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Genome-wide analysis reveals the expansion of Cytochrome P450 genes associated with xenobiotic metabolism in rice striped stem borer, *Chilo suppressalis*

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ABSTRACT

The Cytochrome P450 (CYP) superfamily is a large group of ancient proteins with enzymatic activities involved in various physiological processes. The rice striped stem borer, *Chilo suppressalis*, is an important insect pest in rice production. Here, we report the identification and characterization of 77 *CYP* genes from rice striped stem borer (SSB) through genome and transcriptome sequence analyses. All these *CYP* genes were confirmed by RT-PCR and direct sequencing. Twenty-eight *CYP* transcripts have full open reading frame (ORF) and four additional transcripts have a nearly full length coding region. The SSB *CYP* genes were classified into four clans, the mitochondrial, CYP2, CYP3, and CYP4. Phylogenetic analysis indicated that there was an apparent expansion of the CYP3 clan in insects. The CYP6AB subfamily of the CYP3 clan had nine members in SSB. Evolutionary analysis showed that this subfamily was expanded only in lepidopteran insects. In this study, we identified a new P450 subfamily, CYP321F, which is unique to SSB and located in the genome as tandem repeats. Our work provided a foundation for future studies on the functions and mechanism of P450s in the destructive rice pest.

1. Introduction

The Cytochrome P450 (*CYP*) gene superfamily is an ancient enzymatic system, and exists in almost all living organisms [5]. P450 (P450) enzymes are defined by the absorption of light at 450 nm by the heme cofactor, and catalyze the oxidation of a wide range of organic substances such as lipids and steroidal hormones, resulting in activation or inactivation of many endogenous and exogenous chemicals [8]. The *CYP* super gene family contains many members in an organism, and different genes are named with a *CYP* prefix, followed by a numeral for the family, a letter for the subfamily, and another numeral for the individual gene [17]. Accordingly, nomenclature and classification are based on amino acid sequence similarity. Any two P450 proteins with sequence identity greater than 40% belong to a family, and any two P450s with 55% belong to a subfamily. Different P450 proteins are also grouped into different clans, with a clan containing all P450 families with a monophyletic origin [18].

In insects, the P450 enzymes are found in virtually all tissues and perform many important functions from the synthesis and degradation of physiologically important compounds such as ecdysteroids, juvenile hormones, and pheromones to the metabolism of foreign chemicals of natural and synthetic origin [4,15,9]. Because of their diverse functions, insect *CYP* genes have attracted a great deal of attentions of scientists in different fields. The first insect *CYP* gene, *CYP6A1*, was cloned from an insecticide-resistant strain of housefly, *Musca domestica* [6]. Since then, lots of *CYP* genes were identified in various insects [1,5,10]. The sequencing of numerous insect genomes has revealed dynamic changes in the number and identity of *CYP* genes in different insects [5]. The number of *CYP* genes in sequenced insect genomes ranges from 37 in the body louse *Pediculus humanus* [12], to 160 in the dengue mosquito *Aedes aegypti* [26]. The variation in the number of genes and sequence diversification in many *CYP* homologues are likely due to functional adaptation to specific insect ecological environments.

In plant feeding insects, P450 enzymes are particularly important in detoxification of exogenous compounds including plant defensive secondary metabolites and insecticides [2,13,23]. Although many other enzyme systems participate in the detoxification of plant

Abbreviations: P450, cytochrome P450 protein; *CYP*, cytochrome P450 gene; SSB, striped stem borer (SSB).

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allelochemicals, P450s play a central role in degradation of these toxic chemicals once they are ingested into the insect digestive system. The essential roles of P450s in detoxification of plant defense chemical can be seen by the fact that silencing of a P450 gene in cotton bollworm (*Helicoverpa armingera*) can enhance plant resistance to this insect [14]. P450s are also the major factors responsible for insecticide resistance in pests [8,24].

The rice striped stem borer (SSB), *Chilo suppressalis* Walker, is one of the most important insect pests of rice worldwide. The SSB larvae feed within plant stems, causing severe crop losses annually in China and other parts of Southeast Asia. At present, the insect pest is mainly controlled through applying insecticides. However, insecticides are not always effective due to the narrow window for damage control between hatching and penetration into the plant stem of rice stem larvae. Extensive application of pesticides also causes environmental contamination, and result in insecticide resistance in insects. The objective of this study is to characterize *CYP* genes in SSB through genome sequencing and transcriptomic characterization. Knowledge of SSB *CYP* genes may lead to novel practical applications in managing this insect pest since many P450 proteins are involved in pesticide resistance and in plant–insect interactions.

2. Material and methods

2.1. Insects

The SSB colony was derived from a collection from rice fields of Wenling county, Zhejiang province, China (28°3′N, 121°3′E) in 2010. The colony was maintained in the laboratory on rice seedlings at 28 ± 1 °C under a 16-h photoperiod and >80% relative humidity.

2.2. Total RNA isolation and cDNA synthesis

Fourth instar larvae (~10 mg for each larva) were frozen with liquid nitrogen and homogenized in a tissue grinder. Then, 1 ml trizol reagent was added to homogenized insects. Total RNA was isolated following the manufacturer's recommended procedure (Invitrogen, USA). Potential genomic DNA contamination was removed from the RNA samples with RNase-free DNase I using a DNA-free kit (Roche Diagnostic, Mannheim, Germany). RNA purity was measured using a Nano-drop spectrophotometer and the integrity was checked on a formaldehyde 1.2% agarose gel. The first-strand of cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Kyoto, Japan).

2.3. PCR

PCRs were used to validate the transcripts of identified *CYP* genes. The primers used for PCR were designed using Primer Premier 5.0 [21]. PCRs were conducted in a 25 μL reaction mixture containing 2.5 μL of 10 × r*Taq* buffer (Mg^{2+} free), 2 μL of $MgCl_2$ (25 mmol/L), 2 μL of dNTP (2.5 mmol/L), 1 μL of forward and 1 μL of reverse gene special primers (10 mmol/L), 0.5 μL of cDNA template, 0.25 μL of r*Taq* polymerase (5 U/μL) (Takara, Kyoto, Japan). The PCR program included an initial denaturation step of 3 min at 94 °C, followed by 31 cycles of 94 °C for 30 s, annealed at 50–60 °C for 30 s (depending on different *CYP* genes), and 72 °C for 1 min; then a final extension at 72 °C for 10 min. PCR products were checked by electrophoresis on a 1.5% w/v agarose gel in TAE buffer (40 mmol/L Tris–acetate, 2 mmol/L $Na_2EDTA{\cdot}2H_2O$). The resulting bands were visualized by ethidium bromide staining. The target bands were purified form the gel and cloned into a pGEM-T easy vector (Invitrogen, USA). Recombinant plasmids were sequenced with primers for both strands (GeneScript, Nanjing, China).

2.4. Rapid amplification of cDNA ends (RACE)

RACE strategy was adopted to obtain the 3′ and 5′ ends of *CYP* genes. The 3′ and 5′ cDNA templates for RACE–PCR were synthesized using SMARTer™ RACE cDNA Amplification Kit (Takara, Kyoto, Japan). RACE–PCRs were performed in accordance with standard procedures with 2 μM of each primer and 2 U Takara Ex-Taq DNA polymerase at annealing temperatures of 55–65 °C, depending on the target gene. Nest RACE–PCRs were carried out to obtain better amplification results. End-to-end PCRs were used to confirm the assembled full-length transcripts. All PCR products were checked and sequenced as mentioned previously.

2.5. Sequence analysis

The putative SSB *CYP* genes were obtained by searching the draft genome sequence and a transcriptome. The genes were annotated with BLASTX [3]. To remove redundancy, all putative *CYP* genes were re-assembled using the Cap3 software [11]. After assembly, the putative genes were validated through end to end PCR amplification. The Standardized Cytochrome P450 Nomenclature Committee assigned specific names for these *CYP* genes [16]. The chromosomal locations and gene structures were obtained by analyzing the draft rice striped stem borer genome sequences.

2.6. Phylogenetic analysis

Evolutionary trees were constructed using two methods. One is the Bayesian inference with MrBayes [22]. Another is the maximum parsimony with MEGA 5 software [27]. The inferred phylogeny was tested by bootstrap analysis with 1000 replicates. To ensure the reliability, only those P450 proteins with >300 amino acids were used for phylogenetic analysis. The sequence alignments were performed using Clustal Omega online (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.7. Molecular weight, pI, and signal anchor sequence prediction

Molecular weight and pI calculation was carried out using a tool in the ExPASy server [7]. Signal anchor sequences were determined using signal [20].

3. Results and discussion

3.1. Genome-wide identification of CYP genes in the rice stem borer

We annotated the draft SSB genome sequence (Gene accession number: ANCD00000000), yielding 86 putative *CYP* genes. In addition, we also generated a SSB transcriptome (SRA060774), and identified 109 *CYP* transcript fragments. The putative *CYP* gene sequences of the two datasets were pooled together and re-assembled using the CAP3 software. After removing redundancy, we obtained 77 unique putative *CYP* genes or gene fragments in the rice stem borer. These putative *CYP* genes or gene fragments were further processed as described in subsequent sections.

The number of *CYP* genes varies widely in different insect species. In the insect species with whole genome sequenced, there are 37, 143, 105, 160, 88, 46, 84, 88, and 78 *CYP* genes identified from *Pediculus humanus*, *Tribolium castaneum*, *Anopholes gambiae, Aedes aegypti*, *Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori*, *Danaus plexippus*, and *Trialeurodes vaporariorum*, respectively [19,28]. Since we performed an exhaustive search from both the

genome and transcriptome sequences, most *CYP* genes in the rice stem borer, if not all, should have been discovered. Therefore, the 77 *CYP* genes reported here should provide a foundation for characterization the network of P450 functions in this insect.

3.2. RACE amplification to clone 5′- and 3′-ends of CYP transcripts

RACE was carried out to obtain full-length transcripts of *CYP* genes. RACE results were confirmed by end-to-end PCRs and sequencing (Fig. 1). Through RACE, 28 transcripts contain full-length open reading frames and five contain nearly full-length open reading frames. The remaining 44 transcripts were still missing a portion of the sequence (Fig. S1).

3.3. Full length or nearly full length putative P450s from the rice stem borer

The 28 putative full length and four nearly full length proteins are listed in Fig. S2. All full length proteins have a putative signal anchor sequence at the N-termini, indicating that these proteins are likely integrated into membrane for functions (Table 1). Full length proteins contain 413 to 558 amino acids with predicted molecular weights 47.5 to 63.4 kDa. The molecular sizes are comparable to P450 proteins reported in other species [5]. The predicted pIs ranged from 6.6 to 9.3, with 26 proteins with pI above 8, indicating that the majority of SSB P450s may perform functions in basic environments. First BLAST hits in GenBank are sequences all from Lepidopterans with 50–89% sequence identity and *E*-values from 0.0 to $4e^{-168}$. The very low *E*-values indicate that these Lepidopteran proteins are orthologs and may perform similar functions. Sequence alignment analysis revealed that all 28 full length and the four nearly full length proteins except CYP4AU11contain the consensus FxxGxxxCxG (Fig. S3), which is characteristic of P450s [25], indicating that all identified SSB P450s are likely functional.

3.4. Phylogenetic analysis of insect P450s

Phylogenetic analysis of SSB P450s and P450s from other insects extracted from GenBank was carried out using Bayesian inference and maximum parsimony, both of the methods yielded identical topologies. The Bayesian tree with all analyzed sequences is shown in Fig. S4, in which branch lengths were proportional to the amounts of changes occurring in each lineage. According to their evolutionary relationship, insect P450s were divided into four clans: Mitochondrial, Clan2, Clan3, and Clan4; and the identified SSB P450s are distributed in all four clans with the majority in clan3.

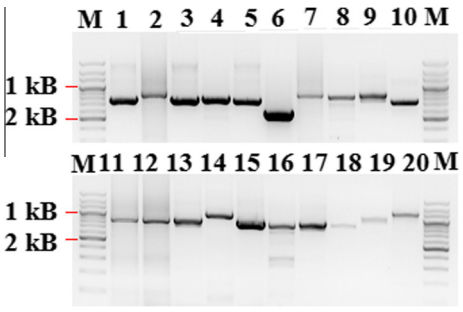

Fig. 1. Confirmation of RACE-identified full length transcripts by end to end PCR. Only representative transcripts are shown. The letter 'M' represents DNA size marker (200 bp ladder). Lanes 1 to 20 are PCR products of transcripts CYP6AE60, CYP9A68, CYP9A69, CYP6CV4, CYP6CV5, CYP6CT1, CYP6AB45, CYP6AB46, CYP6AB49, CYP6AB52, CYP4G90, CYP4G91, CYP4L27, CYP4M38, CYP4M39, CYP367A9, CYP341A15, CYP306A1, CYP307A2, and CYP18A1, respectively.

The mitochondrial clan of P450s is only found in animals. In the rice striped stem borer, nine identified P450s belong to the mitochondrial clan, which can be further classified into seven families and nine subfamilies (Fig. 2). Seven P450s belong to Clan 2. Clan3 is the largest, containing 29 members, which are further classified to seven families and 11 subfamilies. Clan 4 was the second largest clan, containing 11 members, consisting of five families and ten subfamilies. Among the members in Clan 4, CYP3051 represented a new family.

To illustrate the relationship between the SSB P450s and P450s from other insects, a portion of the phylogenetic tree (Fig. S4) containing the subfamily CYP6AB is shown in Fig. 3. Nine member of this subfamily from the rice striped stem borer are distributed in three branches. Two branches each contains four P450s from the rice striped stem borer as a single cluster, indicating that these P450s in a cluster were originated after the rice striped stem borer speciation likely through gene duplications and diversification.

The expansion of genes in the CYP6AB subfamily in clan 3 is interesting. An analysis of clan 3 in other lepidopteran species revealed a similar expansion phenomenon, with 10, seven, and six genes in *Heliconius melpomene, Amyelois transitella*, and *Lymantria dispar*, respectively. No expansion of this subfamily has been observed in other non-lepidopteran insect species (data not shown). This expansion may indicate the adaption of lepidopterans to common ecological and/or physiological environments.

3.5. Tandem repeat of CYP321F genes

A new subfamily, named CYP321F, was identified in clan3 among the identified P450s in the rice striped stem borer genome. This subfamily contained four members, CYP321F1–4. Three members, CYP321F1, CYP321F2 and CYP321F4, were located adjacently in the genome (Fig. S5). Phylogenetic analysis indicated that the new subfamily CYP321F is closely related to CYP321B1 of *B. mori* and CYP321A1 of *Helicoverpa zea* (Fig. S6). Sequence alignments showed that 37 amino acids in the SRS regions were completely conserved and 20 amino acids were highly conserved (Fig. S7). The newly identified multi-member subfamily may represent an unique adaption for the rice striped stem borer since it only shares less than 55% amino acid sequence identity with P450s from other insects (Table 1), indicating functional diversification after the rice striped stem borer speciation. The exact functions of this new subfamily remain to be delineated. One possible function is involvement in detoxification of rice defense chemicals, a mechanism formed during the co-evolution between the stem borer and rice plants.

In summary, we identified 77 *CYP* genes or gene fragments in an important rice insect pest, the rice stem borer. Phylogenetic analysis indicated that there was an apparent expansion of clans in this insect as well as in other insect herbivores. We hypothesize that the formation and expansion of the *CYP* genes in the rice striped stem borer have been a result as adaption to rice defense secondary metabolites during the long course of co-evolution. The insects may have benefited from this expansion by obtaining effective tools to combat toxic secondary metabolites from host plants.

Insecticides are made by human being to control insect pests, which are the substrate of P450 enzymes. It has been reported that the overexpression of some CYP genes conferred insecticide resistance in many insects. In the CYP3 clan, the CYP6 and CYP9 family were reported to participate in the oxidation of chemical pesticides ([29,24]. However, insecticide has been used for about only seventy years. In such a short time, insecticides should have little influence on the evolution of insect CYP genes at genome level. It is unlikely to be an important factor driving the expansion of the CYP genes in insect herbivores. A reasonable explanation is that co-evolution of plant defense and herbivores adaptation induces

Table 1
Characteristics of full or nearly full length P450 proteins.

Protein name	Gene ID	Genbank code	Amino acids	Signal anchor	Molecular weight	pI	Genbank first hit code	Identity (%)/*E*-value	Protein name, organism
CYP4AU10	CSUCYP55	KF701166	498	Yes	58119.57	9.31	AGO62005	50/9e^{-180}	CYP321A7, *Spodoptera frugiperda*
CYP4AU11	CSUCYP56	KF701167	414*	?	–	–	XP_004931776	69/0.0	CYP4C1, *Bombyx mori*
CYP4G90	CSUCYP46	KF701159	558	Yes	63449.35	8.73	ADE05582	87/0.0	CYP4G4, *Manduca sexta*
CYP4G91	CSUCYP47	KF701160	540	Yes	62068.78	8.82	ACZ97414	73/0.0	CYP4G48, *Zygaena filipendulae*
CYP4L27	CSUCYP49	KF701162	491	Yes	56473.79	8.78	CAX94851	61/0.0	CYP4L18, *Cnaphalocrocis medinalis*
CYP4M38	CSUCYP50	KF701163	521	Yes	60260.14	8.33	AAM54723	57/0.0	CYP4M7, *Helicoverpa zea*
CYP4M39	CSUCYP51	KF701164	502	Yes	57782.09	7.16	ADE05575	59/0.0	CYP4M1, *M. sexta*
CYP6AB45	CSUCYP9	KF701129	512*	Yes	–	–	AFP20592	68/0.0	CYP6AB14, *S. littoralis*
CYP6AB48	CSUCYP12	KF701132	491*	?	–	–	AFP20592	65/0.0	CYP6AB14, *S. littoralis*
CYP6AB46	CSUCYP10	KF701130	514	Yes	58464.72	8.61	AFP20592	66/0.0	CYP6AB14, *S. littoralis*
CYP6AB47	CSUCYP11	KF701131	514	Yes	58808.21	8.31	BAM73813	68/0.0	?, *B. mori*
CYP6AB49	CSUCYP13	KF701190	527	Yes	60478.00	8.92	BAM73813	64/0.0	?, *B. mori*
CYP6AB50	CSUCYP14	KF701133	485	Yes	56166.56	9.13	AFP20592	57/0.0	CYP6AB14, *S. littoralis*
CYP6AB51	CSUCYP15	KF701134	509	Yes	58734.05	8.94	AFP20592	62/0.0	CYP6AB14, *S. littoralis*
CYP6AB52	CSUCYP16	KF701135	508	Yes	59183.65	9.18	BAM73813	61/0.0	?, *B. mori*
CYP6AB53	CSUCYP17	KF701136	464	Yes	53739.29	6.74	ABL60877	56/0.0	CYP6AB7, *Depressaria pastinacella*
CYP6AW1	CSUCYP20	KF701139	494	Yes	56525.94	9.18	XP_004930467	73/0.0	CYP6J1, *B. mori*
CYP6CT1	CSUCYP21	KF701140	509	Yes	59646.11	8.85	EHJ78442	65/0.0	CYP6CT1, *Danaus plexippus*
CYP6CV4	CSUCYP22	KF701141	503	Yes	58166	8.86	CAZ65618	61/0.0	?, *C. medinalis*
CYP6CV5	CSUCYP23	KF701142	507	Yes	58208.95	8.93	CAZ65618	66/0.0	?, *C. medinalis*
CYP9A68	CSUCYP26	KF701145	531	Yes	61832.06	8.64	BAM73827	65/0.0	?, *B. mori*
CYP9A69	CSUCYP27	KF701146	532	Yes	61372.61	8.39	ACJ05915	63/0.0	?, *B. mandarina*
CYP18A1	CSUCYP1	KF701122	541	Yes	61848.25	8.98	NP_001077078	79/0.0	CYP18A1, *B. mori*
CYP301A1	CSUCYP73	KF701181	468*	?	–	–	EHJ78478	89/0.0	?, *Danaus plexippus*
CYP306A1	CSUCYP7	KF701127	478	Yes	54757.25	6.58	ACM45975	80/0.0	CYP306A1, *S. littoralis*
CYP307A2	CSUCYP8	KF701128	540	Yes	61370.54	7.62	ACY92457	78/0.0	CYP307A1, *S. littoralis*
CYP321F1	CSUCYP35	KF701152	498	Yes	57872.18	9.21	AGO62009	50/2e^{-178}	CYP321B1, *S. frugiperda*
CYP321F2	CSUCYP36	KF701153	498	Yes	58119.57	9.31	AGO62005	50/9e^{-180}	CYP321A7, *S. frugiperda*
CYP321F3	CSUCYP37	KF701154	497	Yes	57494.54	8.58	ADA68175	54/0.0	CYP321B1, *S. litura*
CYP338A1	CSUCYP41	KF701157	413	Yes	47493.59	8.61	AFP20586	59/4e^{-168}	CYP338A2, *S. littoralis*
CYP341A15	CSUCYP60	KF701170	517	Yes	59425.23	9.08	XP_004926524	65/0.0	CYP4V2-like, *B. mori*
CYP367A9	CSUCYP67	KF701176	506	Yes	58037.89	8.97	AFP20601	59/0.0	CYP367A6, *S. littoralis*

* A few residues truncate at the N-terminal according to Genbank alignment with known proteins. The symbol '–' represents 'not determined due to sequence truncation.

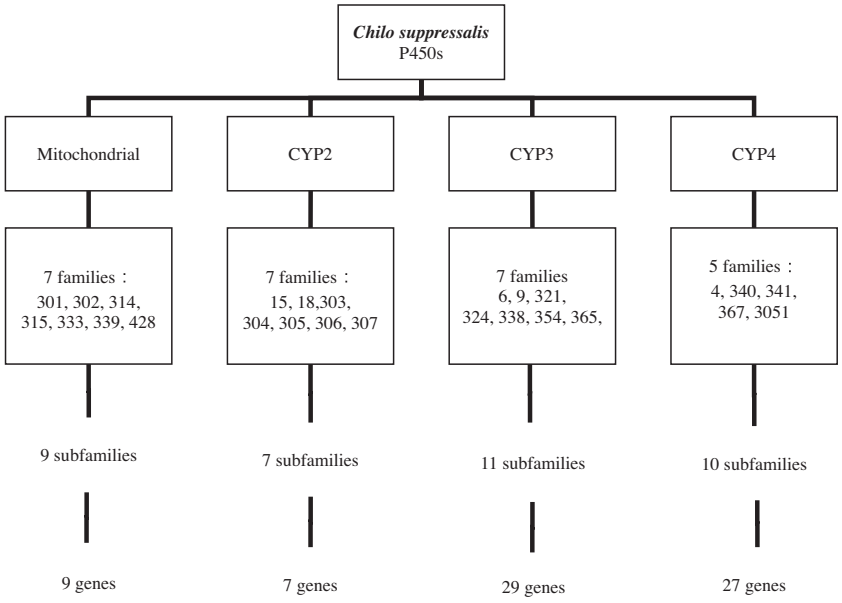

Fig. 2. Distribution of P450s from the rice striped stem borer among four clans: mitochondrial, Clan2, Clan3, and Clan4. The four clans can be further subdivided into 26 families, and 37 subfamilies.

the expansion of some insect CYP genes. Among these positive selected CYP genes, some happened to can metabolize insecticides because they have broad substrates. A strong evidence to support this hypothesis is that CYP6, CYP9 family and CYP6AB subfamily

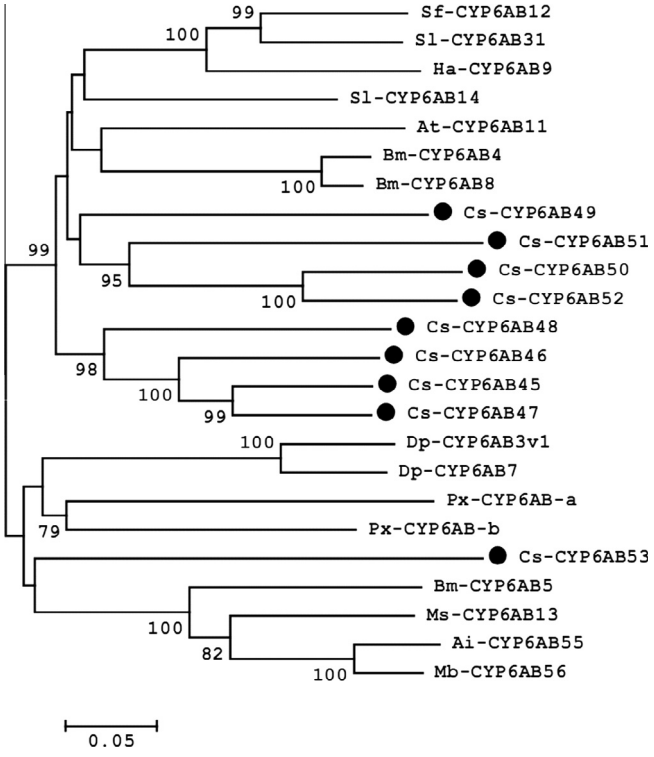

Fig. 3. Phylogenetic relationship of subfamily CYP6AB from different species. Nine members from the rice striped stem borer (●, Cs-before the name), three from *Bombyx mori* (**Bm-**), three from *Depressaria pastinacella* (**Dp-**), two from *Plutella xylostella* (**Px-**), two from *Spodoptera littoralis* (**Sl-**); one from *Manduca sexta* (**Ms-**), one from *Agrotis ipsilon* (**Ai-**), one from *Mamestra brassicae* (**Mb-**), one from *Helicoverpa armigera* (**Ha-**), one from *Amyelois transitella* (**At-**), and one from *Spodoptera frugiperda* (**Sf-**).

involve in metabolizing of both plant secondary metabolites and insecticides [2,30]). The identification and characterization of the *CYP* genes from the rice striped stem borer provide a foundation for future studies on the functions and biological significance of specific *CYP* genes in this destructive pest of rice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.045.

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