DNA extraction techniques for DNA barcoding of minute gall-inhabiting wasps

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ABSTRACT

DNA extraction from minute hymenopterans and their larvae is difficult and challenging due to their small size indicating a low amount of starting material. Hence, eleven DNA extraction methods were compared to determine their efficacy in isolating DNA. Success of each method was scored on a 2% agarose gel after PCR of the *cox 1* mitochondrial locus. A silica-membrane based approach was the most successful, followed by a method using a combination of incubation buffers and a method using magnetic beads. The method using buffers was the most cost and time effective. Using this method, larvae from *Eucalyptus* seed capsule galls could be assigned a role (parasitoid, gall-former or inquiline) in the gall-inhabiting complex.

Key words: DNA extraction; minute insects; mtDNA barcoding; *cox 1*

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Running title: Comparison of DNA extraction methods

INTRODUCTION

Molecular tools are becoming increasingly common for a suite of applications in the field of entomology, including species identification (Roques *et al*. 2008), identification of immature life stages (Dittrich-Schröder *et al.* 2009), identification of pest insects such as fruit flies (Armstrong *et al.* 1997), identification of forensically important insects such as sarcophagid flies (Wells *et al.* 2001), identification of medically important insects such as mosquitoes (Besansky *et al*. 2003) and, more broadly, the establishment of deoxyribonucleic acid (DNA) barcoding libraries (Hajibabaei *et al.* 2005). DNA extraction is a crucial initial step for these molecular applications. Traditional methods of DNA extraction, such as phenol/chloroform extraction, use toxic chemicals and are time consuming (Chen *et al*. 2010, Hajibabaei *et al.* 2005). These have in many instances been replaced by commercial DNA extraction kits that use fewer chemicals and are generally much more rapid. But they can be expensive and include drawbacks such as long incubation times (Ball & Armstrong 2008).

When specimens are particularly small or when only a part of a specimen can be used because other portions need to be retained as voucher specimens, it is difficult to obtain sufficient quantities of high-quality DNA for further molecular work. This is for example true in a study of a complex of gall-inhabiting hymenopterans that attack *Eucalyptus*. In this case, the seed capsule galls are inhabited by five hymenopteran species at different times and frequently, two larvae are present in the gall at the same time (Klein 2009). In order to understand the roles (primary gall former, inquiline, parasitoid or hyperparasitoid) of these hymenopterans, a tool was required to link the larvae to the adults and thus to determine their respective behaviours in the same gall. For example, when a gall is dissected, a larva feeding on another larva might be classified as a parasitoid or hyperparasitoid (Klein 2009). Morphological identification of the immature stages is not possible and the most direct

method is to use DNA barcoding in an attempt to link all the unidentified larvae present in a single gall to adults of one of the five hymenopteran species. The difficulty in doing such barcoding is, however, to routinely obtain sufficient DNA from the minute and fragile hymenopteran eggs, larvae and pupae.

The yield, quality, suitability for *cox 1* mtDNA amplification, cost and time were considered in the comparisons. The success of each method was scored based on the presence and intensity of bands after PCR using the standard DNA barcoding primer. The most effective method was subsequently tested for DNA extraction from larvae and pupae of minute Hymenoptera from galls in *Eucalyptus* seed capsules. The resulting PCR products were sequenced and linked to sequences of identified adult specimens.

The aim of this study was to compare eleven different DNA extraction methods that are commonly used and readily available, for their utility in routine DNA barcoding experiments such that the best method could be applied to link adult and larval specimens for minute *Eucalyptus* gall-inhabiting wasps.

MATERIALS & METHODS

DNA extraction methods

The eleven DNA extraction methods tested in this study included (i) PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Carlsbad, California, USA); (ii) G1N350, GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, California, USA); (iii) DNAzol Reagent (Life Technologies, Carlsbad, California, USA); (iv) Charge Switch gDNA Micro Tissue (Invitrogen, Carlsbad, California, USA); (v) Wizard Genomic DNA Purification Kit Trial size (Promega, Madison, Wisconsin, USA); (vi) ZR Insect/Tissue DNA kit (Zymo Research, Irvine, California, USA); (vii) Nucleospin Tissue XS (Macherey-Nagel, Düren,

Germany); (viii) ZyGEM DNA extraction using *prep*GEM™ Insect (ZyGEM, Hamilton, New Zealand); (ix) Genomic DNA from yeast (Nexttec, Leverkusen, Germany) (x) Chelex; (xi) Phenol/chloroform.

PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) is a homogenous solution free of chelex, resin and matrix and requires the sample to be boiled, spun down and supernatant removed**.** This method had been used for extraction of DNA from insects at the Forestry and Agricultural Biotechnology Institute (FABI) and was, therefore, included as a basis for comparison. G1N350, GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) makes use of a chaotropic salt-containing buffer, which denatures molecules. The DNA binds to the membrane of a spin column and is then eluted after removal of cell debris. DNAzol Reagent (Life Technologies) is a reagent containing guanidine, which acts as a lysing agent. Genomic DNA is subsequently precipitated using ethanol. Charge Switch gDNA Micro Tissue (Invitrogen) uses positively charged magnetic beads to bind to negatively charged nucleic acids, thereby first allowing the elution of unwanted proteins followed by the elution of the DNA. Wizard Genomic DNA Purification Kit (Promega) is a solution-based approach lysing cells and nuclei, followed by salt precipitation to remove cellular proteins and concentration of genomic DNA, and then desalting using isopropanol. ZR Insect/Tissue DNA kit uses "bashing beads" to lyse cells from which the DNA is later isolated and purified using a column-based approach. Nucleospin Tissue XS (Macherey-Nagel) is especially designed for very small samples utilising a silica-membrane approach to yield a high concentration of DNA. ZyGEM DNA extraction using *prep*GEM™ Insect (ZyGEM) is considered a "lossless" method of DNA extraction by Ball & Armstrong (2008) as all of the sample is retained and a combination of incubation and buffers are used to lyse cells. This reaction occurs in one tube. Genomic DNA from yeast (nexttec™) functions

conversely to most column-utilising protocols as here the cell debris is retained in the column and the DNA is eluted. Although this kit is not specific for insects, it was included because a similar kit, intended for use on fungi, had been successfully used to extract DNA from insects in the research group. The protocol used for Chelex DNA extraction was adapted from Walsh *et al* (1991). This method was developed for use in forensics. Samples are boiled in a chelex solution after which a portion of the Chelex supernatant is used for PCR. The high temperatures and alkalinity of Chelex ensure rupturing of the cell membranes and denaturing of DNA. In Phenol/Chloroform DNA extraction method phenol and chloroform are added to the sample, subsequently centrifuged yielding two phases, namely a lower organic phase (containing the protein) and a less dense aqueous phase (containing nucleic acids). Nucleic acids are obtained by ethanol precipitation (Walsh *et al*., 1991).

Isolation of DNA from insects

Five *Leptocybe invasa* (Hymenoptera: Eulophidae) adult specimens and five unidentified Eulophid adult wasp specimens from *Syzygium* (Accession numbers AcSN3023, AcSN3024), of the same species, were used to test each of the eleven DNA extraction methods (n=10). Specimens from the *Eucalyptus* seed capsule galls were too rare to sacrifice for comparison of molecular techniques and thus two other specimens from the same family and forming galls on Myrtaceae were selected. These specimens were also present in large numbers. Specimens were 2-3 months-old and were stored in 100% ethanol, and air dried prior to use. Prior to DNA extraction, the length in millimetres of the air dried hymenopterans was measured using electronic vernier callipers and the mass in milligrams of each specimen was recorded using a microscale. A single specimen was used per reaction. A total of ten reactions were performed for each DNA extraction method. The manufacturer's instructions for each method (Supplemental on-line material) were followed with the exception of (1)

PrepMan Ultra Sample Preparation Reagent: 30µl of PrepMan Ultra Sample Preparation Reagent was used; (2) GenElute^{TM} mammalian Genomic DNA Miniprep kit: Section 1 Step 4: Samples were digested for 4 hours; Step 5: 20µl Rnase was added; (3) DNAzol Reagent: Section 1: the wasps weighed 0.08-0.4 mg and therefore 100 µl of DNAzol Reagent was used; Section 3: 50 µl of absolute ethanol was used for the isolation step, samples were stored at room temperature for 10 minutes; Section 4: DNA precipitate was washed with 500 µl 75% ethanol; Section 5a: 50 µl 8 mM NaOH was added; (4) Wizard genomic DNA Purification Kit: Section 2 (Animal Tissue (Mouse Liver and Brain)) Step C samples were incubated at 55ºC overnight, Step I Samples were centrifuged for 5 minutes (5) Nucleospin Tissue XS: Section 2 Samples were incubated overnight.

Polymerase chain reaction (PCR) was accomplished using the universal barcoding primers LCO1490 (C1-J-1514) (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (C1-N-2173) (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994) to yield a 658bp fragment of the cytochrome oxidase (*cox 1*) region of the mitochondrial DNA. PCR amplifications were performed on a BioRad iCycler under the following conditions: 95°C for 7 minutes, 35 cycles of (95°C for 30 seconds, 61°C for 45 seconds, 72°C for 45 seconds) 72°C for 10 minutes, 4°C hold. Each 25 µl PCR reaction mix was prepared using 10xPCR Buffer, 25 mM $MgCl_2$, 10 µM of each dNTP, 30 pmol of each PCR primer, 1 unit of Roche Fast Start Taq DNA Polymerase, and 4µl of genomic DNA/RNA mix. Contamination was mediated using negative controls. PCR products were examined by electrophoresis on a 2% agarose gel. The presence/ absence as well as the intensity of bands from the resulting PCR was evaluated when assessing the success of each method. The time taken to execute each DNA extraction method was recorded as hours per method per 10 specimens.

Linking larvae and pupae to identified adult specimens

Based on the comparisons of eleven DNA extraction methods, *prep*GEM™ Insect was used to extract DNA from three *Quadrastichodella nova* adult specimens, three *Leprosa milga* adult specimens, three *Megastigmus zebrinus* adult specimens and five unidentified larval specimens. These sequences were taken from a larger collection of samples analyzed to illustrate the value of the technique. Adult hymenopteran specimens were obtained by picking mature seed capsules from *Eucalyptus camaldulensis* trees, placing them in emergence boxes and collecting emerging hymenopteran adults (Klein 2009). Larvae were dissected out of seed capsule galls of the same plant species (Klein 2009).

Polymerase chain reaction (PCR) was accomplished using the cytochrome b primers CP1 (5'-GAT GAT GAA ATT TTG GAT C -3') (Harry *et al*., 1998) and CB 2 (5'-ATT ACA CCT CCT AAT TTA TTA GGA AT -3') (Jermiin & Crozier, 1994) to yield a 716 bp fragment of the cytochrome b (*cyt b*) region of the mitochondrial DNA. These primers were chosen due to their success at amplifying DNA from dry specimens belonging to the genus *Megastigmus,* (Auger-Rozenberg *et al*., 2005). PCR amplifications were performed on a BioRad iCycler under the following conditions: 95°C for 7 minutes, 35 cycles of (95°C for 1 minute, 48°C for 1 minute, 72°C for 1 minute) 72°C for 10 minutes, 4°C hold. Each 25 µl PCR reaction mix was prepared using 10xPCR Buffer, 25 mM $MgCl_2$, 10 µM of each dNTP, 30 pmol of each PCR primer, 1 unit of Roche Fast Start Taq DNA Polymerase, and 4µl of genomic DNA/RNA mix. Contamination was mediated using negative controls. PCR products were examined by electrophoresis on a 2% agarose gel. PCR products were purified using the Roche High Pure PCR Product Purification Kit (Version January 2008, Cat. No. 11732676001, Page 7) following the manufacturer's protocol. Quantities of solutions used

were adjusted accordingly. DNA sequencing was performed using an ABI PRISM® BigDyeTM Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) under the following conditions: 95 °C for 1 min, 35 cycles of (95 °C for 1 minute, 48 °C for 1 minute, 72 °C for 1 minutes), 4 °C hold. Sequencing products were cleaned using the manufacturer's Ethanol/Sodium Acetate /EDTA precipitation protocol. Sequences were visualized on an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems).

Chromatograms were edited using the Staden package (Staden 1996). Alignment and editing of sequences was conducted using ClustalX version 1.81 (Thompson *et al.* 1997) and BioEdit version 7.0.1 (Hall 1999), respectively. A Neighbour Joining (NJ) tree, with 1000 bootstrap replicates and the Kimura 2-parameter substitution model, was constructed using MEGA version 5.0 (Tamura *et al.,* 2011) Uncorrected pairwise DNA distances were calculated using MEGA version 5.0 (Tamura *et al.,* 2011).

RESULTS

Comparison of DNA extraction methods

The average weights and sizes for *L. invasa* and the unidentified Eulophid adult wasp specimens from *Syzygium* were 0.11 mg and 1.46 mm (n=55) and 0.39 mg and 2.52 mm (n=55), respectively. Results showed that 60% of the methods tested resulted in successful DNA amplification (Figure 1). Only the silica-membrane based approach using a specialised column (Nucleospin Tissue XS) gave 100% success in obtaining a PCR product. Both the method using magnetic beads (Charge Switch) and the method using a combination of incubation buffers (*prep*GEM™) had a 90% success rate followed by the method using a silica-based membrane (Gen Elute (60%)), Chelex (40%) and the guanidine-detergent lysing solution method (DNAzol Reagent (20%)).

The time taken to perform the various DNA extraction methods ranged from 0.5 – 16 hours. The most time efficient methods included the use of the homogenous solution (PrepMan Ultra Sample), the bashing bead method (ZR Insect/Tissue DNA kit), the silica-membrane based approach with a specialised column (Nucleospin Tissue XS) as well as the method using a combination of incubation buffers (*prepGEM*™ Insect) with a duration of 0.5 hours (Table 1). The guanidine-detergent lysing solution method (DNAzol Reagent), solution-based method (Wizard Genomic DNA purification kit), Genomic DNA from yeast and Chelex methods all grouped in the time category ranging from 1 – 3 hours. The method using a silica-based membrane (GenElute), Phenol/chloroform and the method using magnetic beads (Charge Switch) were the most time consuming methods.

Linking larvae and pupa to identified adult specimens

The method using a combination of buffers (*prepGEM*™ Insect) was effective in extracting DNA from the two *Quadrastichodella nova* adult specimens, three *Leprosa milga* adult specimens, three *Megastigmus zebrinus* adult specimens and six unidentified larval specimens. These were selected from a larger set of 144 specimens, from which 31% gave positive amplifications during the first PCR attempt, possibly influenced by their state of degradation. The majority of amplifications was represented by a strong, clear band on the gels. Further optimization might thus be needed depending on the condition of the sample, but this was outside the scope of the focus of this study. PCR products were utilised for cycle sequencing. The resulting NJ tree, using 716 bp sequences from fourteen specimens, clearly grouped the six unidentified larvae with identified adult *Quadrastichodella nova, Leprosa milga* and *Megastigmus zebrinus*, respectively, with high bootstrap support (100%) (Figure 2).

DISCUSSION

Results of this study showed that the silica-membrane based approach with a specialised column (Nucleospin Tissue XS) results in DNA amplification for 100% of the samples. This result is consistent with that of Hajibabaei *et al* (2005) who compared five DNA extraction methods and found that the silica-membrane method was most effective. Likewise, research conducted at the DNA bank Network (Zetzsche *et al.* 2008) suggest that overall, silica membrane methods provide the best results for routine DNA extractions.

Application of the silica-membrane method to columns, has seen substantial modification of the columns by reducing the diameter of the silica-membrane. A large silica-membrane diameter generally results in a large amount of eluate of very low concentration, which cannot be used directly for PCR. Nucleospin has thus designed a column having a small diameter and unique shape allowing small volumes of elution buffer to be dispensed accurately, thereby increasing the final DNA concentration. The cost per reaction was high, but the short time required to use this approach (0.5 hours) justifies this expense, especially where only a small number of highly valuable specimens are available for study.

The methods using magnetic beads (Charge Switch) and a combination of incubation buffers (*prep*GEM^{TM}) were very effective with 90% of samples yielding a PCR product. This is also consistent with the study of Ball & Armstrong (2008) who, using ChargeSwitch, showed an equivalent success rate to prepGEM^{TM} Insect when using ethanol-preserved specimens. Charge Switch makes use of magnetic beads to isolate DNA whereas *prep*GEM^{TM} makes use of two solutions to isolate the DNA. *prep*GEM^{TM} is a novel method of DNA extraction as the entire reaction occurs in a single tube and the product from these reactions can be used directly for molecular analyses such as PCR. This is an added advantage,

especially where small initial samples have DNA of low concentration due to the loss of some of the DNA in the process of extraction and elution. This can be problematic as most methods require a substantial amount of starting material for the eluate to be of a high DNA concentration. This is evident when comparing the intensity of the bands resulting from these two methods. Using the *prep*GEM^{TM} method, this problem is curtailed because reactions are performed in a single tube and thus, no material is lost during the extraction procedure.

Although the Charge Switch and *prep*GEM^{TM} methods were equally effective, *prep*GEM^{TM} is substantially less expensive than the Charge Switch method and it is also most time efficient. An added advantage of *prep*GEM^{TM} is that the quantities of the reagents used can be reduced according to the size of the initial sample, thus extending the use of the reagents. The silica-based membrane method (GenElute), guanidine-detergent lysing solution (DNAzol) and Chelex methods yielded acceptable PCR results but the cost per reaction and/ or success rates made them less desirable than other methods tested for extracting DNA from minute insects.

The homogenous solution (PrepMan Ultra Sample), solution-based method (Wizard Genomic DNA), bashing bead method (ZR Insect Tissue kit), genomic DNA from yeast method (nexxtec^{TM}) and phenol/chloroform extractions did not yield. Various explanations might account for the lack of success of some of these methods. The homogenous solution method (PrepMan) was developed for DNA extraction from bacteria, yeast, filamentous fungi and has recently been use for mammalian tissue smears, human cells and blood (Applied Biosystems, Carlsbad, California, USA) and it might not be well-suited to small insect specimens. The bashing beads (ZR Insect Tissue), although designed for small specimens, were not small enough to lyse the cells and in many instances the specimen remained between the bashing

beads without actually being lysed by the beads. The genomic DNA from yeast extraction method (nexxtec^{TM}) was not expected to work, as it is a kit specific for yeast. The phenol/chloroform method for DNA extraction is one of the original methods and it is still widely used. It was, therefore included for comparative purposes

A comparison of the efficiency of the three DNA extraction methods and the cost per sample, showed no correlation between efficiency and cost. The silica-membrane-based approach with specialised column (Nucleospin Tissue XS) showed the highest efficiency, but it also had the highest price per reaction, whereas the method utilising a combination of buffers (*prep*GEM^{TM} Insect) and magnetic bead method (Charge Switch) was slightly less effective but the cost per sample varied substantially. In terms of time efficiency, the silica-membrane based approach with specialised column (Nucleospin Tissue XS) and the method utilising a combination of incubation buffers (*prep*GEM^{TM}) required the least time to perform. These methods were superior to the magnetic bead method (Charge Switch) which required a substantially longer time to perform.

Based on the comparison of eleven techniques, the method using a combination of incubation buffers (*prep*GEM^{TM}) was chosen to assess the opportunity to link larvae and adults of minute *Eucalyptus* gall-inhabiting wasps. Using this approach, DNA was successfully extracted from fresh specimens. Larvae of gall-formers are difficult to identify and rearing larvae through to adults outside their galls is often unsuccessful (Klein 2009). Thus, matching mtDNA from unidentified larvae to identified adult hymenopteran specimens is the most practical method of identification. When dissecting the gall, information can be obtained about the behavioural characteristics of the larva, such as whether it is a parasitoid, feeding on another larva or whether it is feeding on gall-tissue. This information then provides some clarity on the role of the hymenopterans in the gall-inhabiting complex.

Application of this approach in the present study made it possible to conclude that *L. milga* and *M. zebrinus* are parasitoids and that *M. zebrinus* and *Q. nova* feed on the gall tissue. These discoveries can now be supplemented by observations regarding the behaviours of the adult hymenopterans.

The results of this study suggest that the most effective method to link larvae to adult insects of high value or that are very small is using the combination of incubation buffers (*prep*GEM™). This technique is not only highly effective, but it is also the most cost and time efficient. When small numbers of particularly valuable specimens are being considered, the tested silica-membrane based approach with a specialised column (Nucleospin Tissue XS) is recommended, but its high cost precludes studies with large numbers of specimens.

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Data Accessibility:

DNA sequences: GenBank accession numbers JF800077 - JF800090

Table 1. A comparison of DNA extraction methods, PCR success, time taken to perform method and cost per reaction per method for the eleven DNA extraction methods/kits

DNA Extraction Method	**Company**	**Efficiency** (% PCR success)	**Measure of DNA amplification** (strong, weak, not detectable)	**Time taken to perform method** (hrs per 10 samples)	**Cost per single reaction** (units per method)*
PrepMan Ultra Sample Preparation Reagent	Applied Biosystems	0	Not detectable	0.5	0.72
GenElute™ Mammalian Genomic DNA Miniprep Kit	Sigma-Aldrich	60	Not detectable - weak	5	2.52
DNAzol Reagent	Life Technologies	20	Not detectable	1	4.08
Charge Switch gDNA Micro Tissue	Invitrogen	90	Weak	16	4.80
Wizard Genomic DNA Purification Kit Trial size	Promega	0	Not detectable	3	5.40
ZR Insect/Tissue DNA kit	Zymo Research	0	Not detectable	0.5	3.00
Nucleospin Tissue XS	Macherey-Nagel	100	Strong	0.5	5.64
DNA extraction using *prep*GEM™ Insect	ZyGEM	90	Weak - strong	0.5	1.08
Genomic DNA from yeast	Nexttec™	0	Not detectable	3	3.24
Chelex	-	40	Not detectable - weak	1	0.24
Phenol/chloroform	-	0	Not detectable	5	0.24

* calculated as a function of the cost of the cheapest technique, namely phenol/chloroform, which cost 0.24 US$ per reaction at the time of this study.

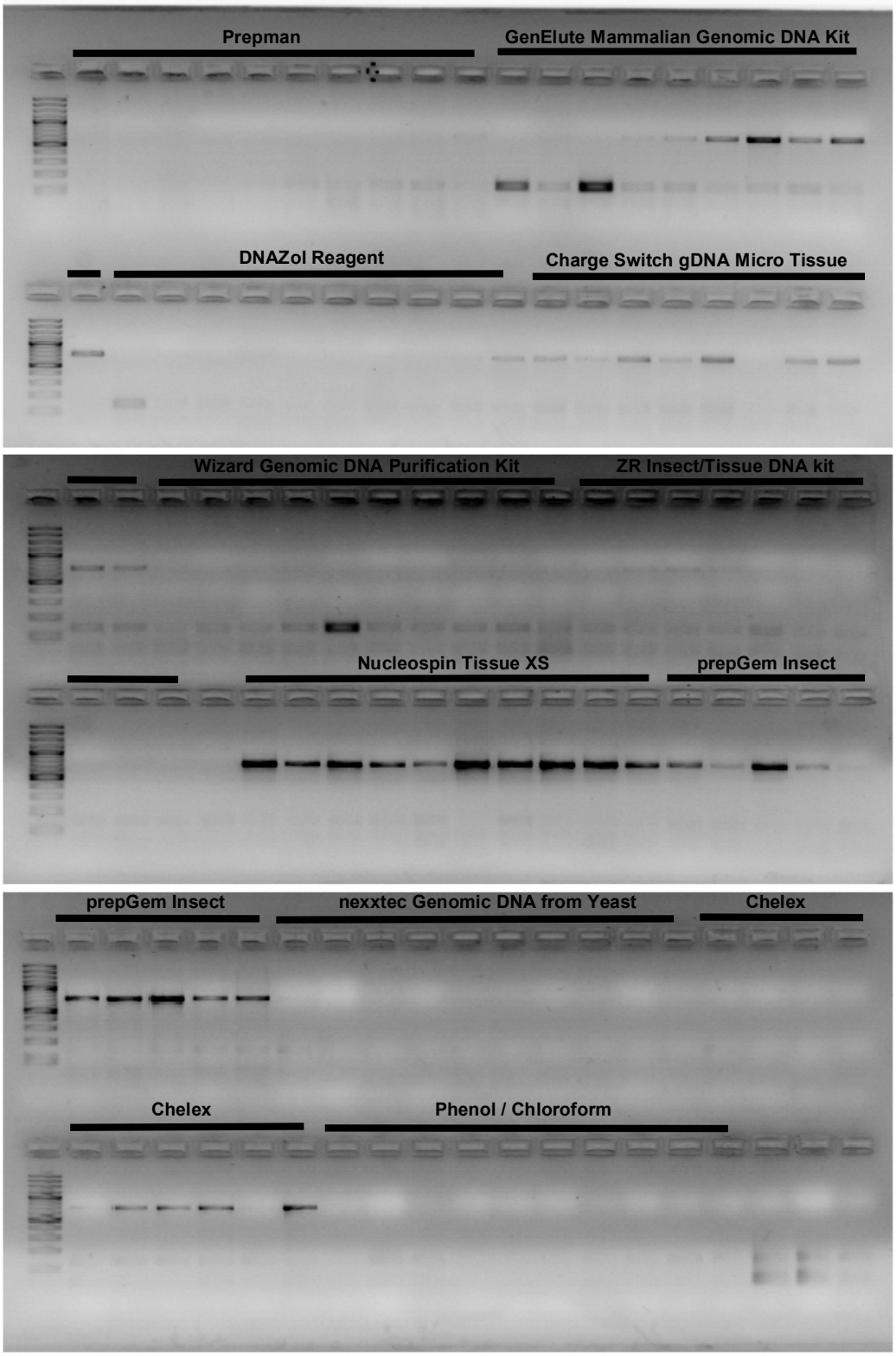

Figure 1. PCR amplification from the eleven DNA extraction methods tested to determine the efficacy of the methods. A 2% agarose gel shows the presence or absence of a band after PCR.

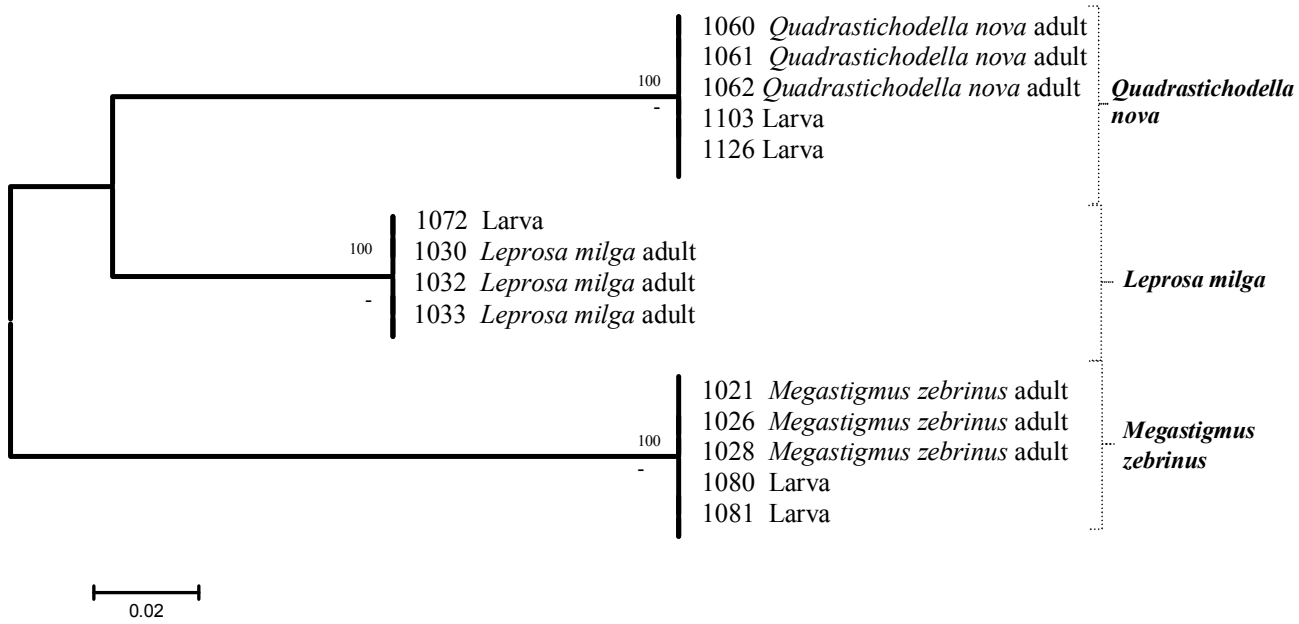

Figure 2. A neighbour joining tree showing the association of unidentified larva and pupae with identified adult specimens. Numbers above branches indicate percentage bootstrap shown only if >70%. Numbers below branches indicate percent sequence divergence.