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## **X-tox: a new family of putative antimicrobial protein specific to Lepidoptera**

Jean-Michel Escoubas, Pierre-Alain Girard, Anne Nathalie Volkoff, Y. Boublik, A. Cousserans, Emmanuelle d'Alençon, K. Mita, P. Taillez, Michel Brehélin

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Colombia. <sup>6</sup> University of Tennessee, USA. marianne.elias@ed.ac.uk

Species-level phylogenetic hypotheses can be used to explore patterns of divergence and speciation. Warningly-colored mimetic butterflies are interesting models, because their wing patterns are under strong selection, and might be involved in speciation. Here we present an almost complete phylogenetic hypothesis for the neotropical mimetic butterfly genus *Napeogenes* (20 species), based on mitochondrial genes (entire cytochrome oxidase I and II) and nuclear genes (tektin, 715bp; and elongation factor 1 $\alpha$ , 1028bp). Whenever possible, we included several subspecies of the same species (with different wing patterns). The results showed good congruence between the different genetic regions. In most cases individuals of the same species clustered together, supporting the current taxonomy. However, *N. larina otaxes* and *N. larina aethra* appeared to be two sister species rather than two subspecies. A combined evidence topology is presented based on a Bayesian analysis of all the genes regions. Using the phylogenetic hypothesis we investigate whether changes in wing pattern were associated with speciation, and whether speciation was dominantly sympatric or allopatric. Using the phylogenetic hypothesis we explore patterns of diversification, and we investigate whether changes in altitudinal range and in wing pattern were associated with speciation.

### **X-tox: a new family of putative antimicrobial protein specific to Lepidoptera**

J.-M. Escoubas<sup>1</sup>, P.-A. Girard<sup>1</sup>, N. Volkoff<sup>2</sup>, Y. Boublik<sup>3</sup>, F. Cousserans<sup>2</sup>, E. D'Alençon<sup>2</sup>, K. Mita<sup>4</sup>, P. Taillez<sup>1</sup>, M. Brehélin<sup>1</sup>.

<sup>1</sup>Écologie Microbienne des Insectes et Interactions Hôte-Pathogène, Institut National de Recherche Agronomique, Université de Montpellier II, CC54, 2 place E. Bataillon, 34095 Montpellier, France; <sup>2</sup>Biologie Intégrative et Virologie des Insectes, Institut National de Recherche Agronomique, Université de Montpellier II, CC101, 2 place E. Bataillon, 34095 Montpellier, France; <sup>3</sup>Plateforme Protéines Recombinantes, C.R.B.M., Centre National de la Recherche Scientifique, 34293 Montpellier, France; Laboratoty of Insect Genome, National Institut of Agrobiological Sciences, Tsukuba,

305-8634, Japan. jmescoubas@univ-montp2.fr

A new family of putative antimicrobial proteins, characterized by imperfectly conserved tandem repeats (up to 11) of cystein-stabilized alpha beta motifs (CS- $\alpha\beta$ ), a structural scaffold characteristic of invertebrate defensins and scorpion toxins, was identified through functional genomic approaches in the lepidopteran species *Spodoptera frugiperda*. Orthologs were also found in ESTs or genomic databases from other lepidopteran species (*Bombyx mori*, *Galleria mellonella*, *Papilio dardanus*), but not in other insect orders or other invertebrate or metazoans, thus suggesting that this protein family is specific to the lepidopteran insects. In *S. frugiperda* as well as in *B. mori*, more than one transcript can be related to this protein family. *In silico* analysis of *B. mori* genome (the sole lepidopteran genome available to date) suggests that the three mRNA encoding X-tox proteins that were characterized in this insect are generated through a mechanism of alternative splicing. This mechanism thus allows the production of different X-tox proteins containing five or six CS- $\alpha\beta$  motifs. *S. frugiperda* X-tox genomic organization is currently under investigation. The role of these proteins in lepidopteran immune response was investigated by transcriptional analyses with one of the *S. frugiperda* cDNAs. Results indicate that the corresponding gene, *spod-11-tox*, is expressed mainly in larval fat body and hemocytes and that transcription is enhanced in both immune tissues upon bacterial challenge. Phylogenetic analyses show that CS- $\alpha\beta$  motifs-containing proteins are divided into three clusters. The first, considered as “ancestral” group, comprises molecules from different invertebrate taxa (odonate insect, arachnids and mollusks) and even a fungus molecule whereas the second group includes defensins isolated from neopteran insects. Finally, the third cluster contains all the CS- $\alpha\beta$  motifs stem from lepidopteran X-tox proteins. According to this phylogenetic analysis and to the position of lepidoptera in insect evolution (one of the latest order in insect speciation), we propose that X-tox proteins evolved from insect defensins to generate a new family of antimicrobial proteins. The main feature of this family is the multi-domain organisation who leads to molecular diversity generated through a mechanism of alternative splicing. The full characterization of this new protein family (gene organization and expression, protein structure and mechanism of action) in several lepidopteran species should contribute to

solve the puzzle of the evolutionary history of arthropod defensins.

### The role of serineprotease homologue 3 (SPH-3) in *Manduca sexta* shown by RNA interference

G. Felföldi<sup>1,2</sup>, I. Eleftherianos<sup>1</sup>, R. H. ffrench-Constant<sup>3</sup>, I. Venekei<sup>2</sup>, S. E. Reynolds<sup>1</sup>

<sup>1</sup>Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK; <sup>2</sup>Department of Biochemistry, Eötvös Loránd University, Pázmány sétány 1/C, Budapest, H-1117, Hungary; <sup>3</sup>School of Biosciences, University of Exeter, Cornwall, UK. bssie@bath.ac.uk

*Photorhabdus luminescens* lives in symbiosis with nematodes that invade insects. Following entry into the insect, the bacteria are released from the nematode gut into the open blood system of the insect. Here they secrete factors, which kill the host and also convert the host tissues into food for the replicating bacteria and nematodes. One of the secreted proteins is an rtx-like zinc metalloprotease PrtA. Using a proteomic approach to determine the natural substrates of PrtA in host haemolymph, we identified a number of *Manduca sexta* haemolymph proteins that are selectively cleaved when plasma was incubated with purified PrtA. One of the PrtA protein targets in *M. sexta* haemolymph included the Serine Protease Homologue Protein 3 (SPH-3). We found that *SPH-3* is not transcribed in the fat-body or haemocytes of naive *M. sexta*, but only when insects are exposed to bacteria, including *Photorhabdus*. We used RNA interference (RNAi)-mediated inhibition of expression through injection of double-stranded RNA of *SPH-3* into *M. sexta* caterpillars and we achieved reduced transcription of *SPH-3* in both fat-body and haemocyte tissues. Knock-down of *SPH-3* dramatically reduced the ability of insects to resist infection to a standard dose of *Photorhabdus*, as measured by the rate at which infected insects die. RNAi of *SPH-3* was also associated with decreased levels of phenoloxidase (PO) activity in haemolymph cell-free plasma from insects infected with either a non-pathogenic strain of *E. coli* or *Photorhabdus*. The reduction in PO activity was also reflected by a reduction in the number of melanotic nodules present in tissues of dissected larvae. Finally, we found that RNAi of *SPH-3* did not affect the transcription of pattern

recognition protein genes (*Hemolin*, *Immulectin-2*, *Peptidoglycan recognition protein*, *Pattern recognition Serine Proteinase*,  $\beta$ -glucan recognition protein-1,  $\beta$ -glucan recognition protein-2), but down-regulated the transcription of certain antibacterial effector genes (*Attacin*, *Cecropin*, *Moricin*). Our findings strongly suggest that *SPH-3* serves specific immune functions and plays a distinctive role in *M. sexta* immune pathways.

### Host plant adaptation and specialization in the Pieridae family

H. M. Fischer<sup>1</sup>, C. W. Wheat<sup>2</sup>, U. Wittstock<sup>3</sup>, D. G. Heckel<sup>1</sup>, H. Vogel<sup>1</sup>

<sup>1</sup>Entomology Dept., MPI for Chemical Ecology, D-07745 Jena, Germany; <sup>2</sup>Dept. of Biological and Environmental Science, University of Helsinki, FI-00014 Helsinki, Finland; <sup>3</sup>Inst. für Pharmazeutische Biologie, Technische Universität Braunschweig, D-38106 Braunschweig, Germany. hfischer@ice.mpg.de

A metabolic diversion of a plant chemical defense caused by an enzyme (Nitrile Specifier Protein, NSP) enables a majority of Pieridae species to feed on Brassicaceous plants. The NSP is a newly recruited detoxifying protein in this family. It shows a specific repeat structure and has no sequence homology to any protein with a known function. We are trying to unravel the molecular mechanisms leading to the evolution of this protein and the subsequent adaptation to a plant defense system. The nucleotide sequence is known for two model species. By generating cDNA libraries of related species and amplifying intron and upstream regions, species and allelic differences are being located. Although NSP shows no sequence homology to any protein of known function, single domains show similarities to the so-called major allergen proteins, found in the gut lumen of many insect species. By establishing heterologous expression systems for both proteins we are trying to investigate the mode of action of NSP and the function of MA.

### The effect of microorganisms in the food on the growth rate and immune system of cabbage looper (*Trichoplusia ni*)

D. Freitag, D. Heckel, H. Vogel