MOLECULAR PHYLOGENETICS OF LEAFROLLERS: APPLICATION TO DNA DIAGNOSTICS

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ABSTRACT

Accurate and rapid diagnosis of taxa, whether they be well-defined species, or biotypes, is of crucial importance to quarantine, pest management and research. Recent developments in DNA technology has resulted in a range of molecular techniques being available for use in such applications. We have employed a phylogenetically focussed approach in the development of a diagnostic key to distinguish a range of leafroller species (Lepidoptera: Tortricidae) using DNA sequence data derived from a 2.3 kb region of the mitochondrial genome containing the genes cytochrome oxidase I and II. Phylogenetic analysis has revealed clear relationships between taxa, although the genus *Apoctena* does not appear monophyletic. The most appropriate diagnostic characters are either those which are phylogenetically informative, either synapomorphies or autapomorphies. A standardised approach to data collection is advocated for future studies.

Keywords: DNA sequencing, phylogenetics, diagnostics, molecular markers.

INTRODUCTION

Pressure on borders to prevent the introduction of exotic pest organisms has increased with globalisation. So too has competition between markets, making export market access a significant issue when the presence of certain organisms on produce is not accepted by importing countries (Export Phytosanitary Standards <http://www.maf.govt.nz/Plants/>).

Over the last decade the use of molecular-based approaches to identifying unwanted pests on both incoming and outgoing produce has increasingly been employed. Primarily this has been due to the often rapid nature of the methods, the ability to identify any life stage of the organism and the uniformity of techniques. There are many examples of DNA-diagnostics being used across a range of different insects, e.g. fruit flies (Armstrong *et al.* 1997), mosquitos (Porter and Collins 1991) and blackflies (Krüger *et al.* 2000).

The task of identifying which of the variable nucleotides in a given DNA-based dataset actually reflect the level of diagnosis required is extremely important. It is possible through these methods to identify molecular markers that differentiate individuals, populations, biotypes, species, genera, families, phyla, etc. (Avise 1994). Which DNA marker for which purpose can be difficult to ascertain, especially in the absence of genealogical analyses (phylogenetics) and a robust taxonomy based on currently valid systematic principles.

Our approach to providing a robust identification tool is to develop DNA diagnostic markers for leafroller moths, which are required for both market access and border control issues. New Zealand's leafroller moth fauna largely consists of many endemic species, which occur on a range of native plants and horticultural crops. Many species have proven difficult to identify using morphological characters, with several

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species only identifiable on the basis of pheromone blends produced by female moths (Foster *et al.* 1990). Our approach to developing diagnostic markers for these leafrollers has been to investigate phylogenetic relationships between species using DNA sequence data derived from the cytochrome oxidase I and II genes of mitochondrial DNA. A subset of our data is presented here to illustrate our approach to developing diagnostic markers.

MATERIALS AND METHODS

Specimens

Moths were either supplied by insect rearing facility at HortResearch, collected using pheromone traps or collected as larvae and reared through to adults. All field collected species were identified by J. Dugdale. Table 1 lists the 20 species used in this study, along with collection details.

DNA data

DNA was extracted from individual adult moths following the protocol outlined in Newcomb and Gleeson (1998). A 2.3 kb region was amplified by Polymerase Chain Reaction (PCR) from the mitochondrial genome, which included both the Cytochrome oxidase I and II genes and the tRNA lysine and tRNA aspartic acid. The amplification primers used were TY-J-1460 and TK-N-3785 (Table 2), as described in the appendix of Simon *et al.* (1994). Amplification reactions were carried out in 50 µl volumes and consisted of 10 pmol of each primer, 10 mM Tris-Cl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl and 0.2 mM of each dNTP with 2 units of *Taq* polymerase (PE Biosystems). Cycling conditions consisted of an initial denaturing step of 94°C for 10 min, followed by 35 cycles of 94°C for 1 min (denature), 50°C for 1 min (anneal) and 72°C for 2 min (extend). A final cycle included a 10 min extension at 72°C.

TABLE 1: Tortricid moth species used and collection details.

Species	Collection Locality
<i>Planotortrix avicenniae</i>	Pollen Island, Auckland 26/3/99
<i>Planotortrix excessana</i>	HortResearch Rearing
<i>Planotortrix octo</i>	HortResearch Rearing
<i>Planotortrix octoides</i>	Pitt Island, Chatham Islands
<i>Planotortrix notophaea</i>	HortResearch Rearing
<i>Planotortrix puffini</i>	Lee Bay, Stewart Island 7/12/99
<i>Ctenopseustis fraterna</i>	Titirangi, Auckland 20/12/98
<i>Ctenopseustis fraterna</i>	Taurewa, Central Plateau 6/12/98
<i>Ctenopseustis obliquana 1</i>	HortResearch Rearing
<i>Ctenopseustis obliquana 2</i>	Pollen Island, Auckland 12/2/99
<i>Ctenopseustis obliquana 3</i>	Hamilton 10/3/99
<i>Ctenopseustis herana</i>	HortResearch Rearing
<i>Ctenopseustis filicis</i>	Horseshoe Bay, Stewart Island 7/12/99
<i>Ctenopseustis servana</i>	Muriwai 14/9/99
<i>Leucotenes coprosmae</i>	Flora Saddle, Mt Arthur Range, Nelson 7/12/99
<i>Apoctena orthopis</i>	Taurewa, Central Plateau 6/12/98
<i>Apoctena conditana</i>	Wenderholm, 6/10/97
<i>Apoctena clarki</i>	Taurewa, Central Plateau 6/12/98
<i>Apoctena orthocopa</i>	Wenderholm, 12/9/98
<i>Apoctena spatiosa</i>	St. Arnaud 26/8/99
<i>Apoctena flavescens</i>	Tophouse, Buller 24/5/99
<i>Apoctena pictoriana</i>	St. Arnaud 13/11/99
<i>Epiphyas postvittana</i>	HortResearch Rearing

The resulting PCR products were purified using the Life Technologies Concert™ Rapid PCR Purification System, following the procedure outlined by the manufacturer. Direct sequencing of purified PCR products was achieved using TK-N-3785 and a

series of 12 sequencing primers, which are listed in Table 2. Both forward and reverse strands were sequenced. All sequences were derived using ABI fluorescent dye terminator chemistry on either an ABI 377 or an ABI 310 automated sequencer (ABI BigDye: Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) according to manufacturer's protocols.

Data analysis

Automated DNA sequence data was analysed using the software Sequencher™ 3.0 (Gene Codes Corporation, Inc.). Sequences were subsequently aligned using Clustal W 1.5 (Thompson *et al.* 1994). Pairwise DNA distance comparisons were calculated using the Kimura 2-parameter distance method (Kimura 1980). Phylogenetic relationships were inferred from the nucleotide sequences using both parsimony and distance based methods, which were both implemented within PAUP 4.0 (Swofford 1998). The PAUP settings used in the heuristic searches for the most parsimonious tree, included 100 random addition sequences with steepest descent and characters weighted according to transitions versus transversions of 2:1. Bootstrap analysis (Felsenstein 1985) was conducted using 10,000 bootstrap replicates implemented in PAUP.

The light brown apple moth, *Epiphyas postvittana*, was used as an outgroup for all phylogenetic analyses.

TABLE 2: DNA sequencing and amplification primers used for tortricid COI and COII genes and their relative 3' position and orientation (F= forward; R = reverse).

Primer Name	Sequence (5' -3')	Position
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	26F
Tort1	AAAGTGGGGGGTAAACTGTTC	368R
Tort2	AATCCTTATTTATTAATAAATTCAATT	1232F
Tort3	AATTGAATTTTAAATAAATAAGGATT	1207R
Tort4	GTTGCCATTTCTAAAAAAAGGAT	1586R
Tort5	CCTTTTTTTAGAAATGGCAAC	1608F
Tort6	CAATGATAYTGAAGTTATGAATA	1929F
Tort7	CAAATTAATTCAATTATTTGTCCTTC	1769R
mt6	GGAGGATTTGGAAATTGATTAGTTCCCA	242F
mt8	ACATTTATTTTGATTTTTTGG	707F
mt9	CCCGGTAAAATTAATAAATAAACTTC	715R
mt11	ACTGTAAATATATGATGAGCTCA	853R
mt15	TCATAAGTTCARTATCATTG	1907R
TK-N-3785	GTTTAAGAGACCAGTACTTG	2325R

RESULTS

A total of 2283 bp were able to be reliably aligned across all taxa used in this study, of which 1722 bp were not variable. Of the 561 variable sites, 393 were parsimony informative with 168 parsimony uninformative, in that these were restricted to single taxa. Parsimony searches resulted in one tree of length = 1681, consistency index = 0.560, retention index = 0.544, which is shown in Fig. 1. The exact tree topology was also achieved using DNA distance data and neighbour-joining.

The phylogeny shows clearly that all species within the genera *Planotortrix* and *Ctenopseustis* each cluster into strongly supported monophyletic groupings, with bootstrap values of 96% and 100% respectively, which is consistent with their taxonomic status. However, members of the genus *Apoctena* do not cluster into a single group. Four species, *A. conditana*, which is regarded as the type of the genus (Dugdale 1990), *A. orthocopa*, *A. spatiosa* and *A. clarki*, clearly cluster together in a monophyletic group with high bootstrap support (100%); these form *Apoctena sensu stricto*, and can be defined on male and female morphological synapomorphies. Of the

FIGURE 1: Phylogenetic tree of the relationships of leafroller moths based on entire cytochrome oxidase I and II nucleotide sequences. Numbers of nucleotides for each branch are given above the branches while bootstrap values are shown below.

remaining three members, *A. pictoriana* and *A. flavescens* do not cluster together, nor other surveyed taxa, have little support for their phylogenetic placing within the tree, and within this context they appear to be terminal taxa. Morphologically, "*A. pictoriana*" and "*A. orthropis*" share several morphological characters, including the *Ctenopseustis*-group longitudinally cleft and inrolled cestum. "*A. orthropis*" on the other hand has significant support for its position with *Leucotenes coprosmae* (bootstrap=99%). The position of "*A. flavescens*" on the tree, isolated from true *Apoctena*, and "basal" to the *Ctenopseustis* + *Planotortrix* grouping may be reinforced by cestum retaining clear vestiges of the *Ctenopseustis*-group cleft and in-rolling. The other species included in *Apoctena* s.s. need further morphological examination to determine whether cestum structure in the above groups represent a clade or a grade.

In the identification of diagnostic markers, those nucleotides which resolve genera and then subsequently species, are those which can be further developed. These data have revealed 36 nucleotides which are unique to members of the genus *Planotortrix* and 64 unique to *Ctenopseustis*. There are 65 nucleotides which are unique to the well-supported *Apoctena* s.s. cluster. Within these genera, numbers of nucleotide substitutions unique to individual species vary. For example within *Planotortrix*, 50 are unique to *P. notophaea* while only seven nucleotides clearly separate the *P. avicenniae* and *P. excessana* cluster from *P. octo*. Whereas among populations of *C. obliquana*, up to six nucleotides are unique to individuals with populations of *C. fraterna* differing by as much as 17 nucleotide positions.

DISCUSSION

Phylogenetic analysis, using cytochrome oxidase I and II mitochondrial genes has provided well resolved lineages of *Ctenopseustis* and *Planotortrix*. However, we have also revealed parafly within the genus *Apoctena*. This has subsequently enabled the identification of appropriate diagnostic markers for each taxonomic level within this group of leafrollers. These data have shown that the identification of the variable nucleotides most appropriate for diagnostic marker development can be overcome by placing the DNA sequences generated into the context of a phylogenetic tree. Through this approach, we are easily able to identify which nucleotides actually reflect evolutionary change. In particular, we can distinguish synapomorphic characters (shared derived character states) and autapomorphic states (unique derived character states) from homoplasies, which do not reflect shared ancestry.

Most major groups of insects have now been included in various molecular systematic studies. A standardised approach to data collection has recently been discussed by Caterino *et al.* (2000). They advocate the use of the mitochondrial genes cytochrome oxidase I and 16S, along with the nuclear gene regions of 18S and elongation factor-1 alpha, which have been shown to resolve relationships across a range of taxonomic levels. We would also advocate their use in insect DNA diagnostics. Since these gene regions can resolve phylogenetic relationships, it then follows that those nucleotides which are synapomorphies and autapomorphies will also be the best diagnostic markers. In cases of closely related taxa, and those still in the process of speciating, the best markers will be ones which are actually involved in the speciation process itself. We are further investigating the utility of the genes responsible for mate recognition within these genera of moths, in particular genes involved in pheromone production and reception.

Once diagnostic markers are identified, there are a range of platforms in which they can be further developed. As opposed to previous restriction fragment length polymorphisms (RFLPs), single nucleotide polymorphisms (SNPs) are becoming much more amenable to diagnostic situations (Chen *et al.* 2000). Previously, RFLPs required identification of appropriate restriction sites, which are not often available as well as separation of DNA fragments alongside appropriate controls. Several reactions are required in order to identify one sample and error is possible, i.e. the same RFLP pattern can be revealed even though the sequences are different. The new SNPs can also take advantage of fluorescent dye chemistry and be processed easily within one day.

Although molecular biology has provided many tools for identification of a range of different organisms, determining the right diagnostic markers remains the most important factor. Our development of markers for tortricid moths has illustrated the utility of a phylogenetic approach. It is our aim to provide a diagnostic key which incorporates both morphological and molecular data in a framework consistent with systematics and taxonomy.

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