



A comparative study of insect odorant protein binding (OBP) genes and isolation of a partial OBP gene from coconut red palm weevil, *Rhynchophorus ferrugineus* F.

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Abstract

The red palm weevil, *Rhynchophorus ferrugineus* F., has become the most important pest of palms in the world. Though the use of pesticides keeps the pest at check, the experience gained during the recent past indicates that improper use of pesticide can cause undesirable side effects. One possible alternative method of pest control could be interference with the specific olfactory signaling pathway. A key component of insect olfactory system is a family of odorant binding proteins (OBPs) which bind to the odorant molecule and form a complex which is capable of activating an odorant receptor. The present study was undertaken to carry out a phylogenetic study of insect OBPs. Based on conserved regions of OBP sequences available in public databases, primers were designed and used to amplify putative odour binding proteins from coconut red palm weevil. Two genomic fragments, which were isolated and sequenced, showed homology to OBPs from other insect species, although at lower levels of identity.

Keywords: Odorant binding protein, red palm weevil

Introduction

The red palm weevil, *Rhynchophorus ferrugineus* F. (Coleoptera: Curculionidae), has been identified as an economically important pest of coconut palm and 17 other palm species worldwide. Although the weevil was first reported on coconut from South Asia, during the last two decades it has gained a foothold on date palm in several Middle Eastern countries from where it has moved to Africa and Europe, mainly due to the movement of infested planting material. Currently, the pest is reported in about 15% of the coconut-growing countries and in nearly 50% of the date palm-growing countries. In young coconut plantations crown, trunk and bole are the natural sites of damage. In older plantations only crowns are infested. Many a time the insect completes several generations inside the crown or trunk feeding on the inner tissues until the trunk or crown becomes hollow and tree gets killed. Infested palms, if not detected early and treated, often die.

Food baited attractants comprising of sugar cane bits, split petioles and other components have been added to enhance attraction of *R. ferrugineus* (Kurien *et al.*, 1984). Weevils rely on a range of senses to interact with their environment and communicate with each other. One of their most developed senses is olfaction. Insects use specific chemical blends to communicate with each other and able to select potential hosts from non-hosts on the basis of olfaction. 4-methyl-5-nonanone and 4-methyl-5-nonanol were identified as aggregation pheromones of *R. ferrugineus* (Hallat *et al.*, 1993). Later it was found that the responses of both males and female weevils were much stronger to 4-methyl-5-nonanol. Synergism between ferrugineol and host palm volatiles was demonstrated and necessitates the inclusion of palm material in traps for maximum trapping efficacy. The peripheral coding of *R. ferrugineus* to host volatiles was established by Gunawardena and Kern (1994).

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In insect olfactory transduction pathway, odorant binding proteins (OBPs) are a key component that bind to the odorant molecule entering the antennal sensillar lymph and form a complex, which is capable of activating an odorant receptor. Insect odorant-binding proteins (OBPs) are small, soluble proteins found in the aqueous lymph that fills the olfactory sensilla on the antenna. OBPs are believed to shuttle hydrophobic family through the sensillar lymph to the underlying receptors (Vogt *et al.*, 1991; Prestwich *et al.*, 1995; Pelosi, 1998). Each insect species contains multiple, distinct OBPs, which are often sequestered within distinct subsets of olfactory sensilla (Steinbrecht *et al.*, 1995). OBPs may display different odorant specificities (Du and Prestwich, 1995; Prestwich *et al.* 1995). OBPs may play a role in olfactory coding, in particular, and OBP present in a particular olfactory sensillum determine the spectrum of odorants to which the underlying receptors have access. The first OBP was discovered in the antennae from the male saturnid moth *Antheraea polyphemus* based on its property to bind (E6, Z11)-hexadecadienyl acetate, which is the major component of the insect's pheromone blend (Vogt and Riddiford, 1981).

Understanding the influence of OBPs on insect olfactory behaviour forms an important study in insect olfaction. OBPs represent interesting targets in strategies aimed at interfering with chemoreception system in insects. Although, the detailed mode of action of OBPs is still not clear, certainly they are the first proteins to come in contact with odorants and pheromones. The present study was carried out to undertake a comparative study of insect OBPs and also to isolate and clone OBP from coconut red palm weevil.

Materials and Methods

Phylogenetic analysis of insect OBP family

Sequences pertaining to insect OBPs were retrieved from the NCBI. Out of these sequences, full length coding sequences from a range of insect species were aligned using CLUSTAL programme (Thompson *et al.*, 1997). The insect OBP alignment was used to create an unrooted distance (neighbour joining) tree (Saitou and Nei, 1987).

Primer design

Nucleotide sequences coding for conserved domain amino acids, based on alignment of insect OBPs, were selected for oligomer designing. Oligomer sequence was selected with minimum degeneracy and high G-C content. The primers were checked for their quality (GC per cent, annealing temperature, presence of dimers) using the software FASTPCR programme.

DNA extraction

DNA was extracted from the grubs of red palm weevil using the DNeasy mini kit (QIAGEN) with slight modifications.

Optimization/Standardization of PCR parameters

Initially, the optimum annealing temperatures were determined for each primer pair using gradient PCR. Once optimized, the PCR reaction was conducted in volumes of 20 μ l containing 50 ng genomic DNA, 0.2 iM each of forward and reverse primers, 50 iM of each dNTPs, 1X buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂) and 0.5 Units of *Taq* DNA polymerase.

PCR amplifications were performed on a gradient thermal cycler with a PCR profile of 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at the different annealing temperatures optimized and 1 min at 72°C with a final extension for 15 min at 72°C.

Agarose gel electrophoresis of amplified products

After amplification, the PCR products were resolved in 1.2 per cent agarose gel using 1X TBE buffer, stained with ethidium bromide, visualized in a gel documentation system and results documented. The sizes of the amplified fragments were deduced using a molecular weight ladder.

Isolation and cloning of DNA fragments

The PCR fragments of expected sizes were excised from the agarose gel and purified using a gel cleanup kit. Cloning of the eluted fragments was carried out using the InsT/A clone™ PCR Product Cloning Kit (M/s MBI Fermentas Inc.). After transformation, the cells were finally plated on pre-warmed LB- agar plates [ampicillin (100 ppm)/IPTG (100 ppm)/X-gal (160 ppm)] and incubated overnight at 37°C. The recombinant clones were identified by blue/white colony selection.

Clone analysis by PCR

Colony PCR was carried out for direct analysis of the positive transformants.

One colony was picked up and resuspended in 20 μ l of the PCR mixture. The reaction mixture was incubated for 5 min at 94°C to lyse the cells and inactivate the nucleases. PCR amplification was carried out and the PCR products visualized by agarose gel electrophoresis. Highly pure plasmid DNA was obtained by means of GenElute™ Plasmid Miniprep Kit (M/s Sigma). PCR was carried out using the recombinant plasmid DNA as template. The PCR products were analyzed on 1.5 per cent agarose gel.

Sequencing of cloned fragments

The recombinant plasmids were sent for sequencing to M/s AXYGEN. The sequencing was done from one end of the vector with M13 primer using BigDye® Terminator v3.1 Sequencing Kit and analyzed on ABI PRISM® 377 Genetic Analyzer.

Sequence analysis

The nucleotide sequences from the M13 forward primer was screened for the presence of vector contamination using VecScreen (Altschul *et al.*, 1997). The contaminated region was cut using the software WINGENE (version 2.31). The resulting sequences were subjected to similarity search using a local alignment search algorithm, tblastx (Altschul *et al.*, 1997). Evolutionary relationships among the sequences were charted out by cluster analysis. The nucleotides sequences were aligned using CLUSTALX programme (Thompson *et al.*, 1997). A genetic matrix was calculated from the alignment data and analyzed with the neighbour-joining method (Saitou and Nei, 1987) and phylograms were constructed using the PHYLIP phylogenetic tree tool (Felsenstein, 1989).

Results and Discussion

Phylogenetic analysis of insect OBP sequences

A total of 3823 protein sequences pertaining to insect OBPs were available in NCBI. OBPs from flies constituted the majority of the sequences (3037), followed by hymenopterans (339), moths (290), beetles (103), bugs (34) and others. Out of these, 94 sequences, which were complete coding sequences, were downloaded and used for multiple sequence alignment. The multiple sequence alignment revealed that the insect OBPs were generally divergent across species and within the same species, with very low percent of conservation. Based on the multiple sequence alignment of insect odorant binding protein, a phylogenetic tree was derived from the full-length OBP sequences, which is given in Fig. 1. In general, the tree revealed the clustering of OBP order wise.

Cloning of OBP genes from coconut red palm weevil

The four primer pairs, designed *in silico*, to amplify putative OBPs from coconut red palm weevil are given in Table 1. Amplification of genes coding for OBP of coconut red palm weevil was done using genomic DNA as template and the four primer pairs. Initially, gradient PCR was carried out to select the optimum annealing temperature for PCR. Only two of the primers *viz.* OBP F3/R3 and OBP F4/R4 amplified fragments of expected

Table 1. Details of primers designed and used to amplify putative OBP genes from coconut red palm weevil

Sl. No.	Primer Name	Sequence	Length
1.	OBPF1	5'-AGGTTTACATGATGTTTGTGTCG-3'	23
	OBPR1	5'-GGGATTAACGTCGTGAAGACA-3'	21
2.	OBPF2	5'-ACAGGAGTTGACGAAGCTC-3'	19
	OBPR2	5'-TATCGCATGGATTTGCTCC-3'	19
3.	OBPF3	5'-ACGAGATGAAGGAATTGGCTCAG-3'	23
	OBPR3	5'-CGTTATCACAAAGCAATTGCTCC-3'	22
4.	OBPF4	5'-TTCGGTTATCCGTGTCAAGT-3'	20
	OBPR4	5'-AGGACGCAATTACCAATGCT-3'	20

sizes. The optimized annealing temperatures were used to amplify putative OBP genes from the weevil using these two primers (Fig. 2). Bands of expected sizes were eluted. The DNA concentration was around 50-75 ng μl^{-1} invariably for all the fragments. The eluted products were ligated with pTZ57R/T. *E. coli* (DH5 α) was transformed with the ligated recombinant pTZ57R/T vectors carrying inserts. Combinations of blue and white colonies were obtained confirming successful transformation.

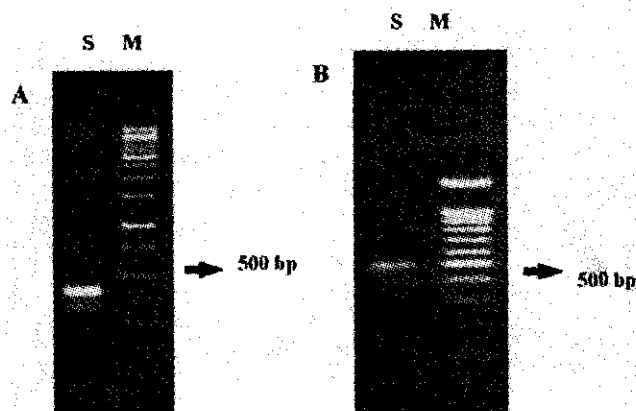


Fig. 2. PCR amplified putative OBP genes from coconut red palm weevil using the primer pairs OBPF3/R3 (A) and OBPF4/R4 (B)

S: Sample; M: Marker

The agar plates containing the transformed colonies were screened for recombinant positive clones. White colonies were picked up and were grown in LB broth containing ampicillin. The recombinant colonies were screened for the presence of inserts by colony PCR. For comparison, genomic DNA samples were also used separately for PCR. After PCR, the amplicons were electrophoresed in agarose gel. There was amplification of expected size in all the recombinant clones.

The plasmids isolated from the recombinant clones were checked for the presence of insert by PCR by amplifying them with specific primers. All the recombinant

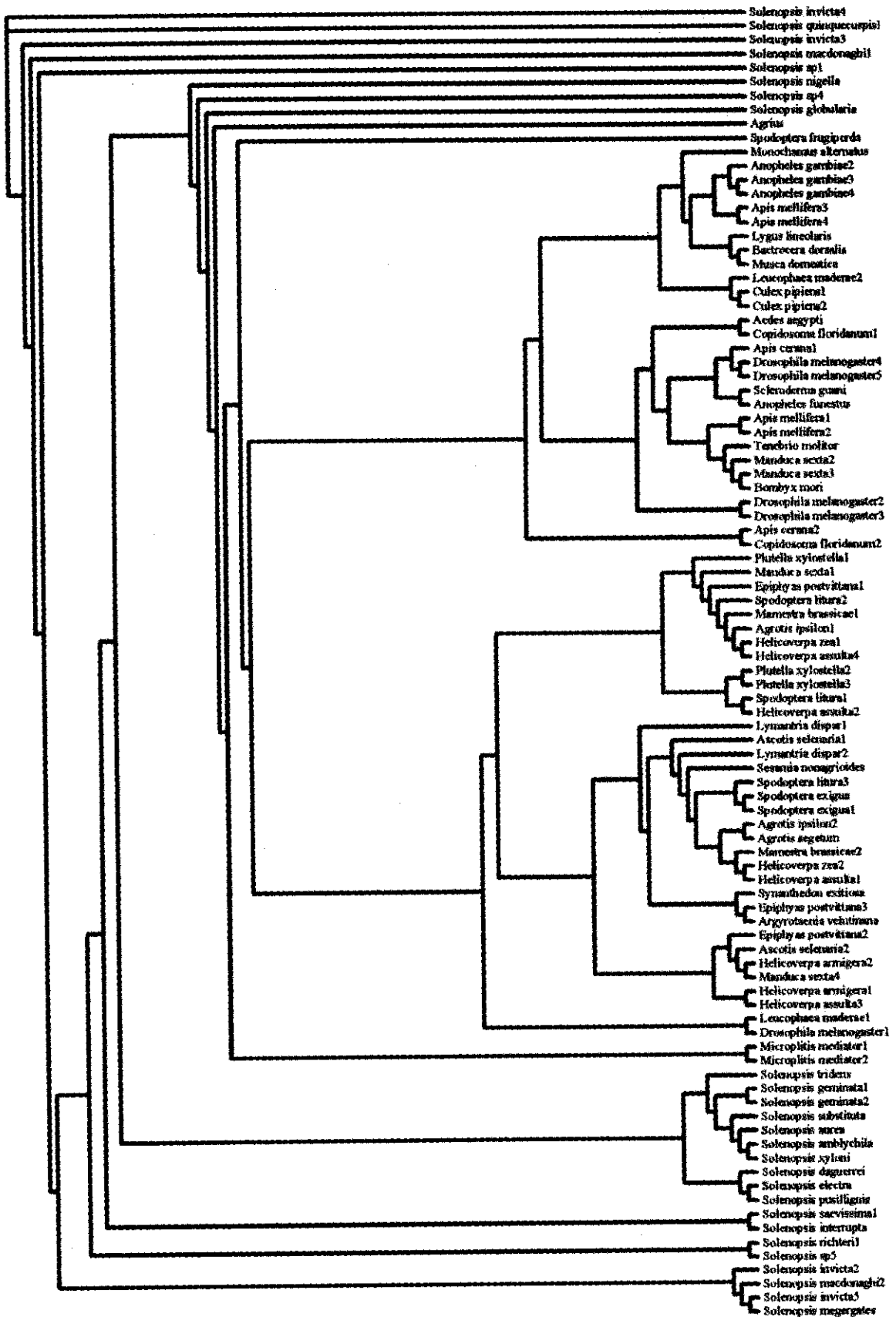


Fig. 1. Phylogenetic tree of family of insect OBPs

clones exhibited specific amplification of expected size confirming the presence of the insert. The recombinant plasmids, for which the confirmation tests were performed, were sent for automated sequencing.

Sequence analysis

The fragment cloned after PCR amplification of red palm weevil genomic DNA by the primer combination OBP F3/R3 after sequencing gave a final sequence of 433 bases after removal of vector backbone and was designated RfOBP1 (*Rhynchophorus ferrugineus* odorant binding protein 1) (GenBank accession no. EU939534). The deduced amino acid sequence of RfOBP1 showed homology to OBPs from *Solenopsis* spp. (34% identity), *Apis mellifera* and *Samia cynthia ricini*. A phylogenetic tree constructed based on neighbour-joining method, after multiple sequence alignment, revealed three major clusters (Fig. 3). RfOBP1 was found to form a distinct cluster.

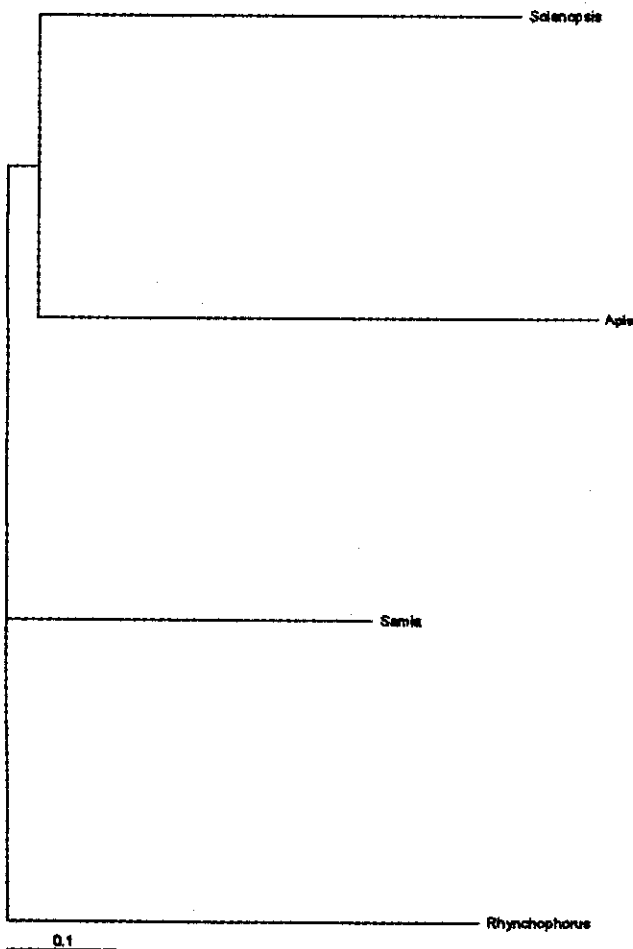


Fig. 3. Phylogenetic tree based on neighbour-joining method of deduced amino acid sequence of RfOBP1 with other related sequences

The scale bar represents a distance of 0.1 amino acid substitutions per site

The fragment cloned after PCR amplification of red palm weevil genomic DNA by the primer combination OBP F4/R4 after sequencing gave a final sequence of 514 bases after removal of vector backbone and was designated RfOBP2 (GenBank accession no. EU939535). The deduced amino acid sequence of RfOBP2 showed homology to *Helicoverpa zea* pheromone binding protein (40% identity), and OBPs from *Tribolium castaneum* and *Solenopsis globularia littoralis*. A phylogenetic tree constructed based on neighbour-joining method, after multiple sequence alignment, revealed three major clusters. Here too, RfOBP2 was found to form a distinct cluster (Fig. 4).

Many important aspects of insect behaviour rely on odours. The molecular mechanism of odour detection in insects has been extensively studied and various extracellular, transmembrane and intracellular molecules facilitate the olfactory signal transduction cascade. The

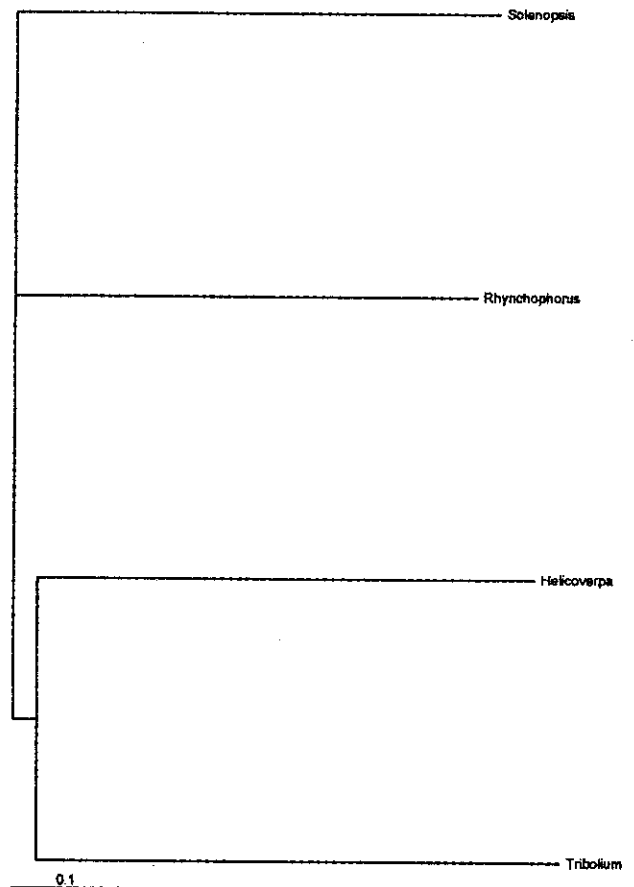


Fig. 4. Phylogenetic tree based on neighbour-joining method of deduced amino acid sequence of RfOBP2 with other related sequences

The scale bar represents a distance of 0.1 amino acid substitutions per site

major molecules, which are involved, are OBPs, G-protein-coupled receptors (GPCRs) and odorant degrading enzymes (ODEs). OBPs are believed to bind to odorants that are typically hydrophobic facilitating their transport through the hydrophilic environment in the haemolymph to GPCRs on cell surfaces. OBPs form a complex with the odorant and only in the complexed form can an odorant bind a receptor. After binding, the odorant is swallowed by the OBP to remove it from further interaction with the receptor (Kaissling, 2001).

Since olfaction profoundly influences insect behaviour, the molecules and processes involved in the olfactory pathway are ideal targets for development of novel, more-effective insect control. Among the molecules involved in olfaction, OBPs represent excellent repellent targets.

The first step towards this direction would be cloning and characterization of OBPs from the insect under study, which would form the base for a powerful new tool for uncovering olfactory genes and allow inferences to be made regarding the potential structure and function of encoded OBPs.

The phylogenetic analysis of insect OBPs revealed that the OBP genes encode a diverse family of proteins. The heterogeneity of the OBPs in *R. palmarum* have been reported by Meillour *et al.* (2004). In contrast to this diversity, only one OBP could be traced in some coleopterans like *Popilla japonica* and *Anomala osakana* (Wojtasek *et al.*, 1998). Insect OBPs have been known to comprise a multi-gene family (Vogt *et al.*, 1999) and are generally divergent across species and within the same species, with percent of conserved residues as low as 8% in some cases (Pelosi *et al.*, 2005).

Candidate olfactory genes can be rapidly detected by sequence similarity to previously discovered olfactory genes from a variety of insects. In the present study, a PCR-based approach using degenerate primers was used to amplify putative OBP genes from coconut red palm weevil. Two genomic fragments, which were isolated and sequenced, showed homology to OBPs from other insect species, although at lower levels of identity. The sequences showed homology to OBPs from *Solenopsis spp.*, *Apis mellifera*, *Samia cynthia ricini*, *Helicoverpa zea* and *Tribolium castaneum*. The homology of OBPs from *R. ferrugineus* with other species described above may be cue for showing their commonality with other insect species which respond to plant volatiles. The peripheral response of ethyl, n-propyl, n-butyl and n-pentyl alcohols, pentanol, nonanal and decenal (plant volatiles) to *R.*

ferrugineus has been reported by Gunawardena and Kem (1994). This study forms the first step towards a detailed study of OBPs from pests of coconut. Understanding OBP diversity and ligand properties can further help in identifying new compounds that fit with these binding sites, but cannot be ejected, hence preventing the other signals from detected which can be of use in pest control measures.

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