



# Molecular cloning and characterization of an $\alpha$ -amylase cDNA highly expressed in major feeding stages of the coffee berry borer, *Hypothenemus hampei*



C.A. Bezerra<sup>a,b</sup>, L.L.P. Macedo<sup>a,b</sup>, T.M.L. Amorim<sup>c</sup>, V.O. Santos<sup>c</sup>, R.R. Fragoso<sup>d</sup>, W.A. Lucena<sup>e</sup>, A.M. Meneguim<sup>f</sup>, A. Valencia-Jimenez<sup>g</sup>, G. Engler<sup>h</sup>, M.C.M. Silva<sup>b</sup>, E.V.S. Albuquerque<sup>b</sup>, M.F. Grossi-de-Sa<sup>a,b,\*</sup>

<sup>a</sup> Catholic University of Brasilia, Brasilia, DF, Brazil

<sup>b</sup> Embrapa Genetic Resources and Biotechnology, Brasilia, DF, Brazil

<sup>c</sup> Federal University of Rio Grande do Norte, Natal, RN, Brazil

<sup>d</sup> Embrapa Cerrados, Planaltina, DF, Brazil

<sup>e</sup> Embrapa Cotton, Campina Grande, PB, Brazil

<sup>f</sup> Agronomic Institute of Paraná, Londrina, PR, Brazil

<sup>g</sup> University of Caldas, Manizales, Colombia

<sup>h</sup> Institut National de la Recherche Agronomique, Sophia-Antipolis, France

## ARTICLE INFO

### Article history:

Received 25 July 2014

Received in revised form 18 September 2014

Accepted 22 September 2014

Available online 28 September 2014

### Keywords:

Curculionidae

Gene expression

Insect pest

Starch hydrolysis

Enzyme modeling

## ABSTRACT

$\alpha$ -Amylases are common enzymes responsible for hydrolyzing starch. Insect-pests, whose larvae develop in seeds, rely obligatorily on  $\alpha$ -amylase activity to digest starch, as their major food source. Considering the relevance of insect  $\alpha$ -amylases and the natural  $\alpha$ -amylase inhibitors present in seeds to protect from insect damage, we report here the molecular cloning and nucleotide sequence of the full-length *AmyHha* cDNA of the coffee berry borer, *Hypothenemus hampei*, a major insect-pest of coffee crops. The *AmyHha* sequence has 1879 bp, containing a 1458 bp open reading frame, which encodes a predicted protein with 485 amino acid residues, with a predicted molecular mass of 51.2 kDa. The deduced protein showed 55–79% identity to other insect  $\alpha$ -amylases, including *Anthonomus grandis*, *Ips typographus* and *Sitophilus oryzae*  $\alpha$ -amylases. In depth analysis revealed that the highly conserved three amino acid residues (Asp184, Glu220, and Asp285), which compose the catalytic site are also presented in *AmyHha* amylase. The *AmyHha* gene seems to be a single copy in the haploid genome and *AmyHha* transcription levels were found higher in L2 larvae and adult insects, both corresponding to major feeding phases. Modeling of the *AmyHha* predicted protein uncovered striking structural similarities to the *Tenebrio molitor*  $\alpha$ -amylase also displaying the same amino acid residues involved in enzyme catalysis (Asp184, Glu220 and Asp285). Since *AmyHha* gene was mostly transcribed in the intestinal tract of *H. hampei* larvae, the cognate  $\alpha$ -amylase could be considered a high valuable target to coffee bean insect control by biotechnological strategies.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

## 1. Introduction

The coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae: Scolytinae), is an insect-pest that causes major economic losses in coffee crops worldwide, reaching annual losses of up to US\$ 500 million (Pava-Ripoll et al., 2008). The coffee berry borer is monophagous, wounding solely on *Coffea* spp. (Gallo et al., 1988). Larvae and adults of the coffee berry borer severely decrease coffee yield and quality (Le Pelley, 1968; Murphy and Moore, 1990; Baker et al., 1992), reducing the commercial grain value (Clifford and Wilson, 1985). This

insect-pest lives and completes its entire life cycle inside coffee beans (Le Pelley, 1968; Murphy and Moore, 1990; Baker et al., 1992), making chemical control difficult. The negative impact of *H. hampei* on coffee crop production makes this insect-pest an important subject of the study.

Coffee grains contain carbohydrate reserves, such as starch (Clifford and Wilson, 1985; Figueiredo, 2011) and mannan (Bradbury and Halliday, 1990), which are broken down by digestive enzymes (Valencia et al., 2000; Acuña et al., 2012).  $\alpha$ -Amylases ( $\alpha$ -1,4-glucan-4-glucohydrolases, EC 3.2.1.1, CAZy family GH13) are essential to hydrolyze polysaccharides present in different plant tissues and are monomeric constituents of a large endoamylase family, responsible for the hydrolysis of  $\alpha$ -1,4 glycosidic bonds of starch, glycogen and other carbohydrates (Terra and Ferreira, 1994) and their conversion into short oligosaccharides.

Abbreviations: *AmyHha*, *Hypothenemus hampei*  $\alpha$ -amylase; TMA, *Tenebrio molitor*  $\alpha$ -amylase;  $\alpha$ -AI,  $\alpha$ -amylase inhibitor.

\* Corresponding author at: Embrapa Genetic Resources and Biotechnology Brasilia, DF, Brazil.

E-mail address: [fatima.grossi@embrapa.br](mailto:fatima.grossi@embrapa.br) (M.F. Grossi-de-Sa).

It is well known that  $\alpha$ -amylases are required for the development and survival of insects, which grow and feed on starch-containing seeds (Grossi de Sa and Chrispeels, 1997; Franco et al., 2002). Starch digestion by intestinal  $\alpha$ -amylases has been reported for several insect species, including *Drosophila melanogaster* (Doane, 1969); *Epiphyas postvittana*, *Planotortrix octo* and *Ctenopseustis obliquana* (Markwick et al., 1996); *Sitophilus granarius* (Silano et al., 1975); *Sitophilus oryzae* (Yetter et al., 1979); *Prostephanus truncatus* (Mendiola-Olaya et al., 2000); *Tribolium castaneum* (Krishna and Saxena, 1962); *Callosobruchus maculatus* (Campos et al., 1989); *Zabrotes subfasciatus* (Lemos et al., 1990); *Tenebrio molitor* (Applebaum, 1964); and *H. hampei* (Valencia et al., 2000). Different  $\alpha$ -amylase isoforms were observed in *C. maculatus*, *Z. subfasciatus*, *Ips typographus* and *H. hampei* (Campos et al., 1989; Silva et al., 1999; Valencia et al., 2000; Viktorinova et al., 2011). Several  $\alpha$ -amylase cDNAs have been cloned and characterized from different insect species, including *Z. subfasciatus* (Grossi de Sa and Chrispeels, 1997), *Diabrotica virgifera virgifera* (Titarenko and Chrispeels, 2000), *Anthonomus grandis* (Oliveira-Neto et al., 2003), *I. typographus* (Viktorinova et al., 2011), *T. molitor* (Strobl et al., 1997) and others. Despite the knowledge of some insect  $\alpha$ -amylase cDNA sequences, the  $\alpha$ -amylase from *T. molitor* (TMA) is the only three-dimensional  $\alpha$ -amylase structure resolved so far (Strobl et al., 1998).

Earlier reports demonstrated that the inhibition of this enzyme by the common bean  $\alpha$ -amylase inhibitors ( $\alpha$ -AI) adversely affects growth, development and reproduction of this insect-pest (Oliveira-Neto et al., 2003; Calderon et al., 2005; Kluh et al., 2005) due to a severe negative effect on energy metabolism. Therefore, the use of  $\alpha$ -AI, abundant in legumes (Franco et al., 2002) and cereals (Iulek et al., 2000), represent a promising biotechnological tool for insect-pest control (Valencia et al., 2000; Oliveira-Neto et al., 2003; Calderon et al., 2005; Valencia-Jimenez et al., 2008). Principles of this strategy with the  $\alpha$ -AI1 were nicely demonstrated in the works on the genetic engineering of peas, azuki beans and coffee plants (Shade et al., 1994; Schroeder et al., 1995; Barbosa et al., 2010). All the successes reported support the use of biotechnology approaches as a promising alternative to generate genetically modified (GM) crops for insect-pest control (Ahmad et al., 2012). Hence, extensive studies involving inhibitors of insect digestive enzymes, such as proteases and  $\alpha$ -amylases, suggest a highly effective defensive mechanism (Carlini and Grossi-de-Sa, 2002; Oliveira-Neto et al., 2003; Valencia-Jimenez et al., 2008).

Recently, aiming to control the coffee berry borer, GM coffee plants (*Coffea arabica*) expressing  $\alpha$ -AI1 gene were developed aiming to prevent coffee grains from the damage caused by *H. hampei* (Barbosa et al., 2010). However, concerning the molecular characterization of the  $\alpha$ -AI1 inhibitor target, i.e. the *H. hampei*  $\alpha$ -amylases, no cognate genes were previously reported. Herein, we have focused on the cDNA molecular cloning, characterization and transcript expression pattern of a new  $\alpha$ -amylase, from *H. hampei*, as well as the structural modeling of the predicted protein. Gathering data about the *H. hampei*  $\alpha$ -amylase is essential for future interaction studies between the AmyHha/ $\alpha$ -AI1 complex. This knowledge will certainly contribute to the design of better approaches for the coffee berry borer control using GM coffee plants expressing  $\alpha$ -AI-1.

## 2. Materials and methods

### 2.1. Insect rearing

Coffee berry borers were reared on Villacorta and Barrera (1993) meridic coffee-based diet, with the following modifications: 150 g of coffee bean powder and 0.5 g of Vanderzant vitamin mixture for insects. The insect rearing was carried out in the laboratory of Entomology of the Instituto Agronômico do Paraná, Londrina, Brasil (25  $\pm$  2 °C, RH 60  $\pm$  10% and complete darkness). The classification of the phases during the *H. hampei* life cycle was based on Fernández and Cordero (2007).

Eggs, larvae, pupae and adult of *H. hampei* were collected, frozen in N<sub>2</sub> and maintained at –80 °C until used.

### 2.2. RT-PCR, 5' RACE and 3' RACE

Total RNA was isolated from the 2nd-instar larvae whole body using Trizol reagent (Invitrogen, California, USA) following the manufacturer's instructions. The cDNA was synthesized from 5  $\mu$ g of total RNA using the SuperScript II kit™ First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo (dT)<sub>30</sub> primer, following the manufacturer's instructions. For partial sequence cloning of the *AmyHha*, degenerate primers (Table 1) corresponding to conserved regions found in the catalytic domains of other insect  $\alpha$ -amylases were used (Oliveira-Neto et al., 2003). Two rounds of PCR using degenerate primers were performed. A first PCR round consisted of the segment amplification that encodes the region between the conserved amino acid residues RPWWERYQ and AFMLAH. A second round of amplification was done using primers corresponding to conserved regions between the amino acids RPWWERYQ and AAKHMMW (Table 1). PCR included the following components: 1  $\mu$ L of cDNA as template, 2  $\mu$ L 10 $\times$  High Fidelity PCR Buffer, 0.8  $\mu$ L MgSO<sub>4</sub> (50 mM), 0.4  $\mu$ L dNTP (10 mM each), 0.4  $\mu$ L of each primer (10  $\mu$ M), 0.2  $\mu$ L (1 U) Platinum® Taq High Fidelity (Invitrogen, California, USA) and 14.8  $\mu$ L ddH<sub>2</sub>O. Incubations were performed using the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s and extension at 68 °C for 1 min. To obtain the sequences of the 5' and 3' ends of *AmyHha* cDNA, gene-specific primers for 3' and 5' RACE were designed (Table 1), following the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech). Specific-PCR was performed using the following conditions: 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and extension at 68 °C for 2 min. All generated PCR products were cloned into pGEM-T easy vector (Promega) and sequenced. The complete sequence of the *AmyHha* was obtained by the overlapping of PCR products derived from the RT-PCR, 5' RACE and 3' RACE.

### 2.3. DNA and protein sequence analysis

The complete sequence of the *AmyHha* cDNA was initially analyzed in BLASTx and BLASTn software (Altschul et al., 1997) to obtain homologous sequences in the NCBI database (National Center for Biotechnology Information). The search for the ORF (open reading frame) was performed using the program ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The nucleotide and amino acid sequences were aligned with the BioEdit software using the ClustalW algorithm (Hall, 1999). The molecular weight and isoelectric point (pI) of the predicted protein were determined by the tool Compute pI/MW available on the ExpASY platform (<http://us.expasy.org>). The sequence of the signal peptide was predicted by the program SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). Prediction of putative O-glycosylation sites was obtained with the program YinOYang 1.2 (<http://www.cbs.dtu.dk/services/YinOYang/>). Putative N-glycosylation sites were predicted by NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) software. Phylogenetic analysis was performed using the MEGA version 5, applying the bootstrap method with 10,000 replications to evaluate the reliability of the topology of the phylogenetic tree obtained (Tamura et al., 2011).

### 2.4. Southern blot analysis

The extraction of *H. hampei* genomic DNA was based on the method described by Sambrook et al. (1989). Three gDNA samples (8  $\mu$ g) were digested with *Swa*I, *Nde*I and *Hind*III restriction enzymes, and separated on a 0.9% agarose gel. Nucleic acids were transferred onto Hybond N + Nylon membrane (Amersham) under alkaline conditions and washed as described by Sambrook et al. (1989). A 1304 bp PCR fragment, between 517 and 1820 bp of *AmyHha* cDNA, was labeled with [ $\alpha$ <sup>32</sup>P] dCTP using the kit Ready-To-Go DNA Labeling Beads (Amersham

**Table 1**  
Primers used for AmyHha cDNA amplification.

Number/primer name	Primer		
	Direction	Type	Sequence (5'–3')
P1 – NV-d(T)30-AP1	R	D	GAATTCACGCGTGCAGTAGTAGCATATGTAC(T)30VN
P2 – amyIsct_Fw_1_RPWVERYQ	F	D	AGACCVTGGTGGARAGRTACC
P3 – amyIsct_Rv_2_AFMLA-AP2	R	D	TCACGCGTGCAGTAGTAGCANGCNARCATRAANGC
P4 – amyIsct_Rv_3_AAKHMW-AP3	R	D	(ATTB2)GGCCACATRTGTTTDCNGC <sup>a</sup>
P5 – AmyHhaFw1	F	S	AGACCATGGTGGGAAAATACC
P6 – AmyHhaRv2	R	S	CCTTGCCAATCGATGTTC
P7 – 5'Primer_SMART_GTW	F	S	(ATTB1)ACCAGACGTCCGATGCCATGGATATCGAATTCGGGG <sup>b</sup>

D: degenerate oligonucleotide, S: specific oligonucleotide, F: forward; R: reverse.

<sup>a</sup> ATTB2: GGGGACCACTTTGTACAAGAAAAGCTGGGCT.

<sup>b</sup> ATTB1: GGGGACAAGTTTGTACAAAAAAGCAGGCT.

Pharmacia) to generate the probe. Unincorporated nucleotides were removed using MicroSpin S-300 HR columns (Amersham Bioscience), following the manufacturer's instructions. The membrane was incubated in hybridization solution ( $5 \times$  SSC/ $5 \times$  Denhardt's solution/0.5% SDS) at 65 °C for 24 h. After the hybridization step, the membrane was washed 3 times with  $1 \times$  SSC solution for 15 min. After the washing step, the membrane was exposed to an imaging plate (BAS-MP, FujiFilm) for 24 h. Images were acquired using a FLA3000 Phosphorimager (FujiFilm).

### 2.5. Quantitative real time PCR analysis

Relative expression of *AmyHha* was evaluated by RT-qPCR for all developmental stages of the *H. hampei* life cycle, including eggs, 1st-instar larvae, 2nd-instar larvae, pupa and adults. Total RNA of the samples was isolated using Trizol (Invitrogen, California, USA) following the manufacturer's instructions. RNA quantification was performed using the ND-1000 spectrophotometer NanoDrop. Aliquots of 1 µg of total RNA were treated with Ambion® DNase I RNase-free™ (Invitrogen) to digest any contaminating genomic DNA. cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, California, USA) with Oligo(dT30)-primer, following the manufacturer's instructions. The real-time PCR amplifications were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Rox plus Sybr Green Master Mix 0.5× (LGC Biotecnologia), 200 nM of each primer (sense and antisense) and 2 µL of 40-fold dilution of cDNA for each reaction, were used with a final volume of 10 µL. All experiments were performed in technical triplicate and biological duplicate. The sequences of the primers used for RT-qPCR analysis were designed using Primer 3 software. *AmyHha* primers used were: forward 5'GGAGTGGCAATTTATGCTG3' and reverse 5'CACGTAATCTTGGCTCTGATCC3'.  $\beta$ -Actin reference gene primers were designed based on *A. grandis* (GABY01014421) and *T. castaneum* actin mRNA sequences (XM\_970777.1), which were forward 5'CCTTCAACACCCCGCCATG3' and reverse 5'TGAGGTAGTCCG TCAAGTCG3'. The PCR cycling conditions were: 95 °C for 15 min to activate the hot-start Taq DNA polymerase, 40 cycles at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s. Finally, the denaturation curve ("melting curve") was established after the amplification to verify the presence of nonspecific products or primer dimers. The cycling conditions for the melting curve determination were 95 °C for 15 s and 60 °C for 15 s, with two degree increments per minute of up to 95 °C. The raw data of fluorescence for all runs were imported into the Real-time PCR Miner software (Zhao and Fernald, 2005) in order to determine the Ct value and the PCR efficiency. The analyses of *AmyHha* expression were completed using qBASEplus program (Hellemans et al., 2007).

### 2.6. Computational modeling

After performing the *AmyHha* amino acid sequence prediction, alignment against the PDB structure database was carried out to obtain

an optimal structural template, with TMA the best mold found (sequence ID: PDB|1JAE). Thus, a structural model of the enzyme *AmyHha* was developed based on the crystal structure of TMA enzyme. The protein sequences were aligned using the ClustalW software. The program, Modeller 9v11, was used to construct 100 three-dimensional models (Eswar et al., 2007). The final step of the modeling process was done to evaluate the different levels of structural organization. At this stage the various levels of overall packing and possible structural errors were analyzed. For validation of the structural model, the program PROCHECK was used in the evaluation of stereochemical parameters (Laskowski et al., 1996). The program 3DSS (<http://cluster.physics.iisc.ernet.in/3dss/>) was used for the calculation of RMSD by superimposing the structure of the built model and that of the mold structure (Sumathi et al., 2006). The three-dimensional structures were viewed and analyzed using PyMOL (<http://pymol.sourceforge.net/>) (DeLano, 2002).

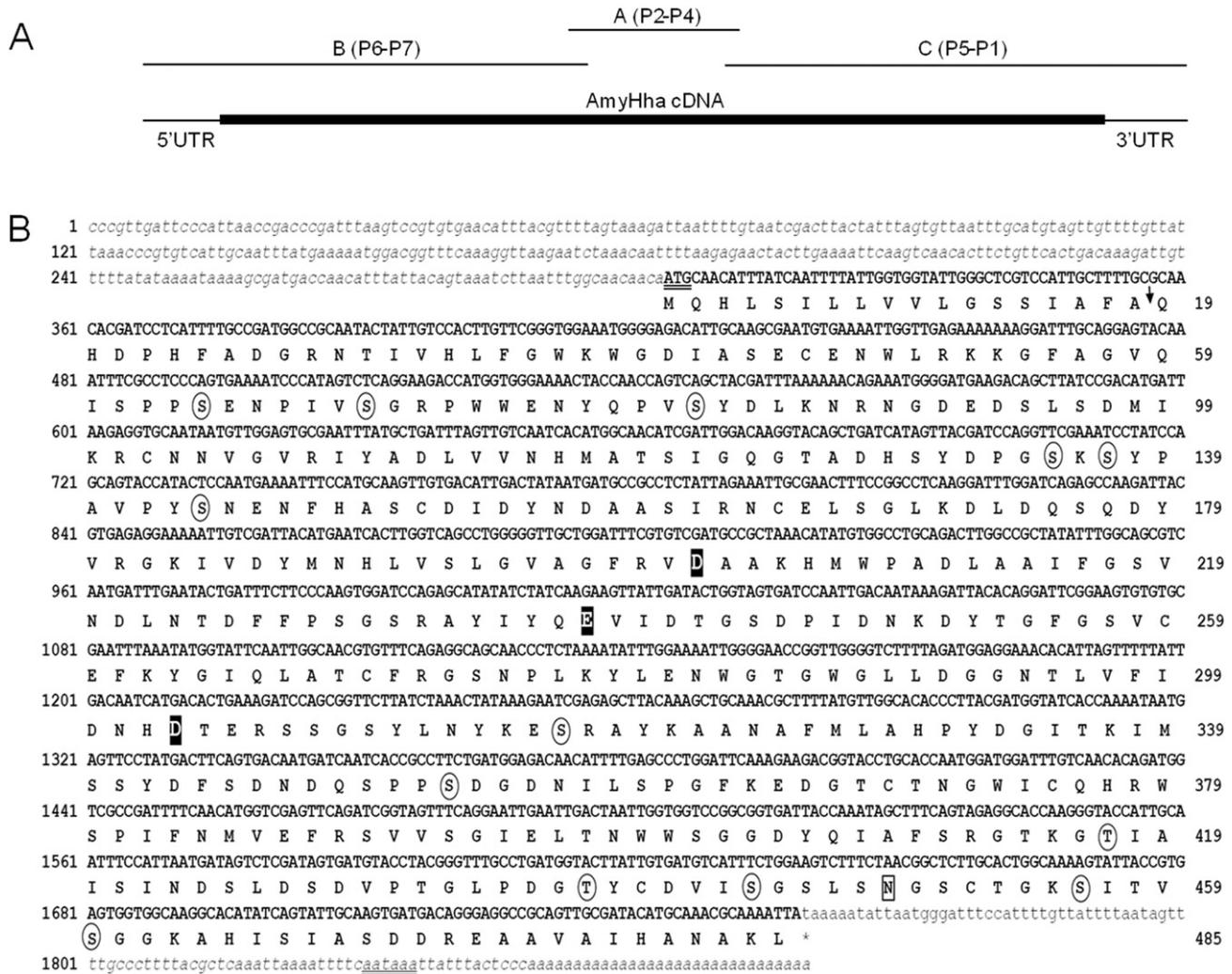
## 3. Results

### 3.1. Isolation and characterization of *AmyHha* cDNA

To clone the *AmyHha* cDNA, total RNA was extracted from 2nd-instar larvae and cDNA was synthesized. Initially, a fragment of 413 bp was obtained by RT-PCR using degenerate primers, whose design was based on conserved regions found in other coleopteran insect  $\alpha$ -amylases. Over 40 colonies were sequenced and only one cDNA sequence was found. Subsequently, two segments of the 5' and 3' ends were amplified by 5'–3' RACE strategies using specific primers. The full-length cDNA sequence of *AmyHha* (GenBank ID: KF963103) was obtained by the overlapping of the three PCR fragments (Fig. 1A).

The 1879-bp *AmyHha* cDNA contains a 1458-bp full-length open reading frame encoding 485 amino acid residues (Fig. 1B). The deduced amino acid sequence of *AmyHha* shows high identity with other insect  $\alpha$ -amylases, presenting 79% identity and 55% identity with isoforms 1 and 2 from *A. grandis*, respectively, 74% identity and 73% identity with isoforms A and B from *I. typographus*, respectively, 69% from *S. oryzae*, 58% from *D. virgifera virgifera* and *T. castaneum*, 57% from *C. chinensis* and *Z. subfasciatus* and 56% from *T. molitor*. The alignment of *AmyHha* with other insect  $\alpha$ -amylases shows a high degree of conservation, particularly in amino acid residues involved in substrate binding and catalytic site (Fig. 2).

The *AmyHha* sequence has a signal peptide composed of N-terminal 18 amino acid residues, predicted by the SignalP program. The molecular mass of the mature *AmyHha* protein was estimated to be 51.2 kDa with an isoelectric point estimated to be 4.88. The catalytic triad is found in *AmyHha* as Asp184, Glu220, and Asp285 (Fig. 1B). In silico analysis predicted thirteen potential O-glycosylation sites in *AmyHha* protein sequence using the program YinOYang, while only one single potential N-glycosylation site was predicted by the program NetNGly (Fig. 1B).



**Fig. 1.** Cloning and protein sequence analysis of *AmyHha* cDNA. A) Cloning strategy of *AmyHha* cDNA. The *AmyHha* full-length cDNA sequence was determined by the overlapping of three PCR fragments. Codes of the used primers in the PCR appear in parentheses, as listed in Table 1. B) Nucleotide and deduced amino acid sequences of *AmyHha* cDNA. Numbers on the left refer to nucleotide sequence, while numbers on the right are for the deduced amino acid sequence. Untranslated regions are in italics and lowercase, the translated region is in uppercase. Start codon (ATG) and putative polyadenylation signal (AATAAA) are double-underlined. The arrow indicates the cleavage site of the signal peptide. The conserved amino acids that participate in the catalytic triad are marked in black box. Potential O-glycosylation sites are circled. Asparagine predicted to be N-glycosylated is boxed.

### 3.2. Phylogenetic analysis of *AmyHha* cDNA

A phylogenetic tree was constructed in order to determine the relatedness of the *AmyHha* ORF sequence with other coleopteran  $\alpha$ -amylase ORFs. In this case, the *Bombyx mori*  $\alpha$ -amylase was used as an out-group to the rooting of the phylogenetic tree. Our data suggest a high phylogenetic relationship between *AmyHha* and the  $\alpha$ -amylase isoform 1 from *A. grandis*, sharing 71.2% nucleotide identity, as well as high identity with the  $\alpha$ -amylases from *I. typographus* isoform A and isoform B (71% and 69%, respectively) and *S. oryzae* (65.5%); all these insects belong to the Curculionidae family. We observed in the 2D-identity matrix that all coleopteran  $\alpha$ -amylases evaluated presented more than 50% nucleotide identity between them (Fig. 3).

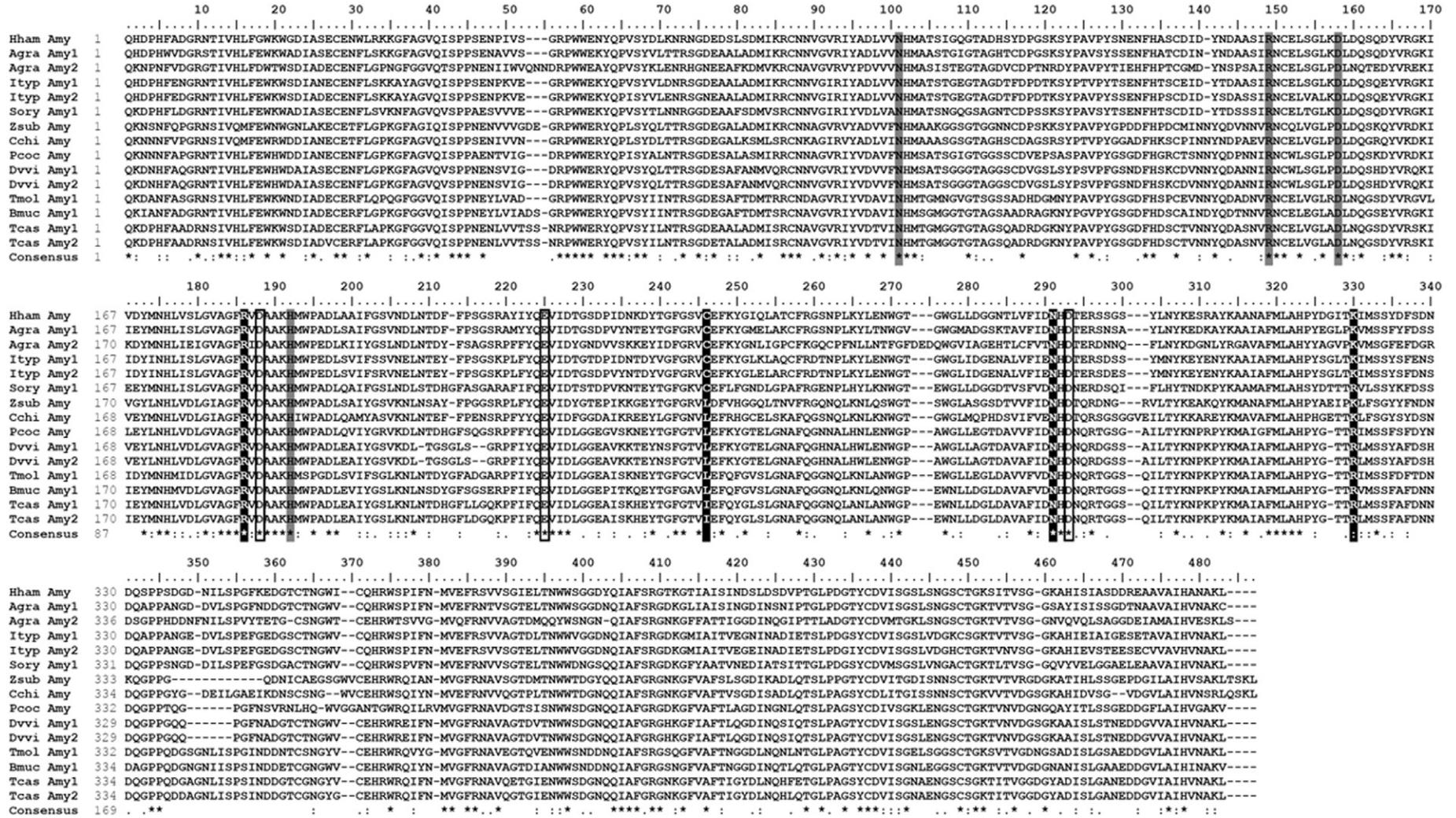
### 3.3. Determination of *AmyHha* gene copy number

The *AmyHha* gene copy number in the *H. hampei* genome was estimated by Southern blot. Among the selected restriction enzymes, *SwaI* and *NdeI* restriction enzymes are expected to cut once the *AmyHha* cDNA and, *HindIII* enzyme does not cut the *AmyHha* cDNA. Considering

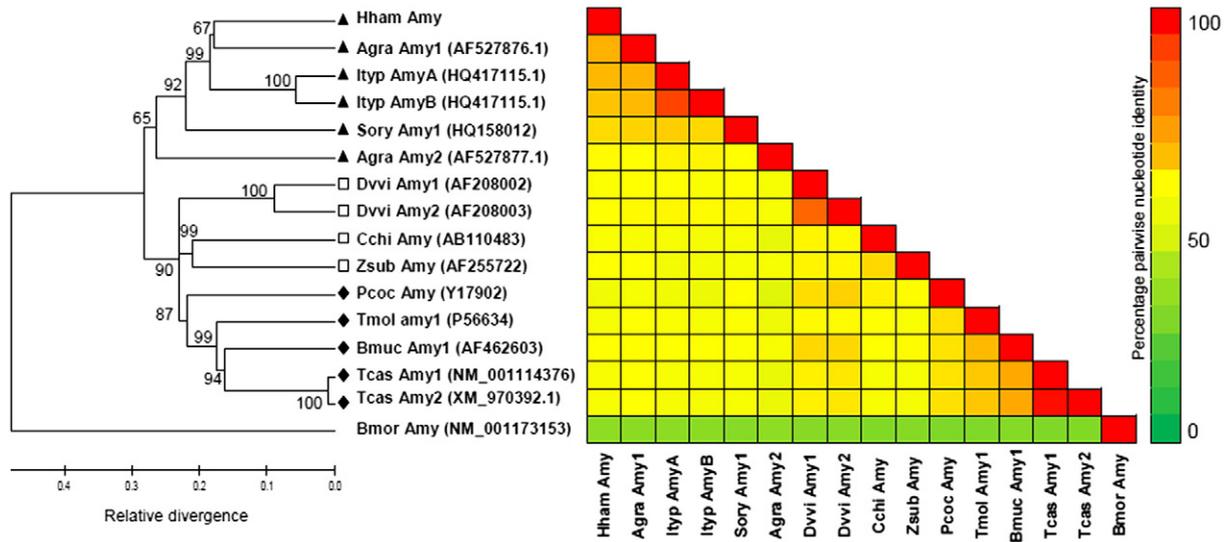
the lines digested with *NdeI* and *HindIII*, hybridization resulted in two and one band(s), respectively (Fig. 4), suggesting that *AmyHha* is a single copy gene in haploid genome. On the other hand, *SwaI* digestion was expected to result in two bands, but only a single darker band is observed, which suggests two overlapping hybridization bands with similar sizes. In addition, some lighter bands could be seen in all lines suggesting the presence of homologous genes belonging to a small  $\alpha$ -amylase family in *H. hampei* (Fig. 4).

### 3.4. *AmyHha* expression profile during the *H. hampei* life cycle

The characterization of the *AmyHha* transcription pattern during the *H. hampei* phenological phases was obtained by quantitative real-time PCR experiment. The level of *AmyHha* transcripts varied during *H. hampei* development (Fig. 5). The low levels of *AmyHha* transcripts were detected in eggs and first instar stage, whereas a 200-fold increase could be observed at the 2nd-instar stage. We noticed a significant reduction of *AmyHha* transcripts during the pupal stage, a period in which insects do not feed. After ecdysis, *AmyHha* transcript accumulation increased again to 200 times during the adult stage which resumes feeding.



**Fig. 2.** Alignment of mature predicted protein sequences of coleopteran  $\alpha$ -amylases using ClustalW. Residues of the catalytic site are boxed; residues involved in calcium ion binding site are marked in gray and residues of the chloride ion binding site are marked in black. *Hypothenemus hampei* (Hham); *Anthonomus grandis*: Agra Amy1 (AF527876.1); Agra Amy2 (AF527877.1); *Ips typographus*: Ityp AmyA (HQ417115.1), Ityp AmyB (HQ417115.1); *Sitophilus oryzae*: Sory Amy1 (HQ158012); *Zabrotes subfasciatus*: Zsub Amy (AF255722); *Callosobruchus chinensis*: Cchi Amy (AB110483); *Phaedon cochleariae*: Pcoc Amy (Y17902); *Diabrotica virgifera virgifera*: Dvvi Amy (AF208002), Dvvi Amy2 (AF208003); *Tenebrio molitor*: Tmol Amy1 (P56634); *Blaps mucronata*: Bmuc Amy1 (AF462603); *Tribolium castaneum*: Tcas Amy1 (NM\_001114376.1), Tcas Amy2 (XM\_970392.1). Symbols in the consensus sequence indicate identity (\*), highly conserved substitutions (:), and conserved substitutions (.).

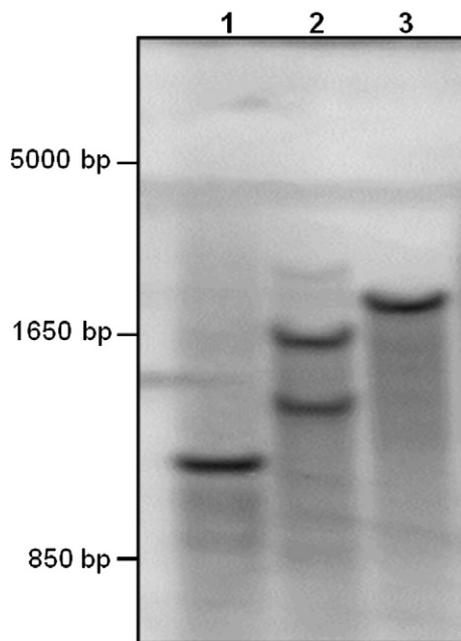


**Fig. 3.** Insect  $\alpha$ -amylase dendrogram and two-dimensional ORF pairwise identity plot illustrating the phylogenetic relationships. The tree was generated by MEGA 5 based on ORF sequences of Coleoptera  $\alpha$ -amylases. The sequences were aligned with the program ClustalW to generate a phylogenetic tree (at the left of the figure). The tree was rooted with *B. mori* (Bmor)  $\alpha$ -amylase. Markers indicate the family of Coleoptera: Curculionidae (black triangles), Chrysomelidae (white squares) and Tenebrionidae (black diamonds). GenBank accession numbers used for phylogenetic analysis are presented. Numbers on the branches represent bootstrap values for 10,000 replicates.

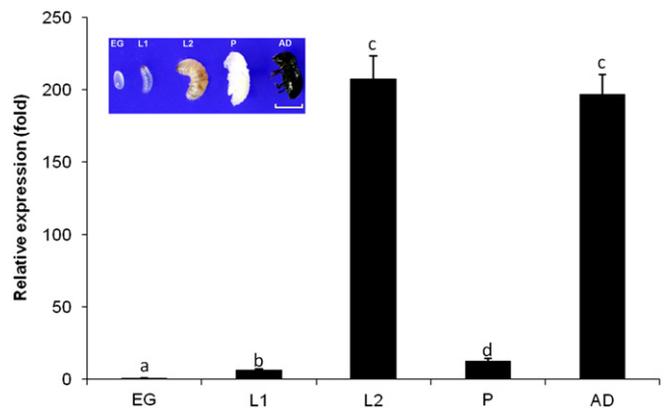
### 3.5. Three dimensional model of the AmyHha protein

The TMA protein sequence shares 56% identity with the AmyHha making it sufficient to yield a reliable model. The structural alignment of the AmyHha against the TMA showed a high conservation of residues involved in the catalytic process and also conserved residues at adjacent sites (Fig. 6A). In addition, the three domains (A, B and C) were observed in AmyHha (Fig. 6B), being easily recognized when compared with the three-dimensional structure of TMA. Domain A is represented as a barrel ( $\alpha/\beta$ )<sub>8</sub>, whereas the B domain is composed of two short  $\beta$ -sheets and

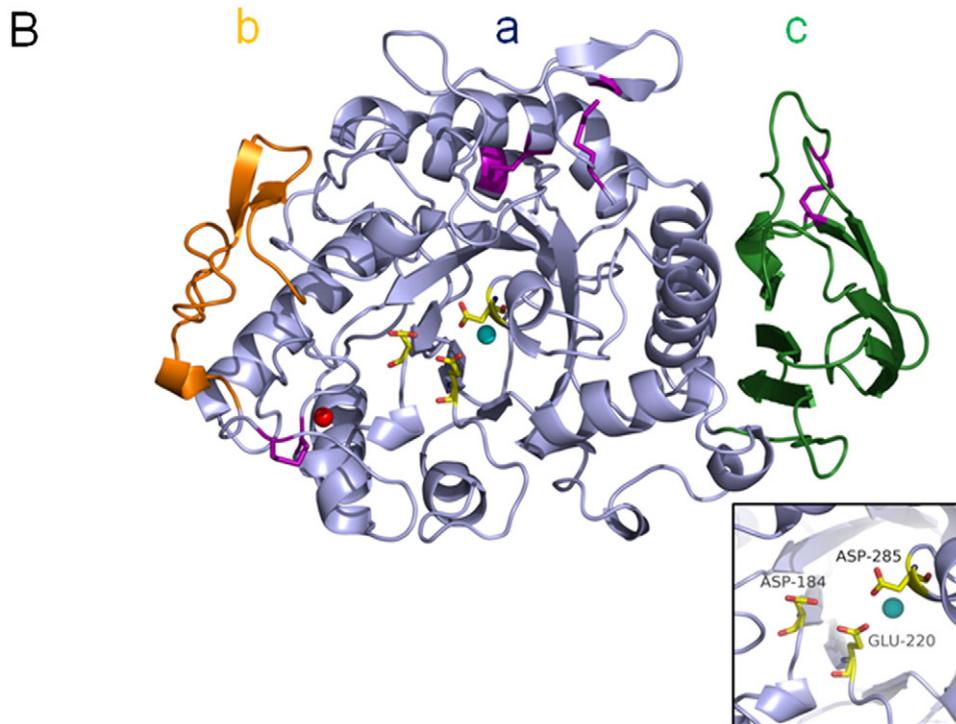
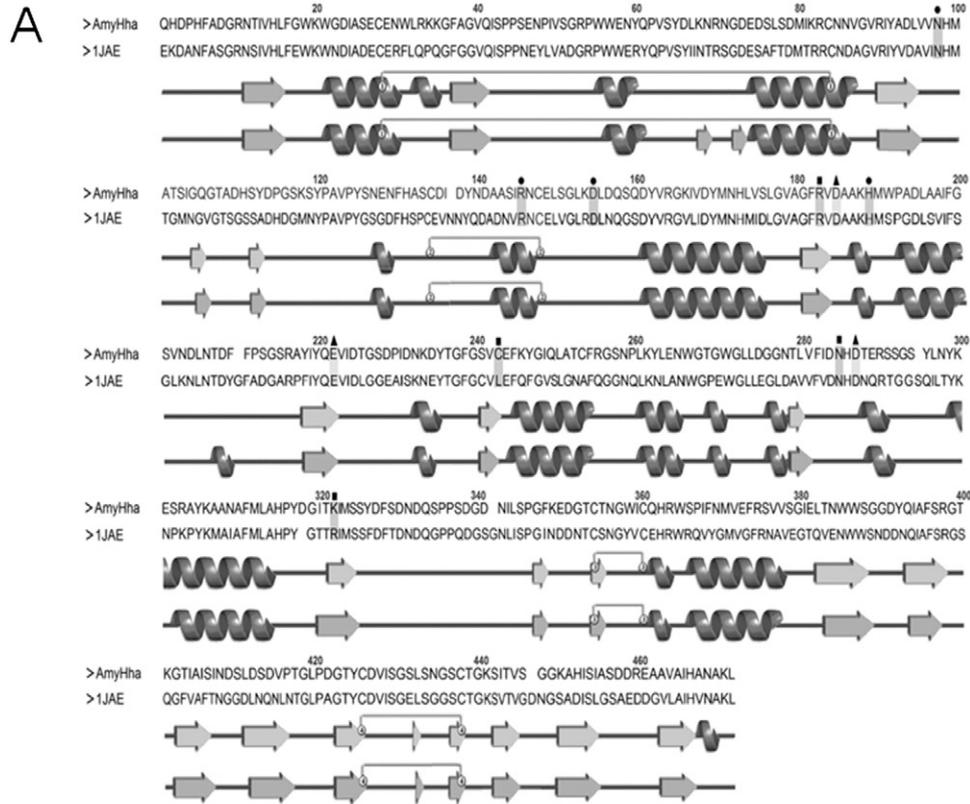
two short  $\alpha$ -helices. The C-domain is formed from a  $\beta$ -sheet set. Furthermore, the possible residues of AmyHha involved in enzyme catalysis (Asp184, Glu220, and Asp285) are highly conserved, forming the catalytic triad of the active site, as well as, the calcium ion ligands (Asn98, Arg145, Asp154, His188) and chlorine ion ligands (Arg182, Asn283) are conserved in other insect  $\alpha$ -amylases (Fig. 2). In the AmyHha model, eight cysteine residues form four disulfide bonds (Fig. 6A). AmyHha protein also contains an additional cysteine residue (Cys241) that appears at the site of interaction with the chloride ion (Fig. 2). The AmyHha model had quality scores within the acceptable range, suggesting that this model represents the native structure of an *H. hampei*  $\alpha$ -amylase (Table S1). In the analysis of torsional angles Phi and Psi in the Ramachandran map, the three-dimensional model proposed for AmyHha showed only 0.5% of residues in disallowed regions. The g-score value, which refers to a total quality of three-dimensional structures, taking into account the physical and chemical parameters



**Fig. 4.** Determination of *AmyHha* gene copy number by Southern blot analysis of *H. hampei* genomic DNA. *H. hampei* DNA (8  $\mu$ g per line) was digested with three restriction enzymes and electrophoresed in line 1 (*Swa*I, one cut on *AmyHha* cDNA), line 2 (*Nde*I, one cut) and line 3 (*Hind*III, no cut). The membrane was probed with a 1304-bp DNA fragment amplified from the *AmyHha* cDNA labeled with <sup>32</sup>P dCTP. DNA size markers are indicated in bp.



**Fig. 5.** Expression pattern of *AmyHha* transcript using whole individuals. Transcript accumulations were determined by qPCR using cDNA from eggs (EG); first- (L1), second-instar (L2), pupa (P) and adults (AD) of *H. hampei* developmental stages, which are illustrated in the insert; bar: 0.5 mm.  $\beta$ -Actin was used as internal reference genes for qPCR normalization. The expression profile was relative to the egg stage, which was considered 1. Different letters above the bars indicate statistically significant differences of *AmyHha* expression along *H. hampei* development based on two biological replications ( $P < 0.05$ , Tukey's test).



**Fig. 6.** Structural model of the AmyHha enzyme. A. Alignment of the AmyHha primary and secondary structures with TMA (PDB|1JAE). Secondary structures:  $\alpha$ -helix (cylinder) and  $\beta$ -sheet (arrow); disulfide bridge (purple line); residues of the catalytic site ( $\blacktriangle$ ); residues binding site with the calcium ion ( $\bullet$ ); residues binding site with the chloride ion ( $\blacksquare$ ); B. The cartoon structure of a three-dimensional model of *H. hampei*  $\alpha$ -amylase – AmyHha. The letters a, b and c indicate the  $\alpha$ -amylase domains; the arrows indicate  $\beta$ -sheet structures; helical structures indicate  $\alpha$ -helices; The catalytic site is illustrated highlighting possible residues (Asp184, Glu220 and Asp285) involved in enzyme catalysis and their respective side chains represented in yellow. The calcium and chloride ions are indicated by red and blue dots, respectively. The disulfide bridges are marked in purple.

such as Phi-psi and Omega distributions, was reliable for the AmyHha model, being within the normal range of acceptance. A further evaluation was conducted to overlap the structural AmyHha model against the mold (1JAE) using the 3DSS program, which RMSD resulted in the value 0.242, indicating little structural variation pointing to high primary sequence conservation between  $\alpha$ -amylases.

#### 4. Discussion

Coffee is one of the most valuable primary products in the world trade and coffee industries are responsible for the employment of nearly 25 million people worldwide (Watson and Achinelli, 2008). Among numerous coffee pests, *H. hampei* is by far the most severe and responsible for large economical losses due to seed weight loss and premature fall of grains (Pava-Ripoll et al., 2008). Both, *H. hampei* larvae and adults live, reproduce and feed into the fruit, digesting proteins and storage polysaccharides of coffee grain using their digestive enzymes (Le Pelley, 1968; Murphy and Moore, 1990; Baker et al., 1992; Acuña et al., 2012).

$\alpha$ -Amylases play a key role in carbohydrate metabolism of various insect-pests, especially those that feed on plant tissues rich in starch. Typical examples are weevils, attacking seeds and the cotton boll weevil feeding on flower buds. Consequently, several insects during their larval and/or adult phases depend on  $\alpha$ -amylase enzymes for survival (Franco et al., 2002; Oliveira-Neto et al., 2003). The presence of amylolytic activity in the *H. hampei* midgut was previously detected and biochemically characterized by Valencia et al. (2000). The presence of active  $\alpha$ -amylases in the *H. hampei* digestive system strongly suggests their participation in starch digestion, a nutrient that is present in coffee beans (Giorgini and Campos, 1992; Figueiredo, 2011).

Here, we describe the molecular cloning and cDNA sequencing of a new *H. hampei*  $\alpha$ -amylase named AmyHha, whose cDNA is predicted to have a 1458-bp ORF encoding 485 amino acid residues. Phylogenetic analysis based on the AmyHha cDNA sequence shows its high identity to the *A. grandis*  $\alpha$ -amylase I (Amylag I), also belonging to the Curculionidae family (Oliveira-Neto et al., 2003). Protein sequence comparison revealed that AmyHha has all the essential features of active  $\alpha$ -amylases (Grossi de Sa and Chrispeels, 1997). An aspartic acid and two glutamic acid residues are known to form the catalytic triad in the TMA (Asp185, Glu222 and Asp287) and in the porcine pancreatic  $\alpha$ -amylase – PPA (Asp197, Glu233 and Asp300). These amino acid residues are also conserved in several other  $\alpha$ -amylases (Strobl et al., 1998). However, it will be necessary to perform the N-terminus sequencing of purified *H. hampei*  $\alpha$ -amylases or to express the recombinant protein in a heterologous organism in order to validate the functionality of AmyHha cDNA to express active  $\alpha$ -amylases.

We determined the AmyHha molecular mass to be 51.2 kDa, which is similar to other insect  $\alpha$ -amylases ranging between 50 and 53 kDa (Grossi de Sa and Chrispeels, 1997; Oliveira-Neto et al., 2003; Viktorinova et al., 2011). In coleopterans,  $\alpha$ -amylase secretion occurs primarily in the anterior portion of the insect midgut (Terra et al., 1985; Cristofolletti et al., 2001). Previous reports show that the pH of the *H. hampei* midgut ranges between 4.5 and 5 (Valencia et al., 2000) that is considered the optimum pH range for  $\alpha$ -amylase activity in many beetle families (Terra and Ferreira, 1994). In most coleopteran families, the midgut content in its anterior part is usually acidic and near neutral or alkaline in the posterior portion (Terra and Ferreira, 1994). High  $\alpha$ -amylase activity in the anterior midgut and high proteolytic activity in the posterior portion are usually associated with this pH gradient. In insects that have a peritrophic membrane, endoectoperitrophic circulation occurs, in which digestive enzymes, such as proteases and  $\alpha$ -amylases, are recycled following a countercurrent flux of fluid from the posterior to the anterior midgut (Terra, 2001). As such,  $\alpha$ -amylases have to be proteolysis resistant and glycosylation may increase the stability of these enzymes in the presence of proteases. Hence, for insect species such as *Acyrtosiphon pisum*, *Apis mellifera*, *B. mori* and *T. castaneum*, it has been shown that their  $\alpha$ -amylases are glycosylated (Vandenborre et al., 2011). In silico analysis

suggest thirteen potential O-glycosylation sites and one potential site for N-glycosylation modification in the AmyHha protein sequence. Although several glycosylation sites have been predicted in the TMA  $\alpha$ -amylase, its crystal structure does not reveal any sugar moiety (Strobl et al., 1998).

Here we determined the transcript expression pattern of the AmyHha during all developmental stages of the coffee berry borer. The highest AmyHha expression was detected during second instar and adult stages corresponding to phases when the insect actively feeds on coffee seeds. Reduced transcript levels were found during non-feeding stages such as egg and pupae. Similar data, reported by Oliveira-Neto et al. (2003) for *A. grandis* using Northern blot analysis, demonstrated higher levels of  $\alpha$ -amylase transcripts in adult insects compared to unfed insects. Using qPCR, comparable fluctuation patterns, tightly correlated with feeding and non-feeding stages, were observed in insect digestive enzymes, like AmyB1  $\alpha$ -amylase from *I. typographus* (Viktorinova et al., 2011) and a trypsin from *Z. subfasciatus* (Magalhaes et al., 2007).

Based on the data obtained here, only one copy of the AmyHha gene seems to be present in the *H. hampei* haploid genome. These data corroborate with data obtained for other coleopteran species, suggesting that the existence of low gene copies of  $\alpha$ -amylase per genome is common in insects (Chen et al., 1992; Stauffer et al., 1992; Viktorinova et al., 2011). In a study performed by Valencia et al. (2000), it was demonstrated by zymography that at least two  $\alpha$ -amylase isoforms exist in *H. hampei*, which probably arose from alternative gene splicing of the AmyHha gene or other homologous genes suggested by lighter hybridization bands observed in this work. In insects, the number of  $\alpha$ -amylase isoforms is significantly smaller than the number of protease isoforms (Oliveira-Neto et al., 2003, 2004; Morris et al., 2009), making the former more attractive as a convenient target for the development of control strategies by expressing  $\alpha$ -amylase inhibitors. Accordingly,  $\alpha$ -amylases became promising targets for biotechnological strategies based on  $\alpha$ -amylase inhibitor expression via transgenesis (Franco et al., 2002; Ahmad et al., 2012).

Previous studies showed that the *P. vulgaris* amylase inhibitor I ( $\alpha$ AI-1) is a promising candidate for *H. hampei* control (Valencia et al., 2000). Thus, coffee plants have been genetically modified to specifically express this inhibitor in seeds (Barbosa et al., 2010). Bioassays demonstrated that coffee seeds expressing  $\alpha$ AI-1 negatively affected the development of *H. hampei* (unpublished data). Despite advanced studies performed on the molecular characterization of  $\alpha$ AI-1 expressed in GM coffee plants (Barbosa et al., 2010), no reports are available in the literature about the molecular and structural characterization of  $\alpha$ -amylases from *H. hampei*. Herein, we propose a model for the AmyHha enzyme, which showed a high structural similarity with the well-known TMA template, to date, the only crystallized insect  $\alpha$ -amylase (Strobl et al., 1998). The domains A, B, C observed in other insect  $\alpha$ -amylases were also detected in AmyHha. The position of the catalytic triad at the bottom of the  $(\alpha/\beta)_8$  barrel is highly conserved between AmyHha and TMA (Strobl et al., 1998). Additionally, the AmyHha model allowed us to predict the existence of four disulfide bonds, conserved in most insect  $\alpha$ -amylases performing important structural functions (Da Lage et al., 2002), and noted for being an important enzyme stabilizer. Calcium and chloride ions in the AmyHha model are shown due to the important role in the substrate binding and catalysis mechanisms, as determined in TMA (Strobl et al., 1998; Franco et al., 2002). Among the predicted amino acid residues involved in the AmyHha catalytic activity, the presence of a cysteine residue Cys241 stands out. This residue is conserved among  $\alpha$ -amylases of the Curculionidae family (Fig. 2), despite the fact that it is apparently not involved in disulfide bonds. In addition, this amino acid residue is located in an important region of the protein found in other  $\alpha$ -amylases and essential for chloride ion interaction (Strobl et al., 1997). Although the functional role of Cys241 is unknown, it suggests that it is associated with adaptive factors of starch digestion in the digestive tract of these insects.

Based on the data obtained, we propose that AmyHha is very likely the main  $\alpha$ -amylase present in the *H. hampei* gut encoded by a single-copy gene in the genome and highly expressed during major feeding stages. This feature makes this  $\alpha$ -amylase a possible target for the generation of more safe biotechnological strategies, such as the RNAi strategy to knock down the crucial digestive enzymes, besides the classic expression of enzyme inhibitors, for insect pest control.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2014.09.050>.

## Acknowledgments

The present work was supported by grants from the Brazilian government (CNPq, CAPES, FAPDF and EMBRAPA). We wish to thank Andrew Gerard Murray for their critical reviews of an earlier draft of this manuscript.

## References

- Acuña, R., Padilla, B.E., Flórez-Ramos, C.P., Rubio, J.D., Herrera, J.C., Benavides, P., Lee, S.-J., Yeats, T.H., Egan, A.N., Doyle, J.J., Rose, J.K.C., 2012. Adaptive horizontal transfer of a bacterial gene to an invasive insect pest of coffee. *Proc. Natl. Acad. Sci.* 109, 4197–4202.
- Ahmad, P., Ashraf, M., Younis, M., Hu, X., Kumar, A., Akram, N.A., Al-Qurainy, F., 2012. Role of transgenic plants in agriculture and biopharming. *Biotechnol. Adv.* 30, 524–540.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Applebaum, S.W., 1964. The action pattern and physiological role of *Tenebrio* larval amylase. *J. Insect Physiol.* 10, 897–906.
- Baker, P.S., Barrera, J.F., Rivas, A., 1992. Life history studies of the coffee berry borer (*Hypothenemus hampei*, Scolytidae) on coffee trees in southern Mexico. *J. Appl. Ecol.* 29, 656–662.
- Barbosa, A.E., Albuquerque, E.V., Silva, M.C., Souza, D.S., Oliveira-Neto, O.B., Valencia, A., Rocha, T.L., Grossi-de-Sa, M.F., 2010. Alpha-amylase inhibitor-1 gene from *Phaseolus vulgaris* expressed in *Coffea arabica* plants inhibits alpha-amylases from the coffee berry borer pest. *BMC Biotechnol.* 10, 44.
- Bradbury, A.G.W., Halliday, D.J., 1990. Chemical structures of green coffee bean polysaccharides. *J. Agric. Food Chem.* 38, 389–392.
- Calderon, L.A., Teles, R.C., Leite, J.R., Franco, O.L., Grossi-de-Sa, M.F., Medrano, F.J., Bloch Jr., C., Freitas, S.M., 2005. Purification of a 6.5 kDa protease inhibitor from Amazon *Inga umbriata* seeds effective against serine proteases of the boll weevil *Anthonomus grandis*. *Protein Pept. Lett.* 12, 583–587.
- Campos, F.A.P., Xavier, J., Silva, C.P., Ary, M.B., 1989. Resolution and partial characterization of proteinases and alpha-amylases from midguts of larvae of the bruchid beetle *Callosobruchus maculatus* (F.). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 92, 51–57.
- Carlini, C.R., Grossi-de-Sa, M.F., 2002. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicol.* 40, 1515–1539.
- Chen, M.-S., Feng, G., Zen, K.C., Richardson, M., Valdes-Rodriguez, S., Reeck, G.R., Kramer, K.J., 1992.  $\alpha$ -Amylases from three species of stored grain coleoptera and their inhibition by wheat and corn proteinaceous inhibitors. *Insect Biochem. Mol. Biol.* 22, 261–268.
- Clifford, M.N., Wilson, K.C., 1985. *Coffee: Botany, Biochemistry and Production of Beans and Beverage*. Croom Helm, London.
- Cristofolletti, P.T., Ribeiro, A.F., Terra, W.R., 2001. Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor* larvae. *J. Insect Physiol.* 47, 143–155.
- Da Lage, J.L., Van Wormhoudt, A., Cariou, M.L., 2002. Diversity and evolution of the alpha-amylase genes in animals. *Biologia* 57, 181–189.
- DeLano, W., 2002. The PyMOL Molecular Graphics System.
- Doane, W.W., 1969. Amylase variants in *Drosophila melanogaster* – linkage studies and characterization of enzyme extracts. *J. Exp. Zool.* 171, 31–41.
- Eswar, N., Webb, B., Marti-Renom, M.A., Madhusudhan, M.S., Eramian, D., Shen, M.Y., Pieper, U., Sali, A., 2007. Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.* 50, 2007, Chapter 2, Unit 2.9., 2.9.1–2.9.31.
- Fernández, S., Cordero, J., 2007. *Biología de la broca del café Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae em condiciones de laboratório. Bioagro, Barquisimeto.
- Figueiredo, S.A., 2011. Caracterização bioquímica e molecular da beta-galactosidase durante a maturação de frutos de *Coffea arabica* (Doctoral Thesis in Genomic Sciences and Biotechnology) Universidade Católica de Brasília, Brasília, DF, Brasil, p. 244.
- Franco, O.L., Rigden, D.J., Melo, F.R., Grossi-de-Sa, M.F., 2002. Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases – structure, function and potential for crop protection. *Eur. J. Biochem.* 269, 397–412.
- Gallo, D., Nakano, O., Silveira Neto, S., Carvalho, R.P.L., Batista, G.C., Berti Filho, E., Parra, J.R.P., Zucchi, R., Alves, S.B., Vendramin, J.D., 1988. *Manual de Entomologia Agrícola*. Agronômica Ceres, São Paulo.
- Giorgini, J.F., Campos, C.A.S.P., 1992. Changes in the content of soluble sugars and starch synthesis and degradation during germination and seedling growth of *Coffea arabica* L. *Rev. Bras. Fisiol. Veg.* 4, 11–15.
- Grossi de Sa, M.F., Chrispeels, M.J., 1997. Molecular cloning of bruchid (*Zabrotes subfasciatus*) alpha-amylase cDNA and interactions of the expressed enzyme with bean amylase inhibitors. *Insect Biochem. Mol. Biol.* 27, 271–281.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8.
- Lulek, J., Franco, O.L., Silva, M., Slivinski, C.T., Bloch, C., Rigden, D.J., de Sa, M.F.G., 2000. Purification, biochemical characterisation and partial primary structure of a new alpha-amylase inhibitor from *Secale cereale* (rye). *Int. J. Biochem. Cell Biol.* 32, 1195–1204.
- Kluh, I., Horn, M., Hyblova, J., Hubert, J., Doleckova-Maresova, L., Voburka, Z., Kudlikova, I., Kocourek, F., Mares, M., 2005. Inhibitory specificity and insecticidal selectivity of alpha-amylase inhibitor from *Phaseolus vulgaris*. *Phytochemistry* 66, 31–39.
- Krishna, S.S., Saxena, K., 1962. Digestion and absorption of food in *Tribolium castaneum* (Herbst). *Physiol. Zool.* 35, 66–78.
- Laskowski, R.A., Rullmann, J.A., MacArthur, M.W., Kaptein, R., Thornton, J.M., 1996. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477–486.
- Le Pelley, R.H., 1968. *Pests of Coffee*. Logmnas Green and Co. Ltd., London, pp. 1–590.
- Lemos, F.J.A., Campos, F.A.P., Silva, C.P., Xavier-Filho, J., 1990. Proteinases and amylases of larval midgut of *Zabrotes subfasciatus* reared on cowpea (*Vigna unguiculata*) seeds. *Entomol. Exp. Appl.* 56, 219–227.
- Magalhaes, C.P., Fragoso, R.R., Souza, D.S., Barbosa, A.E., Silva, C.P., Finairdi-Filho, F., da Silva, M.C., Rocha, T.L., Franco, O.L., Grossi-de-Sa, M.F., 2007. Molecular and structural characterization of a trypsin highly expressed in larval stage of *Zabrotes subfasciatus*. *Arch. Insect Biochem. Physiol.* 66, 169–182.
- Markwick, N.P., Laing, W.A., Christeller, J.T., Reid, S.J., Newton, M.R., 1996.  $\alpha$ -Amylase activities in larval midgut extracts from four species of Lepidoptera (Tortricidae and Gelechiidae): response to pH and to inhibitors from wheat, barley, kidney bean, and *Streptomyces*. *J. Econ. Entomol.* 89, 39–45.
- Mendiola-Olaya, E., Valencia-Jimenez, A., Valdes-Rodriguez, S., Delano-Frier, J., Blanco-Labra, A., 2000. Digestive amylase from the larger grain borer, *Prostephanus truncatus* Horn. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 126, 425–433.
- Morris, K., Lorenzen, M.D., Hiromasa, Y., Tomich, J.M., Oppert, C., Eldipina, E.N., Vinokurov, K., Jurat-Fuentes, J.L., Fabrick, J., Oppert, B., 2009. *Tribolium castaneum* larval gut transcriptome and proteome: a resource for the study of the coleopteran gut. *J. Proteome Res.* 8, 3889–3898.
- Murphy, S.T., Moore, D., 1990. Biological control of the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae): previous programmes and possibilities for the future. *Biocontrol News Inf.* 11, 107–117.
- Oliveira-Neto, O.B., Batista, J.A., Rigden, D.J., Franco, O.L., Falcao, R., Fragoso, R.R., Mello, L.V., dos Santos, R.C., Grossi-de-Sa, M.F., 2003. Molecular cloning of alpha-amylases from cotton boll weevil, *Anthonomus grandis* and structural relations to plant inhibitors: an approach to insect resistance. *J. Protein Chem.* 22, 77–87.
- Oliveira-Neto, O.B., Batista, J.A., Rigden, D.J., Fragoso, R.R., Silva, R.O., Gomes, E.A., Franco, O.L., Dias, S.C., Cordeiro, C.M., Monnerat, R.G., Grossi-de-Sa, M.F., 2004. A diverse family of serine proteinase genes expressed in cotton boll weevil (*Anthonomus grandis*): implications for the design of pest-resistant transgenic cotton plants. *Insect Biochem Mol Biol* 34, 903–918.
- Pava-Ripoll, M., Posada, F.J., Momen, B., Wang, C., St Leger, R., 2008. Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene. *J. Invertebr. Pathol.* 99, 220–226.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, N.Y.
- Schroeder, H.E., Gollasch, S., Moore, A., Tabe, L.M., Craig, S., Hardie, D.C., Chrispeels, M.J., Spencer, D., Higgins, T.J.V., 1995. Bean alpha-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). *Plant Physiol.* 107, 1233–1239.
- Shade, R.E., Schroeder, H.E., Pueyo, J.J., Tabe, L.M., Murdock, L.L., Higgins, T.J.V., Chrispeels, M.J., 1994. Transgenic pea seeds expressing the alpha-amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio-Technology* 12, 793–796.
- Silano, V., Furia, M., Gianfreda, L., Macri, A., Palescandolo, R., Rab, A., Scardi, V., Stella, E., Valfre, F., 1975. Inhibition of amylases from different origins by albumins from the wheat kernel. *Biochim. Biophys. Acta* 391, 170–178.
- Silva, C.P., Terra, W.R., Xavier-Filho, J., Grossi-De-Sá, M.F., Lopes, A.R., Pontes, E.G., 1999. Digestion in larvae of *Callosobruchus maculatus* and *Zabrotes subfasciatus* (Coleoptera: Bruchidae) with emphasis on alpha-amylases and oligosaccharidases. *Insect Biochem. Mol. Biol.* 29, 355–366.
- Stauffer, C., Leitinger, R., Simsek, Z., Schreiber, J.D., Führer, E., 1992. Allozyme variation among nine Austrian *Ips typographus* L. (Col., Scolytidae) populations. *J. Appl. Entomol.* 114, 17–25.
- Strobl, S., Gomis-Ruth, F.X., Maskos, K., Frank, G., Huber, R., Glockshuber, R., 1997. The alpha-amylase from the yellow meal worm: complete primary structure, crystallization and preliminary X-ray analysis. *FEBS Lett.* 409, 109–114.
- Strobl, S., Maskos, K., Betz, M., Wiegand, G., Huber, R., Gomis-Ruth, F.X., Glockshuber, R., 1998. Crystal structure of yellow meal worm alpha-amylase at 1.64 Å resolution. *J. Mol. Biol.* 278, 617–628.
- Sumathi, K., Ananthalakshmi, P., Roshan, M.N., Sekar, K., 2006. 3DSS: 3D structural superposition. *Nucleic Acids Res.* 34, W128–W132.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Terra, W.R., 2001. The origin and functions of the insect peritrophic membrane and peritrophic gel. *Arch. Insect Biochem. Physiol.* 47, 47–61.
- Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes – properties, compartmentalization and function. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 109, 1–62.

- Terra, W.R., Ferreira, C., Bastos, F., 1985. Phylogenetic considerations of insect digestion – disaccharidases and the spatial-organization of digestion in the *Tenebrio molitor* larvae. *Insect Biochem.* 15, 443–449.
- Titarenko, E., Chrispeels, M.J., 2000. cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the alpha-amylases of the Western corn rootworm, *Diabrotica virgifera virgifera*. *Insect Biochem. Mol. Biol.* 30, 979–990.
- Valencia, A., Bustillo, A.E., Ossa, G.E., Chrispeels, M.J., 2000. Alpha-amylases of the coffee berry borer (*Hypothenemus hampei*) and their inhibition by two plant amylase inhibitors. *Insect Biochem. Mol. Biol.* 30, 207–213.
- Valencia-Jimenez, A., Arboleda Valencia, J.W., Grossi-De-Sa, M.F., 2008. Activity of alpha-amylase inhibitors from *Phaseolus coccineus* on digestive alpha-amylases of the coffee berry borer. *J. Agric. Food Chem.* 56, 2315–2320.
- Vandenborre, G., Smagghe, G., Ghesquiere, B., Menschaert, G., Rao, R.N., Gevaert, K., Van Damme, E.J.M., 2011. Diversity in protein glycosylation among insect species. *PLoS One* 6.
- Viktorinova, I., Kucerova, L., Bohmova, M., Henry, I., Jindra, M., Dolezal, P., Zurovcova, M., Zurovec, M., 2011. Characterization of two closely related alpha-amylase paralogs in the bark beetle, *Ips typographus* (L.). *Arch. Insect Biochem. Physiol.* 77, 179–198.
- Villacorta, A., Barrera, J.F., 1993. Nova dieta meridica para criação de *Hypothenemus hampei*(Ferrari, 1867). *An. Soc. Entomol. Bras.* 22, 405–409.
- Watson, K., Achinelli, M.L., 2008. Context and contingency: the coffee crisis for conventional small-scale coffee farmers in Brazil. *Geogr. J.* 174, 223–234.
- Yetter, M.A., Saunders, R.M., Boles, H.P., 1979. Alpha-amylase inhibitors from wheat kernels as factors in resistance to postharvest insects. *Cereal Chem.* 56, 243–244.
- Zhao, S., Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J. Comput. Biol.* 12, 1047–1064.