Comparison of Preservation Techniques for Silkworm (*Bombyx mori L.*) DNA Based on Polymerase Chain Reaction (PCR) Products

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ABSTRACT

There are many ways to preserve insect specimens that will protect its DNA from degradation for the period of time from collection to use in a molecular genetic study. However, techniques vary among groups of insects, suggesting that the determination of preservation should be done before working at the molecular level with large number of individuals from a particular group. This work compares four preservation techniques for the Thai native silkworm, Bombyx mori L. (UB1XNangnoi Srisaket 1): (1) killed and stored at -20˚C, (2) killed and stored at -80˚C, (3) killed and stored in 70% ethanol, and (4) rapid hot-air drying at 60˚C. All specimens were stored for six months. DNA of each specimen was extracted by two different methods: (1) freezing in liquid nitrogen and transferring to lysis buffer for grinding and (2) grinding directly in lysis buffer. The integrity of DNA from those were determined and compared to live specimens using amplification of COI-COII DNA as a target region and genetic mapping with RAPDs. The method using 70% ethanol was found to be most practical and could prevent degradation of silkworm DNA for at least six months.

Key words: Silkworm, *Bombyx mori* L., Preservation, PCR

INTRODUCTION

Since the polymerase chain reaction (PCR) procedure was discovered, the study of insect molecular genetics has developed and increased dramatically (Brower and DeSalle, 1994). Molecular systematics is one area of insect molecular genetics that has been revolutionized by the implementation of PCR. Molecular techniques provide an understanding of relationships among insects in terms of their evolution and genetic diversity. Techniques such as PCR-RFLP, RAPDs, microsatellites, AFLPs, and DNA sequences have allowed comparison of nucleotide sequences and fragments produced from the DNA of many kinds of insects (Hoy, 1994). In the past decade, there have been many such studies on the systematics of insects focusing on both higher and lower taxonomic levels such as Campbell et al., (1994), Dowton and Austin (1994), Tuda et al., (1995), Langor and Sperling (1997), Hwang et al., (1999), Aoki et al., (2001).

The best source of insect DNA for molecular study is live specimens. Insects frozen at -20˚C or -80˚C, dried, and preserved in 70% or 100% ethanol can be a substitute when collection of live insects is not possible due to costs, remoteness, population decline or extinction (Zhang and Hewett, 1998). However, the quality and amount of isolated DNA from preserved specimens may be reduced or degraded and may not be appropriate for PCR. Therefore, the preservation technique is one of the crucial steps for the whole molecular process (Dick et al., 1993; Post et al., 1993). Since the best preservation technique varies from one group of insects to another, it is important to try several and select the best one for the available material.

We are working on the DNA of the Thai native silkworm. Samples can be collected live from many parts of Thailand. However, collection and transportation may be limiting factors for live specimens for molecular study. We need a preservation technique for the time from collection until DNA extraction. Preservation in 70% ethanol and use of dry specimens are techniques commonly used for other insect molecular studies and are especially attractive for this study because they are readily available. Therefore, the integrity of silkworm DNA from specimens preserved in 70% ethanol and dried was determined by using PCR techniques for the COI and COII gene regions and RAPDs. Results were compared to those from frozen specimens at -20˚C and -80˚C and live specimens. In addition, we also examined grinding methods. Normally insects are ground in liquid nitrogen. However, liquid nitrogen is not available in many areas so we compared grinding in liquid nitrogen and grinding directly in the buffer.

MATERIALS AND METHODS

Sample collection and preservation techniques

Representatives of newly-emerged silkworm adults (UB1xNangnoi Srisaket 1) were collected from silkworm cultures in Chiang Mai, Thailand in 2001. Moths were stored under the following conditions for six months: (1) -20˚C, (2) -80˚C, (3) in 70% ethanol and (4) dry after rapid hot-air 60˚C drying. DNA extracted from the stored moths was compared to DNA extracted from live specimens.

DNA extraction

Total genomic DNA was extracted from the thoraces of adult silkworms, using methods modified from the procedures of Tuda et al. (1995). The thoraces of individual silkworm moths was either ground in liquid nitrogen and then transferred to lysis buffer or ground directly in lysis buffer. Following grinding, an individual sample was homogenized in 500 µl of lysis buffer (50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 0.5% SDS, 400 µg/ml Proteinase K, 100 µg/ml RNase A) and kept at 55˚C for 3 hours. Then the lysate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). After adding 40 µl of 3 M sodium acetate, DNA was precipitated by absolute ethanol, separated by centrifugation, washed twice with chilled 70% ethanol and dissolved in 50 µl of TE buffer (10 mM Tris-HCl buffer, pH 8.0, 0.1 mM EDTA).

Primer and PCR amplification

COI and COII amplification

Primer COI-RLR: 5'-TTG ATT TTT TGG TCA TCC AGA AGT-3' and primer COII-Croz: 5'-CCA CAA ATT TCT GAA CAT TGA-3' (Gibco BRL, Rockville, MD) were used for PCR amplification of the COI-COII region. The annealing site of the COI-RLR primer is in the coding region of cytochrome oxidase I gene at the nucleotide position 2,171-2,194, while that of the COII-Croz primer is in the cytochrome oxidase II gene at the nucleotide position 3,663-3,684 based on the sequence of *Drosophila yakuba* (Roehrdanz, 1993).

PCR reactions were carried out by a MJ Research (PTC 100) thermocycler and consisted of 30 ng DNA template, 200 µM of dNTP (Bio 101), 0.2 µM primer, and 3 mM $MgCl_2$, 0.2 µM of each primer, 0.75 U Taq DNA polymerase, and 1X QIAGEN PCR buffer.

The temperature cycle began at 94˚C for 1 minute and was followed by 35 cycles each of which consisted of the following: 92˚C for 1 minute, 55˚C for 1 minute, and 72˚C for 2 minutes. After the 35 cycles, an additional 2 minutes of 72˚C was added. Then the reaction was cooled and held at 4˚C. Amplification products (3 µl) were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under UV illumination (Sambrook et al., 1989).

RAPDs

The random primer for RAPDs was OPM-18: 5'-CAC CAT CCG T-3' (Operon Technologies Inc., Alameda, CA). The PCR amplification was performed in a total volume of 10 µl containing: 10 ng of template DNA, 1X QIAGEN PCR buffer, 1X Q solution, 0.4 mM RAPD primer, 200 µM dNTPs, and 0.06 U/µl Taq DNA polymerase. Forty-five cycles of PCR were performed on an Applied Biosystem (GENEAMP PCR system 2400) using the following conditions: 96˚C for 1 minute, 95˚C for 45 seconds, 35˚C for 45 seconds, 72˚C for 2 minutes 30 seconds, followed by a final extension of 72˚C for 2 minutes 30 seconds.

The DNA fragments produced by RAPDs technique were separated on 4% polyacrylamide gel and detected by staining with ethidium bromide under UV illumination (Sambrook et al., 1989).

RESULTS

DNA extraction

DNA extracted by the method modified from Tuda et al. (1995) with silkworm moth thoraces (UB1xNangnoi Srisaket 1), preserved by the four different methods for six months, showed similar amounts of DNA compared to that extracted from live moths. The grinding methods, i.e., using liquid nitrogen and direct grinding in the lysis buffer provided the same results.

Precipitation of DNA in 100% ethanol from fresh and frozen (-80˚C and -20˚C) specimens exhibited cotton-fiber-like bundles whereas those preserved by hot-air drying at 60˚C and 70% ethanol did not have these bundles. When total genomic DNA was analyzed for those treatments using agarose gel electrophoresis, large amounts of high-molecular-weight

DNA were detected with small amounts of smear bands of low-molecular-weight DNA. Specimens preserved in 70% ethanol provided smaller amounts of DNA than other preservation techniques in this study (Figure 1).

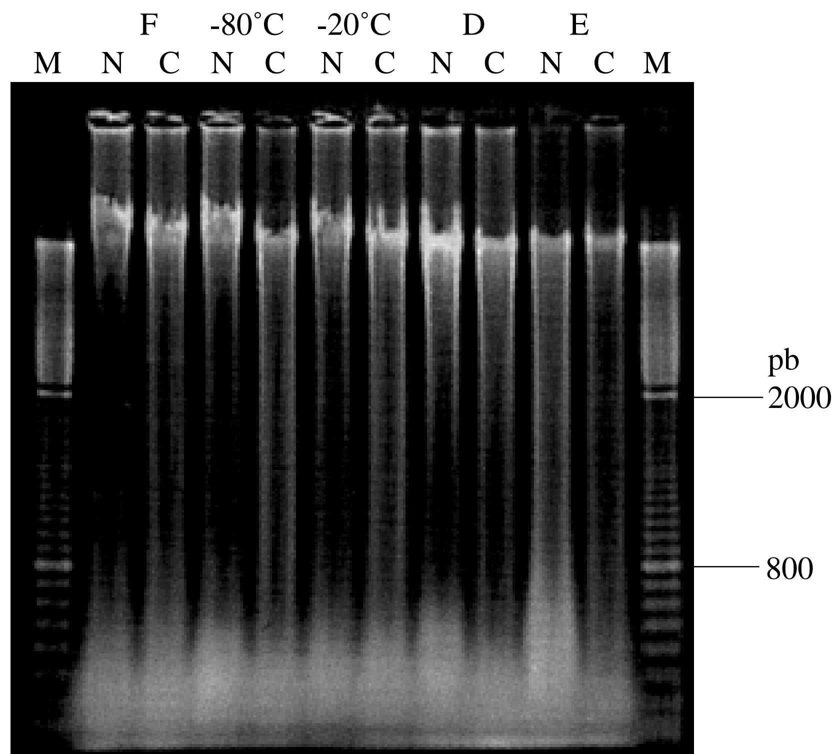

Figure 1. Total genomic DNA extracted (5 μl) from silkworms preserved by different techniques with two different grinding methods on 1% agarose gel, F: fresh live sample, -80˚C, -20˚C, D: hot air drying, E: 70% ethanol, M: 100 bp ladder (Pharmacia), N: grinding in liquid nitrogen, C: grinding direct in the lysis buffer.

Examination for DNA quality by PCR procedures

The quality of DNA from various treatments was examined by using the extracted DNA as a template for: (1) amplification of a specific DNA target, the COI-COII regions and (2) RAPDs to produce multiple bands of DNA.

COI-COII target region was successfully amplified from DNA in all treatments. The specimens preserved in -80˚C and -20˚C yielded a similar quantity to fresh specimens, whereas, hot-air dried specimens and those preserved in 70% ethanol yielded lower amounts of DNA product, although the amount of DNA template for each treatment was equal. Specimens extracted with or without liquid nitrogen generated similar results in all treatments (Figure 2).

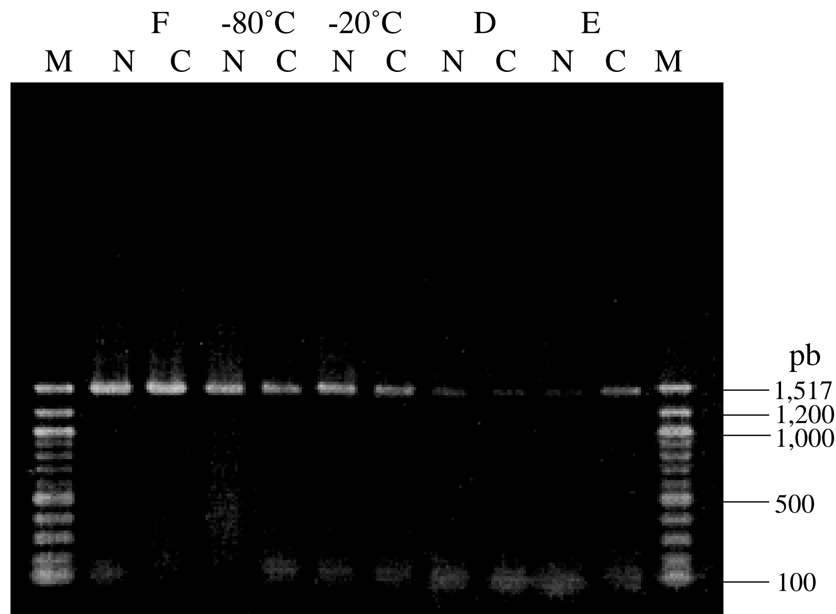

Figure 2. COI-COII gene region amplified from DNA of silkworm preserved in different techniques on 1% agarose gel, F: fresh sample, -80˚C, -20˚C, D: dry sample and E: 70% ethanol, M: 100 bp ladder (New England labs), N: grinding in liquid nitrogen, C: grinding direct in lysis buffer.

RAPDs technique was applied to determine if the quality of DNA template from all treatments was sufficient to produce multiple bands for genetic mapping analysis. The result showed the pattern of random DNA fragments generated from all treatments by the RAPDs reactions to be identical. This infers that DNA templates from all treatments had the same quality. If DNA template from any of those treatments was of low quality, damaged or sheared, then the random primers might anneal to damaged or sheared parts and could cause either failure of DNA fragment amplification or produce different RAPDs amplification products, resulting in dissimilar genetic maps. Yields of 900 bp DNA fragments were lower from specimens preserved at -20˚C, hot-air dried, and in 70% ethanol than live specimens and those frozen at -80˚C. Since the genetic map and cluster analysis is based on the number of bands rather than the intensity, the genetic map and cluster analysis of these treatments should be the same. Grinding with or without liquid nitrogen also produced the same results (Figure 3).

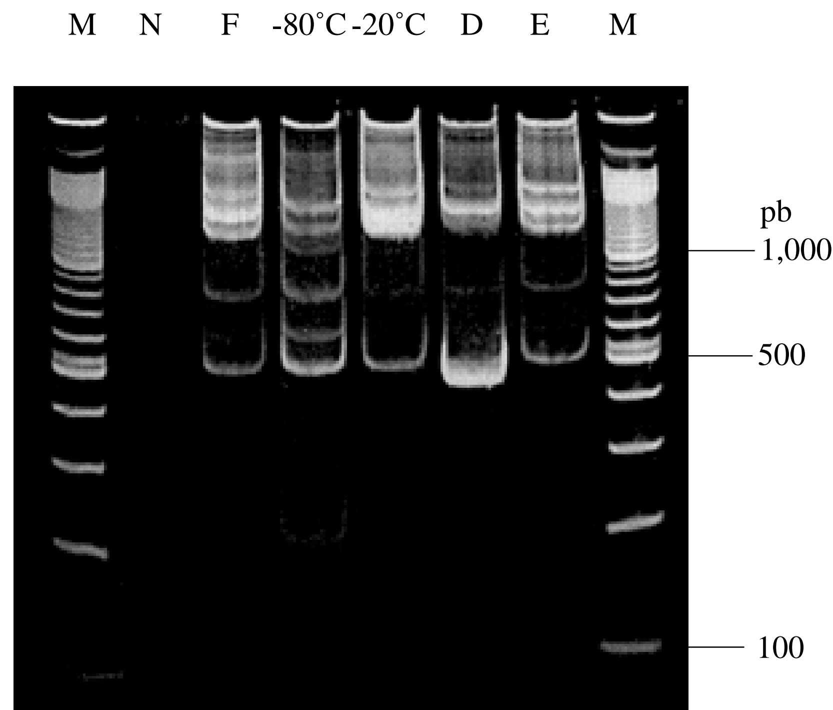

Figure 3. RAPD patterns generated from NDA of silkworm preserved in different techniques using OPM-18 as RAPD primer on 4% polyacrylamide gel, N: Negative control, F: fresh sample, -80˚C, -20˚C, D: dry sample and E: 70% ethanol, M: 100 bp step ladder (Promega), N: grinding in liquid nitrogen, C: grinding direct in lysis buffer.

DISCUSSION AND CONCLUSIONS

Standard preservation techniques such as freezing specimens at -80˚C and preservation in 100% ethanol are commonly used in many groups of insects to protect DNA from degradation (Dessauer et al., 1990; Post et al.,1993; Danforth, 1999). However, these preservation techniques may not be practical for all situations, especially in the field where a -80˚C freezer, liquid carbondioxide, liquid nitrogen or 100% ethanol are not available. The two most convenient and available methods are hot air drying and 70% ethanol. Museums and collections commonly preserve insects by hot-air (60˚C) drying and in 70% ethanol. However, quantity and quality of extracted genomic DNA from collections varies and may yield little or no DNA for PCR amplification (Zhang and Hewett, 1998; Danforth, 1999). Many factors influence the quantity and quality of DNA from museum specimens including: condition and timing of collection, preservation method, and various substances in the body of the insect that may tightly fix and degrade DNA (Dessauer et al., 1990; Pääbo, 1990; Hagelberg and Clegg, 1991; Poinar et al., 1992; Cooper, 1994). In this study silkworm adults were not only caught and preserved carefully with hot air drying but also kept for only six months. Therefore, DNA from these specimens was of sufficient quality and quantity for COI-COII region and RAPDs analyses.

Refrigeration of specimens in 70% ethanol is routinely used in many collections for not only taxonomic work but also molecular study (Dessauer et al., 1990; Post et al., 1993). However, DNA quality from these specimens varies among insect groups. For example, DNA of beetles stored in ethanol will be degraded in six weeks (Reiss et al., 1995) whereas hymenopterans kept in ethanol for twenty four months still provides DNA in good condition (Dillon et al., 1996). Our silkworm adults preserved in 70% ethanol for six months provided results equal to live specimens. Although the amount of genomic DNA was less and its 900 bp DNA band fragment in the RAPDs genetic map was lighter than that from other preservation techniques in this study, this did not affect the analysis. It confirms that silkworm adults can be preserved by hot air drying and in 70% ethanol for at least six months without DNA degradation. Of these two preservation techniques, 70% ethanol is probably more practical because it is commonly available everywhere. Therefore, 70% ethanol will be a basic preservative of silkworm specimens for our future molecular studies. Furthermore, grinding techniques did not affect the recovery of DNA for PCR, therefore, silkworm samples will be ground directly in lysis buffer instead of using liquid nitrogen.

In conclusion, although we found a suitable way to do short term preservation of silkworm adults using 70% ethanol and a simplified grinding method, pilot studies for the whole procedure are still recommended for other insect studies before preserving large number of specimens, especially if the samples are difficult to collect or have limited availability. No method can be guaranteed to work universally well and exceptions may exist. For example, some beetle species preserved in absolute alcohol immediately after collection have yielded DNA that is seriously compromised and useless for amplification by PCR. On the contrary, the extracted DNA from those beetles would have been good if they had been kept alive and starved for a few days before preservation (Zhang and Hewett, 1998).

ACKNOWLEDGEMENTS

We would like to thank Faculty of Agriculture, Chiang Mai University who kindly provided an equipment for this study from the subproject of graduate study and research in agricultural biotechnology (Ag-Biotech).

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