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Isolation of sperm vesicles from adult male mayflies and other insects to prepare high molecular weight genomic DNA samples

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Abstract

We describe here a simple and efficient protocol for genomic DNA isolation from adult males of insects: e.g., Ephemeroptera, Odonata, Orthoptera and Dictyoptera. To minimize contamination of external DNA source, the sperm vesicles were isolated from male individuals from which high molecular weight genomic DNA was extracted. According to this protocol, the genomic DNA samples obtained were high quality (intact), and abundant enough for genotyping analyses and molecular cloning. The protocol reported here enables us to process a huge number of individuals at a time with escaping from cross-contamination, and thus it is quite useful for conducting genetic studies at least in some species of insects. The large yield of high molecular weight DNA from single individual may be advantageous for non PCR-based experiments. As a case study of the protocol, partial coding sequences of histone H3 and EF-1α genes are determined for some insects with PCR-amplified DNA fragments.

Introduction

Isolation of genomic DNA from wild animals is a key step for genetic analyses. Quality of genomic DNA is especially critical for constructing DNA library and also for genotyping using DNA markers such as RFLP, AFLP and RAPD to keep reproducibility and reliability of the results. Contamination of exogenous DNA, e.g., parasitic organisms and the stomach contents, should be avoided. Generally, muscle tissues were widely used as a source of genomic DNA for winged insects [1], and it is a simple and reliable way to prepare DNA samples from muscle tissues of insects for PCR-based analyses. It is, however, time-consuming to dissect individual body to harvest large amount of the muscle tissue suitable for non PCR-based studies such as genomic DNA library construction. In this report, we describe a simple protocol to prepare high molecular weight genomic DNA for genetic analyses from the sperm vesicles of adult males of some species of winged insects. The protocol consists of two steps: firstly, the sperm vesicles are isolated from posterior abdomen of adult male individuals, and secondly, the total DNA is extracted by conventional method as used before [2–5]. Here, a practical method for isolating sperm vesicles from adult male mayfly is demonstrated, and some results drawn from these DNA samples are shown.

Methods

Dissection of male individuals

The sperm vesicles of male adult insects were taken out of the live or fresh individuals using two pairs of sharply pointed tweezers in the binocular image. In case of a mayfly, *Ephemera strigata* Eaton, the ninth abdominal sternites and tergites (Figure 1a) were separated by pulling the pair of claspers to the ventral direction (Figure 1b) before taking out the sperm vesicles. Then a pair of the penis lobes was exposed and the sperm vesicle was visible at the base of each penis lobe (Figure 1b). The sperm vesicle (Figure 1c) was picked out one by one using a pair of sharply pointed tweezers. It takes usually less than 1 min per individuals for mayflies. After the dissection, genitalic portions were kept with individuals for vouchering species identification. To avoid cross-contamination of samples, the pairs of tweezers (washed and preferably autoclaved) are needed as many as numbers of individuals to be processed. In case that enough number of tweezers are not available, washing the tweezers with distilled water twice and ethanol once followed by wiping with Kimwipe® gives successful results as mentioned below.

Isolation of high molecular weight genomic DNA

The sperm vesicles were suspended in 50 μl of ice-cold buffer solution (10 mM Tris–HCl/ 100 mM NaCl/ 10 mM EDTA, pH 7.6; hereafter termed TNE10) in 1.5 ml microtubes. The suspensions were mixed vigorously by vortex to make the sperms released from vesicles. Then, equal volume (50 μl) of TNE10 containing 0.2% SDS and 0.2 mg/ml proteinase K were added to the solution, and the solution was mixed by repeating gentle inversion of the tube. Instead of the TNE10 solution, high salt buffer without EDTA (10 mM Tris–HCl/ 150 mM NaCl, pH 7.6) has also shown satisfactory results. The mixtures were incubated at 50 °C for 1–16 h (overnight). After the incubation, an aliquot (100 μl) of H_2O-saturated phenol was added to each reaction mixture and the genomic DNA was extracted by repeating inversion of the reaction tube followed by centrifugation. Aqueous phase (containing DNA) was transferred to the new microtube and the genomic DNA was precipitated by adding 100 μl of isopropyl alcohol. The precipitate of genomic DNA was recovered and suspended in 50–200 μl (depending upon the visible size of DNA precipitate) of TE buffer (10 mM Tris–HCl/ 1 mM

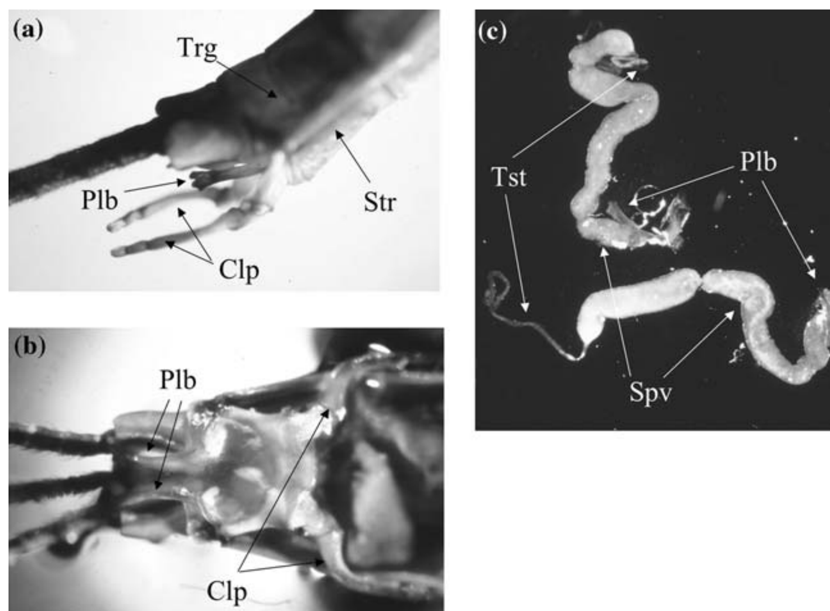

Figure 1. Morphology of the male mayfly, *Ephemera strigata* Eaton collected in the Kibune River, Kyoto, Japan. (a) Terminal abdomen in the lateral view, (b) reproductive organs in the ventral view and (c) penis lobes and sperm vesicles taken out of the male individual. Clp, clasper; Plb, penis lobe; Spv, sperm vesicle; Str, sternite; Trg, tergite; Tst, testis.

EDTA) followed by dialysis against TE buffer. Dialysis step can be omitted for the template for PCR amplification. Usually the DNA samples obtained are ready for restriction enzyme digestion. Because the yields of DNA are quite high and the DNA samples are usually used after appropriate dilution (1/10∼1/100), the carry-over of phenol is very small and thus it does not prevent activity of enzymes.

PCR amplification and DNA sequencing

To evaluate a risk of cross-contamination during the preparation of genomic DNA samples, PCR amplification and DNA sequencing of certain genes are performed. Partial coding sequences of histone H3 and polypeptide chain elongation factor 1 alpha (EF-1α) genes were amplified by PCR with appropriate DNA primers. The DNA primers used are listed in Table 1. The PCR amplification was performed in 25 μl of the reaction mixture containing 1× Taq polymerase buffer (supplied by manufacturer), 0.2 mM each of dNTP, 0.4 μM each of primers, 2 mM $MgCl_2$, 0.5 ng of genomic DNA and 0.5 unit of Taq DNA polymerase (Takara-Bio, Kusatsu, Japan). After preincubating the reaction mixture for 2 min at 94 °C, a cycling protocol was applied entailing 30 cycles of 94 °C for 30 s, 60 °C or 62 °C for 30 s and 74 °C for 2 min, followed by incubation at 74 °C for 7 min. The PCR products were purified by agarose gel electrophoresis with Sephaglas® BandPrep Kit (Amersham Bioscience, Piscataway, NJ, USA), and subjected to DNA sequencing using a model ABI377 DNA sequencer with BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Table 1. DNA primers used for PCR amplification

Gene	Sequence
EF-1α	5′-CGACAAGAGAACCATCGAGAAG TTCGAGAAG-3′
	5′-TTCTTGATGAAGTCTCTGTGTCCAG GGGCAT-3′
Histone H3	5′-ATGGCTCGTACCAAGCAGACGGC-3′
	5′-ATATCCTTGGGCATGATGGTGAC-3′

Reconstruction of phylogenetic tree based on sequence data

The nucleotide sequences were compared to estimate the phylogeny of histone H3 genes in mayflies. The neighbor-joining phylogenetic tree [6] was drawn by a program Neighbor based on the distance matrix, which was obtained by a program Dnadist according to F84 substitution model [7]. Both programs Dnadist and Neighbor are included in PHYLIP ver. 3.6 [8].

Results and discussion

For the first instance, we have shown the results of genomic DNA preparation according to this protocol from mayflies (*Rhithrogena japonica* Uéno, *Ephemera strigata* Eaton and *Epeorus ikanonis* Takahashi), grasshopper (*Euconocephalus thunbergi* (Stal)), and mantis (*Tenodera angustipennis* Saussure). *Epeorus ikanonis* DNA was prepared by using high salt buffer and others were prepared by using TNE10 buffer. The electrophoretic profiles of some of genomic DNA samples on the 1% agarose gel are shown in Figure 2. Although the actual average size of DNA was unknown due to

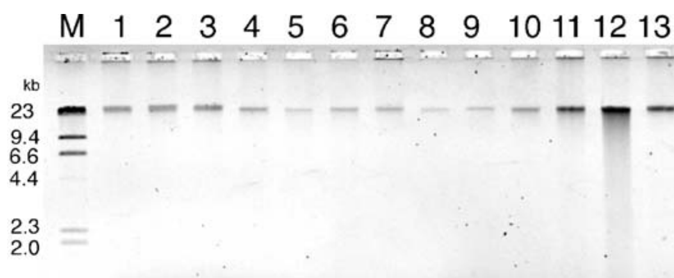

Figure 2. Electrophoretic mobilities of genomic DNA samples. Some of genomic DNA preparations are shown as examples. The aliquots (2 μl) of genomic DNA samples and *Hin*dIII-digested lambda DNA size markers (80 ng in total) are applied on the gel. Lane M, DNA size marker; lanes 1–3, *R. japonica* DNA; lanes 4–6, *E. ikanonis* DNA; lanes 7–9, *E. strigata* DNA; lanes 10–12, *E. thunbergi* DNA; lane 13, *T. angustipennis* DNA. Genomic DNA from each individual was loaded on the respective lanes.

the limited resolution of agarose gel electrophoresis, the majority of DNA migrated as same as the 23 kb band of DNA size marker (*Hin*dIII digests of lambda phage DNA) meaning that the genomic DNA is high molecular weight enough for RFLP and AFLP analyses and construction of genomic DNA library. The recovery of DNA extraction was quite high: in general, 2–30 μg of DNA (estimated by UV absorbance measurement) were obtained from each individual depending upon the size of sperm vesicles. The genomic DNA samples are stable in TE buffer at 4 °C for several years. DNA preparation was performed in May 2004 for *R. japonica*, May 2002 for *E. strigata*, October 2001 for *E. thunbergi* and *T. angustipennis*, and May 1995 and April 1997 for *E. ikanonis*. No serious degradation was observed on the electrophoretic patterns.

The protocol described here has been adopted for an ecological investigation on mayfly *E. ikanonis* using RAPD analysis [9] and also for DNA sequencing of specific genes that would be applicable to molecular systematic studies. A part of the genes coding for polypeptide chain elongation factor 1 alpha (EF-1α) and histone H3 has been amplified, respectively, with specific set of DNA primers by PCR, and their nucleotide sequences have been determined (Table 2). Although the sperm vesicle of *Ameletus costalis* (Matsumura) was prepared with the tweezers that were washed but not autoclaved after the dissection of *E. ikanonis* in April 1997, the nucleotide sequences of EF-1α and histone H3 were clearly defined (AF461030 and AF461031; AY870289 and AY870291) and no problem was encountered in PCR and sequencing reaction. In the molecular phylogeny of histone H3 genes of mayflies (Figure 3), *A. costalis* (AY870291) has clustered with *Ameletus* sp (AY336832), and *E. ikanonis* (AY870289) has clustered with *Epeorus latifolium* Ueno (AY870290) as expected, meaning that virtually no cross-contamination has occurred between *A. costalis* and *E. ikanonis*.

Although we have used high molecular weight genomic DNA for PCR-based sequencing of EF-1α and histone H3 genes as mentioned above, the DNA preparation can be much more simple in occasion that the genomic DNA samples are only used as the template for PCR amplification of specific DNA regions. For the preparation of genomic DNA for PCR, the sperm vesicles were ripped into small pieces and a piece was immersed in aliquot (100 μl) of TNE buffer (10 mM Tris–HCl/ 100 mM NaCl/ 1 mM EDTA) in 1.5 ml microtubes. After incubation at 90 °C for 5 min, they were centrifuged and the supernatants were ready for PCR after appropriate dilution. Genomic sequences of the histone H3 genes had been successfully amplified with this simple template preparation by PCR (data not shown).

We are currently trying to isolate mini- and micro-satellite loci from genomic DNA of the respective species by constructing genomic DNA

Table 2. Nucleotide sequences determined and source information

Accession number	Definition	Locality	Year
AF461030	*Epeorus ikanonis* EF-1alpha gene, partial cds	Kibune River, Kyoto	1997
AF461031	*Ameletus costalis* EF-1alpha gene, partial cds	Kibune River, Kyoto	1997
AF461032	*Pantala flavescens* EF-1alpha gene, partial cds	Onoshiba, Sakai, Osaka	2001
AF461033	*Tenodera angustipennis* EF-1alpha gene, partial cds	OPU Campus, Sakai, Osaka	2001
AF461034	*Euconocephalus thunbergi* EF-1alpha gene, partial cds	OPU Campus, Sakai, Osaka	2001
AY773002	*Euconocephalus thunbergi* histone 3 gene, partial cds	OPU Campus, Sakai, Osaka	2001
AY788897	*Mnais nawai* histone 3 gene, partial cds	Kurama River, Kyoto	2002
AY850372	*Rhithrogena japonia* histone 3 gene, partial cds	Nyu River, Wakayama	2004
AY860841	*Ephemera strigata* histone 3 gene, partial cds	Kurama River, Kyoto	2002
AY866428	*Tenodera angustipennis* histone H3 gene, partial cds	OPU Campus, Sakai, Osaka	2001
AY870289	*Epeorus ikanonis* histone 3 gene, partial cds	Kibune River, Kyoto	1997
AY870290	*Epeorus latifolium* histone 3 gene, partial cds	Nyu River, Wakayama	2004
AY870291	*Ameletus costalis* histone 3 gene, partial cds	Kibune River, Kyoto	1997
AY870292	*Pantala flavescens* histone 3 gene, partial cds	Onoshba, Sakai, Osaka	2001

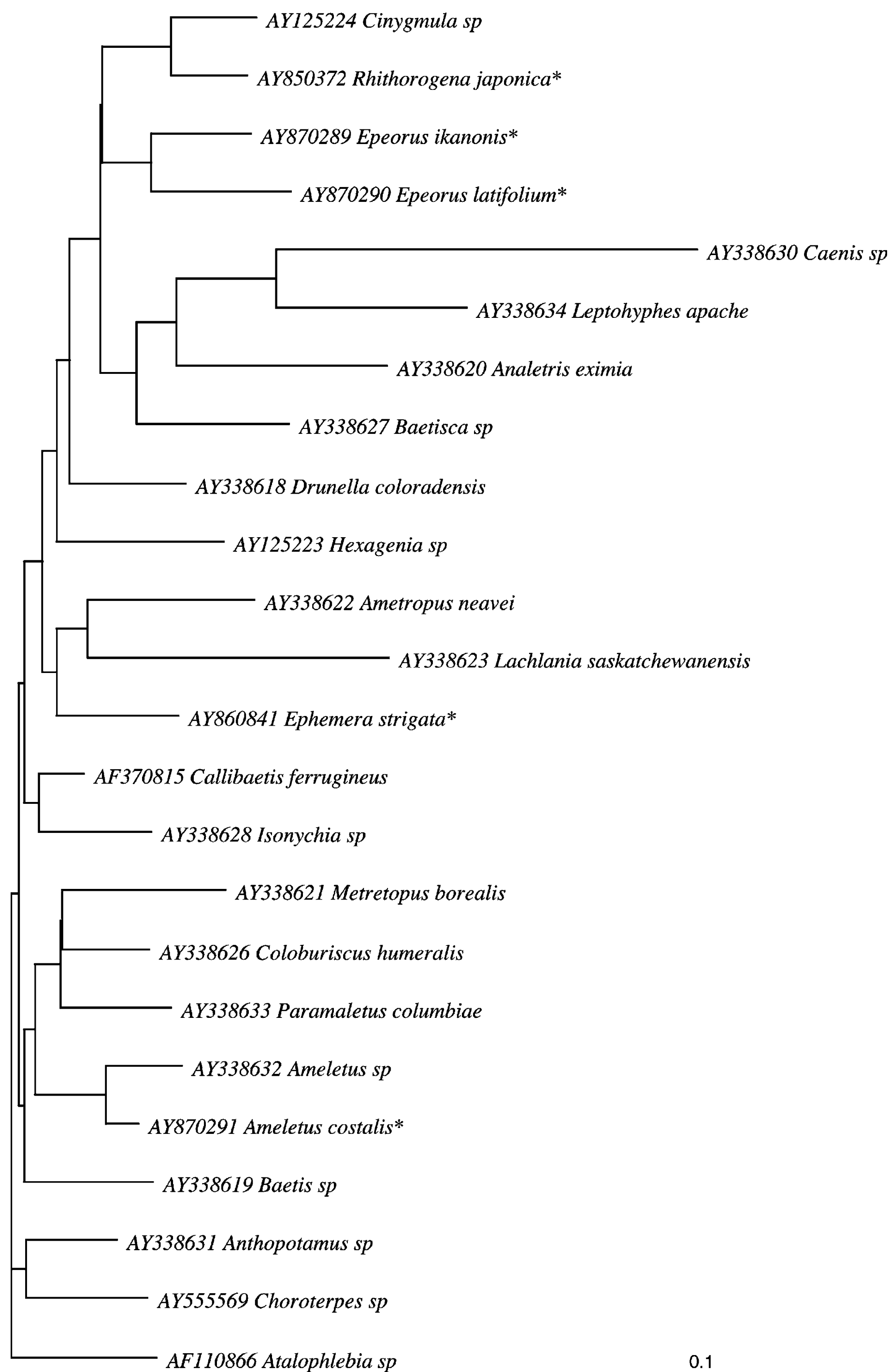

Figure 3. Neighbor-joining phylogenetic tree of histone H3 genes in Ephemeroptera. Asterisks indicate the species that we have determined the nucleotide sequences. Other sequence data were retrieved from GenBank Nucleotide Sequence Database. The distance scale is given at the bottom. Note that the phylogenetic tree reconstructed by neighbor-joining method is unrooted.

library. Collection of high molecular weight genomic DNA from a single individual may be good sources for conducting several kinds of genetic analyses on intra- and inter-population variation using DNA markers as well as genomic cloning and sequencing analysis.

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