FINAL PROJECT REPORT

Project Title: Molecular characterization of taste, smell and feeding in codling moth

PI:	Stephen F. Garczynski	**Co-PI(2)**:	Laura Lavine
Organization:	USDA-ARS	**Organization**:	Washington State University
Telephone:	509-454-6572	**Telephone**:	(509) 335-7907
Email:	steve.garczynski@ars.usda.gov	**Email**:	Lavine@wsu.edu
Address:	230 Konnowac Pass Rd	**Address**:	Department of Entomology
Address 2:		**Address 2**:	P.O. Box 646382
City:	Wapato	**City**:	Pullman
State/Zip:	WA 98951	**State/Zip**:	WA 99164-6382

Cooperators: Drs. Tom Unruh, Pete Landolt and Kevin Wanner

Other funding Sources

Agency Name: USDA-CSREES NRI
Amount awarded: $189,804 for 4/2008 - 3/2010
Notes: This award was obtained using data generated with the funds provided by WTFRC. For that we are most grateful

Total Project Funding: $80,000

Budget History:

Item	Year 1: 2007	Year 2: 2008	Year 3:
Salaries			
Benefits			
Wages	6,240	6,490	
Benefits	150	156	
Equipment			
Supplies	28,110	27,854	
Travel	500	500	
Miscellaneous	5000	5000	
Total	40,000	40,000	

ORIGINAL OBJECTIVES

1) Construct cDNA libraries from codling moth sensory organs and neuroendocrine tissues.
2) Sequence cDNA libraries and perform searches to identify target receptors.
3) Clone target receptors into expression systems suitable for analysis in insect cell lines.
4) Initiate assays to identify receptors for pheromones and kairomones used for codling moth control.
5) Convert cell based assays for practical use in high-throughput screening programs.

Revised Objectives converting original objectives from a 3 year to 2 year time line as requested by Jim McFerson.

Year 1
1) Prepare cDNA from male and female codling moth antenna.
2) Generate antennal ESTs via pyrosequencing services offered by 454 Life Sciences Corp.
3) Generate cell lines expressing codling moth proteins involved in odorant signal transduction.
4) Clone full length cDNAs encoding odorant receptors identified from EST sequences

Year 2
1) Validate cell-based assays that will be used to identify codling moth pheromone receptors.
2) Clone potential codling moth pheromone receptors into cells used for assays.
3) Initiate assays to identify receptors for codling moth-active pheromones and kairomones.

SIGNIFICANT FINDINGS

1) Discovered conserved amino acid sequence and developed a procedure that could be used to identify and clone chemosensory receptors from the codling moth. This was the first time this had been accomplished for an insect without a sequenced genome. Furthermore the procedure is applicable to almost all insects of the Order Lepidoptera (moths and butterflies) as evidenced by our ability to identify and clone "pheromone" receptors from other insect pests of tree fruit including Obliquebanded leafroller, Light Brown Apple Moth, and Apple Clearwing Moth. Additionally, the value of the technique developed here has transcended tree fruit pests and been used to identify and clone "pheromone" receptors from lepidopteran pests of corn and cotton (European corn borer and the corn earworm/cotton bollworm).

2) Nine full-length odorant receptors have been cloned from codling moth, including five "pheromone" receptors, one ubiquitous receptor, and three general odorant receptors. These receptors have been cloned into cell expression vectors and are being prepared for use in cell-based assay screens.

3) One antennal specific and one nervous system specific G-Protein have been cloned and are being assayed for use as a potential target for new pesticide development. The antennal specific G-protein is a critical component for use in high-throughput assays to determine odorant receptor ligands.

4) cDNA has been prepared from male and female chemosensory organs (antennae, legs and mouthparts) and neonate and 5^{th} instar larvae and is being sequenced by Dr. Amit Dhingra at Washington State University. The results from the sequencing will allow us to identify other chemosensory receptors expressed in those codling moth life stages.

RESULTS

Because the genome sequence of the CM is not yet available, three different approaches were proposed to identify chemosensory receptors: A) PCR amplification of chemosensory receptors using degenerate primers designed from conserved regions of previously identified receptors in other insects from cDNA prepared from mRNA derived from CM chemosensory organs of newly emerged adults; B) direct pyrosequencing of cDNA prepared from male and female chemosensory organs; and C) screening libraries constructed from cDNA prepared as above with DNA probes generated from receptors cloned from other lepidopterous insects.

A) PCR amplification of chemosensory receptors using degenerate oligonucleotide primers.

The main focus of this part of the project has been to identify pheromone receptors expressed in antennae from CM males. The results presented in the original proposal remain unchanged and to date we have identified and cloned sequences encoding five full length members of the pheromone receptor family. Additionally, CM cDNA sequences encoding receptors corresponding to Ors 2, 10, 20, 21 and 35 from *Bombyx mori* and *Heliothis virescens* have also been identified and cloned. Or2, the ubiquitous receptor, will be used in developing the high-throughput cell-based assay in objectives 2 and 3.

A major benefit of the degenerate oligonucleotide approach to identify members of the pheromone receptor family from CM is its applicability to other lepidopteran pest species. The degenerate primers developed for this project were used in 3' RACE reactions to amplify cDNAs encoding members of the pheromone receptor subfamily from total RNA extracted from male antennae from *Cydia pomonella*, *Choristoneura rosaceana*, *Epiphyas postvittana*, *Helicoverpa zea*, *Manduca sexta*, *Ostrinia nubilalis*, and *Trichoplusia ni*. PCR products of ~ 200 - 700 bp were visualized on agarose gels stained with EtBr and bands were excised and TA cloned (data not shown). Twenty five transcripts encoding putative members of the pheromone receptor family were identified, five from *H. zea*, two from *M. sexta*, two from *C. rosaceana*, four from *E. postvittana*, five from *O. nubilalis*, two from *T. ni*, and five from *C. pomonella*. The 25 transcripts ranged in size from 238 - 642 bp (data not shown) and encoded 49 - 54 amino acids with high levels of similarity to the lepidopteran pheromone receptor subfamily (Figure 1). With the exception of *M. sexta*, the transcripts from the other species appear to be from individual genes based upon the uniqueness of the DNA sequences of their 3' untranslated regions (UTRs; data not shown). Further characterizations, including cloning full length transcripts and the genes encoding them, will be needed to determine the actual number of unique pheromone receptor genes.

The deduced amino acid sequences from the 25 transcripts cloned from seven different lepidopteran species were used along with the C-terminal regions of reported lepidopteran Ors to construct a phylogenetic tree. Significantly, all 25 sequences cloned using degenerate PCR primers grouped with other members of the pheromone receptor subfamily and not with other general Ors such as female-biased silkworm Ors19, 30, 45,46,47 and 48 (Figure 2). These results indicate that the degenerate primers are specific to the pheromone receptor subfamily and that the C-terminal region yields sufficient sequence information to assign the peptides to this subfamily. To determine the utility of the 3' RACE approach to identify Ors, degenerate PCR primers were designed against a region conserved between BmOrs 35, 37 & 38. An Or transcript was amplified from *Cydia pomonella* antennae, termed CpOr38, and the deduced amino acid sequence of CpOr38 groups with BmOrs 35, 37 & 38 and not with the pheromone receptor subfamily or other Ors (Figure 2), a further demonstration of the utility of this approach. However, these results will need to be verified using amino acid sequences of full-length receptors allowing for more detailed analyses of the phylogenetic relationships.

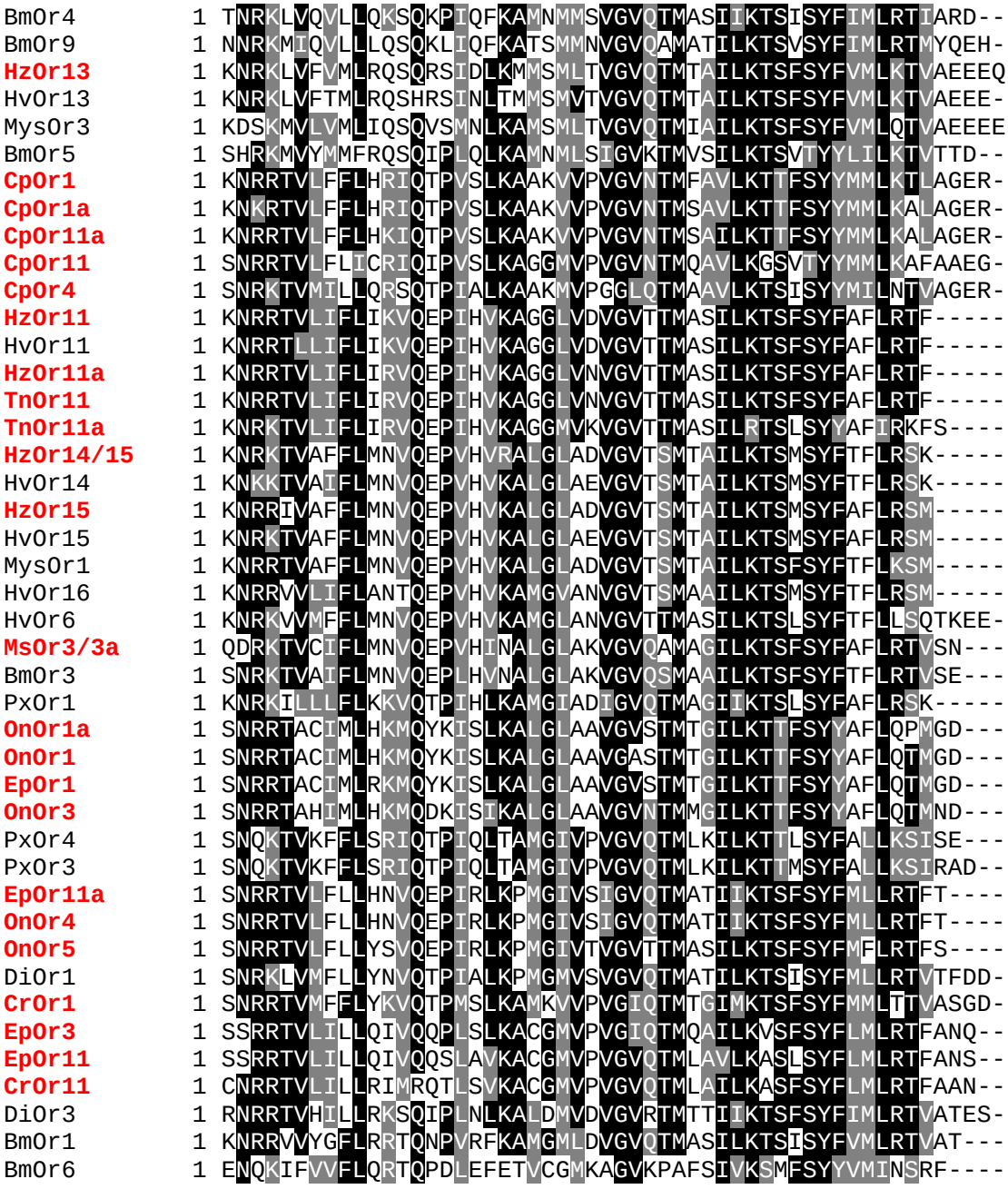

Figure 1. Boxshade of Clustal alignment of the carboxy terminal amino acids of 43 putative members of the lepidopteran pheromone receptor family of proteins. Black background indicates majority of the amino acids are identical; grey background majority conserved. Bm = *Bombyx mori*, Cp = *Cydia pomonella*, Cr = *Choristoneura rosaceana*, Di = *Diaphania indica*, Ep = *Epiphyas postvittana*, Hv = *Heliothis virescens*, Hz = *Helicoverpa zea*, Ms = *Manduca sexta*, Mys = *Mythimna separata*, On = *Ostrinia nubilalis*, Px = *Plutella xylostella* and Tn = *Trichoplusia ni*. Sequence names in red were generated in this study.

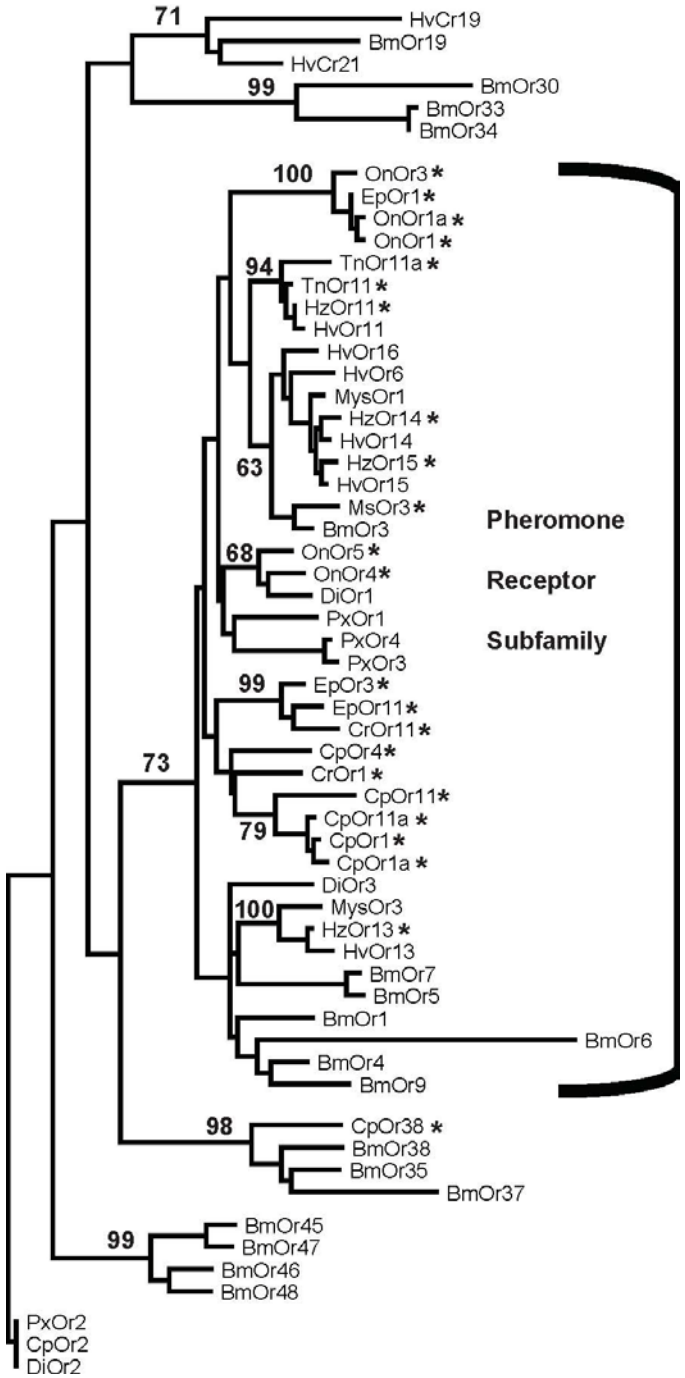

Figure 2. Phylogenetic tree illustrating the relationship of the 25 putative pheromone receptors reported herein (indicated by an *) and 19 previously published pheromone receptors. The tree is rooted using *Drosophila melanogaster* Or83b orthologs PxOr2, CpOr2 & DiOr2 (see Wanner *et al*., 2007). CpOr38 was identified using degenerate PCR primers designed against BmOr38. Bootstrap values are indicated at significant branch points as a percentage of 10 000 replicates. Bm = *Bombyx mori*, Cp = *Cydia pomonella*, Cr = *Choristoneura rosaceana*, Di = *Diaphania indica*, Ep = *Epiphyas postvittana*, Hv = *Heliothis virescens*, Hz = *Helicoverpa zea*, Ms = *Manduca sexta*, Mys = *Mythimna separata*, On = *Ostrinia nubilalis*, Px = *Plutella xylostella* and Tn = *Trichoplusia ni*.

B) Direct pyrosequencing of cDNA prepared from male and female chemosensory organs.

Four cDNA pools prepared from CM chemosensory organs (antennae and pooled legs and mouthparts from males and females) have been sent to Dr. Amit Dhingra (Washington State University) for 454 type pyrosequencing. The pyrosequencing runs will be completed soon and then the sequences will be assembled and annotated. Once the assembly and annotation are complete, the sequences that encode chemosensory receptors will be cloned from CM and readied for ligand determination once the cell-based assay system is fully developed. Annotated sequences will be deposited with GenBank with unidentified single read sequences deposited in dbEST, an expressed sequence tag database.

DISCUSSION

The results from this study have provided not only a method to identify chemosensory receptors in insects without a sequenced genome (which is the majority of insect species), but also has laid the foundation for tools to elucidate the mode of action on pheromones. While these results do not make any immediate products for orchardists to use, we anticipate that in the targets identified in this study will have potential impact on the future discovery of new agrochemicals that will be specific to codling moth control.

EXECUTIVE SUMMARY

Pheromone and kairomone communication, and more generally odor and taste perception, are crucial aspects of codling moth biology and the basis for mating disruption, attract and kill, and monitoring strategies used in the orchard. The codling moth's ability to perceive odors (including pheromones and kairomones), taste food sources, and produce hormones that regulate feeding, digestion and reproduction are all controlled by the brain. Specifically, the senses of smell and taste often regulate feeding and reproductive behaviors. The nerve receptor networks that are involved in the regulation of feeding and reproductive behaviors have been characterized only in insect systems where the genome of that organism has been sequenced (this does not include the codling moth). Therefore, the *goal of our project* was to gain an understanding of how the senses of smell and taste in codling moths lead to the regulation of host finding, feeding, and reproductive behaviors. This fundamental study should provide insight into the molecular basis and the components involved in the codling moth's ability to perceive odorants and enhance our understanding of mating disruption and attraction technologies currently used in the orchard. Identification of the molecular components involved in odor and taste perception will also provide targets and assays that will allow for the rapid screening of potential stimulants and inhibitors of codling moth sensory receptors allowing for the development of more potent lures or disruptants of codling moth's ability to perceive pheromones, kairomones, or feeding stimulants.

Significant progress was made on this project in which a conserved amino acid sequence was identified and a procedure developed that could be used to identify and clone chemosensory receptors from the codling moth. This represents the first time odorant receptors have been identified for an insect without a sequenced genome. The procedure developed using codling moth as a model system was found to be applicable to almost all insects of the Order Lepidoptera (moths and butterflies) as evidenced by our ability to identify and clone "pheromone" receptors from other insect pests of tree fruit including Obliquebanded leafroller, Light Brown Apple Moth, and Apple Clearwing Moth. Additionally, the value of the technique developed here has transcended tree fruit pests and been used to identify and clone "pheromone" receptors from lepidopteran pests of corn and cotton (European corn borer and the corn earworm/cotton bollworm).

The tools generated in this project have also laid the groundwork for development of a high-throughput cell based assay system that will enable us to determine the pheromone or kairomone that interacts with each of the receptors identified from codling moth. The funding obtained from the WTFRC has enabled us to generate the preliminary data to secure enough outside funding to complete this ambitious project. We hope that the results generated from the cell assay system will again make the codling moth a model for studies in other lepidopterous pest insects.

Future directions for this project include the development of the cell based assay system mentioned above, and adapting that system so that it will be able to be used by researchers and chemical companies as an inexpensive tool for the discovery of new semiochemicals that can be used to control codling moth in the orchard. We also hope to these advances to help make codling moth recognized as a model organism for the development of biorational means of pest control.