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SHORT COMMUNICATION

Jhe in *Gryllus assimilis*: Cloning, sequence-activity associations and phylogeny

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Abstract

The 458 amino acid sequence of a mature JHE protein from the cricket *Gryllus assimilis* was identified after isolating the partial cDNA sequence encoding this protein from a fat body and midgut cDNA library. This hemimetabolous JHE sequence shows over 40% amino acid similarity to the known JHE sequences of several holometabolous insects. It also includes previously determined peptide sequences for *G. assimilis* JHE as well as two other motifs associated with JHE enzymes in holometabolous insects. The predicted molecular weight of the protein agrees with that of the JHE previously purified from *G. assimilis*. Partial genomic sequence encoding the *Jhe* contains two large (1330 and 2918 bp) introns. No coding DNA sequence variation was observed over a 1293 bp region between selected lines differing six to eight-fold in hemolymph JHE activity. However, a 19 bp indel was found in one of the introns; the insertion was strongly associated with elevated hemolymph activity, both in the selected lines and in the F₂ progeny of crosses between them. Phylogenetic analyses localised the *G. assimilis* JHE to a clade containing dipteran and coleopteran JHEs, with lepidopteran JHEs occurring in a separate clade.

Keywords: Juvenile hormone esterase gene, *Gryllus assimilis*, Hemimetabolous insect, Indel polymorphism, Phylogeny

1. Introduction

Juvenile hormone esterase (JHE) is an enzyme that hydrolyses and partially regulates the titre of juvenile hormone (JH), a key developmental and reproductive hormone in insects. Because of its importance in endocrine regulation, JHE has been extensively studied from physiological, biochemical and molecular perspectives (Hammock, 1985; Goodman and Granger, 2005; Oakeshott *et al.*, 2005). In a number of insects, especially lepidopterans, the purified JHE enzyme has been characterised kinetically and structurally, and the *Jhe* gene has been sequenced and its molecular regulation investigated (Hammock, 1985; Venkataraman *et al.*, 1994; Oakeshott *et al.*, 2005; Wogulis *et al.*, 2006; Bai *et al.*, 2007).

JHE also has been one of the most extensively studied insect endocrine regulators from an evolutionary genetic perspective, particularly in *Gryllus* crickets (Zera, 2006; Zera *et al.*, 2007).

Thus far, these evolutionary genetic studies have focused exclusively on biochemical and physiological aspects of JHE.

Four hemolymph JHE isoforms were purified to homogeneity from the cricket *Gryllus assimilis*; these isoforms differed among themselves in up to four of 13–20 residues at the N-terminus of the mature protein (Table 1; Zera *et al.*, 2002). We used these amino acid sequences to design a probe that was used in a hybridisation screen of a *G. assimilis* fat body and midgut cDNA library. By these means, we recovered a presumptive *Jhe* gene. We show that this gene encodes a protein with diagnostic JHE sequence motifs, and that there is strong covariation between the sequence of an intron of this gene and hemolymph JHE enzyme activity in artificially selected lines of *G. assimilis*. Phylogenetic analysis indicates that the inferred protein product of the cloned gene sits within the same clade as dipteran and coleopteran JHEs, with lepidopteran JHEs lying in a separate clade.

Table 1.

N-terminal amino acid sequences of the isoforms of the mature JHE protein of *G. assimilis* reported in Zera *et al.* (2002)

| Isoform | Sequence |
|---------------|--------------------------------|
| JHE-1 (minor) | AEAPD EVEKA xGx (K) |
| JHE-2 | AEAAP EVEVA QKRMR AAVV |
| JHE-3 | AEADP EVEVA QxRMR GAVVP |
| JHE-4 (minor) | AEAAP EVEVA QKRMR AAVVP (L) |

“x” refers to an unidentified residue; letter in brackets indicates ambiguity in the identity of the amino acid at that site, which could be either the residue designated by the letter above the brackets or within the brackets; “minor” means that band intensity of the isoform was much less than the JHE-2 and JHE-3 isoforms on an IEF gel (see Figure 4 of Zera *et al.*, 2002).

2. Materials and methods

Mass-reared lines of *G. assimilis* previously selected for high or low hemolymph JHE activity (differing six to eightfold in JHE activity; Zera and Zhang, 1995; Zera, 2006), or maintained as unselected controls, were used in the present study. JHE had been previously purified to homogeneity and characterised from the high activity and control lines (Zera *et al.*, 2002). In the current study, a cDNA library from *G. assimilis* was constructed from fat body and midgut tissues taken from a mixture of 50 mid-last stadium (days 4–7, post moult) individuals from high activity, low activity and unselected control lines. Inbred lines used in pair crosses were produced from the mass-selected high and low activity lines by five generations of brother–sister mating. Crickets were reared, staged and hemolymph obtained as in previous studies (Zera and Zhang, 1995). JHIII hydrolytic activity was tested routinely using the standard radiochemical assay of Hammock and Sparks as applied to *G. assimilis* (Zera and Zhang, 1995).

To construct a cDNA library from *G. assimilis*, total RNA, and subsequently mRNA, were isolated using the Totally RNA isolation kit (Ambion, Austin, TX) as per the manufacturer’s instructions. A combined random and oligo(dT) primed cDNA library was made by Clontech (Palo Alto, CA). The cDNA library was initially screened by PCR to verify that a *Jhe* cDNA was contained within it, and to obtain an amplicon that could be used for further library screening. Degenerate oligonucleotides (5′-CNGARGCNGCICCNARGT-3′, 5′-GCNGCICKCATNCKYTTYTG-3′) (nomenclature as per Sambrook *et al.*, 1989) that bracketed the ends of a 20 amino acid sequence of the N-terminus of one isoform (JHE-3) of the mature JHE protein (Table 1; Zera *et al.*, 2002) were used to initially screen ~10³ pfu (1 µl) of the unamplified library. The PCR reactions were performed in 50 µl and contained the following final concentrations: 1×PCR buffer, 1.5 mM MgCl₂, 2 mM each dNTP, 0.25 µM each primer and

0.5 units Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR cycles comprised an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 3 s, and a final extension step of 7 min at 72 °C. An amplicon of expected size was sequenced using BigDye terminator sequencing (Applied Biosystems, Foster City, CA).

A highly degenerate probe based on the amplicon obtained in the initial library screen (5′-CWGARGCIGCGC-CIGARGTSGARGTIGTICARAARMGIATGMGCGCIGC-3′) was used to subsequently screen 210,000 pfu of the unamplified cDNA library according to the manufacturer’s instructions (Clontech, Palo Alto, CA). A single positive plaque that hybridised was purified and converted to a pTriplex clone (according to manufacturer’s instructions, Clontech, Palo Alto, CA) and sequenced as above.

Rapid amplification of cDNA ends (RACE) was used to amplify the 3′-end of the *Jhe* cDNA. Total RNA was isolated from the head and thorax of a single *G. assimilis* adult using the SV Total RNA Isolation System (Promega, Madison, WI). 3′-RACE was performed using the 3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA) with the gene specific oligonucleotides: 3′-GSP1.602U (5′-TGTCGCCGCTCTCCAAAGGT-3′) and 3′-GSP2.1292U (5′-TCTTCGGCGGCGGGCTCAAC-3′). The amplicon was cloned into the pGem-T Easy vector (Promega, Madison, WI) and sequenced as above. Attempts to amplify the 5′-end of the cDNA (*i.e.*, encoding the untranslated leader and the codons for the presumptive signal peptide) using 5′-RACE were unsuccessful.

Genomic DNA, used to amplify *Jhe* introns by PCR, was extracted from the head and hind legs of a cricket from a line previously selected for high hemolymph JHE activity and a cricket from a line selected for low hemolymph JHE activity (Zera and Zhang, 1995) as follows: tissues were homogenised in cold 0.1 M Tris (pH 9), 0.1 M EDTA using a glass tube pestle, after which an equal volume of 2% SDS (w/v) solution was added. Samples were then incubated at 65 °C for 1 h, cooled to room temperature, and 0.86 M potassium acetate (8 M) added to each tube to precipitate proteins. After incubation on ice for 30 min, samples were centrifuged for 20 min at 16,000×g. The supernatant was extracted once with an equal volume of phenol, then once with 0.5 volume of phenol and chloroform. Samples were treated with 10 µg RNase (Sigma, St. Louis, MO) at 37 °C for 20 min, then with 10 µg of Proteinase K (Invitrogen, Carlsbad, CA) at 37 °C for 20 min. Samples were again extracted with 0.5 volume of phenol/chloroform, and DNA was precipitated with 0.4 volume isopropanol for 10 min at room temperature and collected after centrifugation at 16,000×g for 10 min. The DNA pellets were washed twice with 70% (v/v) ethanol and resuspended in 10 mM Tris (pH 8), 1 mM EDTA.

Oligonucleotides designed to different regions of the *Jhe* cDNA sequence were used to screen *G. assimilis* genomic DNA for introns. The oligonucleotide GaJHE1160U [5′-CC-GGAGCTCATGGAGTTGGA-3′, cDNA (Figure 1) co-ordi-

nates 1036–1055) was coupled with either GaJHE1249L (5'-cggtagccatctcgtagac-3', cDNA co-ordinates 1249–1268) or GaJHE1306L (5'-ACACGCCGTAGTCGTTGAG-3', cDNA co-ordinates 1306–1324]. PCR reactions contained 60–70 ng genomic DNA in 50 µl with final concentrations of the following: 60–70 ng genomic DNA, 1×PCR buffer, 1 mM MgCl₂, 0.2 mM each dNTP (Invitrogen, Carlsbad, CA), 0.2 µM each primer, 2.5% (v/v) DMSO (Sigma, St. Louis, MO) and 1U Taq DNA polymerase (BullsEye, MidSci, St. Louis, MO; or Taq DNA polymerase, Invitrogen, Carlsbad, CA). PCR cycles comprised an initial denaturation step of 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, and a final extension step of 7 min at 72 °C. The oligonucleotide gwalker79U (5'-ATCTACGCCTTCCGCAGCATCCCCTTC-3', cDNA co-ordinates 79–105) was coupled with gwalker205 (5'-GTACGGCGGCACCAAGTAGTTCTTCTG-3', cDNA co-ordinates 205–231) in 50 µl reactions containing the same final concentrations as above. PCR cycles comprised seven cycles of 94 °C for 25 s, 72 °C for 3 min followed by 30 cycles of 94 °C for 25 s, 67 °C for 3 min and a final extension step of 7 min at 67 °C. The PCR

reactions were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and cloned into the pGem-T Easy vector following manufacturer's directions (Promega, Madison, WI). Clones were sequenced as above.

Routine rapid screening of crickets for a 19 bp insertion/deletion (the *Jhe*^{L/S} indel) within an intron was performed using genomic DNA extracted from one hind leg using Chelex DNA Extraction. In brief, each leg was homogenised in a 1.5 ml centrifuge tube, using a motor-driven pestle, in a total of 207 µl containing 0.01 g Chelex 100 Resin (BioRad, Hercules, CA), 0.02 mg Proteinase K (Invitrogen, Carlsbad, CA), 0.34 M dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO). The homogenates were incubated at 56 °C for 1 h, 95 °C for 10 min, vortexed for 15 s, centrifuged at 14,000×g for 1 min, and the supernatant was removed. The supernatant was diluted 1/10 and 1 µl was used as template for PCR. Each PCR reaction was performed in a total of 50 µl and contained the following final concentrations: 1×PCR buffer, 1 mM MgCl₂, 0.2 mM each dNTP (Invitrogen, Carlsbad, CA), 2.5% (v/v) DMSO (Sigma, St. Louis, MO) 0.2 µM each primer [3'-in181U 5'-CACCCCCTTATCGCTTCAT-3', EU0579709

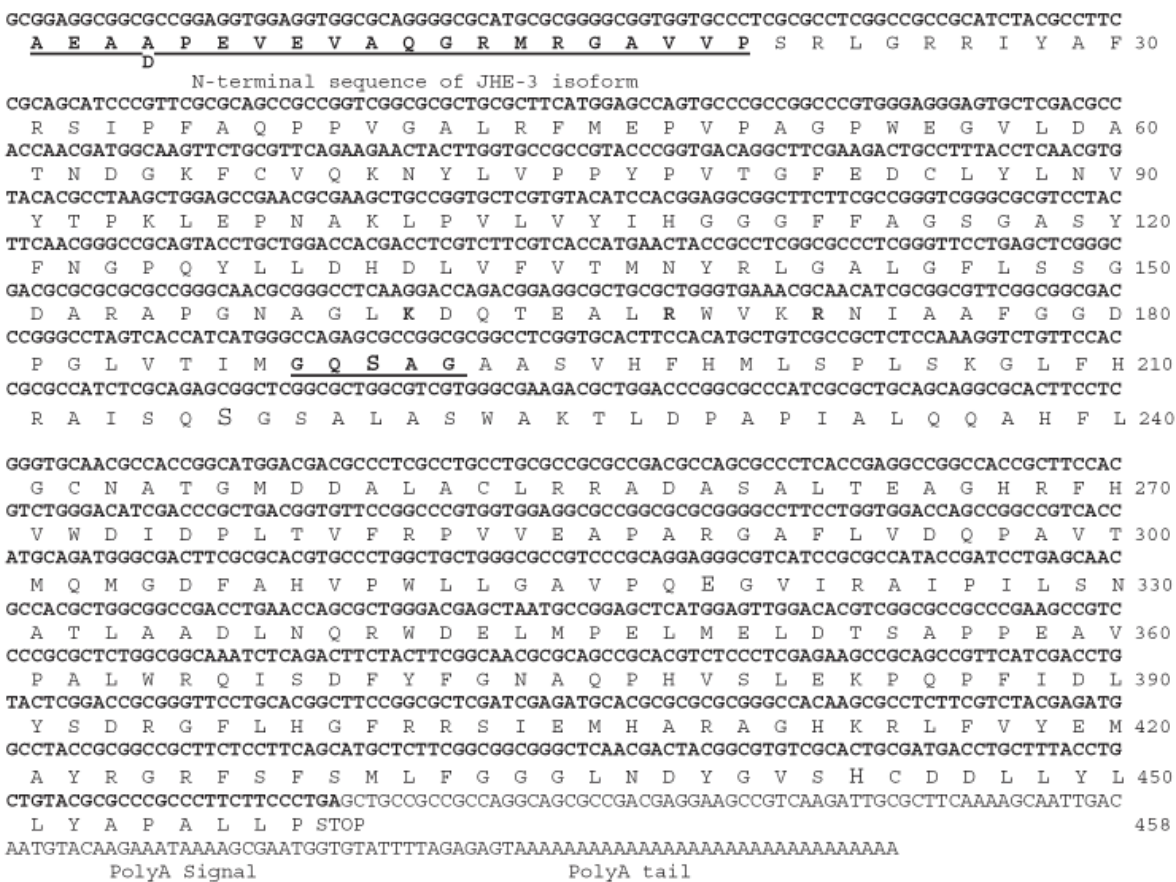


Figure 1. Nucleotide sequence and amino acid translation of *G. assimilis* *Jhe* cDNA (Genbank accession no. EF558769). The residues which make up the 20 amino acid N-terminal sequence of the mature protein are in bold type face and underlined. With one exception (alanine at position four compared to an aspartic acid), the translated amino acid sequence corresponds to the amino acid sequence of the previously reported JHE-3 isoform (Zera *et al.*, 2002). The GQSAG motif, which is an uncommon variant of the general esterase active site motif GXSXG, but which is found in all known JHEs, is in bold type face and underlined. The three amino acids that comprise the amphipathic helix also are in bold type face.

(see Section 3) co-ordinates 1762–1781; GaJint.259L 5'-CTTGGTGTGGCGTGTGTAAC-3', EU057970 coordinate 1953–1972); and 1U Taq DNA polymerase (BullsEye from MidSci, St. Louis, MO; or native Taq DNA polymerase from Invitrogen, Carlsbad, CA). The PCR protocol comprised an initial denaturation step of 2 min at 94 °C; followed by 35 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 30 s; and a final extension step of 72 °C for 7 min and 25 °C for 30 s. PCR products were separated on a 4% (w/v) agarose gel with 1×TAE buffer. DNA banding patterns were visualised using ethidium bromide.

To determine whether the JHE coding sequence differs between crickets selected for high or low hemolymph JHE activity, total RNA was isolated individually from four high activity and four low activity *G. assimilis* using Trizol (Invitrogen). RNA was treated with DNAase, converted into cDNA using 200u SuperScript II (Invitrogen, Carlsbad, CA) and an oligo(dT18) primer following manufacturers instructions. JHE amplicons were produced using the following primers: gwalker79U (sequence given above; cDNA co-ordinates 79–105) and GaJHE1348 (5'-GAAGAAGGGCGGGCGCGTACAG-3'; cDNA co-ordinates 1350–1372 bp). PCR conditions were the same as those described above using gwalker79U, except that the 72C extension step was 1 min. Amplicons were cloned into the pGen-T Easy vector and sequenced as described above.

Phylogenetic analyses were conducted using Neighbor-Joining (Felsenstein, 2004) and Maximum Likelihood methods (Felsenstein, 2004) to determine the relationships among *G. assimilis* JHE and other JHEs. The analysis was performed on the data set that included sequences for JHEs and catalytically active carboxyl/choline esterases reported in Claudianos *et al.* (2006), JHE for *G. assimilis* (this study), and several JHE and carboxyl/cholinesterase sequences reported subsequent to the study of Claudianos *et al.* (2006) (see legend of Figure 3). The amino acid sequence alignment was performed using MUSCLE multiple alignment software (Edgar, 2004), and phylogenetic analyses were conducted in MEGA3 (Kumar, *et al.*, 2004).

3. Results and discussion

A cDNA library from *G. assimilis* was constructed using mid-last-stadium fat body and midgut tissues, two tissues that contain high JHE activity in this (Zera, unpublished data), and other insect species (*e.g.*, Bai *et al.*, 2007). Previously, two major and two minor JHE isoforms were found in *G. assimilis*, which differ by one to several amino acids at the N-terminus of the mature protein (Table 1; Zera *et al.*, 2002). Degenerate primers were designed to the 3'- and 5'-ends of one of these sequences (JHE-3). The presence of *Jhe* cDNA in the cDNA library from *G. assimilis* was established by the finding of PCR amplicons which contained the diagnostic nucleotide sequence encoding three amino acids in the middle of the known peptide sequence that were not encoded by the oligonucleotide primers. The library was then screened by hybridisation using a degenerate probe based on the amplified sequence.

A single partial cDNA clone of 1445 bp that did not contain start codons, and which contained diagnostic JHE motifs, was identified. The 5'-end of the clone encoded the N-terminal sequence of the JHE-3 major isoform of the mature JHE protein (Table 1; Zera *et al.*, 2002), but with an alanine rather than aspartic acid in the fourth position. Two of the other three JHE isoforms, JHE-2 and JHE-4, also contained alanine in this position (Table 1; Zera *et al.*, 2002).

The 3'-end of the *G. assimilis Jhe* cDNA was obtained using RACE. Attempts to obtain additional 5'-sequence by RACE and inverse PCR (data not shown) were unsuccessful suggesting that there may be significant secondary structure in this region. The additional 67 bp of sequence generated by the 3'-RACE, together with the original cloned cDNA provided a total of 1512 bp of cDNA sequence which begins at the codons specifying the residues at the N-terminus of the mature protein determined earlier (Zera *et al.*, 2002) and ends in a 3'-untranslated sequence and polyadenylated tail (GenBank accession no. EF558769). The mature protein encoded by this gene has a predicted molecular weight of 50 kDa, which corresponds to the subunit molecular weight determined for the mature protein (52 kDa; Figure 1; Zera *et al.*, 2002).

The protein sequence has 51% similarity to the JHE of the cerambycid beetle *Psacotheta hilaris*, 49% similarity to *Drosophila melanogaster* JHE and 45% similarity to *Heliothis virescens* JHE. The *G. assimilis* JHE contained two particular sequence features which are found in all known JHEs to date (Figure 1; see Oakeshott *et al.*, 2005 and references therein). The first of these is a **GQSAG** motif around the serine residue of the catalytic triad, which is an uncommon variant of the general esterase active site motif (**GXSXG**). The second of these is a predicted amphipathic helix about 18–29 amino acids upstream of the catalytic serine triad residue which has three or four basic residues along one face.

No differences in 1293 bp of the *Jhe* coding sequence were observed among eight amplicons each of which had been derived from a different *G. assimilis* (four from a high activity line, four from a low activity line). This result suggests that line differences in JHE activity are not due to allelic differences in catalytic efficiency, at least for the *Jhe* gene isolated in the present study. This finding is consistent with a previous analysis of *G. assimilis* hemolymph JHE, which did not identify any kinetic difference between enzymes from these high and low activity lines (Zera, 2006). High and low activity lines differ in JHE transcript abundance (Crone, Zera and Anand; unpublished data), implicating variation in *Jhe* gene expression as a cause of activity differences between selected lines.

Two introns within the *Jhe* gene were located at 138 and 1167 bp (cDNA numbering), and were 1131 and 2918 bp in length, respectively; both contained a variant of the *Drosophila* exon/intron splice recognition sequence (*Drosophila* splice donor A₆₉G₅₉ (exon) [G₁₀₀T₁₀₀A₅₅A₈₁G₈₄T₆₈ (intron)/splice acceptor C₇₆A₉₇G₁₀₀ (intron)] (exon)). Only seven sin-

gle nucleotides differed between the selected lines in the intron at 139 bp, while a 19 bp insertion/deletion polymorphism (5'-GTCGGCCCCAATCCGCTCT-3'; indel sequence found 187 bp from start of intron) was observed in the other intron (partial genomic DNA sequence GenBank accession no. EU057970).

To determine whether the frequency of the 19 bp indel within the 3'-intron differed between the lines of *G. assimilis* selected for high or low hemolymph JHE activity, a fast screening method was developed using genomic DNA isolated from one hind leg of a cricket, PCR amplification, and visualisation by agarose gel electrophoresis (Figure 2). The frequency of *Jhe*^L (insertion) in a high JHE activity line was 0.91 ($n=32$), while the frequency of this insertion in a low activity line was 0.00 ($n=27$).

To more firmly establish the extent of association between JHE hemolymph activity and indel phenotype, pair crosses were performed between inbred lines derived from the high or low JHE activity lines. An individual from a high JHE activity inbred line homozygous for *Jhe*^L was crossed with an individual from a low activity inbred line homozygous for *Jhe*^S. This cross was replicated using another pair of parents from a different set of lines. Individual F₁ progeny from each cross were then pair-crossed and hemolymph JHE activity and indel phenotype (electrophoretic band size) were measured in the F₂ progeny. JHE activities (mid-last stadium; day 4–5 post moult) did not differ between the same indel phenotypes in F₂ progeny of the two crosses, and thus data for the two crosses were pooled. The indel phenotypes differed substantially in JHE activity ($P<0.008$, one-way ANOVA). Individuals homozygous for the *Jhe*^L indel (*Jhe*^{LL}, $n=13$) had significantly higher JHE hemolymph activity (31.8 ± 2.7 nmol JHIII acid/min/ml) than either heterozygous individuals (24.1 ± 1.9 nmol JHIII acid/min/ml; *Jhe*^{LS}, $n=22$; $p<0.05$) or individuals homozygous for the *Jhe*^S indel (20.5 ± 2.4 nmol JHIII acid/min/ml; *Jhe*^{SS}, $n=16$; $p<0.005$). In other words, hemolymph JHE activity and indel phenotype co-segregated strongly in the F₂ progeny.

This strong co-segregation between the indel phenotype and JHE activity does not formally prove that the cDNA cloned in this study encodes a physiological JHE enzyme. What it shows is that the cloned gene is at least very closely linked genetically to the source of the activity variation. In view of the four esterase isoforms with JHE activity in vitro that Zera *et al.* (2002) obtained from *G. assimilis*, it would be plausible to suggest that there are multiple closely linked *Jhe* or *Jhe*-like genes in this species. Importantly, however, there is also evidence for parallel differences in the abundance of transcript encoded by the cloned *Jhe* gene between the high and low selected JHE lines (Crone, Zera and Anand, unpublished data). Given also its possession of the diagnostic JHE sequence motifs and the co-segregation of indel and activity variation observed here, we conclude that the *Jhe* gene described here is, at the least, a significant contributor to physiological JHE activity.

Results of the phylogenetic analysis (Figure 3) placed JHE from *G. assimilis* within the clade containing JHEs from cole-

opterans and dipterans with very strong support (99/85% bootstrap). This clade did not include lepidopteran JHEs, which all clustered on a separate branch of the tree with strong support (99/100% bootstrap), in agreement with the tree presented by Claudianos *et al.* (2006). The tree in Figure 3 provides support for the hypothesis forwarded by Claudianos *et al.* (2006) that there are two separate origins of JHE in the insect esterases.

To more formally test the hypothesis of multiple vs. single origin of JHEs, the likelihood scores were compared for the tree presented in Figure 3 (consistent with two independent origins of JHE), compared with a tree where JHEs were forced into a monophyletic clade (constructed using Treefinder (Jobb *et al.*, 2004). The multiple-origin tree ($-\ln L=-76912.2$) was significantly better supported ($p<0.002$) than the single-origin tree ($-\ln L=-77142.5$) in a Shimodaira–Hasegawa topology test (see Jobb *et al.*, 2004).

The close clustering between JHEs from *G. assimilis* and *Tenebrio molitor* is consistent with biochemical data. For example, *G. assimilis* JHE exclusively exists as dimer while *T. molitor* JHE exists as a partial dimer (Hinton and Hammock, 2003). All other insect JHEs are reported to be monomers (Zera *et al.*, 2002). At present, the esterase tree is not well enough resolved to determine which JHE clade is ancestral.

Other lines of evidence suggest that there may be more than two independent origins of JHE in insects. In the honey bee *Apis mellifera* there are two candidate *Jhe* genes (Claudianos *et al.*, 2006), which sit in different clades, both from

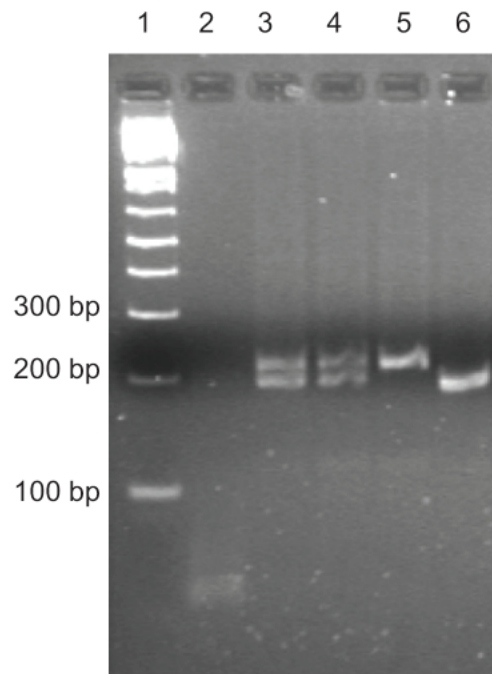


Figure 2. Agarose gel showing separation of amplicons containing an insertion/deletion polymorphism within the 3'-intron of the *Jhe* gene of *G. assimilis*. Lane 1, 1Kb PLUS DNA ladder (Invitrogen, Carlsbad, CA); lane 2, no template control reaction; lanes 3 and 4, show heterozygous individuals (*Jhe*^{LS}); lane 5, homozygous individual for *Jhe*^{LL}; lane 6, individual homozygous for *Jhe*^{SS}. *Jhe*^L=191 bp; *Jhe*^S=172 bp.

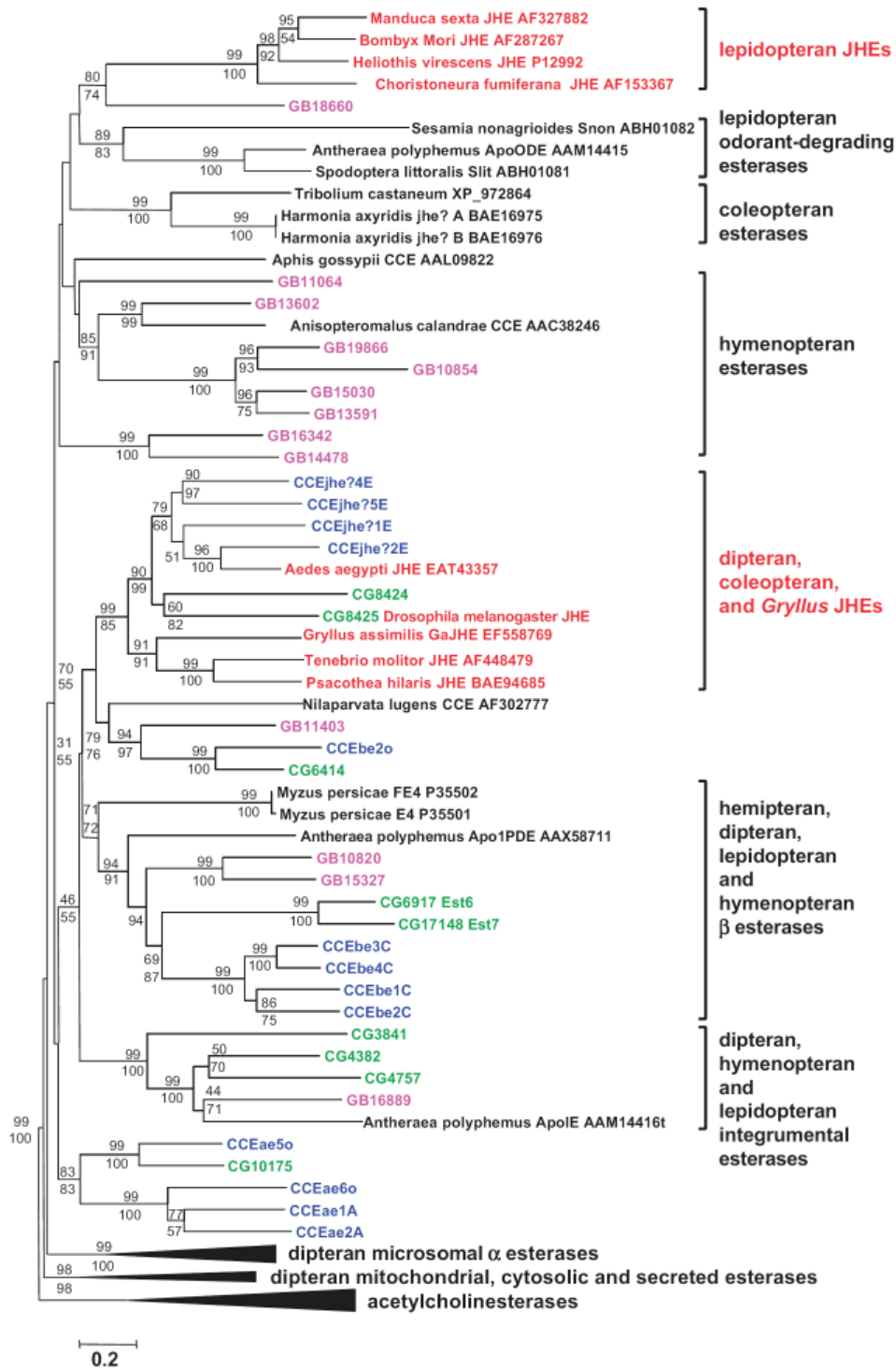


Figure 3. Evolutionary relationships of JHEs and other insect carboxyl/cholinesterases. The tree was obtained by applying the Neighbor-Joining method (Saitou and Nei, 1987; cited in Felsenstein, 2004) to the alignment of catalytic carboxyl/cholinesterases in Claudianos *et al.* (2006) updated with the *G. assimilis* sequence from the present paper; JHE sequences from Hinton and Hammock (2003), Merlin *et al.* (2007), Munyiri and Ishikawa (2007), Bai *et al.* (2007); and recent Genbank entries for one *Tribolium castaneum* and two *Harmonia axyridis* esterases. The optimal tree with the sum of branch length=47.968 is shown. Some large clades have been collapsed. For ease of viewing, all JHE sequences are coloured in red. In addition, *Drosophila melanogaster* (green), *Anopheles gambiae* (blue) and *A. mellifera* (magenta) sequences have also been colour-coded. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to produce it. Distances were computed using the Dayhoff matrix-based method and the units are the number of amino acid substitutions per site. Positions containing alignment gaps and missing data were only eliminated in pairwise sequence comparisons (pairwise deletion option). There were a total of 598 positions in the final dataset. A Maximum Likelihood analysis (Felsenstein, 2004) produced a tree with topology congruent with this Distance-based tree. The percentages of replicate trees in which sister groups clustered together in the bootstrap test in >50% of 1000 replicates (Felsenstein, 2004) are shown for both the Distance and Maximum Likelihood (normal font and italicised, respectively) analyses. Note that six sequences in the tree are shown as 'jhe?' because we have been unable to find published evidence that they encode functional JHEs; the two *H. axyridis* sequences were annotated as JHEs in their Genbank entries while the four *A. gambiae* sequences were simply annotated as candidate JHEs by Ranson *et al.* (2002; cited in Claudianos *et al.*, 2006) on the basis of their sister group status with the known *D. melanogaster* JHE. These six 'jhe?' sequences were not included in the maximum likelihood test of multiple vs. single origin of JHEs described in the text.

each other and from the lepidopteran and dipteran/coleopteran JHEs. Several *D. melanogaster* carboxylesterases have measurable JHE activity in vitro (Crone *et al.*, 2007) further suggesting the potential for repeated JHE evolution from carboxylesterases. With respect to enzyme structure, it may not have been a major evolutionary challenge to evolve physiological levels of JHE activity from a range of potential carboxylesterase starting points: JH is a sesquiterpenoid ester of methanol and as such has a long acyl group but a very small alcohol group. Because the long acyl group can be accommodated along the length of the active site gorge, with little extra space needed at the base of the gorge to accommodate the alcohol moiety, carboxylesterases may not require a highly elaborated pocket structure in their active site to accommodate JH productively (Wogulis *et al.*, 2006).

Isolation of this hemimetabolan cDNA will allow a broader understanding of basic molecular aspects of *Jhe* structure, regulation and phylogeny in insects. The *G. assimilis* *Jhe* indel polymorphism that strongly co-segregated with JHE hemolymph activity also will be useful in studies examining the molecular causes of JHE activity variation in natural populations. Such studies will be particularly important since the molecular basis of naturally occurring endocrine variation in insects is poorly understood (Zera, 2006; Zera *et al.*, 2007).

Acknowledgments

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