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Noninvasive molecular methods to identify live scarab larvae: an example of sympatric pest and nonpest species in New Zealand

M.-C. LEFORT,* S. BOYER,† S. P. WORNER* and K. ARMSTRONG*
*Bio-Protection Research Centre, PO Box 84, Lincoln University, Lincoln 7647, Christchurch, New Zealand, †Department of Ecology, PO Box 84, Lincoln University, Lincoln 7647, Christchurch, New Zealand

Abstract

Despite the negative impact that many scarab larvae have on agro-ecosystems, very little attention has been paid to their taxonomy. Their often extremely similar morphological characteristics have probably contributed to this impediment, which has also meant that they are very difficult to identify in the field. Molecular methods can overcome this challenge and are particularly useful for the identification of larvae to enable management of pest species occurring sympatrically with nonpest species. However, the invasive collection of DNA samples for such molecular methods is not compatible with subsequent behavioural, developmental or fitness studies. Two noninvasive DNA sampling and DNA analysis methods suitable for the identification of larvae from closely related scarab species were developed here. Using the frass and larval exuviae as sources of DNA, field-collected larvae of *Costelytra zealandica* (White) and *Costelytra brunneum* (Broun) (Scarabaeidae: Melolonthinae) were identified by multiplex PCR based on the difference in size of the resulting PCR products. This study also showed that small quantities of frass can be used reliably even 7 days after excretion. This stability of the DNA is of major importance in ecological studies where timeframes rarely allow daily monitoring. The approach developed here is readily transferable to the study of any holometabolous insect species for which morphological identification of larval stages is difficult.

Keywords: *Costelytra*, exuviae, frass, larval identification, multiplex PCR, noninvasive DNA sampling

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Introduction

Many larvae of root-feeding insect species are regarded as pests (Blossey & Hunt-Joshi 2003; Dittrich-Schröder *et al.* 2009). Among these, scarabs are generalist root feeders (Cowles *et al.* 2005) of which several species affect a wide variety of economically important pasture, agricultural and horticultural plants (McPeak *et al.* 2006, Romero-López *et al.* 2010). Most often it is the larvae of these species that cause the damage and consequently research to improve their management tends to focus mainly on that life stage. However, surprisingly, and as highlighted by Dittrich-Schröder *et al.* (2009), very little attention has been paid to the taxonomy of scarab larvae, and so the ability to identify them morphologically to species level is very difficult. This is compounded by the larvae of closely related species looking very similar in size, colour and shape (Bain 1980), and taxonomic keys to distinguish them are underdeveloped and generally rely on the use of minute morphological characteristics often only accessible by dissection. Therefore, the likelihood of misidentification of field-collected larvae particularly where two or more species co-occur (e.g. Miller *et al.* 1999) is high. Unfortunately, accurate species identification of scarab larvae is crucial to prioritize the correct species-specific treatments for pest management, especially when only one of the species present is regarded as a threat and when laboratory studies on live specimens are required.

One way of identifying field-collected larvae of scarabs is to conduct molecular analyses that can link larval genetic profiles to those of identified adult specimens. In the past, this approach has been successfully achieved using the convenience of RFLP analysis for many scarab (e.g. Miller *et al.* 1999) and other species (e.g. Armstrong *et al.* 1997). More recently, an improved approach has been to use DNA barcoding (Hebert *et al.* 2003) for various invertebrates (Armstrong & Ball 2005; Waringer *et al.* 2008; Zhang *et al.* 2008; Zhang & Weirauch 2011)

Correspondence: Marie-Caroline Lefort, Fax: +64 3 325 3864;
E-mail: marie-caroline.lefort@lincoln.ac.nz

including scarabs (Miller *et al.* 2005; Dittrich-Schröder *et al.* 2009). However, this usually requires invasive tissue sampling for DNA extraction purposes, which is clearly not compatible with subsequent behavioural, developmental or fitness studies requiring physiologically unaffected live specimens. The alternative is to use noninvasive DNA sampling methods such as those developed to comply with animal welfare (e.g. Beja-Pereira *et al.* 2009) or for the conservation of endangered species (e.g. Gregory & Rinderer 2004; Beja-Pereira *et al.* 2009; Monroe *et al.* 2010). The above methods, however, were mostly developed for rather large animals, with, for example, the use of shed hair and feathers, or saliva (Taberlet & Luikart 1999), and are generally not appropriate for much smaller animals such as insects. As a consequence, noninvasive DNA sampling methods for the identification of living insects are not common and have been rarely attempted (Feinstein 2004).

Here, we propose two noninvasive DNA sampling and DNA analyses methods suitable for the identification of closely related scarabs. These methods were tested using two New Zealand endemic species, *Costelytra zealandica* (White) and *Costelytra brunneum* (Broun) (Scarabaeidae: Melolonthinae) for which populations can occur in sympatry. Accurate distinction between these two species is important because *C. zealandica* is a significant pest in pastures for which early detection at the larval stage is crucial for its control. Unfortunately, taxonomic keys are only available for the adult life stage of this genus (Given 1952, 1966). Therefore, distinguishing the presence of *C. zealandica* from that of the nonpest *C. brunneum*, which does not reach damaging population densities, is difficult and leads to the potentially unnecessary implementation of expensive management strategies.

Materials and methods

Insect samples

All samples were collected in New Zealand's South Island. Adults were sampled by light trapping or opportunist catching, and *C. brunneum* were collected from Cass (n = 3) and *C. zealandica* from Christchurch (n = 2), Kaikoura (n = 2) and Picton (n = 1). Their morphological identification, based on Given's (1952, 1966), was performed with the assistance of J. Marris, taxonomist and curator of the Entomological Museum of Lincoln University, Canterbury, New Zealand. Larvae were collected from the soil in exotic pastures at Lincoln (n = 10) and Hororata (n = 15) and from native grassland at Cass (n = 89) and Castle Hill (n = 28). All larvae were identified as *Costelytra* based on Given's key of New Zealand Melolonthinae (1952) and in accordance with external characters such as body length and relative head capsule size (Given 1952).

Species-specific primer design and diagnostic assay by multiplex PCR

DNA extractions were performed with the Zymo Research Insect/Tissue DNA Kit-5 using one leg from each of the three morphologically identified adults of *C. brunneum* and five of *C. zealandica*. Universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) were used to amplify a 658-bp fragment of the cytochrome oxydase 1 gene (COI) by polymerase chain reaction (PCR), using Expand High Fidelity Enzyme Mix (Roche Applied Science) and reaction conditions according to the manufacturers protocol. The PCR products were prepared for DNA sequencing with BigDye terminator kit according to the manufacturers protocol (Applied Biosystems, Foster City, CA, USA) plus the same primers as used for the PCR. The sequence products were analysed on an ABI 3130xl (Applied Biosystems) DNA sequencer. Forward and reverse sequences from the same specimen were aligned using MEGA 4 (Tamura *et al.* 2007) (GenBank Accession nos JN793483-JN793487 and JN793498-JN793500). BLASTn searches (Karlin & Altschul 1990) of the GenBank database were performed to confirm that the obtained sequences most closely matched those of other Melolonthinae species. Multiple sequence alignments were performed, and a consensus sequence was constructed for each of the two species studied using MEGA 4 (Tamura *et al.* 2007) (Fig. 1).

Based on the consensus COI sequences, species-specific reverse primers were designed, COI_Czeal_FolB (5′-GTGATAGCTCCTGCTAATACAGGTAAA-3′) for *C. zealandica* and COI_Cbrun_FolB (5′-ACCGGCTCCGG TTTCGAT-3′) for *C. brunneum*, both to be used with the forward primer LCO1490 (5′-GGTCAACAAATCA TAAAGATATTGG-3′) (Folmer *et al.* 1994). These primers pairs were designed to amplify fragments of distinct sizes, so that the respective amplicons (Fig. 1) could be distinguished in a single multiplex PCR. Multiplex PCRs were performed using the GoTaq Green Master Mix (Promega). Each reaction contained 1× GoTaq® Green Master Mix, 0.3 μM of each primer, 2 μL of DNA template and made up to 20 μL with nuclease-free water. PCRs were prepared under a sterilized UV hood, filter tips were used to avoid cross-contamination, and negative controls (i.e. water + PCR mix) were included in each PCR.

The species-specific primers and multiplex assay were subsequently tested on DNA extracted (as above for the adult specimens) from single legs of 122 unidentified *Costelytra* larvae (from Lincoln, Cass and Castle Hill). DNA of the morphologically identified adult specimens

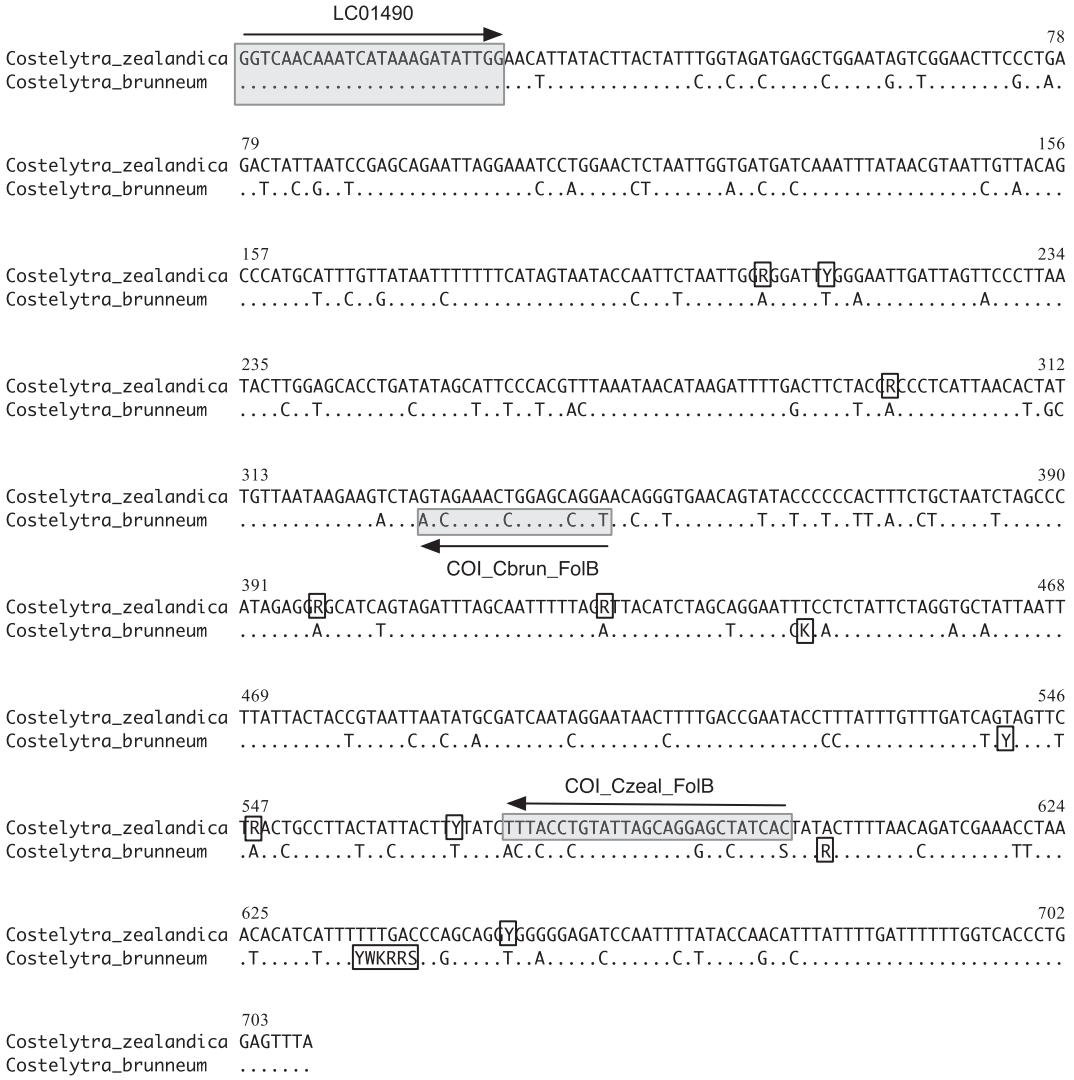

Fig. 1. Consensus sequences alignment of the mitochondrial region cytochrome oxydase 1 gene (COI) for adult specimens of *C. zealandica* (n = 5) and *C. brunneum* (n = 3). Intraspecific variabilities are highlighted by degenerate positions in clear boxes. Grey boxes indicate the location of the species-specific primers designed and used for this study (LC01490 as forward primer and COI_Czeal_FolB and COI_Cbrun_FolB as reverse primers).

was used as positive controls. Size of the PCR products, as an indicator of species identification, was measured after electrophoresis on 2% agarose gels. A total of 20 PCR products (10 of each amplicon size) were sequenced (GenBank Accession nos JN793488-JN793497 and JN793501-JN793510) following the method mentioned earlier and compared to those of identified adults.

Noninvasive DNA sampling methods

Frass experiment. Ten unidentified third instar larvae of *Costelytra* (from Hororata) were used to provide frass over the experimental period. Larvae were placed individually in a compartment of a 12-well ice-cube tray with a 1 cm^3 piece of carrot as per standard culturing conditions (Villalobos *et al.* 1997). Over a 48-h period, all frass produced were collected, individually weighed and stored in 1 mL plastic vials. These were kept at room temperature (~20 °C) for a range of experimental time periods. The average weight of a frass pellet produced by a larva was 0.199 ± 0.048 mg. To determine the minimum amount of frass required to successfully identify each larva, DNA extractions were performed on samples made of various frass quantities [12, 8, 4, 2, 1 and ½ frass pellet(s)]. For each of these quantities, five larvae were selected and their frass were analysed at day 1, 3 and 7 to check the stability of DNA over time. DNA extraction and multiplex PCR were conducted as above, with 0.2 μg/μL of purified bovine serum albumin (BSA) added to each PCR.

Exuviae experiment. Ten unidentified second instar larvae (from Hororata and Castle Hill) were placed individually in a compartment of a 12-well ice-cube tray with soil from the sampling site and a 1 cm^3 piece of carrot. Larvae were checked daily and exuviae collected as soon as they were physically detached from the larvae. Exuviae were kept at −20 °C in 1 mL individual plastic vials. DNA extraction was performed on the whole exuviae chopped into small pieces. Protocols for DNA extraction, amplification and analysis were the same as for the frass experiment.

Results

Specific primers diagnostic assay by multiplex PCR

Single PCR products were produced using the combined species-specific primer pairs with DNA from identified adult specimens of the two species and were clearly distinguishable between the two species by ~240 bp (Fig. 2). This same multiplex assay successfully amplified DNA from each of the 122 larvae. Of these, a total of 96 specimens displayed the 546-bp COI fragment of *C. zealandica*, while 26 specimens displayed the 304-bp COI fragment of *C. brunneum* (Fig. 2). Ten examples of each were sequenced and confirmed that these were the targeted amplicons (GenBank Accession nos JN793488-JN793497 and JN793501-JN793510).

Frass experiment

Considering all treatments combined, more than 88% of the frass DNA amplifications successfully produced a single product from the multiplex PCR (Fig. 3). Results are summarized in Table 1. Unsuccessful amplifications only occurred for low quantity of frass (i.e. 1 and ½ frass pellet, which correspond to quantities inferiors at ~0.2 mg of excrement) and particularly after the longest period of 7–8 days of storage (Table 1).

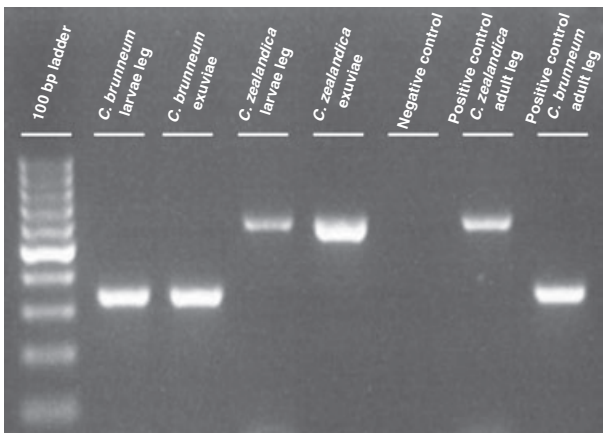

Fig. 2. Two per cent agarose gel depicting PCR amplification of mitochondrial DNA extracted from the leg and exuviae of *C. zealandica* and *C. brunneum* larvae together with negative and positives controls. Three microlitres of each PCR product were loaded on the gel.

Exuviae experiment

The DNA of all 10 exuviae used in this assay were successfully extracted and amplified. PCR products from seven of those were equivalent to the 546-bp fragment (Fig. 2), indicating that they were from *C. zealandica*. PCR products from the remaining three exuviae were equivalent to the 304-bp fragment (Fig. 2) and were consequently considered to be *C. brunneum*.

Discussion

A convenient two-pronged noninvasive approach has been developed here to distinguish live larvae from two sympatrically occurring scarab species. Firstly, a multi-

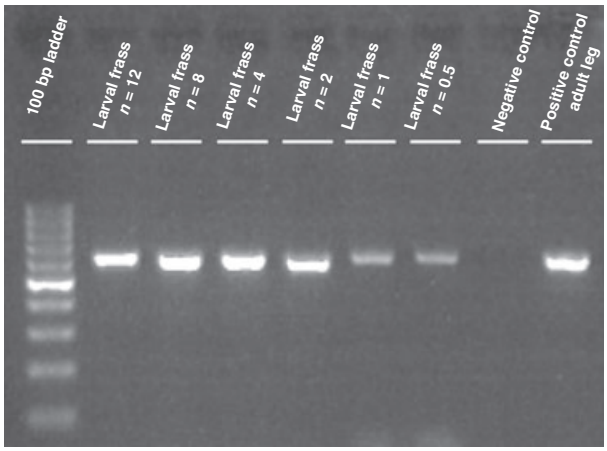

Fig. 3. Two per cent agarose gel depicting PCR amplification of mitochondrial DNA extracted from the variables quantities of *C. zealandica* frass (n = 0.5–12) together with negative and positives controls. All the frass were between 3 and 4 days old. Three microlitres of each PCR product were loaded on the gel.

Table 1 Number of positive DNA amplifications (n = 5 for each treatment) for different frass quantities after 1–2, 3–4 and 7–8 days of storage at room temperature

Number of frass	1–2 days old	3–4 days old	7–8 days old
12	5	5	5
8	5	5	5
4	5	5	5
2	5	5	5
1	5	5	1
0.5	4	5	0

plex PCR analysis, utilizing a species-specific modification of the generic DNA barcode region (Hebert *et al.* 2003), was highly successful in differentiating *C. zealandica* and *C. brunneum* larvae. Unlike DNA barcoding, which requires additional post-PCR sequencing for each individual, the assay was well suited to the effective screening of large numbers of samples. Incorporating three primers in a single multiplex reaction instead of two separate species-specific reactions also made the procedure much simpler and more efficient in both design and deployment. Secondly, noninvasive DNA sampling approaches were successfully applied enabling accurate species differentiation of live specimens.

Because scarabs have complex and long life cycles (Ritcher 1957), their culturing under laboratory conditions can be very challenging (Dittrich-Schröder *et al.* 2009) and is often disregarded in favour of extensive field collections of larvae. This requires prior nonharmful and nondisruptive methods of identification to enable subsequent analyses of ecological, behavioural and fitness data, without undermining the validity of the results obtained. In such context, combining the above rapid diagnostic PCR method with our noninvasive approach to DNA sampling is key.

Noninvasive DNA sampling methods for insects are rare and often limited to specific circumstances. For example, the whole specimen soaking in extraction buffer, specifically developed for the conservation of precious museum-type specimens (Gilbert *et al.* 2007), is not compatible with live specimens. Another method proposed by Katoh *et al.* (2008) involving using the nucleotide-acid binding property of silica particles to retrieve DNA from the reflex bleeding of ladybeetles and leaf beetle also cannot be applied to scarab larvae.

Although excrements are increasingly used as source of DNA in molecular and ecological studies (Zhang *et al.* 2006), such approach has rarely been applied in genetic studies that focus on arthropods. Reasons for that include (i) the presence of only small quantities of DNA (Piggott & Taylor 2003; Zhang *et al.* 2006), compounded by the use of small specimens, (ii) the unwanted cross-amplification of DNA (Fumanal *et al.* 2005) from endogenous and exogenous sources and (iii) the possible presence of highly interfering substances such as PCR inhibitors (Kohn *et al.* 1995; Monteiro *et al.* 1997).

Our results showed that, in the case of scarab species, as little as half a frass pellet can be sufficient to successfully amplify the target DNA. In addition, DNA amplification was successful even up to 4 days following the excretion and in the majority of cases still after up to 8 days, although a higher quantity of frass (>1 pellet) was required. This latter discovery is extremely valuable, as timeframes in ecological studies rarely allow for daily monitoring. Undoubtedly, the time-bound limit over which such sources of DNA can be used will be dependent on the ambient temperature and moisture conditions that determine the rate of degradation of the DNA (Murphy *et al.* 2007). However, this can easily be countered by controlled and monitored laboratory conditions and experimental design where relatively cold and dry conditions are known to be more favourable for the preservation of DNA samples (Piggott & Taylor 2003). Moisture was probably a very important parameter for the quality of the retrieved faecal DNA and the success of its amplification in the present study, as scarab larval frass appeared as being consistently dry (personal observation).

An alternative method for noninvasive sampling of immature life stages is the use of exuviae shed after each instar moult. Insect exuviae are essentially composed of chitin and chitosan (Zhang *et al.* 2000) with no nucleated cells that contain DNA. Consequently, nonmolecular methods, such as the cuticular hydrocarbon composition of exuviae, have been used to identify insect larvae (e.g. Ye *et al.* 2007). However, some living cells are often shed during the moulting process (Bertholf 1925) such that the low quantities of DNA can then be amplified by PCR. This constitutes an easier and cheaper alternative to cuticular hydrocarbon analyses. Several studies have successfully use exuviae as source of DNA. For example, Gregory & Rinderer (2004) obtained similar PCR results from DNA extracted from the exuviae of honey bee (Hymenoptera) larvae as from the tarsi, showing that the content of DNA in bee exuviae was enough to perform successful DNA amplification. The same year, Feinstein (2004) confirmed the utility of caterpillar (Lepidoptera) exuviae as potential source of DNA. The current study now expands these findings to coleopteran larvae. Conversely, an example of unsuccessful use of shed exuviae as source of DNA was reported by Monroe *et al.* (2010) from dragonfly larvae. However, these were collected directly from water, which was likely to have resulted in degradation of any residual DNA. Therefore, the environmental conditions under which the exuviae are recovered are of prime importance to ensure high-quality DNA for molecular analyses. This was not an issue with the species model of the present study. However, in scarabs, one of the main factors potentially restricting the use of this method is that some larvae eat their own exuviae after shedding (personal observation). Therefore, recovering the exuviae before they have been ingested is likely to require considerable vigilance.

Recommendations

The methods developed and described here significantly improve the feasibility of using accurately identified scarab larvae in ecological and/or behavioural studies. The approach is very generic and, therefore, should be

readily transferable to other holometabolous insect species where morphological identification of larval stages is difficult. Requirements for successful application of the methods are:

1 Minimizing the number of species-specific primers in a multiplex PCR is likely to be more robust than compromising on optimal PCR conditions when a large number of primers are involved.
2 Primers that target short fragments of DNA are more likely to amplify using degraded DNA (Idaghdour *et al*. 2003), such as that might be expected from exuviae and frass.
3 Where possible, frass should be used rather than exuviae because it is produced daily and not eaten by the larvae.
4 In this case, a minimum of half a frass pellet (i.e. ~0.1 mg) is required for analysis after 3–4 days after excretion, or alternatively a minimum of two frass pellets (i.e. ~0.4 mg) for analysis after 7–8 days. These parameters need to be considered on an individual study basis with respect to the species tested, ambient humidity and temperature conditions.

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Data Accessibility

GenBank Accessions JN793483-JN793510 (refer to Table S1, Supporting information).

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 GenBank Accession nos for all unique DNA sequences of adults and larvae of *C. zealandica* and *C. brunneum*.

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