RAPD ANALYSIS ON SUBPOPULATIONS OF A MAYFLY SPECIES, *Epeorus ikanonis* (Heptageniidae: Ephemeroptera)

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ABSTRACT
We have characterized genetically six subpopulations of the mayfly *Epeorus ikanonis* TAKAHASHI in northern Kyoto City by comparing electrophoretic patterns of RAPD (random amplified polymorphic DNA). The genomic DNA samples prepared from *E. ikanonis* individuals were subjected to amplification with a single 12-mer DNA primer, and the resultants were fractionated by the agarose gel electrophoresis. The pairwise comparison of amplified DNA bands on the gels performed for each subpopulation indicated that subpopulations at the lower-stream locations retained higher heterogeneity of DNA band patterns. In addition analysis on the similarity of RAPD bands among subpopulations from two neighbouring streams was resulted in higher values among upper-stream subpopulations than those between upper- and lower-stream ones in each stream. These results suggest that the upper-stream subpopulations are maintained with less genetic exchange with the lower-stream subpopulations and the former of each tributary functions as a source of genetic heterogeneity of the latter.

Key words: RAPD, genetic heterogeneity, genetic distance, mayfly population.

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INTRODUCTION

The spatial distribution patterns of genetic variability within a population may relate to how often an individual can travel between areas within the population range and to spatial heterogeneity in the resultant gene flow among subpopulations (1, 2). It is, thus, predicted that the genetic difference between subpopulations will increase when there are barriers hindering individual movement between areas within the population range (1, 2, 3). In this respect, stream benthic animals offer a good material for the subject since their populations are usually distributed across several stream basins which give more or less a chance of isolation for the aquatic inhabitants.

In the present paper, the genetic heterogeneity of subpopulations of a mayfly *Epeorus ikanonis* TAKAHASHI was compared among 5 locations along 2 branches of a stream based on electrophoretic patterns of RAPD (random amplified polymorphic DNA). Polymerase chain reaction (PCR) with a short DNA primer often generates some polymorphic DNA fragments detectable on the gel electrophoresis (4). These polymorphic DNA, called RAPD, have an advantage of detecting genetic variations conveniently among individuals over RFLP (5), and thus, are vital to characterize genetic relationships among intra-specific populations (6, 7). In spite of this advantage of RAPD, however, most previous works have used only some part of RAPD in order to test their possibility as markers for fingerprinting (6, 7). We examined, instead, all of the RAPD obtained from the genomic DNA in order to analyze genetic variability and similarity among subpopulations of the mayfly along a stream and between streams.

MATERIALS AND METHODS

Collection of mayfly individuals

The mayfly, *Epeorus ikanonis* TAKAHASHI, is an inhabitant of the upper to middle reaches of Japanese mountain streams (8,9). In western Honshu it has a univoltine life cycle emerging in early April (10,11). Male adults of the mayfly aggregate in a huge number at the waters edge along streams for mating at the oviposition sites (10,12). This mating habit allows us to collect a large number of males easily at one location. The male mayflies were collected using a insect net at a total of 6 stations in Kibune Stream and Kumogahata Stream which are branches of the Kamo River running through Kyoto City (Fig. 1). The collection was carried out at Azo-dani (St.E), and Yuyaga-dani-deai (St.D) in Kibune Stream, and at Oiwa (St.K) in Kumogahata Stream on 15 April 1995, and, at Matsuo-dani (St.O) in Nakatusu-gawa Stream, and at Ichinose (St.L) and at Oiwa (St.K) in Kumogahata Stream on 13 April 1997. The collected materials were carried in an ice-cooled box to the

laboratory and stored alive at 5°C before extracting the sperm vesicles within two days.

Preparation of genomic DNA
Genomic DNA was prepared from the sperm vesicles of the male mayflies. The sperm vesicles were individually extracted using a pair of sharply pointed tweezers, and were suspended in the solution containing 10 mM Tris-Cl (pH7.6), 150 mM NaCl, 1% SDS, and 0.1 mg/ml proteinase K. They were incubated for overnight at 50°C, then extracted with phenol and precipitated with ethanol. The DNA precipitate from each individual was dissolved in 100μl of 10mM Tris-Cl (pH7.6)/ 1 mM EDTA, and stored at 4°C.

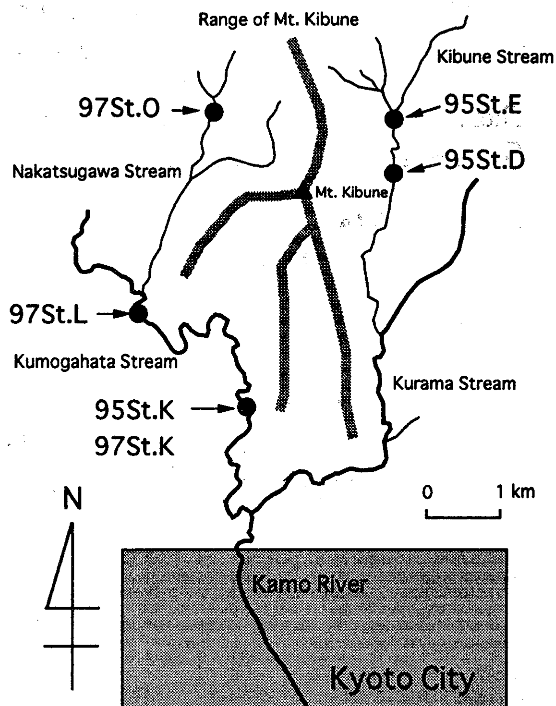

Fig. 1. A map of the study area in Kyoto City.

DNA primer
A potential core for repetitive DNA was used as the primer for DNA amplification. The sequence of the DNA primer was 5'-CGGAAAATCAGT-3'. This sequence exists in a wide range of eukaryotic organisms (13).

Amplification of DNA
The reaction mixture (25 μl) contained 1 μl of genomic DNA solution, 0.2 mM each of dNTP, 1X reaction buffer supplied by the manufacturer, 0.4 μM of 12-mer DNA primer, and 0.5 unit of *Taq* DNA polymerase (Toyobo). The amplification was carried out by GeneAmp PCR System 2400 (Perkin Elmer) under the following condition; 94°C-2 min X 1 cycle, 94°C-30 sec/ 35°C -30 sec/ 74 °C -1 min X 40 cycles, and 74 °C -7 min X 1 cycle.

Detection of RAPD
Aliquots of the reaction mixtures were applied onto the 1.2% agarose gel, and after the electrophoresis, the gel was stained with ethidium bromide. The DNA bands were analyzed by an image analyzer FMBIO-100 (Takara). The respective DNA bands were distinguished by three persons as shown in Fig. 2. RAPD markers not identified by all three persons were considered as non-scorable.

RAPD similarity within a subpopulation
A measure of genetic similarity (SI) between two individual samples was defined as follows;

SI_{ij} =(total number of common DNA bands shared by individuals i and j)/(total number of bands observed in individuals i and j)

Then, SI(X) was defined as the average of the SI_{ij} in the subpopulation X. This measure was used for estimating genetic idéntity of the subpopulation.

Genetic distance among subpopulations

A measure of genetic distance termed DI(X,Y) was defined as follows;

$$DI(X,Y) = \sum_{i,j=1}^{m,n} (1 - SI_{XiYj}) / m \cdot n$$

where m and n were the number of individuals in the subpopulations X and Y, respectively, and SI_{XiYj} was a similarity between the individual i in the subpopulation X and the individual j in the subpopulation Y.

Statistical significance of the differences in SI(X) and in DI(X,Y) among subpopulations were tested by t-test with Welch's correction as mentioned (14).

RESULTS

Patterns of RAPD on the gel

A total of 31 RAPD bands were identified on the agarose gels from a total of 109 mayflies, and each individual showed 8 to 14 DNA bands. Typical electrophoretic patterns are shown in the Figure 2, and all of raw data (gel images in computer files on Macintosh™ format) are available upon request. Table I summarized the number of individuals which showed respective DNA bands.

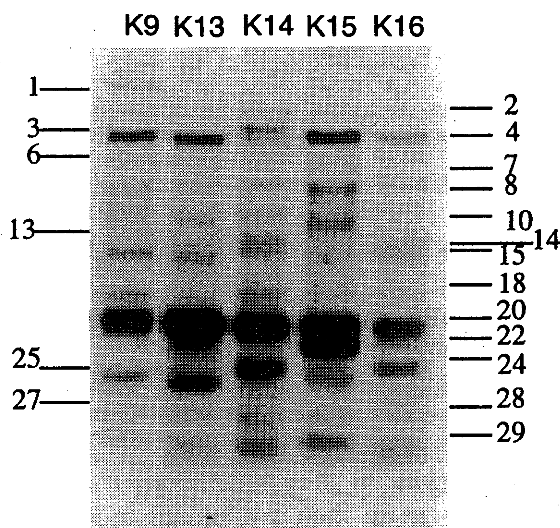

Figure 2. An example of RAPD pattern. The RAPD obtained from individuals in the station K are shown. Only the visible DNA bands in these lanes are indicated by arabic numerals.

Table I. Frequency of DNA bands identified on the gel.

DNA band	97St.K	97St.L	97St.O	95St.D	95St.E	95St.K
1	0.083	0.059	0.056	0	0	0.034
2	0.083	0.235	0.278	0.2	0.167	0.138
3	0.083	0	0	0	0	0.069
4	1	1	1	1	1	1
5	0	0.059	0.056	0	0	0
6	0.083	0	0.056	0.133	0.056	0.069
7	0.083	0.059	0.056	0.267	0.056	0
8	0.833	0.882	0.667	0.133	0.556	0.31
9	0	0.059	0.222	0.8	0.556	0.448
10	0.917	0.706	0.778	0.933	1	0.897
11	0	0.471	0.167	0.733	0.778	0.31
12	0	0	0.056	0	0	0.483
13	0.083	0	0.833	0.733	0.833	0.414
14	0.333	0.118	0.222	0.267	0	0.207
15	0.833	0.941	1	1	1	0.931
16	0	0	0.111	0	0.056	0
17	0	0.176	0	0.133	0.056	0.138
18	0.583	0.353	0.833	0.867	1	0.862
19	0	0	0	0.067	0.056	0.276
20	1	1	1	1	1	1
21	0	0.059	0	0	0.222	0.069
22	0.333	0	0.333	0.467	0.444	0.069
23	0	0	0	0	0	0.31
24	0.333	0.176	0.5	0.467	0.333	0.241
25	0.75	0.882	0.833	0.933	0.778	0.897
26	0	0	0.111	0	0.056	0.034
27	0.167	0.529	0.556	0.533	0.889	0.207
28	0.25	0	0	0	0	0.034
29	0.583	0.706	1	0.667	0.944	1
30	0	0	0	0.067	0	0.034
31	0	0	0	0	0.056	0
number of individuals tested	12	17	18	15	18	29

RAPD similarity within a subpopulation

The pairwise comparison of band patterns were performed and the values of SI are listed in Table II. The result shows that the subpopulation at St. K at lower-stream in Kumogahata Stream had significantly higher heterogeneity than the others both in 1995 and 1997 ($p<0.01$, t-test with Welch's correction). On the other hand, the station E at upper-stream in Kibune Stream had significantly lower heterogeneity than the others ($p<0.01$, t-test with Welch's correction). The upper-stream St. O in Nakatsugawa Stream also had significantly lower heterogeneity than St. K ($p<0.01$, t-test with Welch's

Table II. SI values and *t* -test with Welch's correction on SI difference.

	SI	s.d.	97St.K	97St.L	97St.O	95St.D	95St.E
97St.K	0.684	0.127					
97St.L	0.716	0.099	1.799*				
97St.O	0.738	0.093	3.135	1.969*			
95St.D	0.735	0.116	2.664	1.372*	0.214*		
95St.E	0.797	0.098	6.436	6.952	5.332	4.420	
95St.K	0.695	0.096	0.696*	2.120	4.825	3.256	10.998

Values of *t* between stations are shown in lower diagonal.
Those marked with asterisks are not significant at 1% level.

correction) and than St.L ($p<0.05$, t-test with Welch's correction). Additionally, the middle-stream St. D had also significantly lower heterogeneity than St. K ($p<0.01$, t-test with Welch's correction). These results indicate that the upper-stream subpopulations of *E. ikanonis* have low genetic variations and the variability increases gradually to the lower-stream subpopulations.

Genetic distance among subpopulations

The genetic distance values calculated as DI(X,Y) among subpopulations are shown in Table III. Comparatively smaller distance values were obtained between 95St.E-95St.D, 95St.E-97St.O, and 95St.D-97St.O (Fig. 3), among which the distance values between 95St.E-95St.D and 95St.E-97St.O were significantly higher than that between 95St.D-97St.O ($p<0.01$, t-test with Welch's correction) , but the difference between 95St.E-95St.D and 95St.E-97St.O was not significant ($p>0.05$, t-test with Welch's correction). These results showed that the subpopulation of St.O was genetically more similar to that of St.E than to the lower-stream stations in the same stream such as St.L and St.K.

Table III. DI distance matrix between stations.

	97St.K	97St.L	97St.O	95St.D	95St.E
97St.L	0.325				
97St.O	0.334	0.333			
95St.D	0.395	0.380	0.304		
95St.E	0.367	0.332	0.257	0.250	
95St.K	0.375	0.369	0.322	0.331	0.313

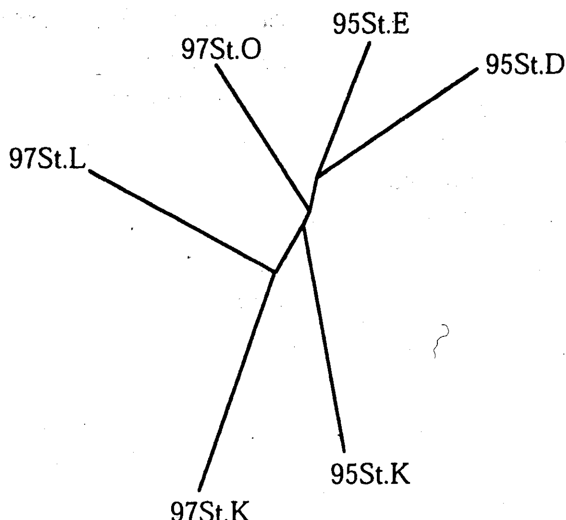

Fig. 3. Genetic distance between subpopulations.
The branch length and the order of branching are determined by the method of Fitch-Margoliash with DI distances using PHYLIP (Phylogeny Interface Package) ver.3.5c (18).

On the contrary, comparatively larger distance values were oberved between 97St.K-95St.D, 97St.L-95St.D, and 95St.K-97St.K (Table III, Fig. 3). Although the distance value between 97St.K-95St.D was significantly larger than that between 95St.K-97St.K ($p<0.01$, t-test with Welch's correction, Table IV), the distance value between 95St.K-97St.K indicated a quite large temporal variability at the station.

Table IV. *t* -test with Welch's correction on DI difference.

	97K-95K	97K-95D	97L-95D	97O-95D	97O-95E
97K-95D	2.606				
97L-95D	0.684*	1.895*			
97O-95D	10.260	11.187	10.279		
97O-95E	17.537	17.297	17.069	6.407	
95D-95E	15.396	15.796	15.252	6.251	0.838*

Those marked with asterisks are not significant at 1% level.

DISCUSSION

RAPD analysis based on sperm DNA

The RAPD prepared from sperm have following advantages for analyzing genetic relations among conspecific groups such as populations and

subpopulations. Firstly, it is easy to extract genomic DNA from an animal of small size since the sperm contains a large proportion of DNA. Secondly, since a sperm vesicle contains large number of sperms which are sibling but not have identical genome organization, the sperm DNA from individual is a kind of population. It means that the sperm DNA itself contains variation. And at last, the RAPD method are more convenient to characterize genetic relationships among groups than the RFLP analysis using specific DNA probes since few efforts are required for finding out an appropriate probe which leads to a high polymorphism at DNA level (5, 6, 7).

Genetic relations among subpopulations of *E. ikanonis*

The pairwise comparison of RAPD bands performed for each subpopulation indicated that the subpopulation at St.K had a higher heterogeneity than the others both in 1995 and 1997 in spite of a quite large annual variation. This may relate to the down-stream drift of nymphs of the mayfly. Stream invertebrates are subjected to drifting particularly at spates (15). Since St.K is located at the down-stream site in the main stream, the subpopulation will receive individuals drifted not only from Nakatsugawa Stream but also from several other branches. The fact of a comparatively larger genetic distance between 95St.K and 97St.K may also be derived from such gene flows at spates during the two years.

On the other hand, the upper-stream subpopulations near the water source such as St.E, St.D. and St.O had significantly lower heterogeneity (higher similarity) than the lower-stream subpopulations. These results indicate that the upper-stream subpopulations of *E. ikanonis* have less chance of genetic exchange between other subpopulations. But, at the same time, the subpopulation at St.O was genetically similar to those of St.E and St.D in spite of a mountain range lying between them. This means that the gene flow of this species is not hindered by a mountain range but the gene flow from lower to upper-stream is rather limited. Coupling with the fact of large genetic distance values between upper- and lower-stream subpopulations, the following conclusion is plausible: *i.e.*, the upper-stream subpopulations are maintained with less genetic exchange with the lower-stream subpopulations and the former of each tributary functions as a source of genetic heterogeneity of the latter.

The spatial pattern of the genetic variability seems to cohere with the behavioral habits in the adult stage of the species. Adults of *E. ikanonis* do not show any distinct up-stream-flight which has been reported for several aquatic insects (15, 16, 17) but subimagines of the species fly far up to the tree

canopies on the mountain side at the emergence (Takemon, unpublished observation) . The lack of up-stream-flight in the life cycle may lead to less genetic exchange between the upper-and lower-stream subpopulations and the vertical migration during the emergence flight may increase a chance of the genetic exchange among upper-stream subpopulations beyond the mountain range.

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