Pertanika J. Trop. Agric. Sci. 36 (4): 373 - 392 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Freezing Method as a New Non-Destructive Modification of DNA Extraction

Yaakop, S.[1]*, C. van Achterberg[2], Idris, A. B.[1] and Aman, A. Z.[1]

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
[2]Department of Terrestrial Zoology, NCB NATURALIS, Postbus 9517, 2300 RA Leiden, The Netherlands

ABSTRACT

A modification of a DNA extraction method by freezing specimens is recognized as one of new non-destructive techniques. In this study, the freezing method has been applied on dried and fresh, tiny and economically important insect samples, i.e. on adults and larvae of wasps, fruit flies and thrips. The modification entails freezing instead of a lengthy incubation of the sample. Most importantly, the sample is not cut into small pieces, but is soaked in a lysis buffer and then frozen in -22°C for a minimum of 20 minutes. After that, the remaining protocols from the manual of DNeasy Blood and Tissue Kit are followed. Several other non-destructive methods also require incubation for at least 20 minutes in a lysis buffer at 55°C. However, the duration of that incubation process is not standard for all insect and arthropod species. This is because the optimization process is based on species size and the thickness of the insect cuticle. With the freezing method, samples are not damaged, and remain available for morphological re-examination. Hence, the sample can also be re-used for taxonomic work with no distortion of samples, no loss of coloration and no phenotypic changes on the external morphology. The complete protocol for the freezing method is described in this paper. With this freezing method, DNA concentration of 0.2-5.61 ng/µl was recovered on various tiny insect species. Furthermore. several specimens of *Bactrocera* and *Heratemis* species were selected as control specimens in analyzing a variety of extraction methods. The freezing method was proven as a new technique to obtain sufficient quantity and a high quality of DNA for molecular work.

Keywords: Freezing method, molecular DNA, non-destructive samples, sufficient DNA, phylogenetic analysis.

ARTICLE INFO

Article history:
Received: 20 December 2012
Accepted: 1 April 2013

E-mail addresses:
salmah78@ukm.my (Yaakop, S.),
Cees.vanAchterberg@ncbnaturalis.nl (C. van Achterberg),
idrisgh@ukm.my (Idris, A. B.),
ameyra.aman@yahoo.com (Aman, A. Z.)
* Corresponding author

ISSN: 1511-3701

INTRODUCTION

Describing a new species based on a single individual is a valid procedure in taxonomy, but should be avoided if possible. For tropical taxa, there is often no other option or opportunity to obtain more specimens. There are several published taxonomic descriptions based on a single specimen or small series of type specimens (Nitz *et al*., 2009; Schmidt-Rhaesa, 2001; Targino & Wild, 2009). This situation becomes more complicated and difficult if that single type specimen is required for molecular studies. From the point of view of a taxonomist who needs to study shape and form, molecular studies are a nightmare as destruction of the whole body or the sacrifice of some parts of a unique specimen for DNA extraction is required. Morphological characters have traditionally been used in defining species; more recently, the use of molecular data has become regular practise (Johnson *et al.*, 2009; Salvo *et al*., 2011; Smith *et al.*, 2003). Both methods seem useful in phylogenetic studies to show similarity or to resolve conflict between two different data (Friedrich *et al*., 2006; Hillis 1987; Whiting *et al*., 1997), and provide an additional approach to resolving conflicts arising from external morphology. Molecular tools are now widely used in species identification (Göker *et al*., 2009) particularly because morphological characters present often limited data for phylogeny, so molecular data is especially helpful and informative in resolving species relationships.

In the molecular process, DNA extraction is the most important technical step to obtain because the procedure affects the quality of DNA. For this reason, the appropriate extraction method and techniques specific to the intended purposes must be identified. One of the main aims of choosing the right extraction technique is to maintain the voucher specimen for taxonomy and to make sure the specimen remains externally as complete as before the extraction process. Although the samples may not be classified as ancient museum samples, the right techniques have to be applied to maintain as complete a structure as possible so as to preserve it for further use. Thus, some modification of the usual extraction method should be considered to maximize the use of the voucher specimen after extraction; this modification will be presented in this study.

Several molecular procedures are available that enable DNA to be obtained from samples without causing morphological damage (Gillbert *et al.*, 2007; Hofreiter, 2012; Rohland, 2012). Usually, the whole insect body or entire body part (e.g hind leg, antenna etc.) are extracted to obtain DNA. Even if a small portion of an insect body is used for extraction, that method is not the best option since a complete voucher specimen is very important and highly necessary for description or re-examination (Yaakop *et al*., 2009, 2010). Furthermore, small and fragile body parts of minute insect samples e.g. thrips, small braconid species (alysiines, opiines) may accidentally be destroyed during the process of removing appendages for DNA extraction.

There are several published papers on non-destructive DNA extraction for insects and other arthropods without any obvious alteration on the morphological characteristics (Castalanelli *et al*., 2010; Dittrich-Schröderet *et al*., 2012; Favret, 2005; Gilbert *et al.*, 2007; Hunter *et al.*, 2008; Pons 2006; Rowley *et al.*, 2007; Thomsen *et al.*, 2009). However, each paper has provided a non-destructive DNA extraction method, but do not seem as efficient as the novel proposed freezing method because the methods provided may require a longer incubation process, invite contamination and require maceration of samples. The freezing method use the commercial kit with some modifications and has proven to be successful for extraction and analysis on several insect samples.

Currently, only limited insect samples deposited in museums are used for extraction. This might be due to the limited number of samples available for similar taxa, or concern regarding damage to the samples and loss of body parts. Very small insect specimens can be damaged during processing or if parts must be removed for molecular work. Therefore, some modification of the typical extraction process is really needed in order to improve and obtain DNA for molecular work. By applying the freezing method, tiny insect samples will remain intact and complete in structure. The extracted voucher specimens can also be kept in the museum repository as a holotype, while the DNA sequences are stored in GenBank. In addition, the voucher specimens can be used again after the extraction process for future studies. The main goal of this study is to document a new modification of the DNA extraction designated as the freezing method, on several minute insects.

MATERIALS AND METHODS

Insect specimens

Fresh insect samples which were preserved in 90% alcohol and dried museum specimens (collected since 1986) were tested with the freezing method . These samples consisted of several insect Orders e.g. adults and larvae of Hymenoptera (braconids), Diptera (tephritids) and Thysanoptera (thrips). A total of 52 individuals of various sizes (0.5-3.0 mm) were used in this study. Three *Bactrocera* larvae which were more or less similar in size were selected as controls. Each specimen was extracted using 1) a destructive method followed by incubation at 55°C until the specimen is completely lysed; 2) a non-destructive method, without freezing and with incubation of the sample at 55°C overnight; and 3) a non-destructive method, with freezing and without incubation at 55°C of the samples. In addition, two individuals of *Heratemis* sp. were selected as control specimens using the first method of DNA extraction.

DNA extraction

The insect samples were completely immersed and soaked in microtubes with distilled water for 3 days in order to wash them (except for thrips samples). [Fresh thrip samples used in this study were washed

several times by rinsing them in absolute alcohol before being soaked it in proteinase K]. Specimens were then dried and DNA extraction was carried out using the DNA isolation Kit, DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, U.S.A.). The manufacturer's steps one and two were modified. According to blood and tissue extraction protocol, samples should be cut into small pieces and placed in 180 ml of buffer ATL + 20 ml of proteinase K, then the sample has to be incubated at 55°C, followed by the remaining general protocol. However, with the freezing method, the sample was soaked with 180 ml of buffer ATL + 20 µl of proteinase K without destroying it (it was not cut into pieces) and then kept in a freezer at -22°C until totally frozen (for a minimum time of 20 min). After that, the remaining general protocol was carried out; with 200 µl Buffer AL and vortex for 15s added to it. 200 µl ethanol (96-100%) was then thoroughly mixed into it again. The mixture was pipetted into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged at ≥ 6000 *x g* (8000 rpm) for 1 min. The flow-through and collection tube were then discarded. The spin column was placed in a new 2 ml collection tube and 500 ul Buffer AW1 was added and centrifuged for 1 min at ≥ 6000 *x g*. The flow-through and collection tube were again discarded. The previous step was repeated, but this time 500 µl Buffer AW2 was added and centrifuged for 3 mins at 20,000 *x g* (14 000 rpm). The flow-through and collection tube were discarded again. Finally, the spin column was carefully removed to ensure that DNA did not come into contact with the flow-through. A new 1.5 ml or 2 ml microcentrifuge tube was transferred to the spin column by adding 200 µl Buffer AE for elution. The sample was incubated for 1 min at room temperature and centrifuged for 1 min at ≥ 6000 *x g*.

DNA concentration measurement and PCR analysis

The DNA concentration was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer after DNA extraction and purification. The extracted samples were then analyzed with PCR. The conditions for PCR analysis varies between species. A total of 25 µl of PCR used contains 0.5 µl of 0.2 mM DNTPs, 10 pmol of each primer, 1.25U of Taq polymerase, and 1µl of 15 mM $MgCl_2$ from Vivantis. The PCR was performed using MyGene MG96G Thermalcycler or Thermocycler Perkin Elmer 240 under different conditions for each primer combination, starting with denaturation for 3 mins at 94˚C, followed by 39 cycles of denaturation for 1 min at 92˚C, annealing for 15 sec-1 min at 45-62˚C, extension for 1 min at 72˚C and final extension for 5 mins at 72˚C. Several sets of primer combinations of 28S, COI and ND1 markers are used in this study. The list of primers, the anneal temperature and duration for PCR analysis are presented in Table 2-3.

Sequencing and BLAST analysis

PCR products for each species were then sent to Macrogen Inc., Korea and First Base Company, Selangor, Malaysia for

sequencing. The status of the species was confirmed using BLAST search and then they were used in the phylogenetic analyses. Prior to that, the sequences were edited using Sequencher 4.8 and aligned using MacClade 4.08.

Quality and Efficiency of the Extraction Process

The quality and efficiency of the freezing method were measured based on comparison of eight DNA sequences of *Bactrocera* (adults and larvae). The phylogenetic analyses used were based on earlier references on constructing phylogeny (Yaakop *et al*., 2009, 2010). In this study, the phylogeny of *Bactrocera* is presented (Fig.4). The DNA used was obtained from the larvae and adult of *Bactroceracarambolae* Drew and Hancock by using the freezing method (MARDI-sample 0E, F, 0I, FF) (Table 1).

For phylogenetic analysis, the maximum parsimony (MP) tree(s) PAUP* 4.0- test version 4.0d63 (Swofford 1998) was used to get the most parsimonious tree(s). A heuristic parsimony search (Hillis *et al.* 1996) was performed using 100 replicates of random addition sequences, including the TBR (tree bisection reconnection) option for branch swapping. Each base was treated as an unordered character with equal weight, with gaps treated as missing data. Statistical support was obtained by bootstrap analysis with 100 replications (Felsenstein, 1985).

Photograph specimens

Extracted specimens of braconids, thrip and larvae of tephritids (Fig.1 to Fig.3) using the freezing method were photographed with a Stereomicroscope Stemi-D4 (braconids and tephritid's larvae) and Olympus BX41 Universal Transmitted DIC microscope (thrips) attached to a Canon camera digital EOS 1000D DSLR.). Photographing is essential to compare the specimens prior to and after DNA extraction. It is also important to have a control in case specimens are lost or misslabelled.

RESULTS AND DISCUSSION

The PCR amplification based on the COI marker of three of the *Bactrocera* samples (controls) that were extracted using the freezing and non-freezing method was successful, but each contained different concentrations of DNA. Of the three methods in the control experiment, we found that the destructive method showed the highest concentration of DNA, 22 ng/µl. However, there were no specimen remains left as vouchers. The second highest concentration obtained was from the specimen that used the non-destructive method and was then incubated at 55°C overnight. That method successfully collected 14.5 ng/µl of DNA. However, it required a long incubation process and was more time consuming. The lowest concentration of DNA (0.07 ng/µl) was obtained with the non-destructive method without incubation at 55°C and without freezing. However, the DNA concentration extract edusing the freezing method was between 1.54-5.61 ng/µl for the *Bactrocera* larvae and 0.2-5.61 ng/µl for the other insect species used in this study (Table 1), and required a minimum

TABLE 1
List of sample used with institute code, locality, accession numbers and DNA concentration after extraction.

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
1.	New genus near *Coelalaysia* SY24*	RMNH100044 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534336	EF535636	EF534349	EF535624	1.3
2.	*Phaenocarpa* sp. 2104*	RMNH100045 (Dried)	Malaysia: Hutan Kuala Lompat	EF534337	EF535637	EF534350	EF535625	0.9
3.	*Cratospila* sp.SY21*	RMNH100046 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534326	EF535626	EF534339	EF535615	0.76
4.	*Heratemis pahangensis* 2147*	RMNH100047 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534334	EF535634	EF535347	EF535622	2.4
5.	*Heratemis pahangensis* 2144*	RMNH100048 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534333	EF535633	EF534346	EF535621	1.6
6.	*Heratemis devriesi*SY7 *	RMNH100049 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534335	EF535635	EF534348	EF535623	2.5
7.	*Heratemis filosa* SY50*	RMNH100050 (Fresh)	Malaysia: Penang, Telok Bahang	EF534338	EF535628	EF534341	EF535617	0.84
8.	*Heratemis filosa*SY6*	RMNH100051 (Fresh)	Malaysia: Pahang, Cameron Highlands	EF534328	-	-	EF535618	0.77
9.	*Heratemis malayensis* SY44*	RMNH100052 (Fresh)	Malaysia: Perak, Bukit Larut	EF534332	EF535630	EF534345	EF535620	0.65
10.	*Heratemis malayensis* SY33*	RMNH100053 (Fresh)	Malaysia: Pahang, Taman Negara Merapoh	EF534331	EF535629	EF534344	EF535619	1.32
11.	*Heratemis malayensis* 2005* [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	RMNH100054 (Fresh)	Malaysia: Johor, Endau Rompin, Selai	EF534329	EF535631	EF534342	-	12.7
12.	*Heratemis malayensis* 2007* [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	RMNH100055 (Fresh)	Malaysia: Pahang, Taman Negara Endau Rompin	EF534330	EF535632	EF534343	-	10.9

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
13.	*Heratemis cubicep* SY46*	RMNH100056 (Fresh)	Malaysia: Pahang, Hutan Kuala Lompat	EF534327	EF535627	EF534340	EF535616	0.55
14.	*Apodesmia irregularis* * SY160	RMNH100187 (Fresh)	The Netherlands: Waarder	HQ416433			HQ416441	0.94
15.	*Aloeides* sp. * SY257	RMNH100188 (Fresh)	Malaysia: Johor, Selai	HQ416431			HQ416436	1.7
16.	*Bracon* sp. * SY318	RMNH100189 (Fresh)	Malaysia: Sarawak, Betong	HQ416427			HQ416435	1.1
17.	*Colastes braconius* * SY152	RMNH100073 (Fresh)	United Kingdom: nr. Bristol organic farm 2005	HQ416430			HQ416439	0.86
18.	*Colastes braconius* * SY280	RMNH100074 (Dried) '?'	Belgium: Prov. Liege, Eben-Emael	HQ416428			HQ416438	0.2
19.	*Gnamptodon* sp. * SY81	RMMH100186 (Fresh)	Malaysia: Pahang, Kuala Lompat	HQ416428			HQ416437	0.45
20.	*Tanycarpa* sp.* SY320	RMNH100126 (Fresh)	Malaysia: Pahang, Cameron Highlands	HQ416432			HQ416440	1.23
21.	*Utetes* sp. * SY74	RMNH100174 (Fresh)	Malaysia: Selangor, Hutan Simpan Bangi	HQ416434			HQ416442	0.64
22.	*Bactrocera carambolae* * FF	UKM0000070 (Fresh)	Malaysia: Selangor, Bangi		JN833638		JN833639	1.54
23.	*Fopius arisanus* * 117	UKM000059 (Fresh)	Malaysia: Selangor, Bangi	JN833636			JN833637	0.96
24.	*Bactrocera carambolae*+ MARDI-0E	UKM000006 (Fresh)	Malaysia: Pahang, Sg. Tekam		X			5.61
25.	*Bactrocera carambolae*+ MARDI-0F	UKM000005 (Fresh)	Malaysia: Selangor, Serdang, DOA		X			2.53
26.	*Bactrocera carambolae*+ MARDI-0G	UKM000004 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			1.75

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
27.	*Bactrocera carambolae*+ MARDO-0I	UKM000002 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			2.39
28.	*Scirtothrips dorsalis* * SN13	UKM000019 (Fresh)	No locality		X			0.78
29.	*Scirtothrips* sp. 1 * SN11	UKM000020 (Fresh)	No locality		X			0.90
30.	*Scirtothrips* sp. 2* SN04	UKM000021 (Fresh)	No locality	X				2.70
31.	*Scirtothrips* sp. 3 * SD03	UKM000022 (Fresh)	No locality	X				2.00
32.	*Scirtothrips* sp. 4 * SD02	UKM000028 (Fresh)	No locality	X				1.56
33.	*Scirtothrips* sp. 5 * SD01	UKM000030 (Fresh)	No locality	X				4.07
34.	*Alysia* sp. * SY94	RMNH000180 (Dried) '1987'	Malaysia: S.W. Sabah, nr. Long Pa Sia (West), c. 1050 m	X				3.5
35.	*Bobekoides* sp. * SY95	RMNH000181 (Dried) '1987'	Malaysia: S.W. Sabah, nr. Long Pa Sia (West), c. 1050	X				2.8
36.	*Aphaereta* sp. *SY94	RMNH000182 (Dried) '1988'	Malaysia: S.E. Sabah, nr. Danum Valle, Field C, c. 150 m	X				3.5
37.	*Psyttalia* sp. * SY101	UKM000069 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	1.53
38.	*Diachasmimorpha* sp. * SY105	UKM000030 (Fresh)	Malaysia: Melaka, D-Paradise (Jambu batu)	X	X			0.5
39.	*Biosteres* sp. * SY109	UKM000066 (Fresh)	Malaysia: Selangor, Serdang, MARDI	X			X	0.38

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
40.	*Heratemis* sp. * SY111	UKM000065 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X	X		X	0.12
41.	*Heratemis* sp. * SY112	UKM000064 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X	X		X	3.29
42.	*Dinotrema* sp. * SY115	UKM000061 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	0.97
43.	*Diachasmimorpha* sp. * SY116	UKM000060 (Fresh)	Malaysia: Melaka, D-Paradise (Jambu Batu)	X	X			4.52
44.	*Bitomus* sp. SY119	UKM000057 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	0.2
45	*Orientopius* sp. SY121	UKM000055 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	5.17
46.	*Asobara* sp. 1 SY122	UKM000054 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	2.35
47.	*Asobara* sp. 2 SY143	UKM000036 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	2.03
48.	*Asobara* sp. 3 SY144	UKM000035 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	2.03
49.	*Aspilota* sp. SY145	UKM000034 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	0.27

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
50.	*Cratospila* sp. 146	UKM000033 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	1.89
51.	*Bactrocera* sp. + [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	UKM000119 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			22.0
52.	*Bactrocera* sp.+ [control] (a non-destructive method, without freezing and with incubation of the sample at 55°C overnight)	UKM000120 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			14.5
53.	*Bactrocera* sp.+ [control] (a non-destructive method, without freezing and without incubation of the sample)	UKM000121 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			0.07

Notes:
(*)= adult; (+)= larva; (H)= Braconidae; (D)= Tephritidae; (T)= Thripidae.
(-)= not successful with PCR
No PCR attempted for the blank boxes.

TABLE 2
List of primers sequences.

Gene	Sequences 5'-3'
28S	28S 3665 (5' AGA GAG AGT TCA AGA GTA CGT G 3') (Forward) (Belshaw & Quicke, 1997)
	28S 4047 (5' TTGGTCCGTGTTTCAAGACGGG 3') (Reverse) (Campbell *et al.*, 1993)
	28S SYR (5' CCGAATAGCCAGTCAGGAAA 3' (Reverse) (Yaakop, 2011)
COI	Ron (5' GGA TCA CCT CAT ATA GCA TTC CC 3') (Forward) (Monteiro & Pierre, 2000; Simon *et al*., 1994)
	Nancy (5' CCC GGT AAA AAT TAA AAT ATA AAC TTC 3') (Reversed) (Monteiro & Pierre, 2000; Simon *et al*., 1994)
	COI SY F (5' CATGGGGGAATTTCTGTTGA 3') (Forward) (Yaakop, 2011)
	D23 (5' TACAATTTATCGCCTAAACTTCAG 3') (Forward) (Han & Ro, 2005)
	D25 (5' CATTTCAAGTTGTGTAAGCATC 3') (Reverse) (Han & Ro, 2005)
16S	16SWb (5'CACCTGTTTATCAAAAACAT 3') (Forward) (Dowton & Austin 1994)
	16S outer (5' CTTATTCAAATCGAGGTC 3') (Reversed) (Whitfield, 1997)
ND1	ND1F (5' ACT AAT TCAG ATT CTC CTT CT 3') (Forward) (Crozier & Crozier, 1993; Smith *et al*. 1999; Smith & Kambhampati, 1999)
	ND1R (5' CAA CCT TTT AGT GAT GC 3') (Reversed) (Crozier & Crozier 1993; Smith *et al.* 1999; Smith & Kambhampati, 1999)
	ND1 SY F (5' GAGCAATTGAGCGGATTGAT 3' (Forward) (Yaakop, 2011)

TABLE 3
PCR procedure (anneal duration and temperature) for each primer combination.

Gene	Anneal duration (sec)	Anneal temperature (˚C)
28S 3665/28S 4047	15	45
28S 3665/ 28S SYR	15	55
Ron/ Nancy	15	45
COI SY F/ Nancy	15	56
D23/D25	60	56
16S SWb/ 16S outer	60	62
ND1 F/ ND1 R	60	50
ND1 SYF/ ND1 R	60	51

of 20 minutes for the freezing procedure. We also compared the DNA concentrations extracted for similar-sized insect bodies after extracting several samples of *Heratemis* sp.by freezing and by the usual method of grinding the entire insect's body. We found that DNA concentrations were relatively higher if we used the whole body (RMNH100054-RM100055, 10.7-12.7 ng/l) than the freezing method (0.55-3.29) ng/µl (Table 1).

However, the 'freezing method' allows the voucher specimen to remain intact. Therefore, if necessary, the specimen can be re-examined and sequenced again. The samples were confirmed and did not show any changes in their morphological feature as proven from the visible morphology noted prior to and after the extraction process. The non-destructive method did not damage the insect cuticle (e.g. scutellum and pronotum), there was no loss of setae (e.g. on the tibia) and did not change the shape and size of the wing, in spite of the shrivelling process. Photographs of the specimens after the extraction process are shown in Fig.1 to Fig.3. In addition, the freezing method was also tested on samples of thrips. Specimens preserved after being mounted on slides are clear, fully macerated the tissue samples and retained their color, which is necessary for identification. Dr. Ng Yong Foo (pers. comm., 2011) confirmed that the freezing method allowed fat tissues of the thrips samples to be taken out during the freezing step without the need to squeeze the body content (Fig.3).

Interestingly, fragile, dried museum specimens were also successfully tested with the freezing method and DNA extraction (Table 1). However, shorter fragments of the DNA are amplified by applying a different set of primer combinations. The short DNA amplification band is assumed to be due to degradation occurring on the dried samples. The dried samples were collected since 1985. Furthermore, the samples were preserved with chemicals using the AXA Alcohol-Xylene-Amylacetate method (van Achterberg, 2009) and probably with empty body tissue. The concentration of extracted DNA was measured before the amplification process and showed lower and sometimes higher concentrations when compared to fresh specimens that are more or less similar in size (0.9-3.5 ng/µl) (Table 1).

The freezing method was successfully scored on 1.5% gel with TAE 1X buffer for 40 min (80 volt) after completion of the PCR process. A minimum amount of DNA, 5 µl was used as template for PCR. The PCR results showed very clear amplification bands. The targeted band sizes are between 300-1300 bp and vary with insect size and type of samples (fresh or dried).

After the extraction process, PCR products are purified before the samples were sent for sequencing. Technically, 5 µl was loaded on the 1.5% TAE 1X buffer for 40 min (80 volt) gel to confirm that there is enough DNA for sequencing. The DNA concentration is measured and between 35-95 ng/µl was obtained. Generally, the DNA band was clearly visible on the gel after purification. This provides an indication of the samples that can proceed for sequencing.

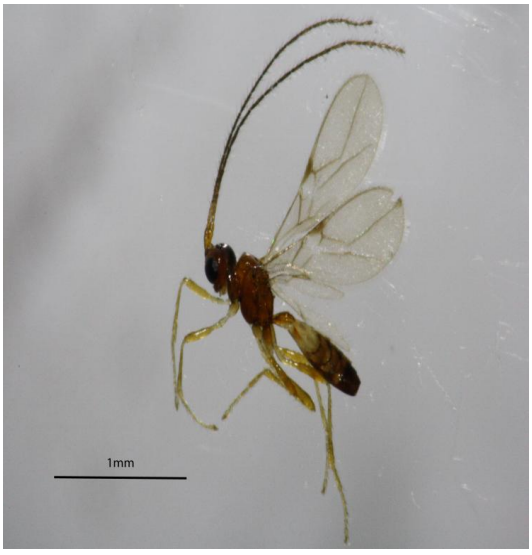

Fig.1: Photograph of extracted specimen of braconid using freezing method

Fig.2: Photograph of extracted specimen of tephritid's larvae using freezing method.

The results from the sequencing process did not show any difference between those that were extracted from body parts or from the entire body. The results showed very nice chromatograms and were very convenient for editing. The edited sequences were aligned and then implemented in BLAST and phylogenetic analyses.

In this study, MP analysis was carried out and implemented on the sequences of the larvae and adults of the *Bactrocera* samples to measure the quality and efficiency of the freezing method by implementing the phylogenetic analysis. The results showed that the freezing method still provides high quality of DNA in a short

Fig.3: Photograph of extracted specimen of thrips using freezing method.

duration. The samples those were were not extracted by freezing method namely *B. occipitalis* and *B. latifrons*+ *B. umbrosa* were successfully separated from the *B. carambolae*, supported by100% and 93% bootstrap values. Besides that, higher length of DNA fragment (approximately 760-1300 bp) obtained from the *Bactrocera* larvae specimens (2-39-5.61 ng/ul) compared to the adult specimen (1.54 ng/ul) and showing DNA obtained from the freezing method still provided enough data for producing a robust phylogeny of *Bactrocera* species (Fig. 4).

In this study, a modification of a commercial manual extraction kit DNeasy Blood & Tissue Kit was carried out. The freezing method does not require a long incubation process, unlike the blood and tissue extraction procedure, which requires a very long process of incubation (Ball & Armstrong, 2008; Thomsen *et al.*, 2009). This is because the freezing method is believed to lead the DNA fragmented and only requires a minimum of 20 minutes to freeze the lysis buffer (ATL buffer and proteinase K) with the whole insect specimen. According to Castalanelli *et al.* (2010), Qiagen Dneasy is often used for the non-destructive method; however no evaluation has been done on that method.

PCR amplification of all insects with selected markers using both the freezing and non-freezing methods (as control) was successful; concentrations were found to be very low to moderately low in the freezing method. In the non-freezing method (method 1-2 in the control experiment), the DNA obtained was high, but there were no remains of the insect body left after the extraction and the process was very time consuming. The DNA amount was found to be very low if there was no incubation after soaking the samples in a lysis buffer and without freezing the samples. After having compared

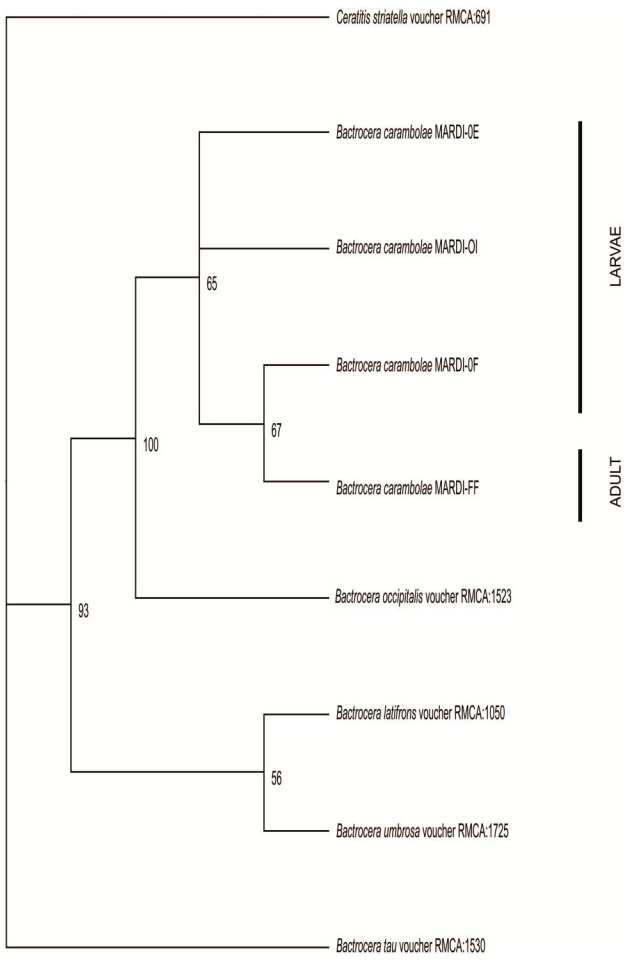

Fig.4: Maximum Parsimony tree of larvae and adults of *Bactrocera* samples using COI markers.

all the methods, we strongly believe that the freezing method provides more benefits and has shown to have a high significance when applied. As a result, we have come up with minor modifications of the normal procedure to ensure that there is no damage of the samples, reduce the duration of the process and avoid contamination.

Interestingly, the mounting process after applying the freezing method on the thrips specimen was easier and more efficient. It is because no maceration was needed after the DNA was extracted from the thrips' body. The method used by Castalanelli *et al*. (2010) without freezing on Eriophyid mites also showed the possibility of sample fragmentation during heating. The process also required short term storage prior to the DNA extraction and mounting to prevent further sample fragmentation. However, this is not necessary when using the freezing method. Furthermore, the method was not standard for several insect species such as mites and beetles, in terms of the duration of

the incubation in order to heat the samples at 99˚C. This is because the duration varies depending on the thickness of the cuticle. On the other hand, when using the freezing method, the process of incubation, in term of the freezing procedure is standard for several species of insects and only requires a minimum of 20 minutes.

DNA concentration was found to be very low when using freezing method compared to the non-freezing methods. In addition, the DNA concentration of dried museum specimens was unstable and sometimes slightly lower or higher compared to the fresh specimens. This might be because the DNA contains too many proteins, phenol and other contaminants; this can be evaluated by measuring the 260/230 ratio. This might also be due to the unpurified DNA samples being measured at that stage (Wilfinger *et al.*, 1997).

We have also proven the quality of DNA obtained in spite of the short time duration required to process the dried and fresh specimens, especially when using the freezing method without a long incubation process (Thomsen *et al.*, 2009). Most importantly, the specimens are are not destroyed. Basically, the ATL Buffer from the isolation kit works similar to the lysis buffer, which functions to dissolve and neutralize cellular components. The ATL buffer functions as a lysis or extraction buffer with the purpose of lysing cells to prepare them for molecular biology experiments. DNA is freed from cellular membranes and becomes soluble using the lysis buffer. Proteinase K is then applied to break down cellular proteins or to digest protein and remove contamination from the nucleic acids. Proteinase K makes nucleases that might degrade the DNA during the purification process inactive. The freezing method is then continued and the remaining protocol is followed through. Through this procedure, it is very clear that it is not necessary for the specimen to be to cut into pieces or to be grinded using liquid nitrogen for DNA collection.

Although several papers have been published on a variety of extraction methods, the freezing method deserves consideration. In other methods, samples may need to be cleaned after the DNA extraction process using ANDE solution, creating the risk of contamination as they have to be pierced with micro pins for the larval specimen (Castalanelli *et al.*, 2010; Rowley *et al.*, 2007). Yet other methods require a long incubation process (Dittrich-Schröderet *et al.*, 2012), which is not necessary for the freezing method. According to Dittrich-Schröder *et al.* (2012), DNA extraction of minute sized insect specimens always results in very low amounts of DNA. It is also very difficult and challenging to obtaining results from the PCR of these specimens. This might be due to the very low amount of DNA templates used. In this method, low amounts of DNA were measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the insect's intact body remained as a voucher specimen. DNA extraction was successful for all the fresh adult samples and higher DNA amounts were retrieved compared to the dried

samples, but this also depends on the insect's body size. The DNA concentration also was not affected only by the specimen size, but was due to the detection of contaminants in the DNA sample. In spite of this, the small amount of DNA could be used as a template and the concept of PCR has been shown by amplifying and duplicating a billion copies of DNA from the available small DNA pieces. This modification technique has been approved after many studies on insect extraction and published in several journal papers (Yaakop et al 2009, 2010).

CONCLUSION

The freezing method is absolutely useful and important in cases where there is a conflict of taxonomical status and only a small sample is available. This method has also been proven to provide very informative data for phylogenetic analyses. This new method would allow tiny insects to be kept intact and available for other purposes even after undergoing the DNA extraction process. This method is also recommended for museum loan specimens, as extraction can also be carried out without the removal of any portion of the sample's body. We would also like to stress that the freezing method provides sufficient quantity or high-quality DNA for molecular work. Besides that the freezing method is highly significant because DNA can be obtained rapidly, it can minimize DNA contamination, does not require a long incubation process and maceration process.

ACKNOWLEDGEMENTS

First and foremost, special thanks to Dr. John Noyes from the Natural History Museum, London (BMNH) for the brilliant idea of freezing tiny samples. The authors would also like to thank Dr. Ng Yong Foo for providing the nice figure of the isolated thrip in this paper and Mr. W. De Prins (Antwerp) for donating Colastes species from Liège, Belgium for molecular extraction. This research was made possible by the research grants UKM-ST-08-FRGS0243-2010 and ERGS grant 320 /1/2011/STWN/ UKM/03/9, Sciencefund 05-01-02SF1024, PTS-2011-032 and GGPM-2012-021.

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