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Nuclear receptors in *Bombyx mori*: Insights into genomic structure and developmental expression

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ABSTRACT

Nuclear receptors (NRs) function as ligand-dependent transcription factors and are involved in diverse biological processes in different animals. The updated assembly of complete genome sequence of the *Bombyx mori* enabled a systematic analysis of the NRs in the five holometabolous insects including *B. mori*, *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera*, and *Tribolium castaneum*. As a result, nineteen NRs were identified in the *B. mori* genome, each of eighteen NRs has 1:1:1:1 ortholog in the other four insects. Interestingly, the average intron number of ligand-binding domain (LBD) of each NR gene in *B. mori* was 2.4, much higher than that in the other four insects; the genomic position of introns in LBDs of all orthologs for each NR presents more diversity. Phylogenetic trees of all NRs from the five insects were consistent or aberrant with classical phylogeny of these insect species. The characteristics in number, genomic structure and phylogeny of all NRs revealed their evolutionary conservation and divergence during insect evolution. The expression patterns of several NR genes displayed temporal specificity similar to that in *D. melanogaster* and may be associated with the key biological processes during silkworm metamorphosis. The RNAi of *BmβFTZ-F1* resulted in abnormality in larva–pupa transition, further suggesting it is also crucial for silkworm metamorphosis. In conclusion, the present study provided new insights into the structure, evolution, expression, and functions of silkworm NRs.

1. Introduction

The nuclear receptor (NR) family functions as ligand-dependent transcription factors in various animals and involves in a variety of biological processes such as cell differentiation, metabolic regulation, insect metamorphosis (Baker et al., 2007; Kastner et al., 1995; Robinson-Rechavi et al., 2001; Thummel, 1995). Most members in this fascinating family share a common structure, which includes the variable modulatory A/B domain, the highly conserved DNA-binding domain (DBD) with two C4 zinc fingers, the hinge region, the less conserved ligand-binding domain (LBD), and an F-domain. The N-terminal DBD and C-terminal LBD are generally used as typical signatures to define this family. Most NRs are transcriptionally activated by binding of small lipophilic molecules, such as steroids, retinoids and thyroid hormones and further regulate the expression of target genes positively and/or negatively.

Recently, many NR gene sets have been characterized from the whole genome sequence (WGS) of many animals. Forty-eight NRs are identified in the human genome (Robinson-Rechavi et al., 2001), and over 270 NRs are found in *Caenorhabditis elegans* (Sluder and Maina, 2001), 49 in mouse and 47 in rat (Zhang et al., 2004). Surprisingly, only 21 NR genes are found in *Drosophila melanogaster*, 20 in *Anopheles gambiae*, 22 in *Apis mellifera*, and 21 in *Tribolium castaneum* (Adams et al., 2000; Bonneton et al., 2008; Velarde et al., 2006). The comparative analysis of NRs from mammalians and insect genomes suggested that the majority of them could be assigned to six well-defined subfamilies and are evolutionally conserved to a certain degree (Maglich et al., 2001; Zhang et al., 2004). Furthermore, the comprehensive studies of NRs

Abbreviations: NR, nuclear receptor; LBD, ligand-binding domain; DBD, DNA-binding domain; SNP, single nucleotide polymorphisms; WGS, whole genome sequence; Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*.

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in *D. melanogaster* have demonstrated that they play key roles in the entire development from embryogenesis to metamorphosis (King-Jones and Thummel, 2005; Sullivan and Thummel, 2003). For example, *DmDHR3* is required for maximal expression of the regulatory genes during *Drosophila* prepupa–pupa transition (Lam et al., 1999). *DmβFTZ-F1* functions as a key regulator for *Drosophila* metamorphosis (Lavorgna et al., 1993). *DmDHR78* can interact with corepressor *Moses* to maintain *Drosophila* growth (Baker et al., 2007; Fisk and Thummel, 1998).

The silkworm, *Bombyx mori*, is regarded as an excellent model for biochemical, molecular genetic and genomic studies of the order Lepidoptera (Goldsmith et al., 2005). Many groups have preformed the studies on the silkworm NRs. The complete or partial mRNA sequences and specific expression patterns of 11 genes encoding silkworm NRs (i.e. *EcR*, *USP*, *HR3*, *βFTZ-F1*, *E75*, *HR4*, *HR38*, *HR39*, *HR78*, *HNF4*, and *SVP*) have been characterized (Charles et al., 1999; Hirai et al., 2002; Kamimura et al., 1997; Matsuoka and Fujiwara, 2000; Niimi et al., 1997; Sun et al., 1994; Sutherland et al., 1995; Swevers et al., 2002; Swevers and Iatrou, 1998; Togawa et al., 2001; Tzertzinis et al., 1994). The different isoforms of five NR genes (i.e. *EcR*, *USP*, *E75*, *HNF4*, and *SVP*) have also been analyzed. The expression profiles of several NR genes indicated that hierarchical regulation in programmed cell death in *B. mori* anterior silk glands at pupal metamorphosis is likely different from that in *Drosophila* salivary glands (Sekimoto et al., 2006).

In 2004, the draft sequence for the *B. mori* genome was independently reported by Chinese and Japanese groups (Mita et al., 2004; Xia et al., 2004); the integration, assembling, and annotation of both WGS data sets with 9× coverage has recently been completed (The International Silkworm Genome Sequencing Consortium, 2008). Approximately 14,623 genes encoding proteins were predicted from new version of the silkworm genome assembly. With the availability of the updated genome sequences for *D. melanogaster*, *A. gambiae*, *A. mellifera*, and *T. castaneum*, it is now possible to perform a systematic, comparative study of NR genes in these five holometabolous insects. In the present study, we identified 19 NRs in *B. mori* genome. The genomic structure and phylogenetic relationships of NRs from *B. mori* and other four insects are also investigated. The expression profiles of NR genes during silkworm metamorphosis further suggest its functional conservation.

2. Materials and methods

2.1. Identification of nuclear receptors in B. mori genome

The protein sequences of 1171 known NRs including 21 representative ones in *D. melanogaster* were downloaded from NucleaRDB (Horn et al., 2001) and GenBank. The protein sequences of DBD and LBD, which are two specific domains to most members of the NR family, were obtained from Pfam (Bateman et al., 2004). The new version of whole genome sequence of *B. mori* became available in 2008, and the updated builds of genome sequences for *A. gambiae*, *A. mellifera*, and *T. castaneum* were downloaded from the Ensembl or NCBI. To identify candidate NRs in *B. mori*, we used the protein sequence of known NRs to query the genes predicted from the updated assembly of the *B. mori* genome by local BLASTP and to search against the *B. mori* genome sequence by TBLASTN, with an *E*-value threshold of 10^{-6}. The known DBD and LBD sequences were also used as queries to perform the same homology search. If a putative DBD or LBD overlaps with other domain types, the questionable domains usually have low scores and high *E*-value. So, we wrote a PERL script to check these overlaps and manually eliminate the questionable domains. All above methods were also used to scan the updated genome sequence for other four insects again. The peptide sequences of the DBD and LBD for further analysis were extracted manually from the predicted NRs in *B. mori* and other four insects using CD-search in NCBI (Marchler-Bauer and Bryant, 2004), which were contained in supplementary data 1.

2.2. Genomic distribution and exon/intron structures of silkworm nuclear receptors

A linkage map of *B. mori* based on single nucleotide polymorphisms (SNPs) can be used to locate the identified NRs in different chromosomes (Yamamoto et al., 2006, 2008). For the other four insects, we searched the chromosomal distribution of their NRs from the resources in NCBI. The same set of NR genes located on a single chromosome across five insect genomes was considered as a syntenic block (Zhang et al., 2004). Since the LBD of a NR is less conserved than DBD and involves in the binding of signal ligands, and ligand binding is acquired during evolution of nuclear receptors (Escriva et al., 2004; Escriva et al., 1997), we further characterized the exon/intron structure of LBDs in each NR that possess a complete LBD and that is found in all five insects by matching the coding sequence of LBDs on the corresponding genomic sequences. Two online programs, BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat; http://www.beetlebase.org/cgi-bin/webBlat) and Sim4 (http://pbil.univ-lyon1.fr/members/duret/cours/inserm210604/exercise4/sim4), were used to perform this analysis.

2.3. Multiple alignment and phylogenetic tree of insect nuclear receptors

Except for the differences of exon/intron structures, the sequence similarity can also regard a characteristic for evaluating the evolution of animal NRs. We also analyzed the phylogenetic relationship of NRs in five insects based on the peptide sequences of their LBDs, DBDs or full coding sequences. The peptide sequences were aligned using ClustalX. The number of complete aligned sites used for the construction of full phylogenetic tree is 148 for LBD and 68 for DBD. The tree was constructed using the neighbor-joining method and with a bootstrap of 1000 replicates, then displayed with MEGA 4.0 (Tamura et al., 2007).

2.4. Developmental expression of nuclear receptors during silkworm metamorphosis

The Chinese silkworm strain *Dazao* was reared at a stable temperature of 25 °C. Under this condition, silkworm individuals stop feeding on day 7 of 5th instar larva (defining this time point as V7), then begin wandering and spinning (W0). They complete spinning at 48 h after just wandering (W48), pupate at W60, and develop into adult (moth) at the end of W240 (Fig. S1). We collected the individuals at twenty time points during metamorphosis from day 3 of 5th instar larva (V3) to adult, to survey the expression profiles of *B. mori* NRs by semi-quantitative RT-PCR method. Total RNA was isolated from the silkworm individuals from each time point using TRIzol reagent (Invitrogen, USA) and was reverse-transcribed into cDNA with MVreverse transcriptase (Promega, Madison, WI) at 42 °C. All cDNA samples were normalized by an internal control of *B. mori* ribosomal protein RpL3 mRNA. All primers for RT-PCR detection were listed in Table S1. RT-PCR reactions in a volume of 25 μl were performed using a common program as follows: initial incubation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 53 °C (in accordance with the primer Tm) for 30 s, and 72 °C for 2 min, followed by 7 min of extension at 72 °C. The PCR products were separated on 1.5% agarose gels and stained with EB.