J. Asia-Pacific Entomol. 8(3): 319~323 (2005)
www.entomology.or.kr

SHORT COMMUNICATION

Restriction Polymorphism of Mitochondrial Cytochrome Oxidase-I in Oriental Mayfly, *Ephemera orientalis* (Ephemeroptera: Ephemeridae)

Young-su Kang[1], Jeonghoon Ko[1], Manwi Han[1], Yeon Jae Bae[2] and Yonggyun Kim*

School of Bioresource Sciences, Andong National University, Andong 760-749, Korea
[1]Korea Minjok Leadership Academy, Hoengseong, Kangwon 225-823, Korea
[2]Department of Biology, Seoul Women's University, Seoul, Korea

Abstract The Oriental mayfly, *Ephemera orientalis* McLachlan, 1875 (Ephemeroptera: Ephemeridae), can be a promising candidate used for monitoring environmental risk in aquatic ecosystem. Its quite large distribution in varying polluted areas raises a hypothesis of its genetic variability due to local selection and adaptation according to environmental hazard gradient. Before addressing the hypothesis, we needed to have polymorphic loci to discriminate individuals and characterize a specific population. For this reason, mitochondrial cytochrome oxidase-I subunit (mtCO-I) was chosen because of its relatively high mutational rate among mitochondrial genome. Two regional *E. orientalis* populations were obtained at Andong and Hoengseong, where few industrial complexes were located and could be regarded as being conserved in genetic variability. The amplified product showed ≈550 bp in all tested samples and digested with *Alu* I and *Rsa* I. *Alu* I cuts one site in wild type (major), but two sites in variants. *Rsa* I cuts one site in both wild and variant samples, but differed in position of the restriction site. With these markers, Andong and Hoengseong populations recorded variant ratios of 1.7% and 30.0%, respectively. However, wide-regional sample including collections from North Korea, China, Japan, and Russia did not show any variant types in this analysis, probably due to quite small numbers of samples (one or two) in each location. In highly polymorphic Hoengseong population, thorax morphological characters reflected the restriction site polymorphism.

Key words cytochrome oxidase-I, *Ephemera orientalis*, mitochondria, PCR-RFLP, population genetics

Introduction

Various biological markers have been used and newly recommended to assess aquatic environmental changes due to various environmental factors (La Point, 1995). These environmental stresses or patchiness can play a role in changing genetic variability of a specific population. Thus, it has been regarded that genetic divergence of different populations can be caused by physical or chemical pollutants in their habitats.

Stream macroinvertebrates have been used to assess ecotoxicities of several potent pollutants. As indicator species, mayfly larvae are members of a suite of potential stream biomonitors (Hare, 1992; Fialkowski *et al*., 2003), especially against heavy metals due to their relatively high tolerance in some species (Aoki *et al*., 1989; Sjøbakk *et al*., 1997).

The Oriental mayfly, *Ephemera orientalis* McLachlan, 1875 (Ephemeroptera: Ephemeridae), is widely distributed in most Oriental regions including Siberia, Okhotsk, China, Japan, and Korea (Chernova, 1973). The fact that *E. orientalis* is found in most streams of various water qualities (Lee *et al*., 2003), suggests that the species can be regarded as a sentinel to monitor a specific stream condition.

In terms of biomarkers, genetic markers can be used to trace genetic relatedness or distance of various populations differentiated through evolutionary processes. In other words, genetic markers can discriminate different populations under various environmental conditions. To develop the genetic markers, polymorphic gene loci should be determined to score individuals in a population. For this matter, mitochondrial DNA genome has been thoroughly screened and used as a promising candidate because it mutates more frequently than nuclear DNA in absence of recombination (Harrison, 1989; Wolstenholme, 1992; Simon *et al*., 1994). This study aimed to determine polymorphic loci from mitochondrial genome of *E. orientalis* using PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism).

*Corresponding author.
E-mail: hosanna@andong.ac.kr
Tel: +82-54-820-5638; Fax: +82-54-823-1628

(Received August 3, 2005; Accepted August 30, 2005)

Materials and Methods

E. orientalis collection

Two adult populations of *E. orientalis* from Andong and Hoengseong (Table 1) were used as reference groups in terms of searching for genetic variability. Collection sites in Andong were chosen in upstream areas (100 m altitude) of the Nakdong river and surrounded with high mountains. Black-light trap was used to collect and monitor adults in Andong (an example in Fig. 1). Sporadic collections were made in Hoengseong during early season (from early June to mid July), where samplings were made in two different altitude sites of Dunnae (504 m) and Ucheon (188 m). International collections were made in North Korea, China, Russia, and Japan including Korea. These insect samples were preserved in 10% formalin or 70% ethanol.

Mitochondrial DNA (mtDNA) extraction

Before DNA extraction, the preservative of ethanol or formalin was replaced with 50 mM phosphate buffer saline (pH 7.4). Mitochondrial DNA extraction followed the method of Kim et al. (1998). Briefly, each insect was homogenized in 200 $\mu\ell$ of homogenizing buffer (250 mM sucrose, 10 mM EDTA, 30 mM Tris, pH 7.5). The supernatant of 1,000 g was centrifuged at 12,000 g for 10 min. The pellet was resuspended with lysis buffer (150 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0) and then mixed with two volumes of a mixture of 0.2 M NaCl and 1% SDS. During incubation on ice for 5 min, 300 $\mu\ell$ of sodium acetate was added and further incubated

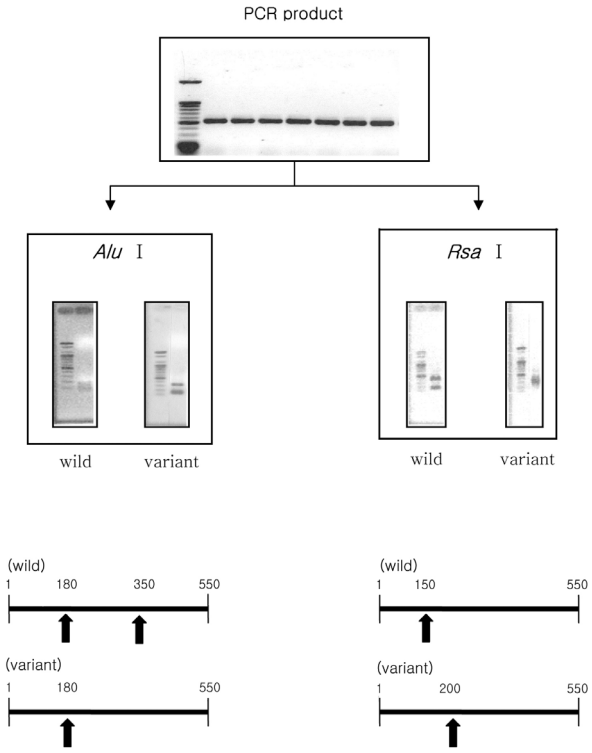

Fig. 1. PCR-RFLP of mitochondrial cytochrome oxidase-I (CO-I) of E*phemera orientalis*. CO-I region was partially amplified as 550 bp. The products were digested with *Alu* I or *Rsa* I. Wild type represents major restriction pattern of either enzyme.

Table 1. Profile of sample collections of *Ephemera orientalis*

Collection sites	Stage	N
Andong, Korea	Adult	60
Hoengseong, Korea	Adult	30
Other regions		24
Chungbuk, Korea	Adult	(1)
Gapyong, Korea	Adult	(3)
Jecheon, Korea	Adult	(2)
Sanchoeng, Korea	Adult	(2)
Seoul, Korea	Adult	(1)
Hwanghai, Prov. Hedju, North Korea	Adult	(2)
Pyungyang Daedong River, North Korea	Adult	(5)
Heirungjang, China	Adult	(4)
Yahagi-gawa, Kunitsuki, Japan	Nymph	(1)
Primosky, Kedrovaya, Russia	Nymph	(3)

for 5 min. After centrifugation at 12,000 g, the supernatant was followed by phenol extraction and ethanol precipitation. The resulting mtDNA was resuspended in deionized H_2O and used for PCR (polymerase chain reaction) reaction.

PCR-RFLP

Partial mitochondrial cytochrome oxidase-I subunit (mtCO-I) was amplified in 1718-2191 site on the basis of the published *Drosophila yakuba* mtDNA sequence (Clary and Wolstenholme, 1985). The primers were designed by the conserved sequences suggested by Simon *et al*. (1994), where the sequence of C1-J-1718 was 5'-GGAGG ATTTG GAAAT TGATT AGTTC C-3' and C1-N-2191 was 5'CCTGG TAAAA TTAA-GATATA AACTT C-3'. PCR was conducted with a DNA Thermal Cycler (PTC-100, MJ Research Inc.) in a total 50 $\mu\ell$ reaction mixture that contained PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.3), 10 $\mu\ell$ of dNTP (each 10 mM), 2 $\mu\ell$ of mtDNA template, 0.5 unit of *Taq* polymerase. Before PCR cycle, the reaction mixture was denatured at 95℃ for 1 min. The PCR program consisted of 35 cycles of a sequential step of denaturation (95℃ for 45 sec), annealing (58℃ for 1 min), and extension (72℃ for 1 min 30 sec. Ten $\mu\ell$ aliquot of PCR product was run at 0.9% agarose gel and visualized with ethidium bromide.

Buffer and primers of the PCR products were cleaned by PCR quick-spin™ (Intron Biotechnology, Sungnam, Korea) and digested with *Alu* I or *Rsa* I (Bioneer, Daejon, Korea) for 3h at 37℃. The digested products were run at 1.5% agarose gel and visualized with ethidium bromide.

Morphological characters

Thorax and wing characters were measured in adult *E. orientalis* of Hoengseong populations. Length of thorax represents a longitudinal distance of mid and hind thorax. Forewing was used to measure its maximal length and width. All measurements were performed with computer images captured under an image analyzer using a program SigmaScan/Image™.

Results and Discussion

Polymorphic loci of mtDNA were investigated in the populations of *E. orientalis* from Andong and Hoengseong in Korea. The adults of *E. orientalis* were attracted to light source from early May to late September in Andong. A morphologically similar species, *E. sachalinensis* Matsumura, 1911, adults were also collected by the light traps during the season. These two species have been known to co-inhabit and can be easily discriminated in adult stage by taxonomic characters of foreleg and male genitalia (Chernova, 1973; Hwang *et al*., 2003). Another collection site was Hoengseong, where natural environment was well conserved likely to make sure genetic variability and the mayflies could be easily caught at night.

Two PCR primers, designed on the conserved CO-I regions of mtDNA (Simon *et al*., 1994), could amplify a specific region (≈ 550 bp) of *E. orientalis* mtDNA (Fig. 1). However, the amplified PCR product was bigger than expected as 523 bp when compared to the sequence of *D. yakuba* (Clary and Wolstenholme, 1985). This difference was also found in *Spodoptera exigua*, where the species had ≈ 530 bp in the region (Kim *et al*., 1998).

All mtCO-I PCR products showed a similar size and did not show any variation. The PCR products were then digested with restriction enzymes recognizing specific 4 bp nucleotide sequences. Among four kinds of restriction enzymes (*Alu* I, *Msp* I, *Nci* I, and *Rsa* I) tested, *Alu* I and *Rsa* I showed polymorphic digestion patterns (Fig. 1). In the *Alu* I locus, most individuals showed two restriction sites, but variants had one restriction site. In *Rsa* I locus, all individuals had one restriction site, but both wild and variant types were different in the position of the restriction site.

Table 2. Gene frequencies of two polymorphic loci in mitochondrial cytochrome oxidase-I (CO-I) of *Ephemera orientalis*, where wild type represents major restriction pattern of either enzyme

Genotype	Gene frequencies according to collection sites[1]		
	Andong (n = 60)	Hoengseong (n = 30)	Other regions (n = 28)
Wild	0.9833	0.7000	1.0000
Variants[2]	0.0167	0.3000	0.0000
Alu I	0.0167	0.0667	0.0000
Rsa I	0.0000	0.0333	0.0000
Alu I/*Rsa* I	0.0000	0.2000	0.0000

[1]See Table 1.
[2]'*Alu* I' and '*Rsa* I' represent individuals showing variant type only in one locus, while '*Alu* I/*Rsa* I' represents those showing variant types in both loci.

Table 3. Morphological variation among Hoengseong subpopulations of *Ephemera orientalis*

	Characters (mm)		
	Thorax length	Wing width	Wing length
Dunnae	3.31 ± 0.59	6.56 ± 0.81	15.13 ± 2.17
Ucheon	3.24 ± 0.41	6.07 ± 0.69	14.43 ± 1.39
Type I error (Dunnae vs Ucheon)	> 0.10	0.06	> 0.10
Variant	3.69 ± 0.70	7.27 ± 1.20	16.84 ± 3.12
Wild	3.19 ± 0.45	6.18 ± 0.51	14.33 ± 1.02
Type I error (Variant vs Wild)	0.07	0.05	0.06

With these genetic markers, we tested individuals collected from different locations (Table 2). The individuals collected from international regions ('other regions' in Table 2) did not show any polymorphism. Lack of polymorphism in the international samples may be more likely due to quite small number of individuals in each location, rather than fixation to wild genotype. Polymorphic restriction sites were detected in two Korean populations, in which more than 30 individuals were analyzed. All mtCO-I PCR products did not show any length polymorphism on 1.0% agarose gel. Restriction site variants could be discriminated after digestion of the PCR products with *Rsa* I or *Alu* I, not with *Msp* I or *Nci* I as described above. Here, Andong and Hoedgseong populations had variant individuals, which were recorded as 1.7% and 30.0% proportions, respectively. The mtCO-I region is relatively variable in mitochondrial genome and used for general markers in population subdivision (Simon *et al*., 1994; Kim *et al*., 1998). The variant ratio of the Hoengseong population suggests that both loci can be regarded as polymorphic on basis of criterion of 5% (Kim *et al*., 2001).

To further examine restriction site polymorphism in Hoengseong population, we analyzed its subpopulations divided by geographical altitude with morphological characters (Table 3). We also compared the morphological characters of restriction site variants and wild type individuals. Interestingly, all restriction site variants were found only in mayflies collected in higher altitude subpopulation ('Dunnae'). However, it was difficult to discriminate these two geographical subpopulations in terms of morphometric characters, though somewhat greater measurements were found in wing width of Dunnae subpopulation. Within Dunnae subpopulation, restriction site variants exhibited bigger sizes in all measurements than wild type individuals. This suggests a speculation that mtCO-I polymorphism is linked to flight activity difference of E. orientalis because thorax-related tissues are rich in mitochondria and CO-I is located in mitochondrial genome. The DNA polymorphism within mtCO-I open-reading frame is likely to induce amino acid sequence change, which then results in polymorphic mitochondrial enzymes and their activities.

This study reports two polymorphic loci in CO-I of *E. orientalis* inhabiting in Korea. The usefulness of these genetic markers can be tested in analyzing various *E. orientalis* populations under differential environmental stress.

Acknowledgments We thank Professor Ohan Kang for his arrangement of summer internship and a genius construction of mini-light trap used to collect *E. orientalis* in Hoengseong. Young-su Kang and Junghoon Kho performed this study for their two summer internships in 2003 and 2004. This research was partially funded to YK as Ecotechnopia 21 project by Korea Institute of Environmental Science and Technology (KIEST).

Literature Cited

Aoki, Y., S. Hatakeyama, N. Kobayashi, Y. Sumi, T. Suzuki and K.T. Suzuki. 1989. Comparison of cadmium-binding protein induction among mayfly larvae of heavy metal resistant (*Baetis thermicus*) and susceptible species (*B. yoshinensis* and *B. sahoensis*). Comp. Biochem. Physiol. 93C: 345-347.

Chernova, O.A. 1973. On palaearctic species of mayflies of the genus *Ephemera* L. (Ephemeroptera, Ephemeridae). Entomol. Obozr. 52: 324-339.

Clary, D.O. and D.R. Wolstenholme. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. 22: 252-271.

Fialkowski, W., M. Klonowska-Olejnik, B.D. Smith and P.S. Rainbow. 2003. Mayfly larvae (*Baetis rhodani* and *B. vernus*) as biomonitors of trace metal pollution in streams of a catchment draining a zinc and lead mining area of Upper Silesia, Poland. Environ. Pollut. 121: 253-267.

Hare, L. 1992. Aquatic insects and trace metals: bioavailability, bioaccumulation and toxicity. Crit. Rev. Toxicol. 22: 327-369.

Harrison, R.G. 1989. Animal mtDNA as a genetic marker in population and evolutionary biology. Trends Esol. Evol. 4: 6-11.

Hwang, J.M., S.J. Lee and Y.J. Bae. 2003. Two co-inhabiting burrowing mayflies, *Ephemera orientalis* and *E. sachalinensis*, in Korean streams (Ephemeroptera: Ephemeridae). Korean J. Limnol. 36: 427-433.

Kim, Y., M.L. Lee and C. Chung. 1998. Study on the genetic variation of the mitochondrial DNA in the beet armyworm, *Spodoptera exigua* (Hübner), using PCR-RFLP. Korean J. Appl. Entomol. 37: 23-30.

Kim, Y., H. Park and M. Chung. 2001. Genetic analysis of three overwintering diamondback moth, *Plutella xylostella* (Linńe), populations in Korea. Korean J. Appl. Entomol. 40: 227-233.

La Point, T.W. 1995. Signs and measurements of ecotoxicity in the aquatic environment. pp. 13-46. in Handbook of ecotoxicology, Eds. D.J. Hoffman, B.A. Rattner, C.A. Burton, Jr. and J. Cairns, Jr. 755pp. Lewis Publishers, Boca Raton, FL.

Lee, S.J., I.B. Yoon and Y.J. Bae. 1995. Altitudinal distribution of *Ephemera strigata* Eaton and *E. orientalis* McLachlan (Ephemeroptera: Ephemeridae). Korean J. Entomol. 25: 201-208.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a ompilation of conserved polymerase chain-reaction primers. Ann. Entomol. Soc. Am. 87: 651-701.

Sjøbakk, T.E., B. Almli and E. Steinnes. 1997. Heavy metal monitoring in contaminated river systems using Mayfly larvae. J. Geochem. Exp. 58: 203-207.

Wolstenholme, D.R. 1992. Animal mitochondrial DNA: structure and evolution. Int. Rev. Cytol. 141: 173-216.