MODIFIED MOLECULAR TECHNIQUES FOR DETECTING RICE ODONATE INSECTS IN THAILAND

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ABSTRACT: Rice odonates are beneficial predators that can help control insect pests in rice, so playing a valuable role in the rice ecosystem. Morphological classification is not reliable at the taxonomic level for some species. Thus, molecular techniques may be used to resolve species more accurately. Normally, genetic DNA sequence amplification is used in molecular identification. This study modified and developed one stage of the DNA extraction process to permit DNA extraction from a single insect leg. After cytochrome oxidase subunit 1 (COI) amplification, nucleotide banding was conducted to determine the efficiency of the extracted DNA. The results showed that this modification to DNA extraction could yield sufficient DNA to amplify the COI gene, and thus be a practical tool for detecting odonates using molecular techniques.

KEY WORDS: Odonate, Morphological classification, Cytochrome oxidase subunit 1 37 (COI), Molecular identification.

Odonates are insects in the Order Odonata, which includes dragonflies and damselflies. There are approximately 6,500 species in over 600 genera scattered all over the world, with most being found in the tropics (Gullan & Cranston, 2000; Trueman & Rowe, 2009). Adult odonates are brightly colored, medium to large in size, slender-bodied aerial predators that hunt by sight (Trueman & Rowe, 2009). They are generally found at, or near freshwater, although some species travel widely and may be found far from breeding sites. The larvae predate on other aquatic organisms, which occur in all types of bodies of water, ranging from streams and rivers, to lakes, to temporary pools, and water-filled tree-holes.

They are common ricefield insects in Thailand. They usually have two or three generations per year, and the nymphal stage is found in stagnant water in and around rice fields (Asahina et al., 1972). They are important predators of rice pests such as planthoppers, leafhoppers, and rice-stem borers (Che Salmah et al., 1999; Li, 1970). Odonate larvae found in rice fields can be used to control mosquito larvae (Watanabe et al., 1968). They are clearly beneficial insects, which play a valuable role in the rice ecosystem (Shepard et al., 1987).

Morphological identification can be unclear for some late-adult odonate insects, so that nowadays molecular techniques are used to detect odonate species. This molecular technique involves DNA extraction and a PCR-based methodology. Many targets have been developed focusing on mitochondrial DNA, COI (Hayashi et al., 2005; Kiyoshi & Sota, 2006), COII (Jordan et al., 2005), 16S rDNA (Groeneveld et al., 2007; Hayashi et al., 2005; Misof et al., 2000) and ND1 (Groeneveld et al., 2007), the nuclear ribosomal DNA region, 18S, ITS1, 5.8S, ITS2 sequences (Weekers et al., 2001), and the nuclear EF-1α gene (Groeneveld et al., 2007; Jordan et al., 2005). The AFLP technique has also been used to detect

odonates, especially damselflies (Wong et al., 2003; Svensson et al., 2004). Most molecular studies used the muscle tissues of the odonate thorax for DNA extraction by either phenol- chloroform extraction or CTAB, and precipitated the DNA using ethanol or a commercial kit. To preserve the reference odonates and to avoid contamination with odonate pathogens, DNA was extracted from their legs.

MATERIALS AND METHODS

Odonate samples and DNA extraction with modification

Five species of rice odonates--*Agriocnemis pygmaea*, *Ischnura a. aurora*, *Ischnura senegalensis* (Zygoptera), *Brachythemis contaminata*, and *Diplacodes trivialis* (Anisoptera) were selected for study because they are most abundant species in rice fields around Thailand, and play a beneficial predatory role to help control the major insect pests of rice (Eak-Amnuay, 1982; Nakao et al. 1976; Thipaksorn et al., 2001, 2003; Vungsilabutr, 1991). Genomic DNA was extracted from 9 Zygopteran and 6 Anisopteran legs. In this study, the QIAamp tissue extraction kit (QIAGEN) was modified for more efficient DNA yield.

In the first modification, only one leg was selected for DNA extraction, to avoiding DNA contamination from odonate insect pathogens harbored in the major body tissues.

In the second modification, because we only used one leg for DNA extraction, the overall amount of buffer could be decreased to half that recommended by the manufacturer, and thereby increase the efficiency of lysis and DNA extraction. Fifteen odonate leg samples were lysed in lysis buffer (Buffer ATL) with proteinase K overnight. The leg samples were not ground. After adding the manufacturer's reagent (buffer AL) and ethanol precipitation, the DNA was attached to a silica membrane in a QIAamp column, then eluted with eluting buffer. After DNA extraction with modification, DNA samples were measured with Nanodrop equipment, and processed in 1-1.5 % agarose gel to separate DNA.

COI amplification

Of each DNA sample, 5 µl were used per 650 base pairs of cytochrome oxidase subunit I (COI) with universal primers: LepF1 forward primer (5' attcaaccaatcataaagatattgg 3') and LepR1 reverse primer (5' taaacttctggatgtccaaaaaatca 3'). Each PCR reaction contained 5 µl of 10x PCR buffer (20 mM Tris-HCl pH8.4 and 500 mM KCl), 2.5 mM $MgCl_2$, 50 µM dNTPs, 0.1 µM each primer, 0.5 U Platinum R Taq DNA polymerase (Invitrogen) and ddH_2O up to 50 µl. The PCR conditions comprised 94°C for 1 min, followed by 5 cycles of 94°C for 30 sec, 45°C for 40 sec, 72°C for 1 min, then 35 cycles of 94°C for 30 sec, 55°C for 40 sec, 72°C for 1 min, and final extension at 72°C for 10 min. Gel electrophoresis was used to separate the DNA product with 1.5 % agarose gel.

RESULTS AND DISCUSSION

After extracting DNA using the modified method, the DNA samples were measured using a Nanodrop spectrophotometer. The amount of DNA was between 0.48-10.15 ng/µl. The group of damselflies--*Agriocnemis pygmaea*, *Ischnura a. aurora* and *Ischnura senegalensis*--had low concentrations of DNA (0.48-5.69 ng/µl), and showed low intensity in agarose gel (Figure 1). The dragonflies--*Brachythemis contaminata* and *Diplacodes trivilis*--had high concentrations of DNA (2.07-10.15 ng/µl) (Figure 2). The intensity of the DNA

bands appearing in agarose gel depended on the quantity of DNA, and the concentrations of DNA depended on the sizes of the odonate leg.

Mitochondrial COI amplification was used to assess the utility of the extracted odonate DNA. Modified DNA extraction, using only one leg, amplified the COI gene successfully from rice odonates, and 650 bp COI of each DNA sample could appear on 1.5 % agarose gel (Figure 3), showing that the DNA derived from this modified extraction method was adequate for amplifying the COI gene, and for DNA barcode sequence identification.

In summary, the modified DNA extraction method reduced the complex extraction process. This study used only one leg in the lysis process and reduced the buffer volume by half. The yield of DNA was adequate for COI amplification. However, the intensity of the band in the agarose gel depended on the size of the odonate leg, and the odonate species.

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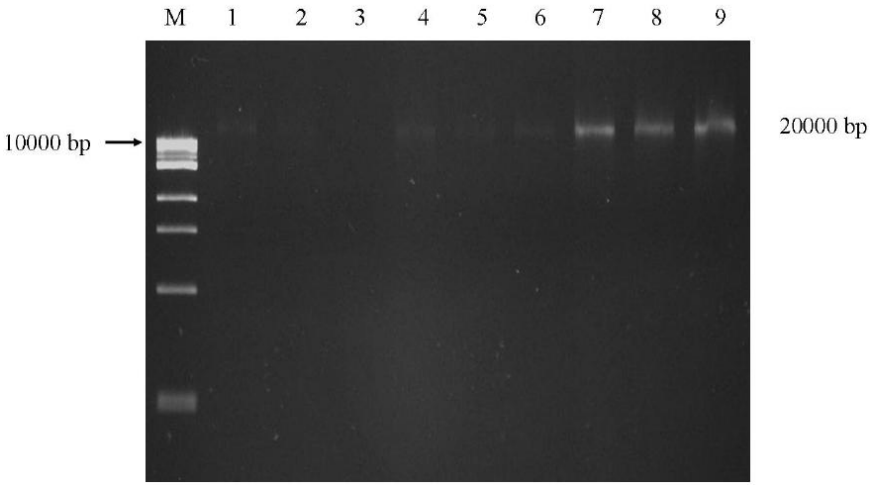

Figure 1. DNA of ricefield damselflies after DNA extraction using the modified method. Genomic DNA extracted from a leg (part of the tibia) shows about 20000 bp. Lane M, 1000 bp DNA ladder (Fermentas); lanes 1-3, *Ischnura a. aurora*; lane 4-6, *Agriocnemis pygmaea*; lane 7-9, *Ischnura senegalensis.*

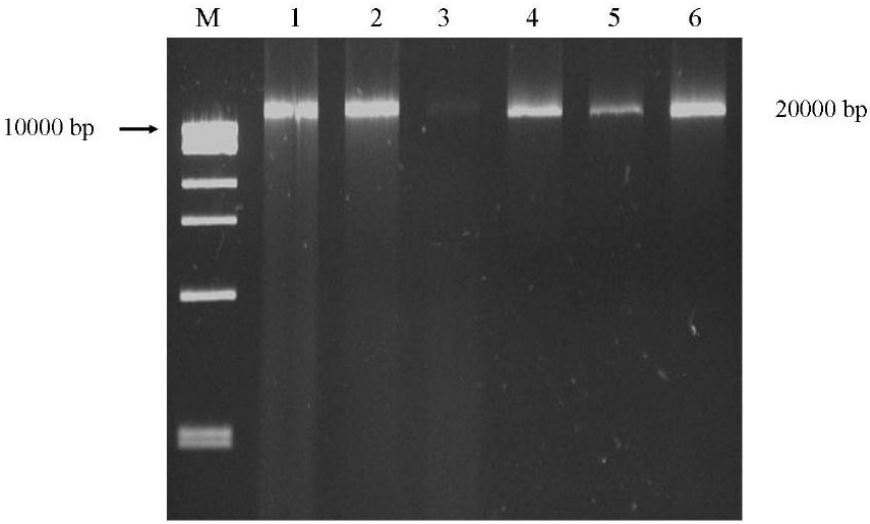

Figure 2. DNA of ricefield dragonflies after DNA extraction using the modified method. Genomic DNA extracted from a leg (part of the tibia) shows about 20000 bp. Lane M, 1000 bp DNA ladder (Fermentas); lane 1-3, *Brachythemis contaminata* and lane 4-6, *Diplacodes trivialis*.

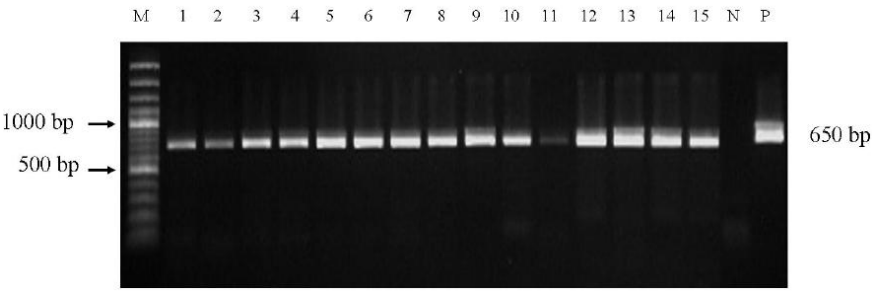

Figure 3. COI amplification of ricefield odonates. 650 bp of COI from 15 odonates were amplified by LepF1 and LepR1 primers. Lane M, 100 bp DNA ladder (Fermentas); lanes 1-3, *Ischnura a. aurora*; lane 4-6, *Agriocnemis pygmaea*; lane 7-9, *Ischnura senegalensis*; lane 10-12, *Brachythemis contaminata*; lane 13-15, *Diplacodes trivialis*; lane N, negative control, no DNA template; lane P, positive control, DNA template of the mosquito, *Anopheles sundaicus*.