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cDNA Sequence and mRNA Expression of a Putative Alcohol Dehydrogenase from the Mole Cricket, *Gryllotalpa orientalis*

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Alcohol dehydrogenases (AHDs) are enzymes responsible for the catalysis of the reversible conversion of various alcohols to their corresponding aldehydes and ketonesis. Until now cDNA sequences of ADH gene is informed exclusively from several diptean species. We describe here the cDNA sequence and mRNA expression of a putative ADH gene from the mole cricket, *Gryllotalpa orientalis*, and phylogenetic relationships among known insect ADHs. The *G. orientalis* ADH cDNA sequences comprised of 798 bp encoding 266 amino acid residues. The multiple sequence alignment of *G. orientalis* ADH gene and known dipteran ADHs shared 100% identity in the nine amino acid residues that are important for the enzymatic activity in *Drosophila melanogaster*. Percent sequence identity ranged from 25% to 32% among all insect ADHs including both types of ADHs. *G. orientalis* ADH gene showed no clear resemblance to any dipteran species and type. Phylogenetic analysis of the deduced amino acid sequences of *G. orientalis* ADH gene with available dipteran ADH genes including both types of ADHs further confirmed that the *G. orientalis* ADH gene is not clearly assigned to either type of ADHs. Northern blot analysis revealed a stronger signal in the fat body than midgut and epidermis, indicating that the fat body possibly is a main site for the synthesis of the *G. orientalis* ADH protein.

Key words: Insect, Mole cricket, *Gryllotalpa orientalis*, Alcohol dehydrogenase, ADH, cDNA sequences, mRNA expression, Phylogeny, Evolution

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Introduction

Alcohols are one of the most essential and common sources for insects, whose survival is linked to the efficient exploitation of alcohol-rich feeds. The alcohol dehydrogenases (ADHs) in insects (EC 1.1.1.1) are responsible for the catalysis of the reversible conversion of various alcohols generated by microbial fermentation in larval or adult feeding sites to their corresponding aldehydes and ketones (Atrian *et al.*, 1998).

ADH genes have been cloned and sequenced from abundant *Drosophila* species, partially because the fruit flies had developed a suitable enzymatic system to cope with the high amounts of alcohols present in the fermenting fruits and rotting roots or leaves (Chambers, 1988, 1991; Winberg and Mckinley-Mckee, 1992). *Drosophila* ADH is a dimmer with molecular weight of 54,800 Da, consisting of two identical subunits (Chambers, 1988, 1991; Winberg and Mckinley-Mckee, 1992). Characterization of *Drosophila* ADH showed that the alcohol metabolizing system in *Drosophila* was not protein homologue to horse liver (Winberg and Mckinley-Mckee, 1992). Instead, characterization of *Drosophila* protein and sequence alignment identified that the *Drosophila* ADH belongs to a large enzyme family, named short-chain dehydrogenase/reductase (SDR) family (Jörnvall *et al.*, 1995; Brendskag *et al.*, 1999).

Drosophila ADH was also popular for the study of evolutionary perspective of gene evolution, because some species of *Drosophila* have been known to contain duplicated genes, named Adh-1 and Adh-2, expressed during different larval stages (Batterham *et al.*, 1984; Fischer and Maniatis, 1985; Mills *et al.*, 1986; Atkinson *et al.*, 1988).

Except for *Droshophila*, genetic information on insect ADH is very limited to a few other species such as the

medfly (*Ceratitis capitata*), the olive fruit fly (*Bactrocera oleae*), and flesh fly (*Sarcophaga peregrine*) (Horio *et al.*, 1996; Benos *et al.*, 2000; Goulielmos *et al.*, 2001). Thus, all available sources of ADH genes including *Drosophila* are confined to Diptera. Biochemical studies have shown that two ADH proteins exist in the *C. capitata* (Gasperi *et al.*, 1992), encoded by two highly linked genes on the second chromosome, probably generated by gene duplication event (Malacrida *et al.*, 1992; Gasperi *et al.*, 1994). In *B. oleae*, the ADH is a dimmer with molecular weight of 48,000 Da, consisting of two subunits (Mazi *et al.*, 1998), and the two ADH genes, named Adh1 and Adh2, consisting of three exons and two introns for a total of 1,981 and 988 nucleotides, respectively, have been reported (Goulielmos *et al.*, 2001).

Until recently insect ADH genes had been cloned extensively from specie of *Drosophila* and a few dipteran species such as *C. captotata* and *B. oleae* (Horio *et al.*, 1996; Ashburner, 1998; Benos *et al.*, 2000). The sequence identity between two ADH genes of the medfly peptide were 83% identical to each other, but shows only ~35% identity to the *Drosophila* ADH genes. Also, one ADH gene cloned from *S. peregrine* is 38% identity to *Drosophila* (Horio *et al.*, 1996), showing a high sequence homology between duplicated genes within species, but low sequence homology between different species.

The mole cricket, *Gryllotalpa orientalis* (Burmeister), is a singly known species of the Family Gryllotalpidae in Korea, and distributed in Asia and many European and African countries (Nevo *et al.*, 2000). In order to obtain molecular information of the mole cricket, therefore, we have previously constructed the cDNA library using *G. orientalis* whole bodies (Kim *et al.*, 2002). The Sec61p γ subunit, chemosensory protein, glutathione S-transferase homologue genes have been identified by screening expressed sequence tag (EST) of the *G. orientalis* cDNA library (Kim *et al.*, 2002, 2003a, b).

In this study, we report the cDNA sequence of the putative alcohol dehydrogenase (ADH) gene from *G. orientalis* and this information is unique excluding dipteran species. The deduced amino acid sequences of the gene were compared to those of dipteran ADHs and the genetic relationship of the gene to other species and evolutionary perspective of the gene were discussed.

Materials and Methods

Animals, cDNA library screening, and nucleotide sequencing

The mole cricket, *Gryllotalpa orientalis* (Burmeister), was collected in Gimhe city, Korea (Kim *et al.*, 2002). *G. orientalis* larval cDNA library (Kim *et al.*, 2002) was screened to generate the ESTs. Sequencing of randomly selected clones harboring cDNA inserts was performed. For DNA sequencing, plasmid DNA was extracted by Wizard mini-preparation kit (Promega, Madison, WI). Sequence of each cDNA clone was determined using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI. GenBank, EMBL, and SwissProt databases were searched for sequence homology using a BLAST algorithm program.

Data analysis

MacVector (ver. 6.5, Oxford Molecular Ltd.) was used to align the amino acid sequence of ADHs. The aligned amino acid sequences of the sixteen GenBank-registered ADH amino acid sequences and putative *G. orientalis* ADH sequence were subjected to phylogenetic analysis by maximum-parsimony (MP) and neighbor-joining (NJ) methods using PAUP* (Phylogenetic Analysis using Parsimony and Other Method*) ver. 4 (Swofford, 2000). In the MP analysis, heuristic search was performed with the options of tree-bisection-reconnection (TBR) for branch-swapping algorithm, steepest descent option not in effect, stepwise addition for starting tree, and initial "MaxTrees" setting for 100. Branches were collapsed if maximum branch length is zero. In the NJ analysis, mean character difference was chosen for distance measure. Negative branch lengths were allowed, but these were set to zero for tree-score calculation. In both analyses, bootstrap analysis was performed for 1,000 replications. Outgroup species was chosen for NAD^+-dependent 15-hydroyprostaglandin dehydrogenase of the bovine, *Bubalus* sp., which is known as an insect homologous of mammals, belonging to the same short-chain dehydrogenases/reductases family (SDR) (GenBank accession number CAA11017) for both analyses. The information and accession numbers of the 16 insect ADH sequences in the GenBank are described in Table 1.

RNA isolation and Northern blot analysis

Total RNA was isolated from fat body, midgut, and epidermis of the *G. orientalis* using the Total RNA Extraction Kit (Promega, Madison, WI). Total RNA (10 μg/lane) from the *G. orientalis* was separated on glyoxalation gel (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a buffer containing 2×PIPES, 50% formamide, 1% sodium dodecyl sulphate (SDS) and blocking agent (Boehringer Mannheim, Mannheim, Germany). The probe used to detect

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-84                                                    ggcgtccccttcgcaggaagaaga
-61  gcaaagaagagcacagcagagcacagcagagctgcacctacctcatccacatacacagtc
  1  ATGGACCCCAAGGGGAAGTACGCTTTGGTGACTGGCGGCGCCACCGGCATTGGTCTGGCC
  1  M  D  P  K  G  K  Y  A  L  V  T  G  G  A  T  G  I  G  L  A
 61  TACGTGAAGGAGCTGCTGCGCCACGGCGTACAGGCTGTGGCTGTCGCTGACCTCGACGAA
 21  Y  V  K  E  L  L  R  H  G  V  Q  A  V  A  V  A  D  L  D  E
121  CGCAAAGGAGACAATTCCGTGAAACAGCTCAACGACGAATTCGGCGCGGGTAAAGCCATC
 41  R  K  G  D  N  S  V  K  Q  L  N  D  E  F  G  A  G  K  A  I
181  TTCATCAAGTGTGACGTCACCAACAAGGACGATCTTGAAGCTACTTTCAGAAAAGCTGCC
 61  F  I  K  C  D  V  T  N  K  D  D  L  E  A  T  F  R  K  A  A
241  GCCACTTTCAAGGGACTGGATATTGTCATTAACAATGCTGGTATCTTGGATGATTCTCGT
 81  A  T  F  K  G  L  D  I  V  I  N  N  A  G  I  L  D  D  S  R
301  TGGGAGACTGAAATCGCCATCAATGTGACGGCAGTGGCACGAGGCACTTGGCTGGCCTTC
101  W  E  T  E  I  A  I  N  V  T  A  V  A  R  G  T  W  L  A  F
361  GATCTGATGGGCAAGAACAAAGGTGGGCGCGGAGGCGTGGTGGTGAACATCGCGTCTATC
121  D  L  M  G  K  N  K  G  G  R  G  G  V  V  V  N  I  A  S  I
421  CTGGGTCTGCAGGCCATGGCTGGCTGTCCAGTGTACGTCGCCACCAAGCACGCCGTGGTC
141  L  G  L  Q  A  M  A  G  C  P  V  Y  V  A  T  K  H  A  V  V
481  GGACTCAGCCGCTCATTCGGGATGCCGTACCACTTTGACCGCACTGGAGTTCGCGTGCTG
161  G  L  S  R  S  F  G  M  P  Y  H  F  D  R  T  G  V  R  V  L
541  ACCATGTGCCCTGGAGTCACAGACACACCTCTTATTTCCGAGGCTCACCACCGCCAGTTT
181  T  M  C  P  G  V  T  D  T  P  L  I  S  E  A  H  H  R  Q  F
601  ACAGACGATTTGGGCAAGGAGTGCGGCCGTGAACTGGATGCTCTTCCCAAGCAGAAACCT
201  T  D  D  L  G  K  E  C  G  R  E  L  D  A  L  P  K  Q  K  P
661  GAAAACGTGGCGAAAGGGATGCTGCACATCATCTGCAAGGCCTCCAACGGAAGCGTGTGG
221  E  N  V  A  K  G  M  L  H  I  I  C  K  A  S  N  G  S  V  W
721  GTGAGCGAGGGGGGGCAGCCTGTCTACGAAGTGGCCATTCCAGAACGGGAGACCATCCGC
241  V  S  E  G  G  Q  P  V  Y  E  V  A  I  P  E  R  E  T  I  R
781  GTGGCAATTAAGCGTCCCTAGtctccagcttctgcactactgttattgctccgctttcac
261  V  A  I  K  R  P  *
841  ttcctaataaagatgattcttcttaaaaaaaaaaaaaa
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Fig. 1. The nucleotide and deduced amino acid sequences of *G. orientalis* ADH. The start codon ATG is boxed and the termination codon is shown by asterisk. The polyadenylation sites are underlined.

Table 1. Alcohol dehydrogenase from various insect species

Common name	Species	Sequence name	Amino acid size	GenBank accession number	References
Orthoptera					
Mole cricket	*Gryllotalpa orientalis*	*Go*ADH	266		This study
Diptera					
Olive fruit fly	*Bactrocera oleae*	BoADH1	257	AJ277835	Goulielmos *et al.* (2001)
Olive fruit fly	*Bactrocera oleae*	*Bo*ADH2	258	AJ277834	Goulielmos *et al.* (2001)
Mediterranean fruit fly	*Ceratitis capitata*	*Cc* ADH1	257	Z30194	Benos *et al.* (2000)
Mediterranean fruit fly	*Ceratitis capitata*	*Cc*ADH2	258	Z30195	Benos *et al.* (2000)
Flesh fly	*Sarcophaga peregrina*	*Sp*ADH	257	D63669	Horio *et al.* (1996)
Fruit fly	*Drosophila hydei*	*Dh*ADH1	254	X58694	Menotti-Raymond *et al.* (1991)
Fruit fly	*Drosophila hydei*	*Dh*ADH2	254	X58694	Menotti-Raymond *et al.* (1991)
Fruit fly	*Drosophila buzzatii*	*Db*ADH1	254	U65746	Unpublished
Fruit fly	*Drosophila buzzatii*	*Db*ADH2	254	U65746	Unpublished
Fruit fly	*Drosophila mojavensis*	*Dmoj*ADH1	254	X12536	Bayer *et al.* (1992)
Fruit fly	*Drosophila mojavensis*	*Dmoj*ADH2	254	X12536	Bayer *et al.* (1992)
Fruit fly	*Drosophila montana*	*Dmon*ADH1	254	U26842	Nurminsky *et al.* (1996)
Fruit fly	*Drosophila montana*	*Dmon*ADH2	254	U26845	Nurminsky *et al.* (1996)
Fruit fly	*Drosophila virilis*	*Dv*ADH1	254	U26846	Nurminsky *et al.* (1996)
Fruit fly	*Drosophila virilis*	*Dv*ADH2	254	U26846	Nurminsky *et al.* (1996)
Fruit fly	*Drosophila melanogaster*	*Dmel*ADH	256	AAF00238	Begun *et al.* (1999)

the ADH transcripts was 798 bp for *G. orientalis* ADH cDNA cloned in this study and labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2×SSC at 65°C, and finally exposed to autoradiography film.

Results and Discussion

A cDNA clone showing similarity to the reported insect ADHs was obtained by screening the cDNA library of the mole cricket, *G. orientalis*. The nucleotide sequence of the cDNA clone was analyzed and its amino acid sequence was deduced (Fig. 1). The sequences of the *G. orientalis* ADH cDNA comprised of 798 bp encoding 266 amino acid residues. The size of *G. orientalis* ADH is longest among both types of known insect ADHs, where size ranges from 254 to 258 amino acid residues (Table 1). The *G. orientalis* ADH gene possesses four internal insertions of one ~ three amino acid residues.

A multiple sequence alignment of the deduced protein sequence of *G. orientalis* ADH gene with other insect ADH sequences is shown in Fig. 2. Alignment of the *G. orientalis* ADH sequences with those of ADHs from several other dipteran species indicates the extent of the identity that exists. Particularly, the nine amino acid residues that were determined to be particularly important for enzymatic activity in *D. melanogaster* (Chen *et al.*, 1993; Cols *et al.*, 1993; Jörnvall *et al.*, 1995) were all conserved in the same positions in all insects including *G. orientalis* (Fig. 2), supporting the significance of these residues for the enzymatic function regardless of ADH type and taxonomic group.

The *G. orientalis* ADH was overall equidistant to other insect ADHs, regardless of species and types, and the percent sequence identity of the *G. orientalis* ADH to other insects ranged from 25% to 32%, the estimates of which are not high (Table 2). On the other hand, the percent sequence identity among *Drosophila* (*D. hydei*, *D. Buzzatii*, *D. mojavensis*, *D. Montana*, *D. virilis* and *D. melagoga*) (78% ~ 100%) and among two species of tephritid (*Bactrocera oleae* and *Ceratitis capitata*) (77% ~ 85%), respectively, were very high, regardless of the ADH type within taxonomic group (Table 2). Considering *G. orientalis* is a member of order Orthoptera and others are Diptera, the low sequence identity of *G. orientalis* to dipteran species appears to be normal. Also, the high sequence identity within two species of tephritid (ranged from 77% to 85%) and within six species of *Drosophila* (ranged from 78% to 100%) appears to be plausible. On

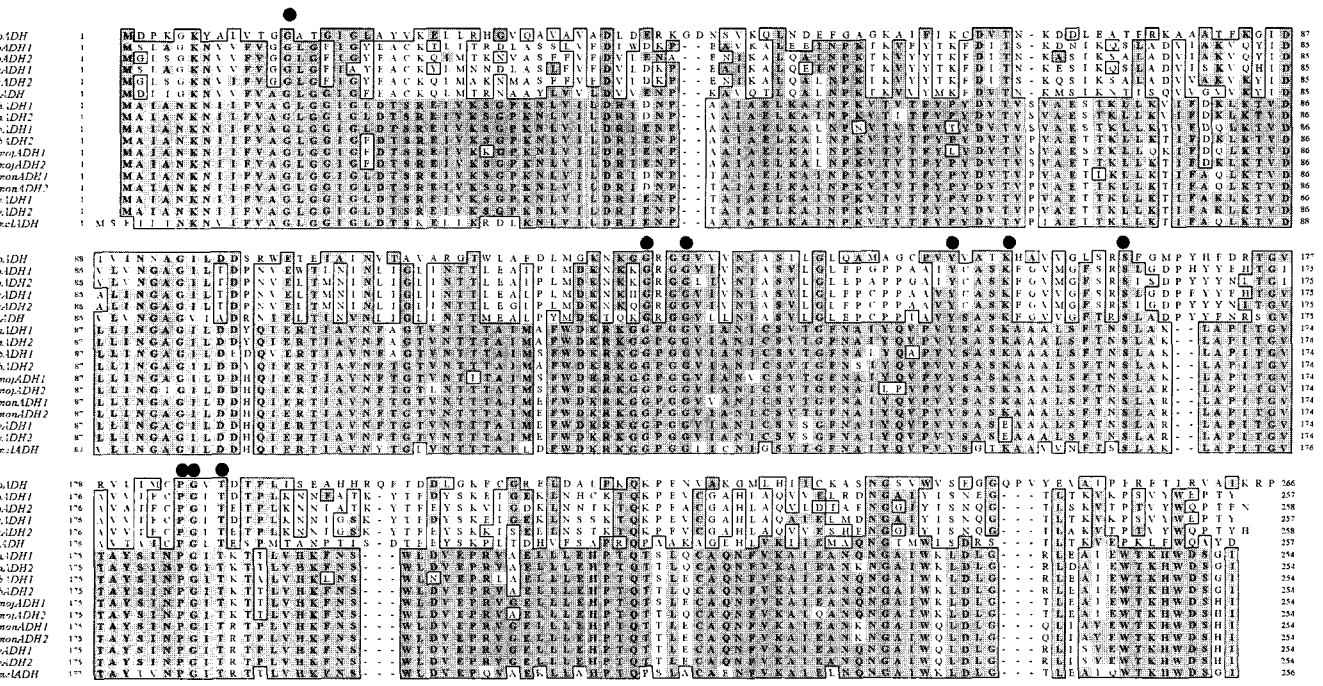

Fig. 2. Multiple sequence alignment of the deduced protein sequences of the *G. orientalis* ADH gene homologue and that of other insects, belonging to Diptera. The solid boxes are the residues that are identical to those of *G. orientalis*. Gaps have been introduced to obtain maximum alignment. Nine residues considered to be essential for the enzymatic activity in *D. melanogaster* are indicated with black circles.

Table 2. Pairwise identities and similarities of the deduced amino acid sequences among *G. orientalis* and other insect ADH genes

		Percent similarity																
Sequence	GenBank no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. ***Go*ADH**	This study		49	48	50	49	50	42	42	41	42	42	42	43	44	44	44	43
2. *Bo*ADH1	AJ277835	32		87	92	89	76	50	50	49	50	52	51	52	51	51	51	54
3. *Bo*ADH2	AJ277834	31	77		88	93	79	51	50	51	52	52	52	52	52	51	51	52
4. *Cc* ADH1	Z30194	33	85	77		90	77	50	51	51	50	51	51	52	52	52	52	52
5. *Cc*ADH2	Z30195	32	77	84	82		76	51	50	51	51	51	53	51	52	51	51	52
6. *Sp*ADH	D63669	30	54	57	56	57		56	56	55	57	57	58	56	56	56	56	58
7. *Dh*ADH1	X58694	26	32	30	34	33	34		99	96	98	96	96	95	95	95	95	90
8. *Dh*ADH2	X58694	26	31	29	34	32	33	98		96	98	95	96	95	96	95	95	90
9. *Db*ADH1	U65746	25	30	29	33	32	34	93	91		96	94	95	93	94	94	94	89
10. *Db*ADH2	U65746	26	31	30	33	33	35	96	95	92		96	97	96	96	95	95	90
11. *Dmoj*ADH1	X12536	26	31	30	33	32	35	93	91	91	93		96	95	94	95	95	90
12. *Dmoj*ADH2	X12536	26	31	30	33	33	35	93	92	90	95	93		95	95	95	95	89
13. *Dmon*ADH1	U26842	27	33	31	35	33	34	90	91	87	91	90	90		99	98	98	90
14. *Dmon*ADH2	U26845	27	33	31	35	34	34	91	92	88	92	90	91	99		99	99	90
15. *Dv*ADH1	U26846	27	33	31	35	33	35	91	90	88	92	90	91	96	97		100	90
16. *Dv*ADH2	U26846	27	33	31	35	33	35	91	90	88	92	90	91	96	97	100		90
17. *Dmel*ADH	AAF00238	29	35	32	37	34	37	81	79	78	81	80	79	79	80	79	79	

Percent identity

the other hand, relatively low sequence identity between tephritid and *Drosophila* species (ranged from 29% to 57%) was observed, and the estimate is somewhat higher or similar than the values obtained when the orthopteran *G. orientalis* was compared with dipteran species (25% ~ 32%). In terms of ADH type, the percent sequence identity was high between two ADH types, regardless of species in some instances, whereas it was high within species, regardless of types in other cases. For example, the two species of tephritids have each two types of ADH genes and these show higher percent sequence identity between types (84% and 85%), rather than within each species (77% and 82%), but *Drosophila* species does not show clear-cut values in terms of species and ADH types clearly (Table 2). Therefore, the values of the percent sequence identity appear not to wholly reflect the taxa-based divergence.

Phylogenetic analysis among deduced amino acid sequences of known insect ADH genes has been performed by maximum-parsimony (MP) and neighbor-joining (NJ) methods (Fig. 3a, b). Overall the topology of the two trees was identical regardless of the method employed. Both analyses further confirmed the complexity of the relationships among species and types of insect ADHs (Fig. 3). *G. orientalis* ADH was unresolved and placed between the outgroup *Bubalus* sp. and dipteran ADHs, possibly due to limited number of orthopteran ADH gene analyzed, and substantial difference of the *Bubalus* sp from other ADH sequences. As predicted from the analysis of sequence identity (Table 2) each type of *B. oleae* and *C. capitata* clustered together with its own type, regardless of species, but two species of *Drosophila*, such as *D. montana* and *D. virilis* clustered together with its own species, regardless of type (Fig. 3a, b). Thus, no simple clustering pattern was obtained. Possibly due to this unexpected complexity, it was not possible to predict the evolutionary type of *G. orientalis* ADH gene with current data at present. The similar result was obtained with limited taxonomic groups in other study. Nurminsky *et al.* (1996) presented both types of *G. orientalis* ADH genes from several *Drosopila*, and found commixed output among species and types. Becuae we currently have only one type of ADH gene and the current available database only provides ADH genes from Diptera, it may not be wise to attempt to assign *G. orientalis* ADH gene to given types. However, the central difficulty in assigning *G. orientalis* ADH to the present types mainly stemmed from duplication event of ADH genes in insects, and timing of the duplication is not certain in connection with speciation event in the dipteran species. Goulielmos *et al.* (2001) critically analyzed available ADH genes of dipteran species with both types and found similar result to our phylogenetic result (*i.e.*, commixed output among species and types). They concluded that a firm choice between the hypothesis of a single duplication event that occurred

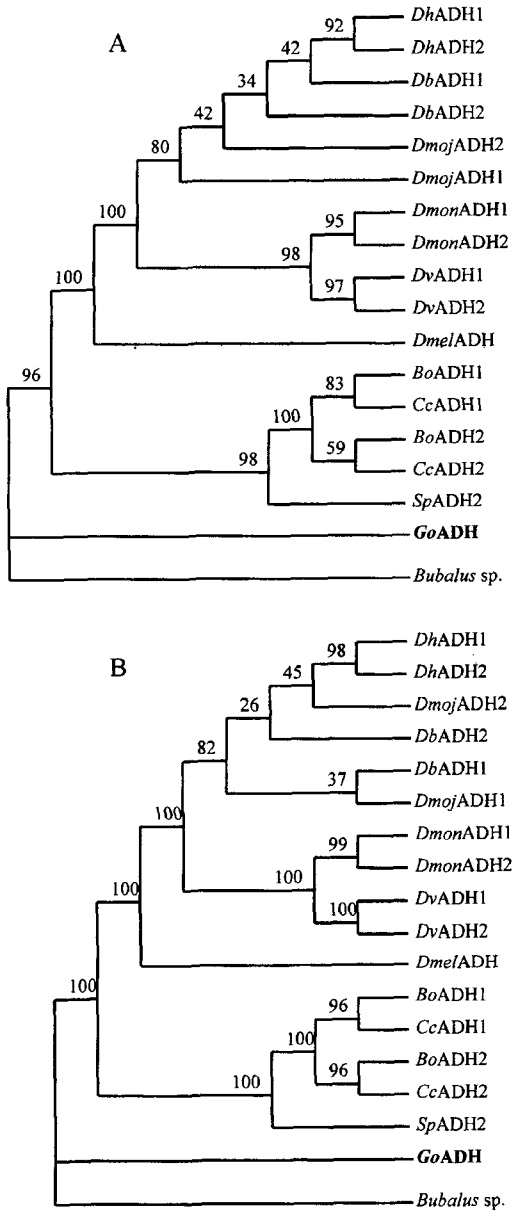

Fig. 3. Relationships among amino acid sequences of the *G. orientalis* ADH and the known ADHs. The information of ADH sequences extracted from GenBank is described in Table 1. The bovine, *Bubalus* sp., NAD^+-dependent 15-hydroprostaglandin dehydrogenase, which is known as an insect homologous of mammal was utilized as an outgroup (GenBank accession number CAA11017) for both analyses. A, MP tree obtained by heuristic search. Among a total of 273 characters 32 are constant, 46 are variable (but parsimony-uninformative), and 195 are parsimony informative. A single most parsimonious tree with the length of 732 was obtained. Consistency index was 0.870 and retention index was 0.875. The numbers on the branches represent bootstrap values for 1,000 replicates. B, NJ tree obtained by neighbor-joining method. Distance measure was obtained by mean character difference. The numbers on the branches represent bootstrap values for 1,000 replicates.

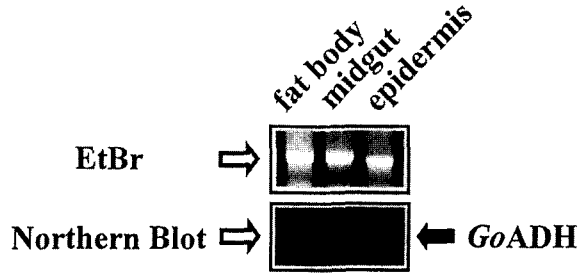

Fig. 4. Northern blot analysis of the *G. orientalis* ADH protein messages. Total RNA was isolated from a fat body, midgut, and epidermis. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred onto a nylon membrane, and hybridized with the appropriate radiolabeled probe (lower panel). *G. orientalis* ADH protein messages are indicated by arrow on the right side of the panel.

before the split of two species of tephritids from their common ancestor and the hypothesis of two independent duplication events, one in each of the two genera could not obtain with current available data set. The same can be applied to our data. Thus, cloning of another type of ADH from *G. orientalis* and information of expression pattern during developmental stages, and illustration of full genomic structure will further enhance our understanding of poorly known insect ADHs.

To confirm the expression of the *G. orientalis* ADH protein gene at transcriptional level, Northern blot analysis was performed using total RNA prepared from fat body, midgut, and epidermis of *G. orientalis* (Fig. 4). Signals were detected from fat body, midgut, and epidermis. Signal was stronger in the fat body than midgut and epidermis, indicating that the fat body might be the main site for the synthesis of the *G. orientalis* ADH protein. The two types of insect ADHs are known to have different expression patterns and tissue-specific distributions in other species. For example, *C. capitata* Adh-1 was expressed in muscle and was separated from third-instar larvae, although Adh-2 was expressed in gut, ovaries, and fat body and can be detected in all stages (Gasperi *et al.*, 1994). From these data, the present *G. orientalis* ADH may be type 2 ADH, although a decisive conclusion can be made after much more information is obtained.

In conclusion, we have cloned a novel cDNA encoding putative alcohol dehydrogenase from the mole cricket *G. orientalis*. The gene was unable to assign currently to known types due to shortage of available information on the insect ADH genes. Nevertheless, this study will expand the understanding of insect ADH genes by including ADH gene of non-dipteran species and is hoped to accumulate the genetic information of *G. orientalis*, which is largely unknown to us.

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