GENETIC VARIATION IN GRASS GRUB, *COSTELYTRA ZEALANDICA*, FROM SEVERAL REGIONS

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ABSTRACT

Grass grub, *Costelytra zealandica*, is a species endemic to New Zealand and the only one of eleven described *Costelytra* species which has become a significant pasture pest. Specimens were collected from throughout New Zealand and the DNA subjected to the polymerase chain reaction using primers specific to a highly conserved region of ribosomal DNA. A surprising degree of genetic variation was revealed when the amplified products were sequenced and compared. Grass grub populations could be split into two geographical groups, North Island and South Island, based on the similarity of ribosomal DNA.

Keywords: grass grub, *Costelytra zealandica*, polymerase chain reaction, genetic variation, ribosomal DNA

INTRODUCTION

Grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae: Melolonthinae), is one of the worst pasture pests in New Zealand, causing up to \$89M in lost production per year (Garnham and Barlow 1993). It is one of the few native insects to become a major pest after intensification of agriculture in the last 150 years.

The genus *Costelytra* was erected by Given (1952) and originally contained six species which were classified on the morphology of the male genital claspers. A further five species were added to the genus in subsequent reviews by Given (1960; 1966). Only two species, *C. zealandica* and *C. brunneum* (Broun), have been found in both the North and South Islands (Given 1966). *C. zealandica*, the only pest species, has a wide distribution throughout New Zealand, including the Chatham Islands, and is found existing from sea level to over 4000 ft, through most soil types and most plant communities (Given 1966). Despite the wide geographical spread and variations in climate, it is recorded as one species, with no obvious morphological differences observed in any regional populations (Given 1952). However, there have been anecdotal comments that 3rd instar larvae from Taranaki are, on average, larger than from other regions (Townsend pers. comm.).

Many molecular biology techniques have proved useful in the study of intraspecific variation. Previously, the study of conserved ribosomal DNA (rDNA) has contributed to understanding the variation in a number of systems including insects (eg. Pfeifer *et al.* 1995) and fungi (eg. Curran *et al.* 1994). These studies used polymerase chain reaction (PCR) amplification of the variable internal transcribed spacer regions (ITS1 and/or 2) and the more conserved intervening 5.8S of the rDNA to compare strains and species.

In this study we compared sequences of rDNA from the 16S/18S to the 5.8S regions encompassing all of the ITS1 region to determine the level of intraspecific variation in a small sample of *C. zealandica*.

MATERIALS AND METHODS

Collection of Specimens

Grass grub larvae were field-collected from around New Zealand (Table 1). Other members of the subfamily Melolonthinae (*C. brunneum*, *Chlorochiton suturalis*, *Pyronota* sp. [*festiva*?] and *Odontria* sp.) and the sand scarab, *Pericoptus truncatus* Fabricius (Dynastinae), were included as outliers for the genetic analyses.

TABLE 1: Scarab larvae used for DNA extraction and analysis.

Proc. 50th N.Z. Plant Protection Conf. 1997: 338-343

Scarab	Code	Region	Site/Farm ¹	Year collected	State
<i>Costelytra zealandica</i>					
	Otago	Otago	McRaes Flat	1990	frozen larva
	Cant1	Canterbury	Winchmore	1996	frozen larva
	Cant2	Canterbury	Murdoch	1996	frozen larva
	Chtls	Chatham Is.	Holmes	1995	frozen larva
	Hawke	Hawkes Bay	Richie	1988	frozen larva
	Waiko	Waikato	Wilson	1994	frozen larva
	Tmac	Taranaki	Mace	1994	frozen larva
	Tcoc	Taranaki	Cockburn	1993	frozen larva
	Tmul	Taranaki	Mueli	1993	frozen larva
<i>C. brunneum</i>					
	Cbrun	Canterbury	Cass	1994	frozen larva
<i>Odontria</i> sp.					
	Odont	Rangitaiki	Kotara Station	1997	fresh larva
<i>Pyronota</i> sp. (<i>festiva</i> ?)					
	Pyno	Nelson	Taihape Rd	1997	frozen larva
<i>Chlorochiton suturalis</i>					
	Chlor	Nelson	Kaiteriteri	1997	fresh adult
<i>Pericoptus truncatus</i>					
	Peric	Nelson	Kaiteriteri	1996	fresh larva

¹ one larva from each site was prepared for sequencing

DNA extraction

DNA was extracted using a modification of the method described by Henry *et al.* (1990). For each specimen, the head capsule was ground in liquid nitrogen in a microcentrifuge tube using a blunted pipette tip. One ml of TENT buffer (10 mM Tris-Cl (pH 7.4), 25 mM EDTA, 10 mM NaCl, 0.5% Triton X-100) was added, the sample mixed and centrifuged at 13000 rpm for 5 min. The resulting pellet was resuspended in 600 µl of TEN buffer (TENT without Triton X-100) containing 1 mg/ml of proteinase K (Gibco BRL). SDS (sodium dodecyl sulphate) was added to give a final concentration of 1% and the sample incubated for 4 h at 37°C. Following the addition of 60 µl of 5 M NaCl, the DNA was phenol/chloroform extracted twice, precipitated with isopropanol and the pellet resuspended in 50 µl of TE buffer. Residual RNA was removed by incubation with DNase-free RNase A (50 µg/ml) for 1 h at 37°C.

PCR amplification of ITS1

To amplify the ITS1 region of rDNA, a combination of previously designed primers were used (Fig. 1). The primers TW81 (5'...GTTTCCGTAGGTGAACCTGC...3') and AB28 (5'...ATATGCTTAAGTTCAGCGGGT...3') were originally designed to amplify the rDNA from the 16S/18S through to the 28S region in fungi (Curran *et al.* 1994). When used with grass grub, these primers produced a band of approximately 1200 bp, which was too large to sequence easily. A third primer, based in the 5.8S region, was used to target the ITS1 region of rDNA. This primer, designated HITR (5'...AATGTGCGTTCGAATGTGCG...3'), was a shortened version of the reversed complementary strand of the HITS1 primer originally designed to amplify ITS2 in gypsy moth (Pfeifer *et al.* 1995). PCR of grass grubs using HITR in conjunction with TW81, yielded a product of approximately 780 bp which included ITS1.

Polymerase chain reaction (PCR) was performed using 0.65 units of *Taq* DNA Polymerase (Boehringer Mannheim) in a Perkin Elmer or Ericomp cyclor. Reactions were carried out in 25 µl volumes including 2.5 µl buffer (Boehringer Mannheim), 0.5 µl dNTPs (10 mM) and 1.0 µl of each primer (10 µM). Typically, 2 µl of a 1/10 dilution of DNA extraction was used for amplification. The cycle profile was as follows: 94°C (1 min), 55°C (1 min), 72°C (2 min) for 30 cycles. A negative, DNA-free control,

FIGURE 1: Location and amplification direction of rDNA primers.

was included in each run. All products were visualised on 1.5% agarose gels alongside a 100 bp ladder (Gibco BRL).

Restriction digests

*Hae*III (GG/CC) restriction endonuclease was used to identify differences in the amplified rDNA. Digests were typically performed in 10 µl volumes using 1 unit of enzyme (Gibco BRL), 1 µl buffer and 5 µl of PCR product. Incubation was performed at 37°C overnight and digests were visualised on 2% agarose gels alongside a 100 bp ladder (Gibco BRL).

Sequencing of PCR products

PCR products for sequencing were amplified using 2.6 units of Expand High Fidelity PCR System (Boehringer Mannheim) in a Perkin Elmer cyclor. Reactions were carried out in 100 µl volumes including 10 µl buffer (Boehringer Mannheim), 2 µl dNTPs (10 mM) and 4 µl of each primer (TW81 and HITR at 10 µM). Prior to sequencing the amplified product was purified using Wizard PCR DNA Purification System (Promega). Sequencing was performed by the Centre for Gene Research, Otago University.

Analysis of sequence data

Sequence data from rDNA of *C. zealandica* and the specimens used as outliers were compared using a Genstat (1993) programme first developed for rDNA by Arnold *et al.* (1994) and adapted for our use by David Baird (AgResearch, Lincoln). The programme constructs a similarity matrix using the number of base pairs in common between the two sequences as a proportion of the sequence length to determine a measure of distance. One output is a dendrogram produced using a cluster analysis which indicates a percentage of genetic similarity.

RESULTS

Amplification of all grass grub samples with TW81-HITR resulted in a band of approximately 780 bp. The size of this product in the other scarabs varied: *C. brunneum* 760 bp; *Odontria* sp. 750 bp; *Pyronota* sp. 750 bp; *Ch. suturalis* 720 bp; *P. truncatus* 975 bp.

Originally, grass grub ITS1 products were analysed using *Hae*III and other restriction enzymes. Two profiles were produced with *Hae*III: 30, 110, 280, 360 bp bands or 140, 280, 360 bp. When these profiles were related back to the geographic location of the specimen, they correlated with a North Island/South Island split, respectively. The Chatham Islands larva was grouped with the South Island samples. Larvae from different farms in the same region (Canterbury x 3; Hawkes Bay x 3; Taranaki x 3; Waikato x 3; Chatham Islands x 2) produced identical *Hae*III results.

The DNA from specimens listed in Table 1 was further analysed by sequencing the whole TW81-HITR fragment. A cluster analysis of a 650 bp segment of the amplified ITS1 sequence (Fig. 2) produced a similar result to the *Hae*III digest. All South Island and Chatham Island larvae formed a group, with sequences over 99% similar (Table 2). This represented approximately 5 bp difference in the 650 bp sequence. Another group, containing larvae from Hawkes Bay, Taranaki and Waikato, showed sequence homologies of 99.7% or greater, except for Tmul (Table 2). Tmul, a single larva from Taranaki

FIGURE 2: Dendrogram generated by cluster analysis showing relationships between larvae based on the ITS1 sequences (numbers represent % genetic similarity of branch).

showed only 98.7% sequence homology to other larvae in the North Island group. Cluster analysis of the sequence homologies between the two groups (Fig. 2) indicates the level of separation.

C. brunneum, *Odontria* sp. and *Pyronota* sp. were clearly separate from *C. zealandica* samples (Table 2; Fig. 2). Although in Fig. 2, *Pyronota* sp. appears more closely related to *Costelytra* than *Odontria*, this is likely to be a product of difficulties in aligning the very divergent sequences of the three genera, rather than a phylogenetic reality. *Chlorochiton suturalis* and *P. truncatus* were found to have so little homology with sequences of the *C. zealandica* ITS1 that they could not be aligned sufficiently for Genstat analysis.

TABLE 2: Similarity matrix (percentage) of homologies between the ITS1 sequences of Scarabaeidae larvae.

Otago	100												
Cant1	99.9	100											
Cant2	99.3	99.2	100										
ChtIs	99.3	99.2	100	100									
Hawke	98.9	98.7	98.7	98.7	100								
Waiko	99.0	98.9	98.6	98.6	99.9	100							
Tmac	99.0	98.9	98.6	98.6	99.9	100	100						
Tcoc	98.9	98.7	98.5	98.5	99.7	99.9	99.9	100					
Tmul	98.3	98.2	97.9	97.9	98.7	98.9	98.9	98.7	100				
Cbrun	80.2	80.0	79.9	79.9	80.1	80.1	80.1	79.9	79.9	100			
Odon	56.1	56.0	55.8	55.8	56.2	56.3	56.3	56.5	56.5	55.1	100		
Pryno	64.8	64.7	64.5	64.5	64.1	64.2	64.2	64.1	64.2	64.3	56.4	100	
	Otago	Cant1	Cant2	ChtIs	Hawke	Waiko	Tmac	Tcoc	Tmul	Cbrun	Pryno	Odon	

DISCUSSION

Morphological comparisons of *C. zealandica* from throughout the country have

suggested that little variation exists from one region to another (Given 1966). However, in this small study using one segment of rDNA, consistent variation was found between larvae from north and south of Cook Strait. Whether this separation is consistent for all sites cannot be determined from this limited study, but it does raise the possibility of the existence of distinct subspecies. It was interesting to note that the larva from the Chatham Islands was similar to those of Canterbury and Otago, suggesting that Chatham Island grass grubs originated from the South Island.

Our results suggest that *HaeIII* digestion of ITS1 amplification products may be a quick method for identifying Melolonthinae specimens at both the genus and species level. However, genetic comparison studies between species within *Costelytra* will be hindered by a lack of specimens. For example, *Costelytra diurna* was first reported in droves of thousands around Timaru in 1955 but has only been located once since then (Given 1966).

Within *C. zealandica* there is evidence of further variation than demonstrated by analysis of conserved rDNA. We have initiated a study using random amplified polymorphic DNA (RAPD) techniques and preliminary results suggest that within the north-south groups, further variation can be detected (Richards and Glare unpubl. data). This appears especially apparent within the Taranaki region where two very distinct patterns have been obtained. Further elucidation of the relationships within *C. zealandica* may not be simple. Species are defined as those individuals which can successfully mate; however, due to the difficulty of mating grass grub in the laboratory, the extent of interbreeding between the geographic groupings may not be easy to investigate.

At present, the level of variation detected within *C. zealandica* is unlikely to be sufficient to define a new species, especially when compared to *C. brunneum*. Yet clarification of sub group distribution within *C. zealandica* may have implications for both the ancestry of grass grub species and control strategies. While a preliminary study by Jackson *et al.* (1989) reported no geographical variation in susceptibility of grass grub larvae to the biological control agent, *Serratia entomophila*, our results call for a closer investigation into this issue. It is also unknown if the genetic variation detected is associated with any ecological or behavioural differences.

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