (1,000 bootstrap replicates). Bootstrap values are on branches; Scale = K2P distance.

Table 3. Intraspecific divergences of *Cricotopus bicinctus* detected in Gwangju Stream and Pungyeongjeong Stream.

	GJ-1	GJ-3	GJ-9	PY-10	PY-4	PY-7	PY-9
GJ-1	0.0						
GJ-3	13.4	0.0					
GJ-9	12.9	16.3	0.0				
PY-10	22.3	28.2	26.1	0.0			
PY-4	10.2	13.7	5.6	23.0	0.0		
PY-7	10.8	15.4	6.3	24.0	4.2	0.0	
PY-9	22.0	28.2	25.5	2.0	23.7	24.6	0.0

investigation of the family Chironomidae and water quality assessment using the species of this family poses difficulties because chironomid larvae cannot be easily distinguished based on their morphology. The method of DNA barcoding uses different sequences and not morphological information. It can be used to identify Chironomidae and investigate their community structure by using continuously accumulated sequence data (Kim *et al*., 2012; Hebert *et al*., 2016).

The BMI, using species and individuals, showed that the biological water quality of the Gwangju Stream was better than that of the Pungyeongjeong Stream (Table 2). The number of chironomid species was three at class A sites, three at class B sites, eleven at the class C sites, and three at the class E sites. Although the relationship between water quality and the species detected is difficult to explain because of the small number of samples from each study site, the number of chironomid species showed a tendency to increase at the sites with a quality below class C. The water quality of class C was moderate, but sites below class C had poor water quality. The survey time for each site varied from January to July. This might also lead to differences in distribution of Chironomid due to water temperature and environmental differences at the time of investigation.

Cricotopus bicinctus was detected at many class B and C sites. The concentrations of BOD, COD, TN, and TP were the highest at class B St.1-1, indicating that *Cricotopus bicinctus* can inhabit a wider range of environmental conditions than can other chironomid species. Surber (2011) found that *Cricotopus bicinctus* was particularly resistant to electroplating wastes containing hexavalent chromium, cyanides, and copper, and to low oxygen levels.

Intraspecific divergences of *Cricotopus bicinctus*, detected in both Gwangju Stream and Pungyeongjeong Stream, were 4.2∼28.2% (Table 3). Intraspecific divergences were the lowest for samples PY-4 and PY-7 and the highest for GJ-3 and PY-10. Mean intraspecific divergence was 16.4% for the three samples from the Gwangju Stream (GJ-1,3,9) and 16.9% for the four samples from the Pungyeongjeong Stream (PY-4,7,9,10). Mean intraspecific divergence among the three samples from Gwangju Stream and the four samples from Pungyeongjeong Stream was 17.9%; no major difference was observed between the mean intraspecific divergences of the two streams. It was concluded that the nine specimens of *Cricotopus bicinctus* detected in Gwangju Stream and Pungyeongjeong Stream were the same species genetically.

CONCLUSIONS

Gwangju Stream and Pungyeongjeong Stream, the tributaries of the Yeongsan River, are urban streams with anthropogenic disturbances. The two streams have had insufficient maintenance flow and multiple kinds of water sources have been used to supplement maintenance flow. Although the two streams are just 3 km apart, the only chironomid species detected in both streams was *Cricotopus bicinctus*. We could identify the species of chironomid larvae in our samples, but could not analyze the differences between the species collected from the two streams or the relationship between the genetic sequences and water qualities. Large quantities of waste water processed at the STP are discharged into the Gwangju Stream, but in the Pungyeongjeong Stream, only water from the Yeongsan River is discharged. *Cricotopus bicinctus* was detected at St.1-1, St.2-1, St.2-2, and St.2-6, where there was no discharge of waste water processed at the STP. We believe that the waste water processed at the STP influences the presence of *Cricotopus bicinctus*. However, the number of species and individuals of benthic macroinvertebrates including Chironomidae, can change according to water quality, streambed structure, and other factors; thus, additional investigations and studies are required to elucidate this situation.

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Author contribution statement Conceptualization,

Sang-Hoon Yoon; Methodology, Sang-Hoon Yoon & Jeong-Wook Park & Jae-Keun Chung; Formal Analysis, Sang-Hoon Yoon & Jeong-Wook Park; Investigation, Sang-Hoon Yoon & Ji-Young Park; Resources, Jeong-Wook Park & Ji-Young Park; Data Curation, Jeong-Wook Park & Jin-Jong Seo; Original Draft Preparation & Writing, Sang-Hoon Yoon; Review & Editing, Jin-Jong Seo & Suk-Kyung Jeong; Supervision, Seok-Jin Bae; Funding Acquisition, Suk-Kyung Jeong & Seok-Jin Bae.

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