TUSSOCK MOTH SPECIES ARRIVING ON IMPORTED USED VEHICLES DETERMINED BY DNA ANALYSIS

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ABSTRACT

Egg masses of tussock moths are frequently intercepted at the border, most commonly on imported used vehicles. These have been assumed to be of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). However, there are six other Lymantriid pest species with similar indiscriminate oviposition and overwintering behaviour that are considered to have the potential to reach New Zealand. Unfortunately there is no accurate record of what arrives, as early immature life stages of tussock moths cannot be reliably identified morphologically to the species level. A molecular diagnostic system was therefore adopted for the identification of all interceptions. During the period 2000–2002, 151 specimens were intercepted on used vehicles from Japan and one on a vehicle from the USA. Of these 82% were identified as gypsy moth, 2% were other high-risk species (nun moth, *L. monacha*, and white spotted tussock moth, *Orgyia thyellina*), 6% were unknown species and 10% had no detectable DNA. This information is interpreted with respect to the quarantine systems in place and the practical role of molecular tools for biosecurity.

Keywords: biosecurity, quarantine, Lymantriidae, gypsy moth, PCR-RFLP.

INTRODUCTION

Around 30 species of tussock moths (Lepidoptera: Lymantriidae) are listed as unwanted organisms under the Biosecurity Act (MAF Biosecurity, Unwanted Organisms Register). The majority of species, such as the browntail moth (*Euproctis chrysorrhoea*), are closely associated with their host plants throughout the life cycle and so have a limited ability to reach New Zealand. However, seven northern hemisphere species have been highlighted as a high risk to New Zealand forestry based on their pest status and polyphagous nature. These are Asian and European gypsy moth (*Lymantria dispar*), nun moth (*L. monacha*), pink or rose gypsy moth (*L. mathura*), vapourer or rusty tussock moth (*Orgyia antiqua*), white marked tussock moth (*O. leucostigma*), Douglas fir tussock moth (*O. pseudotsugata*) and white spotted tussock moth (*O. thyellina*) (Armstrong 2000). Specific life history strategies, especially a long overwintering phase in the egg stage, could enable them to arrive here and remain alive after crossing the equator. In addition, indiscriminate oviposition observed on vertical inanimate surfaces, such as containers and ship superstructures, could aid their arrival, especially during population outbreaks (P. Walsh, pers. comm.). Of the gypsy moths, the Asian form is particularly well equipped to invade as the females are capable of sustained flight (Keena et al. 2001) and are attracted to lights (Wallner et al. 1995). This can result in egg deposition on forest equipment, cars and trucks, which facilitate their spread. Gypsy moth also has variable occlusion cues enabling hatching to coincide with favourable environmental conditions. Establishment of a population may therefore be possible if mating can occur between

individuals of unrelated egg masses that arrive at different times (D. Bejakovich, pers. comm.). In practice, although gypsy moth has been found capable of crossing the equator and arriving in New Zealand as a viable organism (Walsh 1993), it has not successfully established. This may be due to unsynchronised physiological cues in the southern hemisphere associated with a single generation per year and diapause (E.R. Frampton, pers. comm.). In comparison, white spotted tussock moth has up to three generations per year and has been capable of establishing here (Hosking 1998).

Egg masses are more commonly intercepted on imported used vehicles than containers (Gadgil 2000) or ships. These have previously been assumed to be the Asian gypsy moth, due to its proven invasive ability (Savotikov 1995). Unfortunately, early life stages of tussock moths cannot be identified morphologically to the species level and so there has been no accurate record of which species actually arrives here. This has implications for the suitability of the post-border quarantine systems that are in place. Consequently a molecular diagnostic method was developed to enable any life stage of the nominated high risk species to be identified (Armstrong 2000). This paper reports on the species identified using that method for all tussock moth interceptions associated with used vehicles imported to New Zealand over a 21 month period.

METHODS

Specimens

Adults of the seven high risk species were obtained as dried specimens from suppliers in North America, Europe and Asia (details listed in Armstrong 2000) and used as positive controls. Over the period September 2000 to June 2002, interceptions at the border, believed by quarantine officers to be "AGM", "gypsy moth", "tussock moth" or "Lymantriid", were placed in 95% ethanol and sent to Lincoln University for identification. These were all associated with on-arrival inspections of used vehicles discharged in the main ports at Auckland, Wellington, Lyttelton, Timaru and Dunedin.

DNA extraction

A proteinase K and modified Prep-A-Gene® (Bio-Rad, USA) method was used to extract DNA from either 1-3 legs of each adult or 10 eggs/egg mass. DNA was resuspended in 100 µl of TE and used directly for PCR. Details are provided in an operational manual available from MAF Policy (Armstrong & McHugh 2000).

PCR-RFLP

PCR amplification of the partial 18S plus complete first internal transcribed spacer region of nuclear ribosomal DNA was carried out using a specific 5' primer, designed from 18S sequence data, together with a universal 3' primer in the 5.8S gene (ITS6) (Armstrong & McHugh 2000). Component and thermal amplification details are given in Armstrong & McHugh (2000) and are based on the manufacturer's protocol for Expand High Fidelity polymerase (Roche Diagnostics, Germany). DNA negative and positive controls were included in each PCR run. An aliquot of each PCR product was checked for presence and quantity by agarose gel (1% TBE) electrophoresis and ethidium bromide fluorescence over UV. Further aliquots were then used in four separate restriction digests with the enzymes *Acc I*, *Cfo I*, *Rsa I* and *Taq I*, incubated at 37°C for 1.5 h. The digestion products were resolved on a 2% agarose gel together with 100 bp molecular weight standards, electrophoresed using 130 V for 1.5 h and detected by ethidium bromide fluorescence over UV light. Patterns for the egg mass samples were compared directly with the positive controls and with the documented patterns for each enzyme and species (Armstrong & McHugh 2000).

RESULTS

The banding pattern after *Rsa I* digestion for each of the seven high risk species is shown in Figure 1a as an example of the restriction patterns used for diagnosis. In Figure 1b an unknown sample collected from the border is compared with three known species. The pattern of the unknown sample is consistent with that for gypsy moth; this diagnosis was confirmed by analysis with the other three restriction enzymes (results not shown).

FIGURE 1: *Rsa* I digests for (a) positively identified controls for all species and (b) an unknown specimen from the border (MAF) compared with three known species. Abbreviations used: gypsy moth (G), Asian (A), European (E), pink gypsy moth (PG), nun moth (N), vapourer moth (V), white marked tussock moth (Wm), Douglas fir tussock moth (Df), white spotted tussock moth (Ws) and 100 bp molecular weight marker (Mw).

Over the period September 2000 to June 2002, 152 suspect tussock moth interceptions were made at the ports of Auckland (n=125), Wellington (n=5), Nelson (n=11), Lyttelton (n=6), Timaru (n=3) and Dunedin (n=2). All but one were from used vehicles imported from Japan. The other was a pupal case found in a used car from the United States. A summary of the species identified from these samples using the molecular technique described above is given in Table 1. The data clearly indicate that the most commonly intercepted species is gypsy moth. On a few occasions other high-risk species (nun moth and white spotted tussock moth) were also intercepted. In addition, a number of specimens gave different restriction haplotypes to those established for the seven high-risk species. These were labelled as “unknown species” and included the specimen from the United States and two that gave the same haplotype as each other. Even specimens that appeared very dry and in poor condition could be identified. However, 10% of the samples failed to PCR-amplify. These appeared to be empty egg, larval or pupal cases that were unlikely to contain any nucleated cells.

TABLE 1: Identification of tussock moth species intercepted at the border.

Species identified	No. Intercepted					% of total
	Egg masses	Larvae	Pupae	Adults ¹	Total	
Gypsy moth	107	9	7	1	124	81
Pink gypsy moth	0	0	0	0	0	0
Nun moth	0	1	0	0	1	1
Vapourer tussock moth	0	0	0	0	0	0
White marked tussock moth	0	0	0	0	0	0
Douglas fir tussock moth	0	0	0	0	0	0
White spotted tussock moth	2	0	0	0	2	1
Other	0	0	0	1 ²	1	1
Unknown species ³	2	4	3	0	9	6
Unidentifiable ⁴	5	2	8	0	15	10
TOTAL	116	16	18	2	152	100

¹Moths identified morphologically and not by DNA analysis.
²*Cifina locuples confusa* Bremer (no common name); identification by P. Schaefer, USDA FS.
³Restriction profiles inconsistent with those for the seven high risk species.
⁴Specimens failed to PCR-amplify.

DISCUSSION

Tussock moths have become of increasing concern to New Zealand quarantine authorities since the first alerts over gypsy moth in the early 1990s (Walsh 1993). Since that time it has been assumed that gypsy moth was the species most commonly intercepted at the border. This study confirms that this assumption is true in the large majority of cases, at least for used vehicles imported from Japan over the past two years. Consequently, the results support the decision by MAF to use gypsy moth as an appropriate species to emphasise in terms of ensuring that effective post border surveillance and response systems are in place. Such systems require knowledge of species-specific pheromones as well as optimal trap types, sites and locations. Access to accurate interception data has contributed to refinement of the existing trapping system for gypsy moth, involving the replacement of a lure formulation capable of attracting both nun moth and gypsy moth with one more specific and sensitive for gypsy moth (Leonhardt et al. 1992).

The predominance of gypsy moth intercepted in this way is consistent with the greater likelihood of it encountering cars in Japan compared to the other species. Not only are they more abundant there and fly during the day as well as at night, but they are less restricted to forest hosts so are commonly found around human settlements (P. Schaefer, pers. comm.). However, the present study also indicates that other high-risk species are arriving on cars imported from Japan. This work demonstrates that a tool capable of identifying these species is now available. It also highlights the need to ensure lures for these other species are available, should they be needed in an incursion response. That there were no systems in place for species other than gypsy moth may in part be responsible for the unanticipated arrival of white spotted tussock moth in 1996 (Hosking 1998) and painted apple moth (*Teia anartoides*) from Australia in 1999 (Frampton 2000). A number of “unknown” species are also arriving on used vehicles. These warrant further attention in terms of their DNA identification, as the consequences of exotic species arriving without warning in ecosystems devoid of competition and natural control are well known.

This is the first time that a molecular method has been used as an operational quarantine tool to identify immature life stages of tussock moths to the species level. Diagnosis of an unknown sample is based on consistency with restriction patterns for known species. Other species, not characterised in this study, may have the same restriction profile, but that likelihood is reduced by inclusion of all those with characteristics enabling arrival by the same pathway, in this case used vehicles. Other methods previously published for identifying tussock moths have been concerned with sub-specific level discrimination of Asian and European gypsy moth races. The methods of Bogdanowicz et al. (1993) and Schreiber et al. (1997) are currently used as a diagnostic tool by the USDA. The method of Pfeifer et al. (1995) also included markers for the nun moth and pink gypsy moth, but it was considered to be not robust enough for quarantine use in New Zealand. This is because other species of interest were not characterised and the clarity of the diagnostic ITS2 PCR-RFLP patterns were compromised by secondary PCR products.

The method employed in the present study is now part of a common approach to identification of insect immature life stages intercepted at the New Zealand border. Other methods are available for fruit flies (Armstrong et al. 1997), mealy bugs (Armstrong 2001) and tortricids (Dugdale et al. 2002). Extending this approach for the identification of other insects threatening New Zealand's biosecurity, such as wood boring beetles within the Cerambycidae and Scolytidae, and mosquitoes within the Culicidae, would be valuable, empowering biosecurity authorities to be more anticipatory and focused. In the expectation that more tests will be required, technological advancements to DNA diagnostics for quarantine application are the focus of current collaborative research led by Landcare Research and the National Centre for Bio-Protection Technologies at Lincoln University.

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