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SHORT COMMUNICATION

The Impact of Storage Times of Museum Insect Specimens on PCR Success: Case Study on Moth Collections in Indonesia

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Museum specimens are vast repositories of genetic information of interests to biological researchers. Since a new method in DNA extraction, a non destructive method, has been reported to be successful in extracting DNA of museum specimens even fossils without any morphological damages, using museum specimens as resources of genetic information for molecular studies is becoming popular recently. However, the PCR success depends on the quality of the specimens. To evaluate the impact of the storage times of museum specimens on PCR success, we conducted DNA extraction of 14 dry museum specimens of the moths collected from 1992 to 2010 by using a non destructive method. The results showed that the DNA specimens museum were fragmented into various sizes (100-1000 bp) depend on the storage times. On the other hand, fresh specimens which were preserved within absolute ethanol were almost not fragmented. The specimens of < 6 years old (2005-2010) succeed to amplify in 650 bp amplicon but for some specimens of 7 years old (2 of 3 specimens) resulted in a very weak amplification. These specimens, however, were able to amplify strongly in 300 bp amplicon. The results also showed that specimens of 1-19 years old were success to amplify in 100 bp amplicon.

Key words: museum specimens, PCR, a non destructive method, DNA extraction

INTRODUCTION

Museum specimens are a vast repository of genetic information of interests to biological researchers. These samples are important to phylogenetic studies or population genetic studies based on museum specimens, including extinct species. A recent review outlines important considerations and guidelines when working with specimens from museums and other natural history collections has been reported by Wandeler *et al*. (2007).

DNA is repaired with a great efficiency in living cells (Stivers & Kuchta 2006), but this repair ceases upon the death of the organisms or preservations of a sample. The DNA in such samples degrades more or less strongly over time and often becomes inaccessible to genetic studies, depending on the conditions of the storage (Lindahl 1993; Lehmann & Kreipe 2001; Wandeler *et al*. 2003; Paabo *et al*. 2004).

A new method of DNA extraction for museum specimens has been reported to be successful to get DNA from only 50 bp to thousands bp without any morphological damages. This method is well known as a non destructive method. It is able to extract DNA from fossil specimens (Gilbert *et al*. 2007). One of the benefits of using this method is saving funds for providing the fresh materials. Collecting fresh materials by doing exploration is not only time and budget consuming. Moreover, a lot of organism was not be able to be recovered again since their habitats have changed or loss. The other benefit of using this method is that it can be used to get the DNA from the extinct creature which is usually their type materials remain in the museums. On the other hand, using conventional method, destructive method, usually need fresh materials and it does not work for all specimen museums. It is often to keep the specimens in good condition for molecular study, researchers preserving them into absolute ethanol (96%). The problem is that not all insect specimens can be preserved in ethanol especially for those which will loss their characters (scales or color patterns). Adults of moths and butterflies always preserve as dry collection by pinning them. Moreover, another disadvantage of using this method is that the specimens will damage and cannot be used for morphological studies. Therefore, we recommend preserving directly an apart of the body specimens into absolute ethanol for molecular studies and the rest deposited in Museum for morphological work or as voucher specimens.

To understand the impact of storage time of museum moth specimens on PCR success, we evaluate the moth specimens deposited in Insect collection, Research Center for Biology from different date of collection by using a

non destructive method and then conducted PCR by using a primer mitochondrial CO I gene (Hebert *et al*. 2010). This mitochondrial gene was chosen due to its size is not too long (about 650 bp) which is possibly appropriate with the age of specimen collection used in this study (Sutrisno 2003; Sutrisno *et al*. 2006). Moreover, CO I gene has been chosen as one of the candidate genes to be applied in DNA barcoding (a novel system designed to provide rapid, accurate, and automatable species identifications by using short, standardized gene regions as internal species tags) (Hebert &Gregory 2005). Almost all requirements that are needed in DNA barcoding can be reached by this gene. This gene can be used to distinguish species in almost all animals. Compared with another mitochondrial gene, CO I gene is more conserved. Therefore, this gene is very suitable to identify a species since its sequence has a low variability (in general less than 1-2%), even for the closely related species its value is less than 1%. Moreover, using CO I gene through a non destructive method in DNA extraction of museum specimens will speed up in populating the genetic identity data for all specimen collections. Thus, the accuracy level of species identification for all specimen collections, especially for cryptic species will increase and no longer each specimen collection having own genetic identity deposited in the data base.

The results of the study will give benefits to the management of museum collections in improving the preservation quality and services. Thus, the museum is not only for serving the collection references but also providing the genetic resources data. A lot of researchers, quarantine staffs, plant protectionist, students and others will gain this huge important data.

MATERIALS AND METHODS

Specimen Museums for a Non-Destructive DNA Extraction. A total of 14 museum specimens of moths collected from 1992 to 2010 were used in this study (Table 1). No chemical was used during preservation except ethyl acetate which has been used to kill the insects. All dry moth specimens were stored in 21 ^{o}C and 50% humidity in Insect collection, Research Center for Biology-LIPI, Indonesia. For comparison, fresh materials of 14 specimens of *Mythimna* was also extracted (Table 2).

A Non-Destructive DNA Extraction. We used a non-destructive method which is modifications from QIAGEN animal tissue protocol kit using spin column. Firstly, the abdomens were removed from the body then was placed into a sterile 1.5 ml microtube and added 0.1 ml proteinase K (PK) 1% Buffer (1% PK buffer = 20 ul proteinase K solution (20 mg/ml) in 180 ul buffer ATL (Qiagen). This

Table 1. Museum specimens from different storage times used for a non destructive DNA extraction

Species	Date of collection	Collector	Locality
Asota heliconia	2-15.iii.1992	Darling, Rosichon, Sutrisno	Semboja Km 38, Balik Papan, East Kalimantan
Asota caricae	2.iv.1996	Hari Sutrisno	Bogor Botanic garden, Bogor, West Java
Asota phapos	15.viii.2002	Yayuk, Cholik, Cahyo	Gua Mimpi, Bantimurung, Maros, South Sulawesi
A. heliconia	2.VII. 2003	Kahono & Kholik	Batang Toru, Tapanuli Selatan, North Sumatra
Neochera marmorea	20-21.iii.2004	Kahono & Kholik	Batang Toru, Tapanuli Selatan, North Sumatra
Asota producta	17-18.iii.2004	Kahono & Cholik	Batang Toru, Tapanuli Selatan, North Sumatra
Asota plana	20-21.iii.2004	Kahono & Cholik	Batang Toru, Tapanuli Selatan, North Sumatra
Asota caricae	30.iv-3.v.2005	Hari Sutrisno, Gianto, Darmawan	Cibiuk, Taman Jaya Girang, Ujung Kulon NP, West Java
Asota heliconia	30.iv-3.v.2005	Hari Sutrisno, Cholik, Darmawan	Andongrejo, Tempurrejo, Meru Betiri, Est Java
Asota albiformis	28.iv.2007	Darmawan & Yadi	Ciapus, Sukamantri, Bogor, West Java
Asota plana	25.xi.2008	Darmawan &Gianto	Baturetno, Wonogiri, Central Java
Asota sp.	25.xi.2008	Darmawan &Gianto	Baturetno, Wonogiri, Central Java
Asota egens	1.xi.2008	Darmawan	Wates, Lebak gedong, Banten, West Java
Neochera privata	16.iv. 2010	Hari Sutrisno, Darmawan, Giyanto	Cibiuk, Taman Jaya Girang, Ujung Kulon NP, West Java

Table 2. Fresh materials of *Mythimna* moth specimens (preserved within absolute ethanol after collecting) for a non destructive DNA extraction; all specimens were collected from Taman Nasional Gunung Halimun-Salak, Cidahu, Sukabumi, West Java

Species	Date of collection	Collector
Mythimna (*Mythimna*) *albomarginata*	2.vii.2009	Hari Sutrisno & Darmawan
Mythimna (*Mythimna*) *albomarginata*	2.vii.2009	Hari Sutrisno & Darmawan
Mythimna (*Mythimna*) *yu*	2.vii.2009	Hari Sutrisno & Darmawan
Mythimna (*Mythimna*) *yu*	2.vii.2009	Hari Sutrisno & Darmawan
Mythimna (Mythimna) *radiata*	26.vi.2009	Hari Sutrisno & Darmawan
Mythimna (Mythimna) *radiata*	26.vi.2009	Hari Sutrisno & Darmawan
Mythimna (Mythimna) sp. A	26.vi.2009	Hari Sutrisno & Darmawan
Mythimna (Mythimna) sp. B	26.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Mythimna*) sp. C	27.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Hyphilare*) *decisissima*	27.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Hyphilare*) *decisissima*	27.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Hyphilare*) *epieixilus*	28.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Hyphilare*) *epieixilus*	28.vi.2009	Hari Sutrisno & Darmawan
Mythimna (*Pseudaletia*) *pallidicosta*	29.vi.2009	Hari Sutrisno & Darmawan

abdomen then was incubated at 55 °C for 2-4 hours and was added a further 0.1 ml PK buffer and incubated at 55 °C overnight. The next morning the abdomen was removed for morphologically work. The tube containing the insect mixture was then treated by following the manual of QIAGEN animal tissue protocol kit using spin column.

DNA Visualization. To elucidate the level of fragmentation of extract DNA and the length of PCR product, the extract DNA and the PCR product was electrophoresised on 2% agarose within 30 minutes on 100 Voltages and stained in ethydium bromate (15 minutes).

DNA Amplification. DNA amplification was conducted by using a mitochondrial CO I gene using three pair primers, to amplify 650, 300, and 100 bp. The complete sequence primers used for 650 bp were LepF1: 5' ATT CAA CCA ATCATA AAG ATATTG G 3', and LepR1: 5' TAA ACT TCTGGA TGTCCA AAA AATCA 3' (Hebert *et al*. 2010). The complete primers for 300 bp were LepF1: 5' ATT CAA CCA ATC ATA AAG ATA TTG G 3' and MLepR1: 5'CCT GTT CCA GCT CCA TTT TC 3'. The complete primers for amplify 100 bp were MLep F1: 5' GCT TTC CCA CGA ATA AAT AAT A 3' and MLep R1: 5'CCT GTT CCA GCT CCA TTT TC 3' (Hajibabaei *et al*. 2006). All amplifications were performed with Taq DNA polymerase (NEB), with an initial denaturation at 95 °C for 5 min., then 35 cycles of 92 °C for 30 s, 47 °C for 60 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min.

RESULTS

DNA Fragmentation. We used moth museum belonged to *Asota* and *Neochera* genus preserved in pinned and not exposed to other preservatives used in non destructive DNA extraction. The fragmented DNA was able to be recovered from the specimens of 1-19 years old (1992-2010) (Figure 1). The raw data showed a considerable spread of sizes, ranging from approximately 100 to 1000 bp. All specimens have a most abundant fragment size of approximately < 200 bp. On the other hand, DNA extraction of fresh specimens that preserved within absolute ethanol after collecting showed almost no fragmentation for all specimens that belong to the genus *Mythimna* (Lepidoptera: Noctuidae) (Figure 2).

DNA Amplification. The specimens of 1-6 years old (collected 2005-2010) were well amplified as indicated by strong bands using LepF1 and LepR1 primers (650 bp)

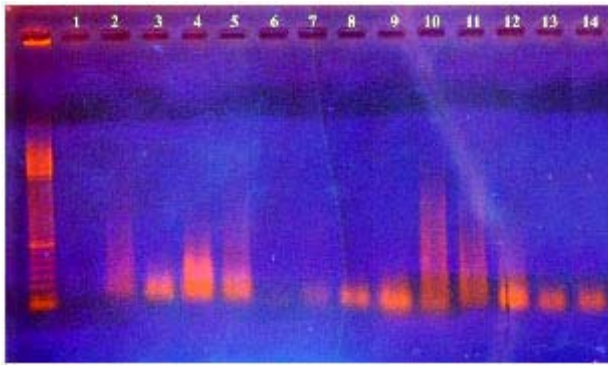

Figure 1. DNA extraction of museum specimens from different storage times. 1: 1992, 2: 1996, 3: 2002, 4: 2003, 5: 2004, 6: 2004, 7: 2004, 8: 2005, 9: 2005, 10: 2007, 11: 2007, 12: 2008, 13: 2008, 14: 2010.

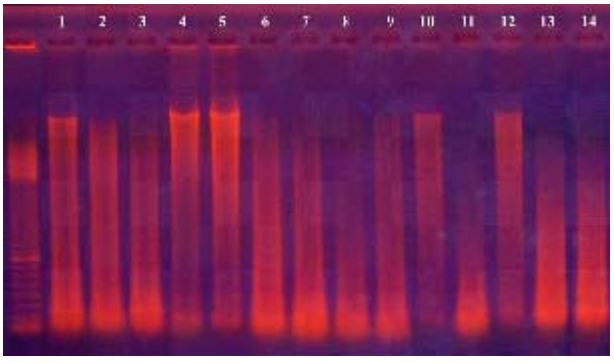

Figure 2. DNA extraction from 14 fresh material specimens of *Mythimna* spp. showed large fragments (almost no fragmentation).

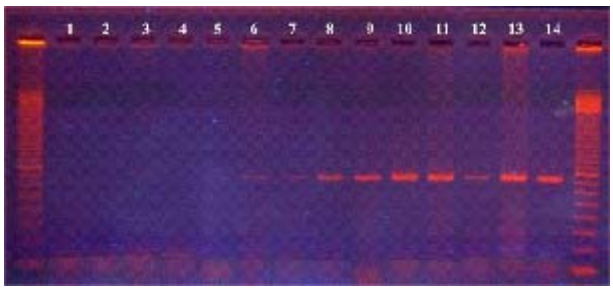

Figure 3. PCR amplification by using a pair primer LepF1 and LepR1 (650 bp) on different storage times. 1: 1992, 2: 1996, 3: 2002, 4: 2003, 5: 2004, 6: 2004, 7: 2004, 8: 2005, 9: 2005, 10: 2007, 11: 2008, 12: 2008, 13: 2008, 14: 2010.

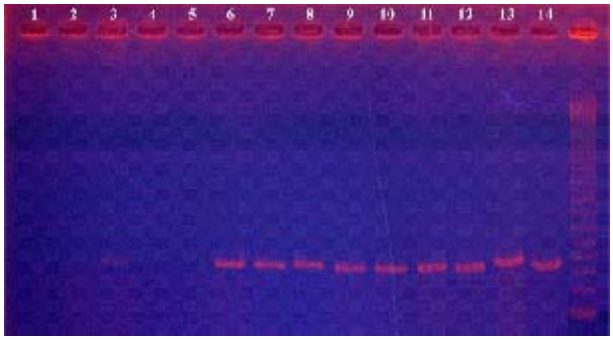

Figure 4. PCR amplification by using a pair primer of LepF1 and MLepR1 (300 bp) on different storage times. 1: 1992, 2: 1996, 3: 2002, 4: 2003, 5: 2004, 6: 2004, 7: 2004, 8: 2005, 9: 2005, 10: 2007, 11: 2008, 12: 2008, 13: 2008, 14: 2010.

(Figure 3). However, low amplicons was resulted from some of specimens of 7 years old (2 of 3 specimens). Moreover, for specimens of > 7 years old were not amplified.

DNA amplification based on LepF1 and MLepR1 primers (300 bp) showed successful results for specimens of 1-7 years old (2004-2010) as indicated by strong bands (Figure 4). However, for the specimens of 7-19 years old (1992-2004) could not be amplified. On the other hand, strong band of the amplicons were resulted from all specimens for small (100 bp) target bands using pair of MLepF1 and MLepR1 primers (Figure 5).

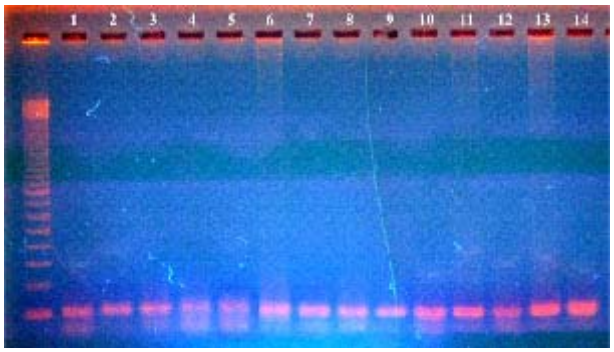

Figure 5. PCR amplification by using a pair primer of MLepF1 and MLepR1 (100 bp) on different storage times. 1: 1992, 2: 1996, 3: 2002, 4: 2003, 5: 2004, 6: 2004, 7: 2004, 8: 2005, 9: 2005, 10: 2007, 11: 2008, 12: 2008, 13: 2008, 14: 2010.

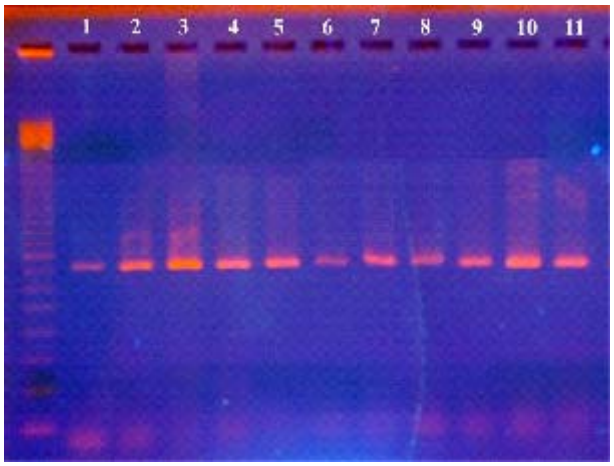

Figure 6. PCR amplification by using primer LepF1 and LepR1 (650 bp) on fresh materials of specimen *Mythimna* no. 1-11 (Table 2).

Based on LepF1 and LepR1 primers (650 bp), all specimens were well amplified with length of amplicons approximately 650 bp (Figure 6). In summary, results of DNA amplification on the different storage times showed that the amplification results were influenced with length of storage times (Table 3).

DISCUSSION

Museum specimens of moths are very fragile, not only due to their ages but also due to their external structures, especially, the scales of the wings that are very easy to damage. Although in the method used in this study was described as a non-destructive extraction, in fact the described method requires physical puncturing, thus damage, of the exoskeleton prior to digestion (abdomen). In contrast to our method, the previous method used in the DNA beetles extraction is truly non-destructive. The buffer liberates DNA through the mouth, anus, spiracles, and possibly through areas of thin cuticle between sclerites, ectodermal glands and possibly broken setae (Gilbert *et al*. 2007). This method was also used to extract DNA from museum samples of mammal teeth (Rohland *et al*. 2004). The extraction buffer enters the sample and liberates DNA through dentinal tubules that perforate the teeth. However, this method would not appropriate to be applied for whole specimens of moths since the extraction buffer will remove all the scales of wings, in which this part providing important characters in identification. Using abdomen only, we also gain benefits not only the external structure of abdomen itself but also the internal structure (genitalia) that can be preserved as slides after DNA extraction without damaging important characters. This method can also be applied on a single leg dissected from

Table 3. Amplification results for different specimens using three different sets of COI gene primers

Specimens	Year of collected	LepF1 + LepR1(650 bp)	LepF1 + M LepR1(300 bp)	MLep F1 + M Lep R1(100 bp)
A. heliconia	1992	-	-	+
A. caricae	1996	-	-	+
A. phapos	2002	-	-	+
A. heliconia	2003	-	-	+
N. marmoria	2004	-	-	+
A. producta	2004	+/-	+	+
A. plana	2004	+/-	+	+
A. caricae	2005	+	+	+
A. heliconia	2005	+	+	+
A. albiformis	2007	+	+	+
A. plana	2008	+	+	+
Asota sp.	2008	+	+	+
A. egens	2008	+	+	+
N. privata	2010	+	+	+

+: amplified, -: not amplified, +/-: weak (low DNA concentration).

the specimen. After extraction the undamaged leg can be replaced on a card with the pinned specimen. If one considers the virtual certainty that DNA extraction and other molecular protocols will improve in the future, limiting the extraction to one leg would ensure that undigested tissue remains in the specimen for future use. The problem with small museum moths, however, it is quite often that the DNA quantity recovered in a small numbers.

The result of DNA extraction showed that there were various sized of fragmentation. The degree of fragmentation in these samples, however, was not clear enough visualized on the agarose gel. Possibly, by using gel electrophoresis is not sensitive enough to evaluate the degradation level under 100 bp amplicon. Previous study showed that capillary electrophoresis is the best for small fragments (Zimmermann *et al*. 2008). Their studies showed that the samples from 2000 (8 years old) show a most abundant fragment size of ~70 bp. All samples from 1990 (> 18 years) have a most abundant fragment size of approximately 50 bp. The distribution of fragment sizes becomes narrower with sample age and is in the range of 20-100 bp for the oldest samples from 1960. Notably, there is a small increase in fragment size for the samples between 30 and 40 years of age (collected around 1970).

DNA degradation in dead tissue correlates with a number of factors including the presence of free water, oxygen, heat and time since death. Many museum specimens, particularly moths, are stored pinned and are not subjected to any further preservation treatment (Dick *et al*. 1993). While the exoskeleton of the moths is stable over many years, the soft tissue soon dries out and decomposes. In a recent study, the effect of different methods of killing and specimen storage on mitochondrial DNA content and PCR success from *Drosophila simulans* specimens was described by Dean and Ballard (2001). The study showed a significant impact of storage time on PCR success, whereas the method of killing and the investigated storage conditions had no marked effect. Main factors affecting DNA during storage are expected to be partial dehydration and exposure to air and light, all potentially leading to diverse types of damage. The deamination of cytidine residues has been identified as a common miscoding lesion in studies of ancient DNA (Mitchell *et al*. 2005).

In the present study, failure to amplify CO I mtDNA (650 bp) from the old specimens of moths (> 7 years old) is likely due to post-mortem degradation of DNA to sub-amplifiable levels (Lindahl 1993). The best amplifications that indicated by strong bands were from the specimens of < 6 years old. The previous study that has been conducted by Zimmermann *et al*. (2008) showed almost the same results. They extracted DNA of a single leg of *Euxoa messoria* moths from different storage times (collected in 1960-2005) using a non destructive method. They conducted PCR using primer CO I mtDNA to amplify 500 bp. The PCR failed to amplify 500 bp from the specimens of > 3 years old (collected 2005). Moreover, in the preset study, some of the specimens of 7 years old (2 of 3 specimens) resulted in very weak bands (650 bp), as well as the specimens of 8-10 years old in the previous study (500 bp) (Zimmermann *et al*. 2008). Indeed, DNA moth specimens of > 7 years old were fragmented to sub-amplifiable level (650 bp). There is no doubt that the oldest specimen of moth (19 years) still able to be amplified at 100 bp. This result was concordant with those found in the previous study on 14 museum beetles (recent specimens up to 94 years old) conducted by Gilbert *et al*. (2007). Beetle specimens within those ranges were able to recovery up to 220 bp amplicon.

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