Eur. J. Entomol. **108**: 164–168, 2011
http://www.eje.cz/scripts/viewabstract.php?abstract=1600
ISSN 1210-5759 (print), 1802-8829 (online)

Universal primers for amplifying the complete coding sequence of cytoplasmic heat shock protein 90 (HSP90) in Lepidoptera

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Key words. Universal primer, Lepidoptera, coding sequence, untranslated region, HSP90, RACE

Abstract. Using sequence alignment, a conserved domain in the 3' untranslated region (UTR) of the cytoplasmic heat shock protein 90 (HSP90) of Lepidoptera was found. This region is highly variable in other insect groups. Furthermore, universal primers were designed to amplify the complete coding sequence (CDS) of HSP90 from total genomic DNA in Lepidoptera, avoiding the commonly used reverse transcription-polymerase chain reaction (RT-PCR) and 3', 5'-rapid amplification of cDNA ends (RACE) methods based on cDNA. These primers amplified a fragment of about 2.25 kb in the 11 species tested, which represent seven different families of Lepidoptera, including moths and butterflies. The results suggest that the conserved domain of 3'UTR is universal in Lepidoptera and these primers successfully amplify the complete CDS of cytoplasmic HSP90 from genomic DNA.

INTRODUCTION

Heat shock proteins 90 (HSPs90) are among the most abundantly expressed stress proteins and are recorded in all life stages. They play significant roles in the activation and regulation of numerous client proteins critical for diverse functions (Itoh et al., 1993; Izumoto & Herbert, 1993; Gass et al., 1994; Rutherford & Lindquist, 1998; Furay et al., 2006; Johnson & Brown, 2009). Previous studies show that HSPs90 are important in the development and adaptability of insects, for example, in morphogenesis (Rutherford & Lindquist, 1998; Gunter & Degnan, 2007) and resistance to pesticides (Skandrani et al., 2006; Eder et al., 2009). Moreover, HSPs90 are used in systematic and population genetic studies of insects (Breglia et al., 2007; Fukuda & Endoh, 2008; Feng et al., 2009).

For molecular studies of HSPs90 it is necessary to identify long stretches of DNA sequences as complete coding sequence (CDS) to draw conclusions. The complete CDS of HSP90 is commonly obtained using reverse transcription-polymerase chain reaction (RT-PCR) and 3', 5'-rapid amplification of cDNA ends (RACE) from cDNA templates (Sonoda et al., 2006; Li et al., 2009; Feng et al., 2010). Both these methods are difficult and costly.

Lepidopteran cytoplasmic *hsp90* is a single-copy gene without introns (Landais et al., 2001; Sonoda et al., 2006). After aligning existing sequences of cytoplasmic HSP90, a highly conserved region was located in the 3' untranslated region (UTR) in Lepidoptera, which is not present in the alignments of Diptera and Hymenoptera (data not shown).

Furthermore, a set of universal primers were designed for amplifying the gene that spanned the complete CDS and partial 3'UTR region. The primers successfully amplified specific cytoplasmic *hsp90* sequences from genomic DNA templates in all of the 11 species, representing seven lepidopteran families, tested.

MATERIAL AND METHODS

Primer design

The following *hsp90* sequences in the GenBank were used in the primer design: *Bombyx mori* (Linnaeus) (Bombycidae) (NM_001043411), *Chilo suppressalis* (Walker) (Crambidae) (AB206477), *Plutella xylostella* (Linnaeus) (Plutellidae) (AB214972), *Spodoptera exigua* (Hubner) (Noctuidae) (FJ524853), *Omphisa fuscidentalis* (Hampson) (Crambidae) (EF523380), *Antheraea yamamai* (Guerin-Meneville) (Saturniidae) (AB176669), and *Mamestra brassicae* (Linnaeus) (Noctuidae) (AB251894). The sequences were aligned using CLUSTAL W as implemented in MEGA 4.0 (Tamura et al., 2007) with default parameters.

DNA extraction and primer evaluation

Three individuals of each of 11 species from seven families of Lepidoptera, including both moths and butterflies, were used to evaluate the primers (Table 1). Genomic DNA was extracted from each individual using EasyPure Genomic DNA Extraction Kit (TransGen, Beijing, China), which purifies DNA using proteinase K digestion and silica-membrane technology.

PCR amplifications, sequencing and sequence confirmation

PCR reagents (25 µl) contained 300 ng of template DNA, 0.5 µl of each primer (10 mM), 3 µl of dNTP mixture containing 2.5 mM of each dNTP, 2.5 µl of 10 × reaction buffer and 1 U of High Fidelity Expand *Taq* polymerase (TransGen). The PCR

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TABLE 1. Lepidopteran species used to evaluate the universal primers and related information on their *hsp90*s.

Family name	Species name	Species origin	Primers	AA	MW (kDa)	pI
Noctuidae	*Helicoverpa armigera*	Laboratory rearing	L90F1/ L90R1	717	82.59	4.71
	Helicoverpa assulta	Laboratory rearing	L90F1/ L90R1	717	82.19	4.85
	***Mythimna separata*	Wild	L90F1/ L90R1	717	82.56	4.71
	Spodoptera litura	Wild	L90F1/ L90R1	717	82.60	4.73
	**Spodoptera exigua*	Wild	L90F1/ L90R1	717	82.62	4.73
Pyralidae	*Ostrinia furnacalis*	Wild	L90F1/ L90R1	716	82.41	4.75
Geometridae	*Exangerona prattiaria*	Wild	L90F2/ L90R1	716	82.28	4.75
Papilionidae	*Papilio memnon*	Wild	L90F1/ L90R1	717	82.44	4.74
Plutellidae	**Plutella xylostella*	Laboratory rearing	L90F1/ L90R1	717	82.37	4.70
Nymphalidae	*Argynnis paphia*	Wild	L90F2/ L90R1	718	82.45	4.76
Pieridae	*Gonepteryx amintha*	Wild	L90F2/ L90R1	718	82.56	4.68

AA – the number of deduced amino acids; MW – molecular weight; pI – isoelectric point; * indicates that the former submission of the *hsp90* sequence to GenBank is the same as the sequence obtained in this study, which was not submitted; ** indicates that the former submission of the *hsp90* sequence to GenBank is different from the sequence obtained in this study, which was submitted.

reaction consisted of an initial denaturation step (94°C for 4 min) followed by 40 cycles of 94°C for 10 s, 50–52°C for 1 min, 68°C for 2.5 min, and a final extension step (72°C for 10 min). Targeted PCR bands were purified and cloned using the pEASY-T3 Simple Cloning Vector (TransGen). Three positive clones of each insect were sequenced using a 3730XL sequencer based on the Sanger method (BioSune, Beijing, China). Nucleotide sequences were translated into amino acids to confirm translation and all sequences identified by BLAST searches implemented in National Center for Biotechnology Information (NCBI) (http://www. ncbi.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Primer design and evaluation

A highly conserved region of aligned lepidopteran cytoplasmic *hsp90* sequences occurred in the 3' UTR (Fig. 1B). Furthermore, the following set of primers were designed: L90F1 (forward primer): 5'-AMAATGCCBGAAGRDATGC-3; L90F2 (forward primer): 5'-AMAATGCCBGAAGRDATGG-3; and L90R (reverse primer): 5'-GAACTAAATCAGTCTTTGG-3. Primer L90F1 or L90F2, located at the 5' end (Fig. 1A), contained the start codon and primer L90R1 was located in the 3' UTR region. Theoretically, these primers could amplify a 2.25 kb fragment that spans the complete CDS and part of the 3'UTR region of *hsp90*.

The genomic DNA of *Helicoverpa armigera* was used as a reference to test for optimum PCR conditions. Because a fragment of approximately 2.25 kb was to be amplified, the cycling conditions were similar to those developed for Long-PCR (Cheng et al., 1994). The results of a range of annealing temperatures and different amounts of genomic DNA are summarized in Fig. 2A, B and the amplification condition detailed in the Material and Methods were chosen. PCR amplification of 33 genomic DNA samples of 11 species from seven families of the Lepidoptera were performed. Although some weak nonspecific amplifications occurred, the targeted fragments in 11 species were successfully amplified (Fig. 2C). A very weak band of the targeted fragment was observed in *Gonepteryx amintha*, probably because of poor PCR amplification efficiency. Nevertheless, the specific sequence was obtained after DNA purification, cloning, and sequencing.

Identification of sequences

Primers were designed and 11 sequences successfully obtained. Their characteristics (molecular weights, isoelectric points, and number of encoded amino acids) are presented in Table 1. BLAST searches were employed to confirm sequence

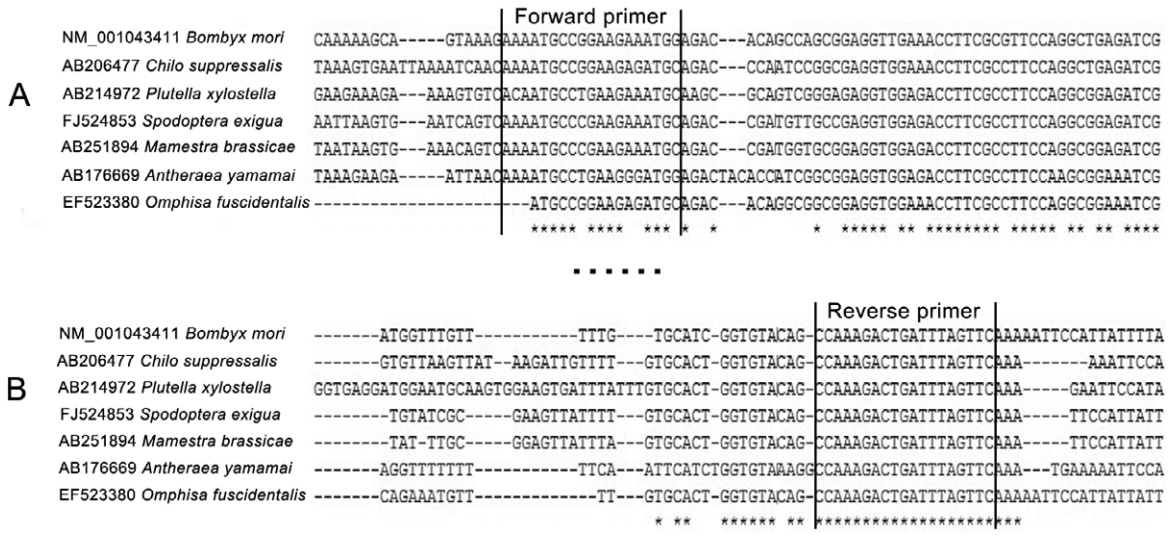

Fig. 1. The alignments of (A) the 5' end and (B) 3' UTR region of the heat shock protein 90 (HSP90) gene from Lepidoptera. Symbols: "–" represents a gap in the alignments, "*" identical sites in the alignments.

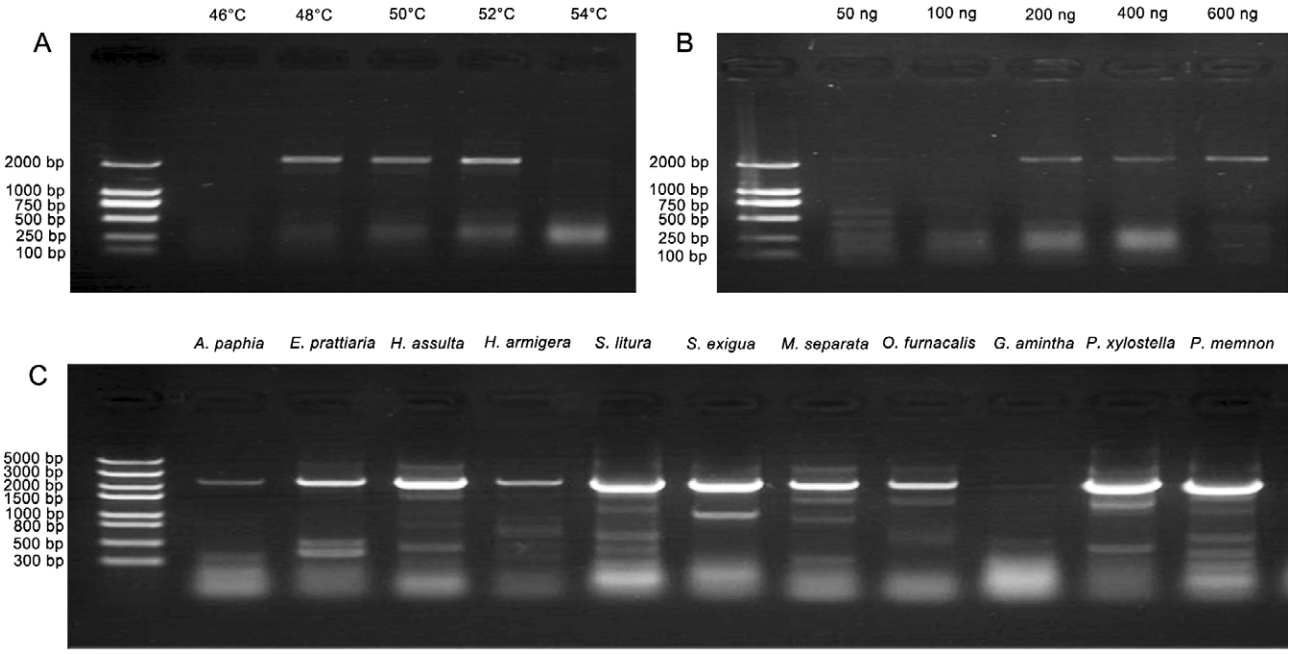

Fig. 2. Photographs of gels showing the effects of annealing temperatures and quantity of template DNA on the efficiency with which the *hsp90* sequence was amplified for 11 lepidopteran species. Analysis of the effect of (A) the different annealing temperatures and (B) the different amounts of DNA in *H. armigera*, and (C) the result of the amplification in the 11 species. Refer to Table 1 for generic names of species examined.

orthology. Both nucleotide and deduced amino acid sequences were tested. All sequences obtained with these primers were very similar to cytoplasmic *hsp90* of other lepidopteran species (Table 2). Moreover, the five conserved signatures of the HSP90 family (Gupta, 1995) and the conserved pentapeptide (MEEVD) of cytoplasmic HSP90 (Terasawa et al., 2005) were found in the alignments of the amino acid sequences (Fig. 3). The above analysis confirmed that the cytoplasmic *hsp90* sequences were amplified by these primers. The nucleotide sequences obtained in this study were deposited in GenBank under accession numbers GU230732–GU230740.

Specificity of Lepidoptera *hsp90* gene and universal primers

One intron is located directly upstream from the start codon of cytoplasmic *hsp90* in Diptera, Hymenoptera, and Coleoptera (Blackman & Meselson, 1986; Benedict et al., 1996; Kurzik-Dumke et al., 1996; Konstantopoulou & Scouras, 1998). This intron regulates the basal transcription of the cytoplasmic *hsp90* at normal physiological temperatures (Lange et al., 1997). However, this intron is missing in the cytoplasmic *hsp90* of Lepidoptera (Landais et al., 2001; Sonoda et al., 2006).

In addition, the cytoplasmic *hsp90* of Lepidoptera has a conserved 3'UTR region, which is not present in at least two other

TABLE 2. Results of BLAST searches of *hsp90* sequences.

Species	Closest species	Query coverage (%)	E value*	Similarity(%)
Helicoverpa armigera	*Helicoverpa zea*[N]	100	0	98
	Helicoverpa zea[P]	100	0	98
Helicoverpa assulta	*Helicoverpa zea*[N]	95	0	97
	Helicoverpa zea[P]	100	0	98
Mythimna separata	*Mythimna separata*[N]	95	0	97
	Mamestra brassicae[P]	100	0	98
Spodoptera litura	*Spodoptera frugiperda*[N]	100	0	96
	Spodoptera frugiperda[P]	100	0	99
Ostrinia furnacalis	*Loxostege sticticalis*[N]	94	0	90
	Chilo suppressalis[P]	100	0	97
Exangerona prattiaria	*Spodoptera frugiperda*[N]	100	0	84
	Mamestra brassicae[P]	100	0	94
Papilio memnon	*Spodoptera frugiperda*[N]	100	0	85
	Spodoptera frugiperda[P]	100	0	94
Argynnis paphia	*Spodoptera frugiperda*[N]	100	0	85
	Spodoptera frugiperda[P]	100	0	96
Gonepteryx amintha	*Chilo suppressalis*[N]	95	0	83
	Bombyx mori[P]	100	0	93

[N]Shown is the BLAST search with nucleotide sequence; [P]Shown is the BLAST search with amino acid sequence; *low E values indicate high reliability of BLAST results.

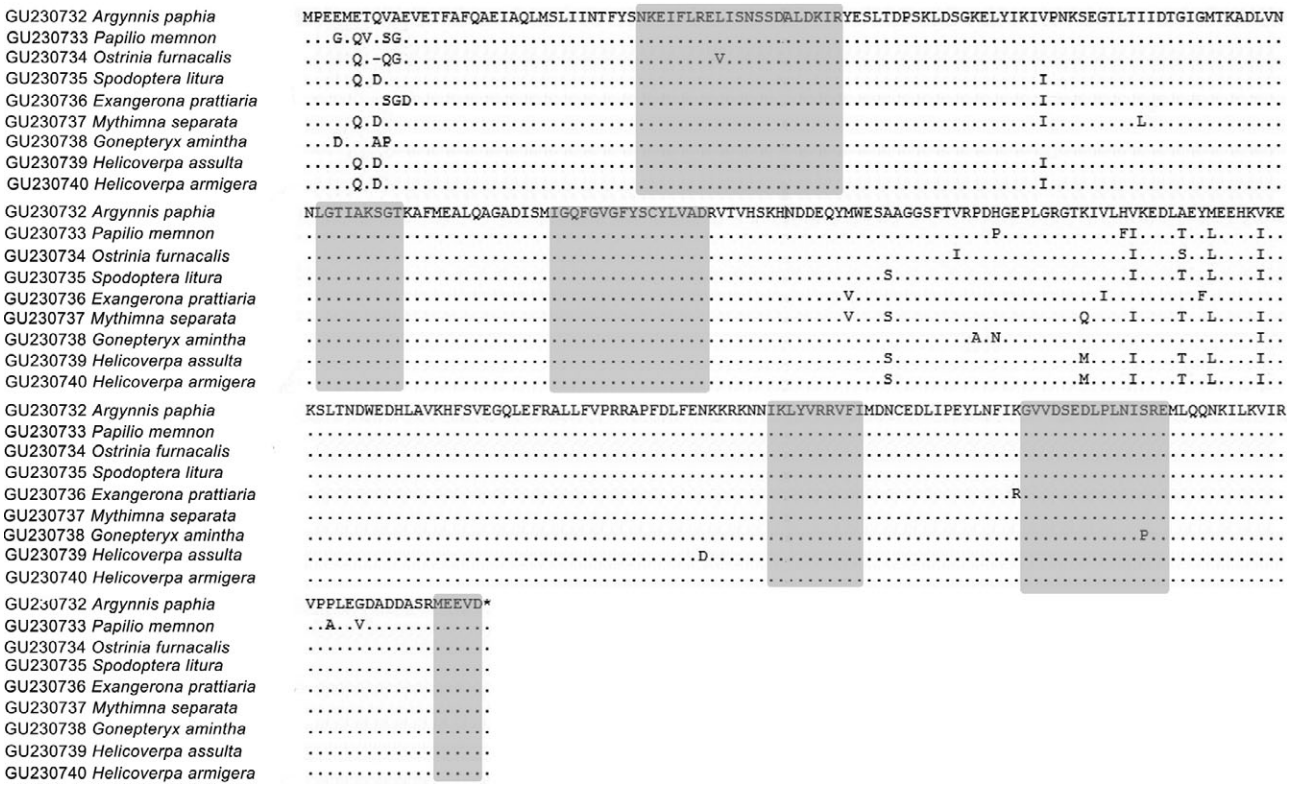

Fig. 3. Partial alignments of the amino acid sequences obtained in this study. The five conserved signatures of HSP90 family and the conserved pentapeptide of cytoplasmic HSP90 are shaded. Symbol "*" indicates the stop codon. GenBank accession numbers are given in left column.

insect orders. However, due to the limited information on the occurrence of cytoplasmic *hsp90* sequences in insects, it is not possible to confirm that this conserved 3'UTR region only occurs in Lepidoptera. Given this difference, this important functional marker could greatly facilitate research in Lepidoptera. The universal primers designed here amplified the complete CDS from genomic DNA, and, consequently, circumvented the use of costly RACE methods.

The amplification yielded some nonspecific bands, which might have resulted from the nonspecific binding of the primers. Nevertheless, identification and purification of the expected fragment for cloning and sequencing proved to be easy. Most of the products were amplified with primers L90F1/L90R and yielded the most consistent results during initial trials.

In conclusion, the results suggest that the cytoplasmic *hsp90* of Lepidoptera has a universally conserved 3'UTR region. The primers described here proved to be very promising for the amplification of the cytoplasmic *hsp90* fragment containing the complete CDS from Lepidoptera.

ACKNOWLEDGMENTS. This project was supported by the National Natural Science Foundation of China (NSFC grant no. 30770302, 30570970), partially by Program of Ministry of Science and Technology of the Republic of China (2006FY110500) and by National Science Fund for Fostering Talents in Basic Research (Special subjects in animal taxonomy, NSFC-J0630964/J0109). We would like to thank W. Xin and TransGen Biotech Company (Beijing) for providing reagents.

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Received August 19, 2010; revised and accepted October 8, 2010