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DNA barcoding: an innovative tool to identify internal lepidopterans in apples

Annabelle Firlej[1], Jean-Philippe Légaré[2], Jean-François Landry[3], Richard Hogue[1], Gérald Chouinard[1], Daniel Cormier[1]
[1]Institut de recherche et de développement en agro-environnement, 335 chemin des 25 Est, Saint-Bruno-de-Montarville (Québec) J3V 0G7, Canada; [2]Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, 2700 rue Einstein, Québec (Québec) G1P 3W8, Canada; [3]Agriculture et Agroalimentaire Canada, 960 avenue Carling, Ottawa (Ontario) K1A 0C6, Canada

Abstract: The identification of Lepidoptera larvae can be problematic when different species occur at the same time in crops and when their identification requires dissection or laboratory rearing. In Quebec, this is the case for three Tortricidae that feed synchronously in apples: the codling moth [*Cydia pomonella* (L.)], the lesser appleworm [*Grapholita prunivora* (Walsh)] and the oriental fruit moth [*Grapholita molesta* (Busck)]. Codling moth larvae can usually be distinguished from the two other species by their larger size and the absence of anal comb, but larvae of the lesser appleworm and oriental fruit moth are nearly indistinguishable.

The objective of our study is to validate DNA barcoding as a method for identifying major lepidopteran larval pest insects occurring in apple orchards in Quebec. This molecular method uses primers common to all Lepidoptera that target a 658 bp gene fragment coding for cytochrome oxydase I, which are specific to each lepidopteran species. 140 adult moths of the three species were collected in 2011 from six apple-producing regions in Quebec to establish a DNA sequence library that would be included in the "Barcode of Life Data Systems". Lepidoptera larvae were sampled in the 2012 summer season in orchards of Quebec to assess the effectiveness of the molecular method for larval identification. When validated, this method will become a useful identification tool for laboratories specialized in plant pest identification.

Key words: barcoding, Lepidoptera, taxonomic tool

Introduction

Identification of internal lepidopteran pests of apple can be problematic due to their similar morphological characteristics. In Quebec, three species of Tortricidae can occur simultaneously in orchards during the fruit-growing season: the codling moth [*Cydia pomonella* (L.)], the lesser appleworm [*Grapholita prunivora* (Walsh)], and the oriental fruit moth [*Grapholita molesta* (Busck)]. Larvae of *C. pomonella* can be distinguished from the other two species by their larger size and absence of anal comb, but the other two possess an anal comb and can have a similar number of crochets on the ventral and anal prolegs (Chapman & Lienk, 1971). We are adapting the method of DNA barcoding developed for animal biodiversity identification (Ratnasingham & Hebert, 2007) to identify larvae of internal apple lepidopteran pests. This method uses a 658 base-pair segment of the 5' end of the mitochondrial cytochrome c oxydase gene as a standardized genome region useful for species identification and delineation.

This project has two objectives: 1) to determine DNA barcode variation within adults of *C. pomonella*, *G. prunivora* and *G. molesta* encountered in different regions of Quebec; and

2) to validate DNA-based identifications of larvae from different apple orchards by comparing them with those obtained from adult moths .

Material and methods

Adult sampling
Adults of *C. pomonella*, *G. prunivora* and *G. molesta* were collected during the summer of 2011 in pheromone traps deployed in 14 apple orchards of Quebec, Canada. To determine variation in DNA barcode sequences, we targeted to obtain 10 specimens of the three species from six regions where these species are presents. Collected specimens were labelled, photographed, mounted and their geo-referenced data recorded. Morphological identification was performed by the diagnostic laboratory at the Quebec Department of Agriculture, Fisheries and Food, and confirmed by Lepidoptera taxonomists from the Canadian National Collection of Insects at Agriculture and Agri-Food Canada.

Larvae sampling
Apples were collected in 2012 in three organic apple orchards from the Monteregian region of Quebec, Canada. Larvae found in apples were extracted, labelled, categorized according to size (small, medium, or large) and kept in a 1.5 ml tube (Eppendorf) at -14 °C. Sampled orchards were known to have populations of at least *C. pomonella* and *G. prunivora*.

DNA barcode analysis
Following previously published protocols (Ivanova *et al*. 2006; de Waard *et al*. 2009), DNA from one or two legs of each adult specimen was extracted on mini-column in individual tubes or plate of 96-tubes and DNA was amplified by PCR with the primers COI LEP F1 (5'-ATTCAACCAATCATAAAGATATTGG-3'), COI LEP R1 (5'-TAAACTTCTGGATGT CCAAAAAATCA-3') and COI LEP R350 (5'-CTTATATTATTTATTCGTGGGAAAGC-3') which are universal for Lepidoptera species. After visualization of the PCR-products on agarose gel, they were purified and sequenced. Putative identification was obtained from the online BOLD (Barcode of Life Data Systems) identification systems taxon search (http:/lepbarcoding.org/organization.php). For larval specimens, molecular analysis was performed from samples of frozen tissues (2-4 mm^3 or 4-6 mm^3).

Results and discussion

Adult sampling
In 2011, 140 specimens representing all three species were collected from the sampled orchards. The first attempt at barcoding adults was unsuccessful because using one leg for the DNA extraction process with Proteinase K lysis buffer resulted in very low efficiency of DNA extraction. The protocol was modified and new attempts were made using two legs and/or abdomen of adults and by using pestles (manual grinding) with Guanidine (GuHCl) lysis buffer. This method showed more promising results and is still under development.

Larvae sampling
A total of 130 larvae were collected in three orchards from the Monteregian region. The first attempt at obtaining PCR-products with *C. pomonella* larvae lab-reared showed positive results when using manual grinding (pestles) with GuHCl lysis buffer. An assay with different

amounts of frozen larvae was also performed and 2-4 mm^3 of frozen tissue gave the best DNA extraction result. Even if it is more time-consuming, the utilization of mini-columns to extract larval DNA is better than using plates of 96-wells, because, in the later case the accumulation of tissue under the silica membrane disrupt washing and elution efficiency and resulted in low DNA recovery. A method of molecular identification for major lepidopteran larval pests in apple orchard will be available soon.

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