



# Genome-based identification and analysis of ionotropic receptors in *Spodoptera litura*

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## Abstract

The ability to sense and recognize various classes of compounds is of particular importance for survival and reproduction of insects. Ionotropic receptor (IR), a sub-family of the ionotropic glutamate receptor family, has been identified as one of crucial chemoreceptor super-families, which mediates the sensing of odors and/or tastants, and serves as non-chemosensory functions. Yet, little is known about IR characteristics, evolution, and functions in Lepidoptera. Here, we identify the IR gene repertoire from a destructive polyphagous pest, *Spodoptera litura*. The exhaustive analyses with genome and transcriptome data lead to the identification of 45 IR genes, comprising 17 antennal IRs (A-IRs), 8 Lepidoptera-specific IRs (LS-IRs), and 20 divergent IRs (D-IRs). Phylogenetic analysis reveals that *S. litura* A-IRs generally retain a strict single copy within each orthologous group, and two lineage expansions are observed in the D-IR sub-family including IR100d-h and 100i-o, likely attributed to gene duplications. Results of gene structure analysis classify the SlitIRs into four types: I (intronless), II (1–3 introns), III (5–9 introns), and IV (10–18 introns). Extensive expression profiles demonstrate that the majority of SlitIRs (28/43) are enriched in adult antennae, and some are detected in gustatory-associated tissues like proboscises and legs as well as non-chemosensory organs like abdomens and reproductive tissues of both sexes. These results indicate that SlitIRs have diverse functional roles in olfaction, taste, and reproduction. Together, our study has complemented the information on chemoreceptor genes in *S. litura*, and meanwhile allows for target experiments to identify potential IR candidates for the control of this pest.

**Keywords** *Spodoptera litura* · Ionotropic receptor · Gene duplication · Gene structure · Expression profile

## Introduction

Chemosensory systems, referring to olfactory and taste systems, are of particular importance for survival and reproduction of insects involved in various vital behaviors, such as host searching, partner recognition, predator avoidance, and food assessment (Dahanukar et al. 2005; Hallem et al. 2006; Hansson and Stensmyr 2011). The olfactory-related behaviors are driven primarily by volatile organic compounds (VOCs),

including sex pheromones, and host or non-host odors (Arimura et al. 2009; Gomez-Diaz and Benton 2013). In addition, behaviors that rely on the sensing of taste are mediated by tastants such as sugars, carbon dioxide, and bitter compounds (Agnihotri et al. 2016; Isono and Morita 2010; Slone et al. 2007; Xu and Anderson 2015). These crucial chemosensory behaviors are associated with three distinctive super-families of chemoreceptors: odorant (ORs), gustatory (GRs), and ionotropic (IRs) receptors. These receptor proteins are expressed in specific chemosensory sensilla harboring one to five gustatory (GSNs) or olfactory (OSNs) sensory neurons that are tuned to various classes of compounds (Ihara et al. 2013; Su et al. 2009; Touhara and Vossahl 2009).

IRs, belonging to a variant sub-family of ionotropic glutamate receptors (iGluRs), were first discovered from *Drosophila melanogaster* genome using bioinformatics-based methods (Benton et al. 2009). Initially, this gene family was proposed to solely detect the VOCs, and thereby was defined as olfactory receptors. Except those IRs involved in olfaction, functions of the remaining IRs were still unknown

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due to no expression in various tissues (Benton et al. 2009). Accordingly, *Drosophila* IRs were distinguished into two sub-families: divergent IRs (D-IRs) and antennal IRs (A-IRs), based on sequence characteristics and phylogenetic analyses (Croset et al. 2010). More recently, a growing number of studies have improved our knowledge on IR functions with involvement in olfaction, gustation, hearing, temperature, and humidity (Chen and Amrein 2017; Enjin et al. 2016; Hussain et al. 2016; Knecht et al. 2017, 2016; Koh et al. 2014; Ni et al. 2016; Prieto-Godino et al. 2017; Senthilan et al. 2012; Silbering et al. 2011; Stewart et al. 2015; Tauber et al. 2017). For example, members of the IR20a clade in *D. melanogaster*, consisting of 35 IR genes, were identified as candidate taste and pheromone receptors (Koh et al. 2014). Moreover, three combinatorial patterns of IR21a/IR25a/IR93a, IR25a/IR40a/IR93a, and IR25a/IR68a/IR93a have been shown to mediate the sensing of cool-temperature avoidance, humidity, and moist, respectively (Knecht et al. 2016, 2017; Ni et al. 2016). Interestingly, two candidate co-receptor IRs, IR25a and IR76b, have dual roles, i.e., participating in the formation of hetero-complexes as co-receptors and sensing compounds or temperature as stimulus-specific IRs (Chen et al. 2015; Croset et al. 2016; Ganguly et al. 2017; Hussain et al. 2016; Knecht et al. 2017; Ni et al. 2016; Rytz et al. 2013; Silbering et al. 2011; Zhang et al. 2013).

Although the IR gene family has been intensively investigated since 2009, especially focusing on dipteran species including *Drosophila* species and *Anopheles gambiae* (Abuin et al. 2011; Benton et al. 2009; Croset et al. 2016; Ganguly et al. 2017; Grosjean et al. 2011; Hussain et al. 2016; Liu et al. 2010; Pitts et al. 2017; van Giesen and Garrity 2017), little information on its functions and evolution in other insect orders is available until now. Apart from the two chemosensory super-families of ORs and GRs, the IR gene repertoire is also important for lepidopteran chemosensory reception. More recently, a study on IR evolution has been conducted in *Heliconius* butterflies (van Schooten et al. 2016), which has enhanced our knowledge of lepidopteran IRs. However, the IR gene family has still been largely unexplored and poorly understood in moth species, which has largely restricted our ability to understand IR evolution and functions within lepidopteran species or across insects. With the increased availability of genome data in Lepidoptera, we are able to explore the problems of IR sequence characteristics, gene structures, gene expansions, phylogenetic relationships, expression profiles, and evolution.

In the current study, we integrated bioinformatics-based approaches and molecular biology strategies to exhaustively identify IR genes from the genome of *Spodoptera litura* (Cheng et al. 2017). This study greatly complements the information on chemoreceptors in *S. litura* and other lepidopteran species, and meanwhile provides reference data for further functional studies and evolution of IRs across insects.

## Materials and methods

### Insect rearing and tissue collection

The larvae of *S. litura* were reared on an artificial diet (Huang et al. 2002) in the laboratory of Southwest Forestry University, under the conditions of 14:10 h light/dark and  $65 \pm 5\%$  relative humidity. Pupae were sexed and kept in separate cages until eclosion. The emerging adults were supplied with 10% honey solution.

Adult tissues were dissected between the 6th and 8th hours of the scotophase from 2-day-old moths of both sexes. These tissues included antennae, proboscises, heads without antennae and proboscises, thorax, abdomens, legs, wings, and reproductive systems (female: accessory gland, bursa copulatrix, ovary, spermatheca, and spermathecal gland; male: accessory gland, ejaculatory duct, seminal vesicle, and testis) of both sexes. Two independent samples of each tissue were collected as two templates.

### Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from collected tissues using TRIzol Reagent (Ambion, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instruction. The quality and concentration of RNA were examined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Synthesis of first-strand cDNA was performed with 1 µg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, Liaoning, China) according to the protocol. Briefly, genomic DNA was first removed by the treatment with gDNA Eraser at 42 °C for 2 min, and then cDNA template was synthesized at 37 °C for 15 min and 85 °C for 5 s. The prepared cDNA templates were stored at –20 °C prior to use.

### Gene identification

The genome assembly and transcriptomes of *S. litura* were downloaded from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/genome/?term=spodoptera+litura>) (Cheng et al. 2017) and NCBI Sequence Read Archive (SRA) Databases (<https://www.ncbi.nlm.nih.gov/genbank/tsa/>), respectively. Candidate IR genes were identified from *S. litura* based on the stand-alone genomic and transcriptomic data, using the IRs of *Bombyx mori*, *Helicoverpa armigera*, *Spodoptera frugiperda*, and *D. melanogaster* (Croset et al. 2010; Gouin et al. 2017; Liu et al. 2014; Pearce et al. 2017; van Schooten et al. 2016) as initial queries in exhaustive TBLASTN and PSI-BLAST searches, with a *E* value cutoff of  $e^{-5}$ . Further, all identified *S. litura* IRs were used as queries to screen the genome in an alternative method, with manual adjustments.

## Gene nomenclature

Conserved *S. litura* IRs were named following the nomenclature conventions of *B. mori*, *H. armigera*, *S. frugiperda*, and *D. melanogaster* IRs (Croset et al. 2010; Gouin et al. 2017; Pearce et al. 2017), together with the orthology. In addition, other IRs were designated as IR100 appended with a lower-case “a-p”-like SlitIR100a to SlitIR100p. Notably, SlitIR100a was specific to moths and had no orthology to *D. melanogaster* IR100a. *S. litura* iGluRs were named with the Arabic numerals from 1 to 12, based on the orthology to *H. armigera* iGluRs (Liu et al. 2014; Pearce et al. 2017) (Table S1 and Additional file 1).

## Sequence and phylogenetic analysis

Transmembrane segments and signal peptides of candidate IRs were predicted using TMHMM Server v2.0 (Krogh et al. 2001) and SignalP 4.1 Server (Petersen et al. 2011), respectively. Multiple alignments of IR protein sequences were generated by MAFFT v7.308 (Kato and Standley 2013) or MUSCLE (Edgar 2004), with the default parameters and necessary manual adjustments. Aligned sequences were edited using JalView 2.8 (Waterhouse et al. 2009). Exon and intron boundaries were predicted and analyzed using GeneWise with the synchronous model and GT-AG rule (Birney et al. 2004). Gene models were generated with Exon-Intron Graphic Marker (<http://www.wormweb.org/exonintron>). For comparison of IR gene numbers across insects, *S. litura* and ten other species were selected including *B. mori*, *Danaus plexippus*, *H. armigera*, *Heliconius melpomene*, *Manduca sexta*, *S. frugiperda*, *Acyrtosiphon pisum*, *Apis mellifera*, *D. melanogaster*, and *Tribolium castaneum*. Gene gains and losses were analyzed between the above-mentioned lepidopteran species.

In the phylogenetic tree, the IRs were selected from *B. mori*, *H. armigera*, *S. frugiperda*, and *S. litura* as well as a model insect *D. melanogaster* (Croset et al. 2010; Gouin et al. 2017; Pearce et al. 2017; van Schooten et al. 2016). *S. litura* iGluRs were used as an outgroup to root the tree. The tree was constructed using PhyML 3.2 (Guindon et al. 2010) based on the aligned protein sequences, under Jones-Taylor-Thornton (JTT) model with Nearest-Neighbor Interchange (NII). Support values were estimated by approximate likelihood ratio test (Chi2). The tree was viewed and edited using FigTree 1.4.3.

## Gene expression profile analysis

Expression profiles of candidate *S. litura* IR genes in various adult tissues and internal reproductive systems of both sexes, were examined using reverse transcription PCR (RT-PCR). Gene-specific primers (Table S2) were designed by Primer

Premier 6.0 (PREMIER Biosoft International, CA, USA), with an expected product size of about 600 bp, containing at least one intron if possible. RT-PCR was conducted with rTaq DNA polymerase (TaKaRa, Dalian, Liaoning, China) under the following conditions: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s, and final extension for 5 min at 72 °C. Ribosomal protein L10 (RPL10) gene (Lu et al. 2013) was used as the reference gene to check the quality and integrity of cDNA templates. Negative control was set using sterile water as the template. For some IR genes, two biological replicates were carried out to acquire accurate data.

## Results

### Identification of candidate IRs in *S. litura*

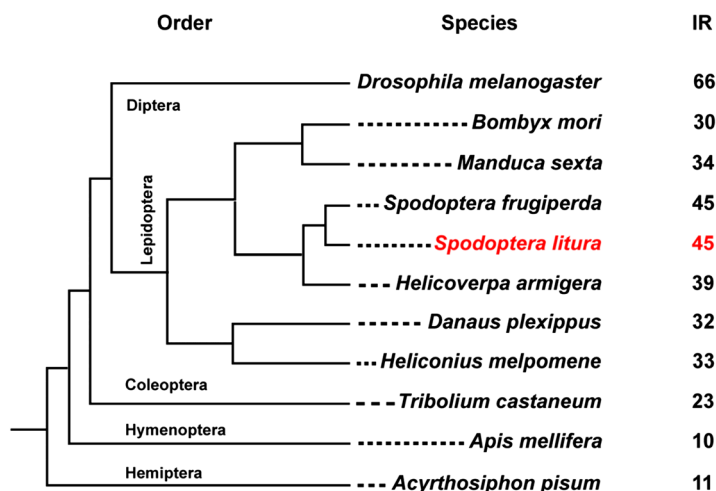
Based on the genome sequences of *S. litura*, coupled with published transcriptomes, 45 genes encoding IRs were identified by combining bioinformatics-based approaches and manual efforts. They contained two putative pseudogenes (IR2 and IR2.1) and one partial sequence (IR68a). The remaining IRs were full-length sequences with various sizes containing 543 to 919 amino acids (AAs). Apart from the IR family, we also identified two  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), two *N*-methyl-D-aspartate (NMDA), and eight Kainate receptors. Of these, iGluR1–iGluR9 were full-length sequences varying from 871 to 985 AAs while iGluR10–iGluR12 were partial sequences (Table S1 and Additional file 1).

In comparison with most of the SlitIRs, the three other iGluR sub-families had longer full-length sequences (871–985 AAs). However, we noticed that for two co-receptors (IR8a and IR25a), the predicted genes encoded protein sequences of 899 AAs and 919 AAs, respectively. By contrast, the third co-receptor, IR76b, showed the shortest open reading frame, encoding 543 AAs. Seven IR genes encoded over 650 AAs, including IR21a, IR40a, IR60a, IR93a, IR100a, IR100b, and IR100b.1. The remaining IR genes (79%) encoded 580 to 643 AAs (Table S1 and Additional file 1).

### Comparison of IRs between Lepidoptera and other insect orders

To compare the differences of IR gene numbers among various lepidopteran species as well as between Lepidoptera and other insect orders, we examined seven lepidopteran species and four non-lepidopteran species. By comparison, the number of IRs in Lepidoptera was larger than in *A. mellifera*, *A. pisum*, and *T. castaneum* but smaller than in *D. melanogaster* (Fig. 1a). To further unravel gene gains or losses in Lepidoptera, another six species were selected. In seven IR orthologous groups (LS-IRs: IR1.2, IR2.1, IR100b, and

**A**



**B**

Gene	LS-IRs							A-IRs							D-IRs						
	1.1	1.2	2	2.1	87a/100a	100b	100b.1	8a/21a	25a/31a	40a/41a	60a	64a/68a	75d	75p	75p.1/p.2	75q.1/q.2	76b/93a	7d.1/d.2	7d.3/85a	7d.4	100c-q/143
<i>B. mori</i>	1	1	1F,P		1/1	1		1/1	1/1	1/1		1/1	1	1	1/1	1/1	1/1	1/1	1/1	1	3/1
<i>H. armigera</i>	1	1	1F,P	1F,P	1/1	1	1	1/1	1/1	1/1	1	1/1	1	1	1/1	1/1	1/1	1/1	1/1	1	8/1
<i>M. sexta</i>	1	1	1F,P		1/1	1		1/1	1/1	1/1		1/1	1	1	1/1	1/1	1/1	1/1	1/1	1	6/1
<i>S. frugiperda</i>	1	1	1F,P	1F,P	1/1	1	1	1/1	1/1	1/1	1	1/1F	1	1F	1F/1F	1F/1F	1/1	1/1	1/1	1	14(1F)/1
<i>S. litura</i>	1	1	1F,P	1F,P	1/1	1	1	1/1	1/1	1/1	1	1/1F	1	1	1/1	1/1	1/1	1/1	1/1	1	14/1
<i>D. plexippus</i>	1	1F	1F,P		1/1			1/1	1/1	1/1	1	1/1	1		1/1F	1/1	1/1	1/1	1/2(1F)	1/1	3/1
<i>H. melpomene</i>	1		1F,P		1/1			1/1	1/1	1/1	1	1/1	1		1/1	1/1	1/1	1/1	1/1	1	7/1

**Fig. 1** Lepidoptera IR gene family. **a** Numbers of ionotropic receptor genes across insect species. **b** Presence and absence of IR orthologous groups within seven lepidopteran species. Numbers in colored boxes represent gene copies identified from the genome. “P” and “F” mean

pseudogene and fragment, respectively. Blank space means gene losses in the corresponding species. A-IRs, antennal IRs; LS-IRs, Lepidoptera-specific IRs; D-IRs, divergent IRs

IR100b.1; A-IRs: IR60a and IR75p; D-IRs: IR7d.4), the orthologous analysis revealed an absence of IRs in some lepidopteran species. Of these, IR2.1 and IR100b.1 were found only in noctuid moths, and IR100b was present in moths rather than in butterflies. In the A-IR sub-family, IR60a was not identified from *B. mori* and *M. sexta*, and IR75p was present only in moths. For the D-IR sub-family, IR7d.4 was not detected in *B. mori*. The remaining 23 orthologous groups were highly conserved among lepidopteran species (Fig. 1b).

**Sequence and phylogenetic analyses of candidate IRs in *S. litura***

To explore three key amino acid residues involved in ligand binding, the protein sequences of 12 iGluRs and 43 IRs (except IR2 and IR2.1) were aligned and analyzed. Only seven iGluRs (iGluR2, iGluR3, iGluR4, iGluR6,

iGluR7, iGluR10, and iGluR12) retained the conserved pattern of R-T-D/E, whereas this conserved pattern was lacking in all IRs. The threonine in the second position was not present in all IRs, and ten IRs exhibited a conserved aspartate or glutamate in the third position (Fig. 2).

Our phylogenetic analysis revealed that twelve A-IR groups and one D-IR group were homologous to *D. melanogaster* IRs with each clade showing a strict single copy. In Lepidoptera, two large lineage expansions were observed in IR7d and IR75 clades. In addition, two expansions were observed in the D-IR sub-family, representing IR100d-h and IR100i-o. These expansions are possibly derived from gene duplications, further supported by IR gene physical location on a single chromosome and relative high identities among the paralogs (Figs. 3 and 4a). Two candidate coreceptors IR8a and IR25a were phylogenetically clustered together with iGluRs. Interestingly, IR60a and IR87a members





**Fig. 2** Alignment of ligand-binding domains of candidate iGluRs and IRs in *S. litura*. Three key residues (R, T, and D/E) that are diverse among

different IRs in *S. litura* are marked in red with blue squares. S1 and S2 mean two lobes of ligand-binding domains

belonging to the A-IR and LS-IR sub-families showed a close phylogenetic relationship to the D-IR family (Fig. 3).

**Structure and organization of candidate IRs in *S. litura***

We mapped all the IRs onto the genome of *S. litura* by TBLASTN searches. These IR genes were oriented onto fifteen chromosomes, three scaffolds, and two contigs, respectively. In particular, six possible gene duplication events were detected including IR7d.1/7d.2, IR75p/75p.2, IR75q.1/75q.2, IR100b/100b.1, IR100d-h, and IR100i-o. In each duplication event, these IR genes were

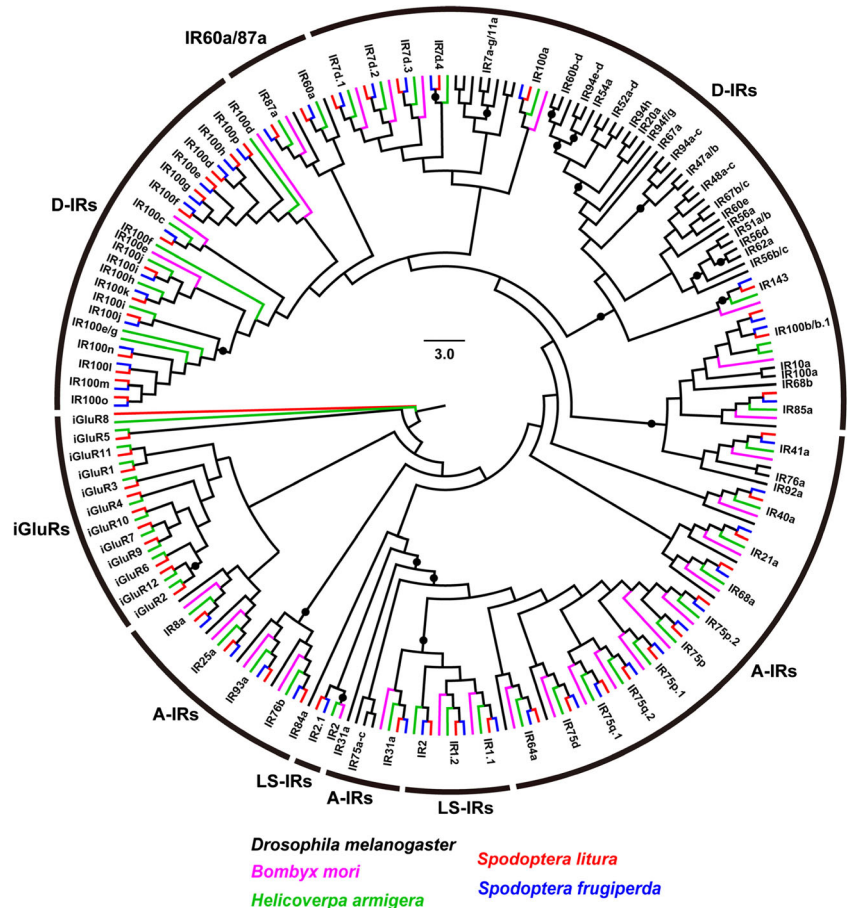
tandemly located on one chromosome. Moreover, except IR75p and IR75p.2 presenting an opposite transcriptional orientation, the IR genes of other five events were closely located on a single chromosome with the same transcriptional orientation (Table S1; Fig. 4a).

To compare exon and intron structures of IR genes in *S. litura*, we first analyzed their intron numbers. Based on the analysis of intron numbers, four types of IR genes could be defined: type I—intronless: the D-IR sub-family (except IR85a), and IR60a and IR87a from the A-IR and LS-IR sub-families, respectively; type II—one to three introns: IR85a/100a/100b/100b.1 from the LS-IR and

D-IR sub-families; type III—five to nine introns: five A-IR groups of IR21a, IR31a, IR41a, IR64a, and IR76b; and type IV—ten to eighteen introns: the remaining IRs including IR1.1, IR1.2, IR8a, IR25a, IR40a, IR93a, and members of IR75 groups (Fig. 4b, c).

Intron size and position were analyzed based on genome and cDNA sequences. Several typical characteristics could be summarized. For example, the type I IRs had similar positions of functional domains and transmembrane segments (one ion channel pore (P), two lobes of ligand-binding domains (S1 and S2), three transmembrane domains (M1, M2, and M3)) due to their similar sequence lengths (Fig. S1). For the type II IRs, intron lengths were usually short, varying from 60 to 444 bp. Notably, IR100b and IR100b.1 shared the same intron numbers, similar intron lengths, and similar positions of functional domains and transmembrane segments. Unlike the type I and type II IRs, two other types of IRs possessed variable intron lengths. Of these, the shortest intron was 67 bp (intron 3 of IR1.1 and intron 9 of IR75q.2), while the longest intron was 7559 bp (intron 9 of IR25a). In addition, we found that members of IR75 groups had similar positions of functional domains and transmembrane segments (Table S1; Fig. S1; Fig. 4c).

**Fig. 3** Phylogenetic relationship of insect IRs. The tree was constructed using PhyML 3.2 under the JTT model of substitution with NNIs, based on the aligned amino acid sequences by MAFFT v7.308. Branch support value was estimated using an approximate likelihood ratio test (Chi2) and indicated (circles < 0.90). The IRs were grouped into various small clades with species-specific color patterns. Three sub-families (A-IRs, LS-IRs, and D-IRs) were distinguished based on the classification of *Drosophila* IRs. iGluRs was used as the outgroup to root the tree

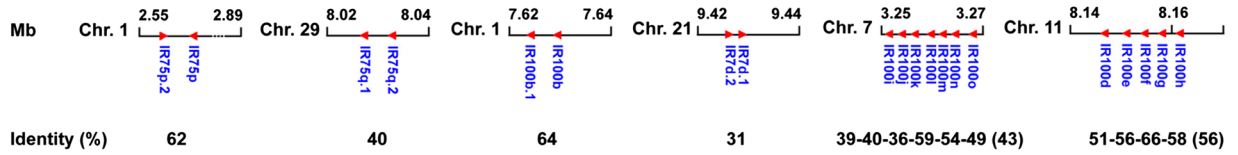


**Fig. 4** Candidate IR gene family in *S. litura*. **a** Possible duplication events of candidate IRs in *S. litura*. Transcriptional orientation of genes is indicated by red arrows. Protein sequence identities between the adjacent IRs are shown, and the identities in the brackets represent a mean value among the paralogs. **b** Phylogenetic tree and sequence lengths of *S. litura* iGluRs and IRs. The tree was constructed using PhyML 3.2, with estimated branch support values (circles < 0.90) using an approximate likelihood ratio test (Chi2). Color-coded branches represent intron numbers of IR genes, of which intron numbers of incomplete IRs are unknown and indicated in black. At the bottom of the tree, ORF length of each gene is presented. **c** Models of candidate IRs in *S. litura*. Gene structures of some representative IRs in *S. litura* are shown. Various colors mean different functional domains and transmembrane segments (S1 and S2, purple; P, green; M1, M2, and M3, red). Scale bars represent a size of 500 bases. Models of the remaining IRs of *S. litura* are shown in Fig. S1

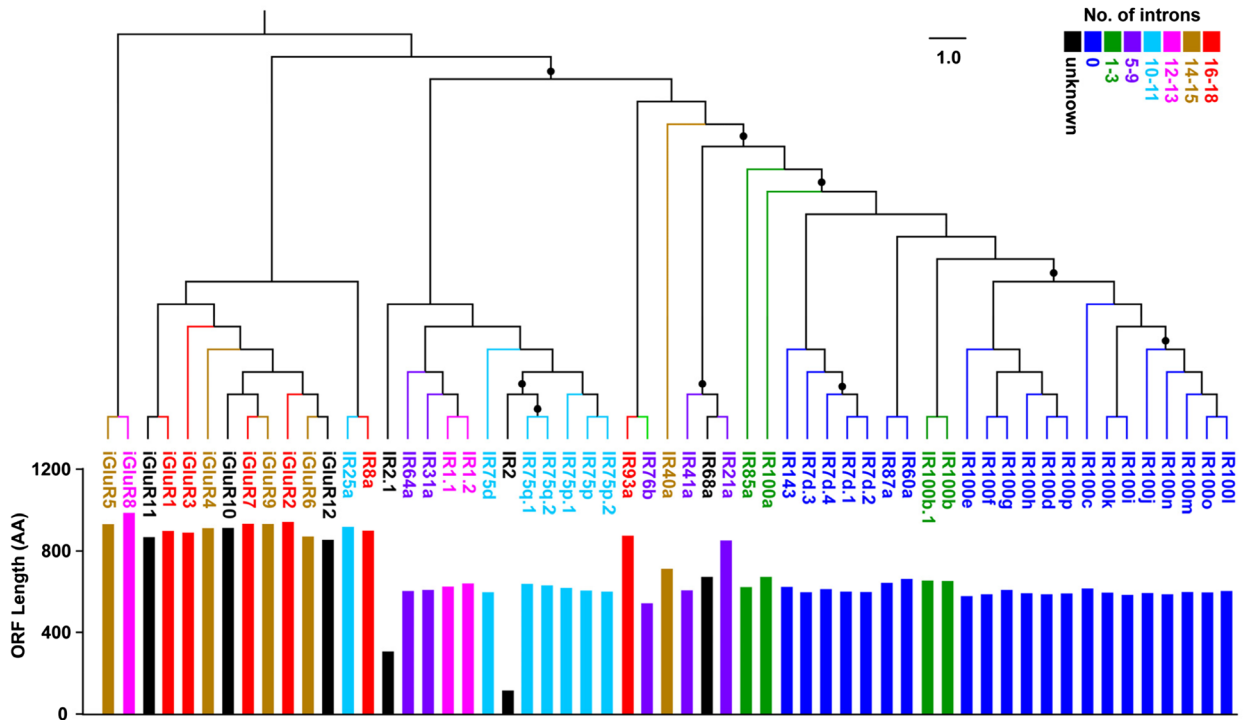
**Expression profile of candidate IRs in *S. litura***

To explore possible functional roles of *S. litura* IRs, we investigated global expression of 43 IRs (except two IR pseudogenes) and 12 iGluRs in different tissues of both sexes. In total, 13 IR genes belonging to the D-IR sub-family were not detected in all tested tissues, which was consistent with the expression of *Drosophila* D-IRs (Benton et al. 2009; Rytz et

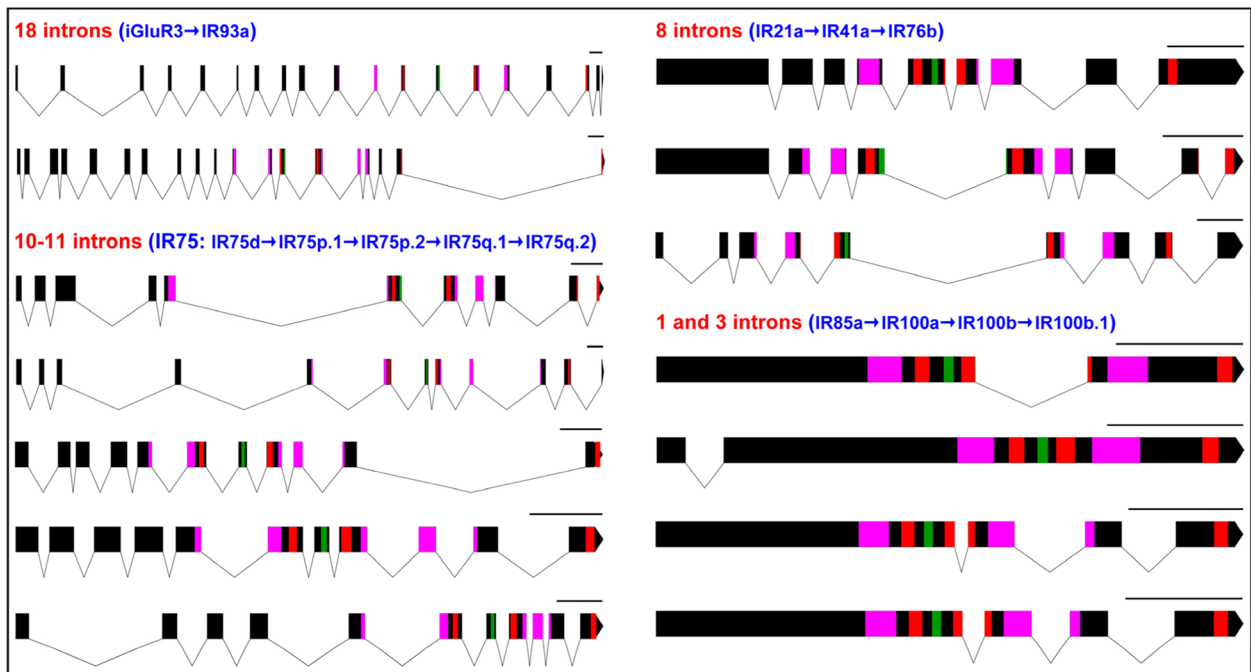
**A**



**B**

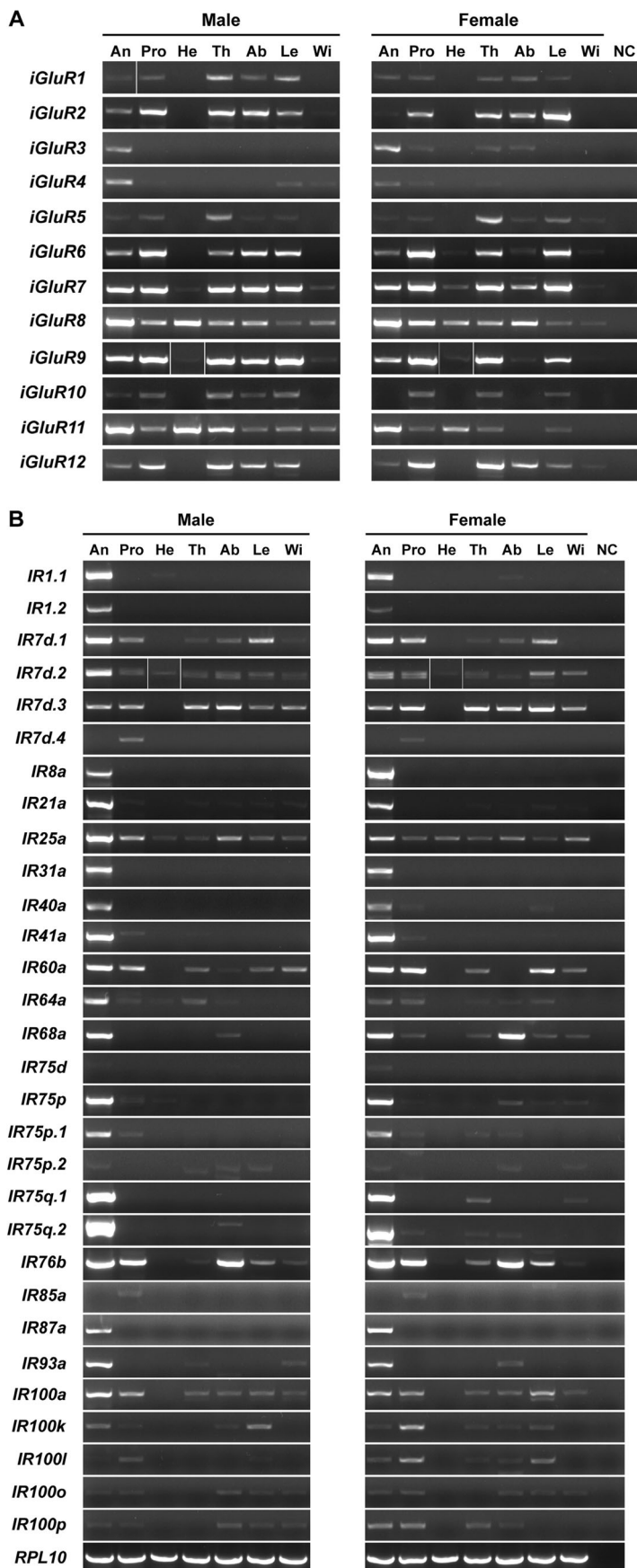


**C**





**Fig. 5** Expression profile of candidate *S. litura* IRs in different tissues of both sexes. **a** Expression of candidate SlitIGluRs. **b** Expression of candidate SlitIRs. *S. litura* RPL10 gene was used as quality and quantity control for all cDNA templates. Negative control (NC) is set using sterile water as the template. An, antennae; Pro, proboscises; He, heads without antennae and proboscises; T, thorax; Ab, abdomens; Le, legs; Wi, wings





al. 2013). Overall, 12 iGluR genes presented a broad tissue distribution, and a similar expression profile was also observed in seven D-IR members (IR7d.1, 7d.2, 7d.3, 100k, 100l, 100o, and 100p), four A-IRs (IR25a, IR60a, IR64a, and IR76b) including two co-receptors and one LS-IR member (IR100a) (Fig. 5a, b). As expected, the majority of IRs were expressed predominantly in adult antennae. Of these, IR1.2, IR8a, IR31a, and IR87a were specifically expressed in the antennae. Over half of the IRs (proboscis, 21/30 and leg, 17/30) were detected in gustatory tissues with IR7d.4 and IR85a being specific to proboscises of both sexes, although some displayed extremely low expression. Notably, one A-IR member IR68a was abundant in female abdomens, but it had low expression in male abdomens. In addition, some IRs were present in non-chemosensory tissues including the thorax, abdomens, and wings. For example, IR7d.1, IR7d.2, IR7d.3, IR25a, IR76b, and IR100a were expressed in all three non-chemosensory tissues. IR60a had expression in thorax and wings (Fig. 5b).

To unravel potential reproductive-related roles of *S. litura* IRs, we further investigated the expression profiles of 43 IRs and 12 iGluRs in reproductive systems of male and female moths, consisting of the accessory gland, ejaculatory duct, seminal vesicle, and testis of male adults as well as accessory gland, bursa copulatrix, ovary, spermathecal gland, and spermatheca of female adults (Fig. 6a). Results showed that 26 IRs and seven iGluRs were detected in at least one reproductive-related tissue. Of these, iGluR6, iGluR7, and iGluR9 as well as IR7d.1, IR7d.2, IR7d.3, IR25a, IR64a, IR76b, and IR100a were broadly expressed in tested tissues. Intriguingly, IR68a and IR75q.2 exhibited relatively high expression in ovary and accessory gland of females, respectively (Fig. 6b). The expression of IR68a was enriched in the ovary, which was consistent with its female abdomen-biased expression characteristics (Fig. 6b). Additionally, IR76b showed abundant expression in bursa copulatrix and ovary, and IR100o was highly expressed in male accessory gland (Fig. 6b).

## Discussion

Outside dipteran species (mainly *D. melanogaster* and *A. gambiae*), our ability to understand IR characteristics, evolution, and functions in other insects has been restricted to the studies on gene identification, gene numbers, phylogenetic analyses, or expression profiles (Dong et al. 2016; Feng et al. 2015; Xu et al. 2015). Little is known about IR functions and evolution in Lepidoptera and other insect orders (Gouin et al. 2017; Harrison et al. 2018; Liu et al. 2014; Olivier et al. 2011; Pearce et al. 2017; Wang et al. 2018). In the current study, we have identified and characterized the IR gene repertoire from *S. litura*, which has been earmarked as potential molecular targets for pest control, in parallel with two other

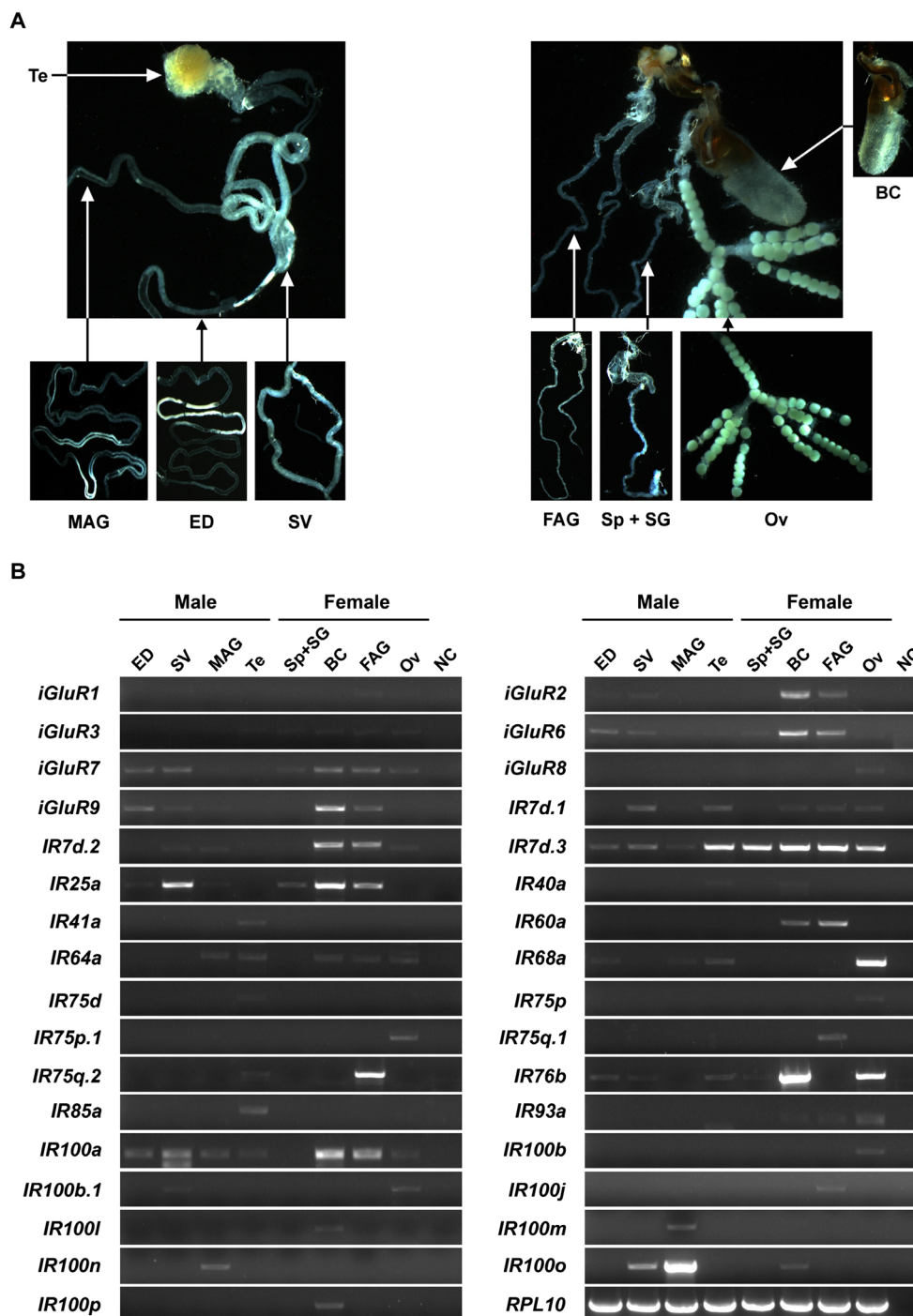
multigene families of ORs and GRs. Together, our work greatly complements the information on the chemoreceptor super-families in *S. litura* (Feng et al. 2015) or other lepidopteran species (Olivier et al. 2011; van Schooten et al. 2016).

The number of *S. litura* IRs (45) is larger compared with that of *A. mellifera* (10), *A. pisum* (11), *B. mori* (30), *D. plexippus* (32), *H. melpomene* (33), *H. armigera* (39), *H. zea* (39), *M. sexta* (34), and *T. castaneum* (23), equal to that of *A. gambiae* (46) and *S. frugiperda* (45), but smaller than that of *Aedes aegypti* (95) and *D. melanogaster* (66) (Croset et al. 2010; Gouin et al. 2017; Pearce et al. 2017; Rytz et al. 2013; van Schooten et al. 2016). Although the species-specific D-IR sub-family generally exhibits relatively low protein sequence identities across insects (Croset et al. 2010; Gouin et al. 2017; Pearce et al. 2017), a typical characteristic of gene structures, i.e., intronless, makes the identification of this sub-family easier. Based on the analyses of gene structures, several typical characteristics such as intron insertion sites, intron numbers, sequence lengths, gene organization on chromosomes, and conserved positions of functional domains, greatly assist in completing manual curation. Notably, we notice that some prior studies, showing IR identification, sequence, and phylogenetic characteristics, have some errors in full-length sequences or conclusion (Bengtsson et al. 2012; Dong et al. 2016; Feng et al. 2015; Koenig et al. 2015; Liu et al. 2014; Xu et al. 2015). Thus, our work would be helpful for the improvement of previous IR work and subsequent IR identification and annotation in other lepidopteran species, especially in moths.

In comparison with the classification of *Drosophila* IRs (Croset et al. 2010), lepidopteran species possess a specific sub-family (namely LS-IRs), described in this study and previous studies in Lepidoptera (Liu et al. 2014; Olivier et al. 2011; van Schooten et al. 2016). Of these, IR100a is Lepidoptera specific, whereas IR100b is present only in moths and IR100b.1 is restricted to noctuid moths (van Schooten et al. 2016). Exon and intron structural analyses indicate that the LS-IR (except IR1.1 and IR1.2) and D-IR sub-families have fewer introns relative to conserved A-IRs. Sequence alignment analysis reveals that *S. litura* IRs possess diverse amino acids in three conserved positions, together with diversities of expression profiles, suggesting IR diverse functional roles in this species. Our phylogenetic results show the relationships between three sub-families and intron numbers. For example, the D-IR (except IR85a) sub-family is intronless, while the A-IR sub-family in general has multiple introns, similar to *Drosophila* IRs (Croset et al. 2010). Interestingly, two groups of IR60a and IR87a that have no introns and respectively belong to the A-IR and LS-IR sub-families are close to the D-IR sub-family in phylogeny, but they show considerable expression in antennae deserving further functional studies.

The broad expression profiles of *S. litura* IRs provide insights into their functional diversities. Most of the A-IR sub-

**Fig. 6** Expression profile of candidate *S. litura* IRs in reproductive tissues of both sexes. **a** Reproductive systems of male (left) and female (right) *S. litura*. The photos were taken by light microscopy. ED, ejaculatory ducts; MAG, male accessory glands; SV, seminal vesicles; Te, testes; BC, bursa copulatrix; FAG, female accessory glands; Ov, ovaries; Sp + SG, spermathecae and spermathecal glands. **b** Expression of candidate SliGluRs and IRs. *S. litura* RPL10 gene was used as quality and quantity control for all cDNA templates. Negative control (NC) is set using sterile water as the template. The abbreviation of tissues is showed in (a)



family (28/43) display antennal expression at a high level and some (*IR1.1*, *IR8a*, *IR31a*, *IR75d*, and *IR87a*) are specifically expressed in the antennae, indicating their potential olfactory roles (Abuin et al. 2011; Benton et al. 2009; Croset et al. 2010). The result is consistent with IR tissue-expression characteristics in the noctuid moths of *H. armigera* and *Spodoptera littoralis* (Liu et al. 2014; Olivier et al. 2011). Of three co-receptors (*IR8a*, *IR25a*, and *IR76b*), *IR8a* expression is restricted to the antennae, possibly indicating that it has

extremely low or no expression in other tissues. The two other co-receptors exhibit broad expression, consistent with their designated co-receptor roles. Similar results have been observed in *H. armigera* *IR8a*, *IR25a*, and *IR76b* (Liu et al. 2014). Intriguingly, some IRs from the LS-IR and D-IR sub-families are also detected in the antennae, possibly guiding olfactory-related behaviors.

Outside olfactory roles driven by antennae, the sensing of taste is mediated primarily by gustatory-associated tissues like

proboscis, leg, mouthpart, labellum, pharynx, or wing margin as previously indicated in *A. aegypti*, *Culex quinquefasciatus*, *D. melanogaster*, *H. armigera*, *H. melpomene*, and *S. littoralis* (Koh et al. 2014; Leal et al. 2013; Liu et al. 2014; Olivier et al. 2011; Sparks et al. 2014; van Schooten et al. 2016). Our current analysis reveals that over half of SlitIRs (22/43) are expressed in proboscises or legs with involvement in taste detection. Notably, IR7d.4 and IR85a are detected only in the proboscises of both sexes, possibly indicating their involvement in feeding behaviors of adults. In addition to chemosensory tissues, SlitIRs are present in non-chemosensory organs including the thorax, abdomens, wings, and reproductive-related tissues, similar to *H. armigera* and *S. littoralis* IRs (our unpublished data; Liu et al. 2014; Olivier et al. 2011). In *D. melanogaster*, IRs have been demonstrated to participate in hearing, and also humidity and temperature sensing (Enjin et al. 2016; Knecht et al. 2016; Senthilan et al. 2012). For example, DmelIR68a expressed in antenna and head mediates moist sensing (Knecht et al. 2017). DmelIR76b is required for the detection of polyamines that are attractive egg-laying substrates and increase reproductive success of *Drosophila* species (Hussain et al. 2016). In our study, an interesting result is the high expression of *S. litura* IR68a in female ovary, as a novel candidate for female reproductive regulation. Likewise, IR75q.2 and IR76b are likely to be involved in female reproductive behaviors, while IR1000 may mediate male reproduction. Together, these results suggest that SlitIRs may have additional functions other than chemoreception, especially reproductive-associated behaviors.

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