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First novel microsatellite marker sets developed for Korean Plecoptera: Development and characterization for *Nemoura geei* (Nemouridae) in South Korea

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Plecoptera, commonly known as stoneflies, play crucial roles in stream ecosystems, either as herbivores or predators, and are one of the key aquatic insects for assessing water quality. A total of 104 species are known from South Korea, and their weak flight ability and habitat specificity make them vulnerable, warranting studies on their population genetic features. However, population-level analyses of genetic diversity and structure using polymorphic microsatellite markers have not been conducted in any of these species. Here, we first developed and characterized novel microsatellite markers for *Nemoura geei*, a dominant stonefly species in Korea, to investigate its genetic diversity and population structure. Using high-throughput sequencing, we developed the first set of microsatellite markers for a Korean Plecoptera species. The markers revealed low genetic diversity across three populations, suggesting small effective population sizes and high conservation risks. These novel microsatellite markers will be highly useful for understanding the population genetics and conservation status of *N. geei* from broader distributions in Asia.

Keywords: aquatic insect, high-throughput sequencing, microsatellites, population genetics, spring stonefly

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Introduction

Plecoptera, commonly known as stoneflies, are a small order of hemimetabolous insects whose nymphs are entirely aquatic, typically inhabiting diverse streams (Stewart *et al*., 2009; DeWalt *et al*., 2015). Most species inhabit mountainous regions at high altitudes, while relatively lower species diversity is found in lowland areas. They mostly live in permanent, oxygenated streams and play crucial roles in stream ecosystems, either as herbivores or predators, depending on their developmental stage (DeWalt *et al*., 2015). Stoneflies are also a key component of the EPT (Ephemeroptera, Plecoptera, Trichoptera) index, which is widely used to the assessment of water quality (DeWalt *et al*., 2015). Approximately 4,500 species of stoneflies have been identified worldwide (DeWalt *et al*., 2024), with 104 species known in South Korea (Hwang and Muranyi, 2020; Hwang *et al*., 2023). Some species are highly vulnerable in lotic ecosystems and are also considered endangered in different regions (Fochetti and Tierno de Figueroa, 2006; 2008). Their vulnerability is amplified by their weak flight ability, which could limit their dispersal to new habitats, making them particularly important for studies on how dispersal ability shapes population genetic structures in aquatic insects (Elbrecht *et al*., 2014; Phillipsen *et al*., 2015). However, the level

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of genetic diversity, conservation status based on genetic data at the population levels or population genetic structure (e.g. gene flow, inter-population differentiation) in any Korean plecopteran species have not been investigated. In addition, misidentification often occurs in Plecoptera species, highlighting species-specific markers being needed for *Nemoura* species.

To address the genetic diversity and population structure of these vulnerable species, microsatellite markers, which are highly polymorphic and co-dominant, can serve as one of the most efficient genetic toolkits (Moritz, 1999; Abdul-Muneer, 2014). These markers can assess polymorphic information content, gene diversity, and heterozygosity (Abdurakhmonov, 2016). They are widely used to investigate the level of genetic diversity, population structures, phylogeography, and phylogenetics of diverse insect taxa (Miller *et al*., 2002; Theissinger *et al*., 2013; Schröder *et al*., 2022). Furthermore, the origin and dispersal route of invasive populations can be analyzed based on this species-specific genetic marker (Kang *et al*., 2023). A recent study on the population genetics of Ephemeroptera, Plecoptera, and Amphipoda species showed that microsatellite datasets are more suited for investigating recent ongoing gene flow and small-scale population structure, whereas mitochondrial haplotype data are better for large-scale phylogeographic patterns (Schröder *et al*., 2022).

In the context of Korean plecopteran taxa, the genus *Nemoura*, belonging to the family Nemouridae, is particularly worth to study since *Nemoura* spp. were reported as one of the most dominant species groups, accounting for 32.24% of total abundance from 412 sampling sites across Korea (Lee *et al*., 2022). The genus *Nemoura* Latreille, 1796, encompasses 226 species worldwide (DeWalt *et al*., 2024), with 16 *Nemoura* species reported in Korea (Hwang and Muranyi, 2020). According to Baumann (1975), the Asian species of the genus *Nemoura* are suggested to form two species complexes based on the structure of the male terminalia. *Nemoura geei* Wu, 1929, originally described from Beijing and widely distributed in Korea, China, Japan, and the Russian Far East (Zwick, 2010; Murányi and Park, 2011), can be a good candidate species for developing the first microsatellite makers of Korean Plecoptera due to its high abundance in Korea.

Here, we developed novel microsatellite markers for *N. geei* using high-throughput sequencing methods for the first time on Plecoptera species in Korea. We also validated the polymorphism of these novel microsatellite markers with Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) analyses. Furthermore, the level of genetic diversity and differentiation among three populations of *N. geei* were tested. This new set of microsatellite loci will be used as an important tool to investigate the level of genetic diversity, population genetic structures, and conservation status for one of the vulnerable and not well-studied stonefly populations in Korea.

Materials and Methods

Sample collection and DNA extraction

A total of 32 *N. geei* adult individuals were collected from four sampling sites in South Korea (Fig. 1). One individual from Mulgok 2 Bridge, Bongsu-ri, Sangmyeon, Gapyeong-gun, Gyeonggi-do 37°49′40.31″N 127°19′5.51″E (alt. 200 m) (2020-III-20) (n = 1) was used for microsatellite development and 31 individuals from three sites were used for population genetic analyses. The species was identified using key characters (e.g., epiproct) by comparison with the original description in Wu (1929) (Fig. 1C). All specimens were stored in 100% ethanol until use. Total genomic DNA was isolated from the thorax

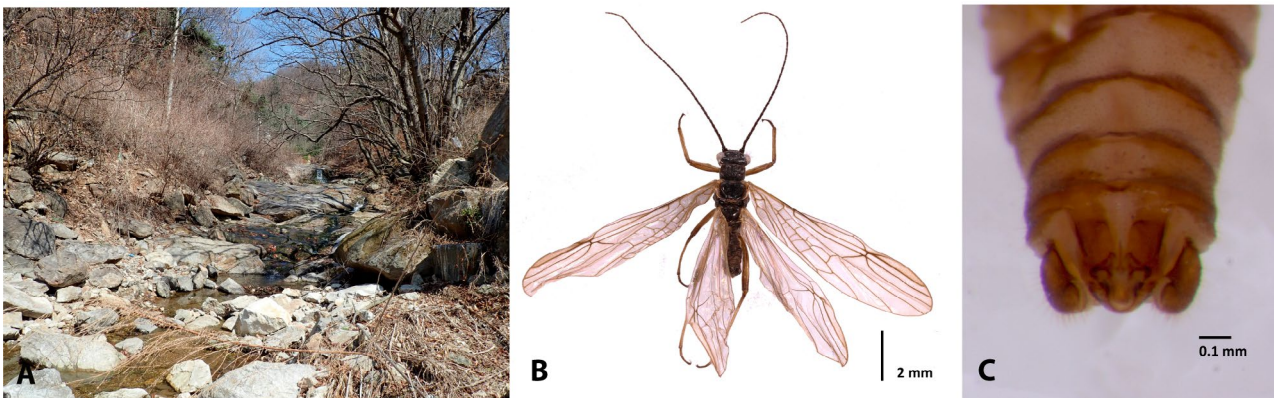

Fig. 1. Habitat (A), adult male (B) and male genitalia (C) of *Nemoura geei* in Korea.

and legs of specimens using DNeasy blood & tissue kit (Qiagen, USA).

Novel microsatellite marker development using high-throughput sequencing

Whole genome shotgun sequencing was performed for a *N. geei* species collected from Gapyeong-gun. Total genomic DNA was extracted from the thorax of one specimen using DNeasy blood & tissue kit (Qiagen, USA) according to the manufacturer's protocol. Integrity, concentration and purity of DNA was assessed using a bioanalyzer 2100 (Agilent, USA). The sheared genomic DNA was processed to construct an MGI paired-end library using a MGIEasy DNA Library Prep Kit and 150 bp of paired-end reads were generated through the MGI MGISEQ2000 platform (Illumina, USA). Sequencing artifact such as sequencing adapters were removed using Trimmomatic v 0.39 (Bolger *et al*., 2014) with custom sequencing adapter information used in MGISEQ library. Genome assembly for microsatellite identification was conducted using megahit v1.2.9 (Dinghua *et al*., 2015) and microsatellite marker identified using Krait v1.3.3. (Du *et al*., 2018). Identified microsatellite markers were filtered using type > 1 and start > 99 for proper primer design and amplification specificity of primers for genotyping were tested to assembled genome using MFEprimer-3.0 (Wang *et al*., 2019). The primers were designed with their lengths ranging from 20−26 nucleotides, annealing temperatures ranging from 50−52°C, and product sizes ranging from 133−204 bp. A set of 20 candidate microsatellite primer pairs was initially tested for polymorphism, of which eight microsatellite markers were chosen. GenBank accession numbers of the eight loci are listed in Table 2.

Microsatellite genotyping

One of each primer pairs for eight novel microsatellite markers were labeled the fluorescent dyes 5'-FAM. The PCR was performed in a 20-μL reaction volume containing 30 ng DNA, 10 mM dNTPs, 100 nM each primer, and 5U HANLAB Taq polymerase (HANLAB, Korea) in an ABI 2720 Thermo cycler (Applied Biosystems, USA). The following PCR cycling conditions were used: an initial denaturation at 95°C for 2 min, 35 cycles of 20 s at 95°C, 30 s at 50°C, and 30 s at 72°C, and a final extension step at 72°C for 5 min. Amplified PCR products were electrophoresed on an ABI 3730x. automated DNA sequencer and fragment sizes were analyzed using GENEMAPPER software 5 (Applied Biosystems, USA).

Genetic diversity of microsatellite markers

Microsatellite diversity was accessed with mean number of alleles per locus (N_A), the polymorphic information content (PIC), allelic richness (*AR*), expected (H_E) and observed heterozygosity (H_O), and inbreeding coefficient (F_{IS}) were estimated using GENEPOP v4.0 (Rousset, 2008) and FSTAT v.2.9.3.2 (Goudet, 2002). Presence of null alleles was assessed using MICROCHECKER v. 2.2.3 (Van Oosterhout *et al*., 2004) with 1000 randomizations at the 95% confidence level. The multi-locus tests for Hardy-Weinberg equilibrium (HWE) and null allele frequency at each locus were estimated using GENEPOP v4.0.

To examine inter-population differentiation, exact tests for population differentiation and calculation of population pairwise of F_{ST} were conducted at both markers using GENEPOP v4.0. The pairwise population comparisons were tested for significance with a Bonferroni correction.

Results and Discussion

Accurate species identification is essential for developing novel microsatellite loci as species-specific markers. Identifying species accurately using larvae from 16 *Nemoura* species in Korea is highly challenging. Therefore, *Nemoura* spp. as a group rather than specifying each species, has been used for ecological studies (Lee *et al*., 2022). Microsatellite marker development based on precise species identification of *N. geei* adult males by examination of distinct male epiproct characteristics is crucial.

Microsatellite loci of *N. geei* were identified using a high throughput sequencing method. A total of 327,418,478 reads with 407,685 scaffolds containing di-, tri- and tetra-nucleotide repeat motifs, with an average length of 22.34 bp were generated. The number of perfect microsatellite sequences, which are suitable for primer designing, was 80,661 containing 8,292 di- (10.28%), 8,627 tri- (10.7%) and 6,435 tetra-nucleotides (7.98%) repeat motif. Eight polymorphic microsatellite makers out of 20 initially screened markers were successfully amplified with stable and reproducible amplicon patterns with distinct peaks in capillary electrophoresis. The numbers of allele for each locus ranged from 3 to 15, and the PIC across loci ranged from 0.243 to 0.865, with its highest value of 0.865 found at the NG1380 locus (Table 1). The H_O per locus ranged from 0.065 to 0.806 with an average 0.501, while H_E was from 0.266 and 0.893 with average of 0.703. Although a single population would be ideal for microsatellite marker assessment to minimize the potential population bias, we used 31 individuals from three different populations as it was difficult to sample more than 20 individuals per population. However, these three populations are not genetically divergent and can thus be considered a single genetic group, as indicated

Table 1. Characteristics of eight polymorphic microsatellite loci developed and validated in of 31 *Nemoura geei* individuals from three sites in this study.

Loci	GenBank accession	Primer Sequence (Fw)	Repeat motif	Tm (C)	Allele size (bp)	N_A	*AR*	PIC	H_E	H_O	F_{IS}	*HWE*
NG246	PP188157	F: ATGTGGATTAAGTGTCCG R:CTCCAACAAGCTATAAGC	(CT)8	51.6	133	11	10.740	0.780	0.823	0.355	0.569	High. sign.
NG256	PP188158	F: CACGTTTTCTAACCTACC R: AGGAAGGATGAGTATTGG	(TGG)5	50.0	201	3	3.000	0.243	0.266	0.065	0.758	0.002
NG445	PP188159	F: GCATATATCACTCAAGAGC R: GTAGTCCTACTTTCTTTAGG	(AC)7	50.5	182	10	10.000	0.733	0.775	0.269	0.652	High. sign.
NG802	PP188160	F: GCTCCGTATAGTACATGG R: TGCTAGATAGATATACGACC	(TG)7	51.3	204	6	5.952	0.637	0.697	0.774	−0.11	0.803
NG808	PP188161	F: ACATCGAATGACACTTCC R: CTCGCAATTCTAAATACTGG	(TCG)5	51.9	186	3	2.839	0.390	0.508	0.516	−0.016	0.996
NG1380	PP188162	F: GACACTTCCAGTATCACC R: ACAGAGAGTTAGAATGAGG	(CT)8	51.6	159	15	14.139	0.865	0.893	0.774	0.133	0.066
NG2062	PP188163	F: GTTTAATGGGTTTGAGTCC R: TATACCATGGGTAAAGTCC	(TGC)10	51.3	171	8	7.677	0.784	0.824	0.806	0.021	0.738
NG3390	PP188164	F: GAATAGAGTAACCGTTTCC R: CTATGCTTGATGCTTATGC	(TG)15	50.3	162	7	6.999	0.787	0.834	0.448	0.462	0.002

F forward primer, *R* reverse primer, N_A number of allele, *AR* average allelic richness, PIC polymorphism information content, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} inbreeding coefficient *HWE* exact *P* value of Hardy-Weinberg Equilibrium (*$P < 0.05$)

Table 2. Summary of genetic diversity statistics in three populations of *Nemoura geei* in South Korea with eight novel microsatellite loci.

Populations	Sites	GPS	*N*	N_A	*AR*	H_E (SD)	H_O (SD)	F_{IS}	H-W tests (*P*)
ST01	Gaeunsan (Mt.), Seongbuk-gu, Seoul	37°35′44.00″N 127°01′42.23″E	10	5.38	5.020	0.702 (0.135)	0.569 (0.327)	0.198	0.007
ST02	Tapdong-dong, Dongducheon-si, Gyeonggi-do	37°52′27.80″N 127°6′39.01″E	9	5.75	5.565	0.700 (0.239)	0.453 (0.253)	0.367	**
ST03	National endangered species restoraton center, 23, Gowol-gil, Yeongyang-eup, Yeongyang-gun, Gyeongsangbuk-do	36°38′21.32″N 129°9′9.70″E	12	5.63	5.105	0.668 (0.272)	0.476 (0.304)	0.298	**

N sample size, N_A number of allele across eight loci, *AR* allelic richness, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} observed inbreeding coefficient, H-W tests (*P*) *P* value for multi-locus tests for Hardy-Weinberg equilibrium (HWE).

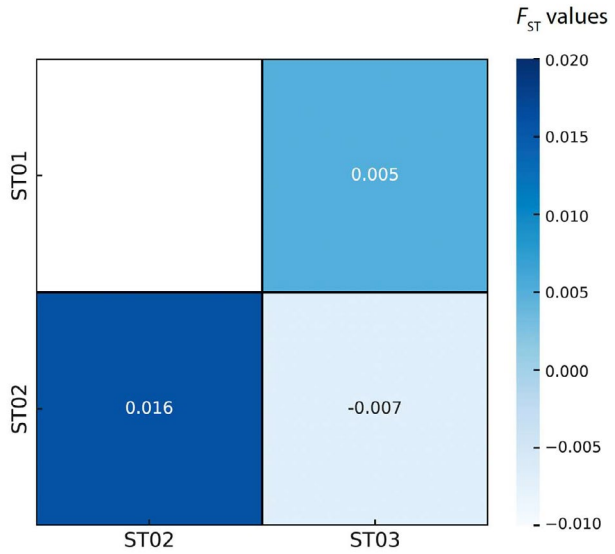

Fig. 2. Pairwise F_{ST} values among *Nemoura geei* populations in South Korea.

by low, non-significant F_{ST} values, making them suitable for marker assessment. Some microsatellite markers (e.g., NG246 and NG445) in this study show significant deviation from Hardy-Weinberg equilibrium, which may indicate homozygote excess potentially due to the presence of null alleles. Additionally, heterozygote deficiency can be influenced by factors such as inbreeding, the Wahlund effect, and selection, though it is generally attributed to non-amplifying PCR null alleles (Selkoe and Toonen, 2006; Wang *et al*., 2014). Nonetheless, all loci in this study were subject to variation in SSR motif number and were identified as reliable and repeatable.

Novel microsatellite markers were successfully validated in 31 individuals from three *N. geei* populations, providing information for the level of genetic diversity and population differentiation. Genetic diversity within populations of Korean *N. geei* was relatively lower with a range of H_E of 0.668−0.702 and H_O of 0.453−0.569. The highest H_E and H_O values were found in ST01 (Table 2). N_A across the eight loci per population ranged from 5.38 to 5.75 (Table 2) and the level of *AR* ranged from 5.020 to 5.565. In particular, two populations (ST02 and ST03) showed significant deviation from HWE (Table 2) indicating nonrandom mating, probably inbreeding within these groups. The level of F_{IS} ranged from 0.198 to 0.367 and its highest value was observed from ST02 (Table 2). All pairwise F_{ST} values calculated among the three populations were statistically non-significant ($p<0.05$) with a range of -0.007 to 0.016 after being corrected for multiple tests with the Bonferroni correction method (Fig. 2). Although Plecoptera species are generally known for their low dispersal ability (Schröder *et al*., 2022), the lack of population genetic structure indicated by small F_{ST} values might reflect the distinct connectivity characteristics of *N. geei* populations in Korea. Despite only analyzing three populations with novel microsatellite markers in this study, the findings can provide valuable insights into the level of ongoing gene flow among the *N. geei* populations. Furthermore, the observed low level of genetic diversity suggests a small effective population size, indicating a high risk of conservation concern or vulnerability for this species. Future research needs to be expanded using these markers with more samples and also more populations from other geographic distributions, including China, Japan, and the Russian Far East in order to understand its current population connectivity and genetic structures covering its whole distribution. The novel microsatellite markers developed for *N. geei* will be versatile in understanding the population genetics related to their ecological characteristics and will significantly contribute to assessing the vulnerability of a Plecoptera species as well in the Asian region.

Conflicts of Interest

The author of this paper have no affiliation with any interests and is solely responsible for the paper.

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