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# Complementary DNA Cloning and Molecular Evolution of Opine Dehydrogenases in Some Marine Invertebrates

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**Abstract:** The complete complementary DNA sequences of genes presumably coding for opine dehydrogenases from *Arabella iricolor* (sandworm), *Haliotis discus hannai* (abalone), and *Patinopecten yessoensis* (scallop) were determined, and partial cDNA sequences were derived for *Meretrix lusoria* (Japanese hard clam) and *Spisula sachalinensis* (Sakhalin surf clam). The primers ODH-9F and ODH-11R proved useful for amplifying the sequences for opine dehydrogenases from the 4 mollusk species investigated in this study. The sequence of the sandworm was obtained using primers constructed from the amino acid sequence of tauropine dehydrogenase, the main opine dehydrogenase in *A. iricolor*. The complete cDNA sequence of *A. iricolor*, *H. discus hannai*, and *P. yessoensis* encode 397, 400, and 405 amino acids, respectively. All sequences were aligned and compared with published databank sequences of *Loligo opalescens*, *Loligo vulgaris* (squid), *Sepia officinalis* (cuttlefish), and *Pecten maximus* (scallop). As expected, a high level of homology was observed for the cDNA from closely related species, such as for cephalopods or scallops, whereas cDNA from the other species showed lower-level homologies. A similar trend was observed when the deduced amino acid sequences were compared. Furthermore, alignment of these sequences revealed some structural motifs that are possibly related to the binding sites of the substrates. The phylogenetic trees derived from the nucleotide and amino acid sequences were consistent with the classification of species resulting from classical taxonomic analyses.

**Key words:** marine invertebrates, cDNA cloning, opine dehydrogenases, molecular evolution.

## INTRODUCTION

Opine dehydrogenases are monomeric pyruvate oxidoreductases that catalyze the NADH-dependent reductive condensation of pyruvate and an amino acid to produce

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**Table 1.** Primers Used in This Study

| Primer | Sequence[a] (5′–3′) | Length (bp) |
|---|---|---|
| TDH-1F | AAYCARCCNAAYATGGARGT | 20 |
| TDH-2F | ATGGARACNAAYGAYTTYAC | 20 |
| TDH-4R | AANGGDATRTCYTCNCCRMRTA | 23 |
| TDH-5R | TGNGCCCAYTCDATDATYTTRTC | 23 |
| TDH-8R | CCTTACCGGTGGCATACTTGATGGTG | 26 |
| TDH-9F | GGCTTCAAGGGTCTCACCCATCCCTG | 26 |
| TDH-11R | CATGCCCAACAGGTGTCCGGAGC | 23 |
| TDH-12F | GGCAGTCAATGGCATGCTCCACCCCTCC | 28 |
| ODH-9F | GCYGACGARGCMGAGMGVTGG | 21 |
| ODH-11F | CGRCAVGCCCARGGRAGRGWYWCRAA | 26 |
| Hdi-1R | TGACCAGGCATTCCAACAATGG | 22 |
| Hdi-2F | GGAGAACGATCTCAGGATTACCGTCG | 26 |
| Hdi-3F | GGCCAAGCAGTGTGTCATCATGTCG | 25 |
| Pye-2F | ATGGCCCCGTATGTACAGGACTCA | 24 |
| Pye-3R | TGGCTTGGAAGGCCTACGATAA | 22 |
| Pye-6F | GTAGGGATATTTTAGGAGACAAGGC | 25 |

[a]D includes A, G, and T; M includes A and C; N includes A, C, G, and T; R includes A and G; V includes A, C, and G; W includes A and T; Y includes C and T.

opines. They play an important physiologic role in the regulation of cytoplasmic redox balance during anaerobic glycolysis and, thus, functionally replace lactate dehydrogenase as the terminal enzyme of anaerobic energy metabolism (Grieshaber et al., 1994). Besides plant pathogenic bacteria, such as *Agrobacterium tumefaciens* (Schell et al., 1979) and *Arthrobacter* sp. (Dairi and Asano, 1995), opine dehydrogenases occur only in invertebrates, preferentially in many marine (Sato et al., 1993) and some freshwater animals (Gäde and Zebe, 1973).

To date 6 opine dehydrogenase activities have been detected in some marine invertebrates. Besides pyruvate and the coenzyme NADH, different opine dehydrogenases use the following amino acids as a secondary substrate: octopine dehydrogenase (D-octopine: NAD oxidoreductase; EC 1.5.1.11; L-arginine); alanopine dehydrogenase (*meso*-alanopine: NAD oxidoreductase, EC 1.5.1.17; L-alanine); strombine dehydrogenase (D-strombine: NAD oxidoreductase, EC 1.5.1.22; glycine); tauropine dehydrogenase (tauropine: NAD oxidoreductase, EC 1.5.1.23; taurine); β-alanopine dehydrogenase (β-alanopine: NAD oxidoreductase, EC 1.5.1.26; β-alanine); and lysopine dehydrogenase (lysine: NAD oxidoreductase, EC 1.5.1.16; lysine). Some opine dehydrogenases have high specificity for pyruvate and the respective amino acid, whereas others are characterized by relatively low specificity for their substrates (Sato et al., 1993; Manchenko et al., 1998).

A deluge of information has been published on the distribution, catalytic properties, and physiological roles of these enzymes (for review, see Grieshaber et al., 1994), but less information is available on the structure and sequence homology of the genes of different opine dehydrogenases. In addition, it has not been elucidated whether species that harbor several different activities of opine dehydrogenases within the same tissue (Kreutzer et al., 1985, 1989) possess more than one gene, each encoding a specific opine dehydrogenase, or whether the expression of one gene results in a single enzyme that catalyzes the formation of different products using multiple substrates.

In order to partially answer these questions, we determined the complete complementary DNA sequence of opine dehydrogenases from *A. iricolor*, *H. discus hannai*, and *P. yessoensis*, and the partial cDNA sequences of *M. lusoria* and *S. sachalinensis*. In the course of these studies, we constructed a primer set that is useful for the amplification of partial sequences from a variety of species. We tried to correlate nucleotide sequences to substrate-binding domains, analyzed their possible evolutionary relationship, and compared the resulting phylogenetic tree with that obtained by classical taxonomic procedures.

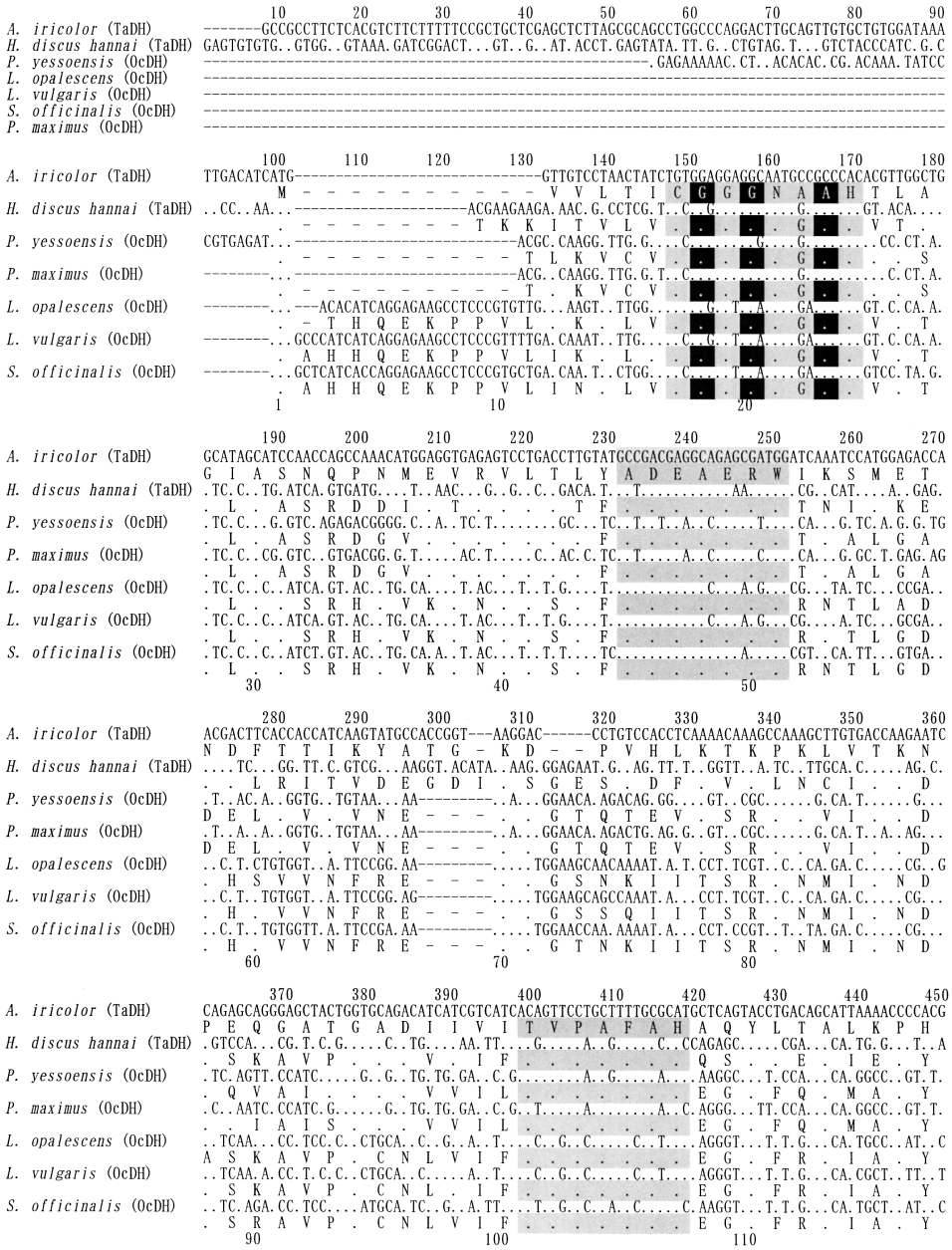


**Figure 1.** Alignment of nucleotide (upper) and deduced amino acid (lower) sequences. Regions of ODH-9F, ODH-11R, polyadenylation signals, and highly conserved segments are shaded. The stop codons are marked with asterisks. White letters on a black background indicate residues of the expected NADH binding sites are indicated sites (continues).

## Materials and Methods

### Animals

Live *A. iricolor* were collected at the seashore of Sanriku (Iwate, Japan), and live specimens of *P. yessoensis*, *H. discus hannai*, *M. lusoria* (Japanese hard clam), and *S. sachalinensis* (Sakhalin surf clam) were purchased from a fishmonger at the local fish market in Sendai, Japan. Messenger RNA was extracted from the body-wall tissue of *A. iricolor*, columella muscle of *H. discus hannai*, and adductor muscles of *P. yessoensis*, *M. lusoria*, and *S. sachalinensis*. The excised tissue samples were frozen in liquid nitrogen and stored at −70°C until use.

### Chemicals

Restriction endonucleases were obtained from Roche Diagnostics. A 100-bp DNA ladder was purchased from Takara Bio. Pfu DNA polymerase was purchased from Promega Biotech. Specific oligonucletotides were custom synthesized by Invitrogen Corp. and Genset KK. Other reagents were the purest grade that was commercially available.

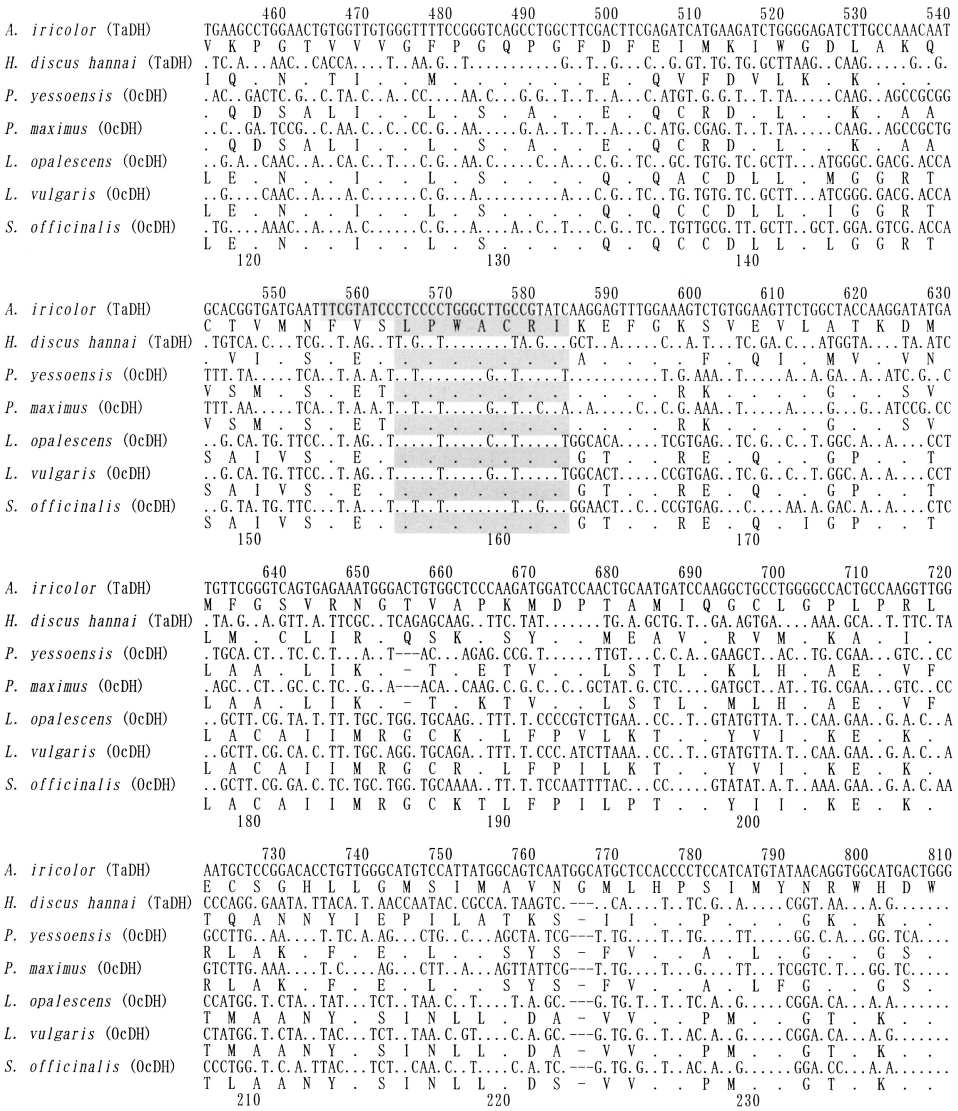


**Figure 1.** Continues.

## Complementary DNA Cloning and DNA Sequencing

Total RNA was extracted from the tissues of each species using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly $(A)^+$ RNA was purified from the total RNA using a microfast track kit (Invitrogen Corp.). First-strand cDNA was constructed using a SMART RACE cDNA amplification kit and following the instruction manual (Clontech).

Oligonucleotide primers specific to *A. iricolor* tauropine dehydrogenase (TDH-1F, TDH-2F, TDH-4R, and TDH-5R in Table 1) were designed from the complete amino acid sequence of this opine dehydrogenase, as reported in the protein database (accession number A59226). In order to obtain partial fragments of cDNA coding for tauropine dehydrogenase, polymerase chain reaction (PCR) was performed using these primers, pfu DNA polymerase (Promega), and first-strand cDNA as a template. The PCR conditions mostly used in this study were one cycle of 94°C for 1 minute, 30 cycles of 94°C for 1 minute, 50°C or 55°C for 1 minute, and 72°C for 2 min/kbp, and a final cycle of 72°C for 5 minutes.

Amplified DNA was separated on a 1.5% to 2.0% agarose gel, extracted using the Rapid Gel Extraction System (Marligen Biosciences), then subcloned into a Zero Blunt TOPO vector following the instruction manual (Invitorogen Corp.). Plasmid DNA was prepared using the alkaline–sodium dodecyl sulfate method (Hattori and Sakaki, 1986), and sequenced using BigDye Terminator (Applied Biosystems), DYEnamic ET (Amersham Pharmacia Biotech) with ABI 311 or 377 Genetic Analyzers (Applied Biosystems).

To determine the complete sequences of cDNA encoding tauropine dehydrogenase from *A. iricolor*, DNA frag-

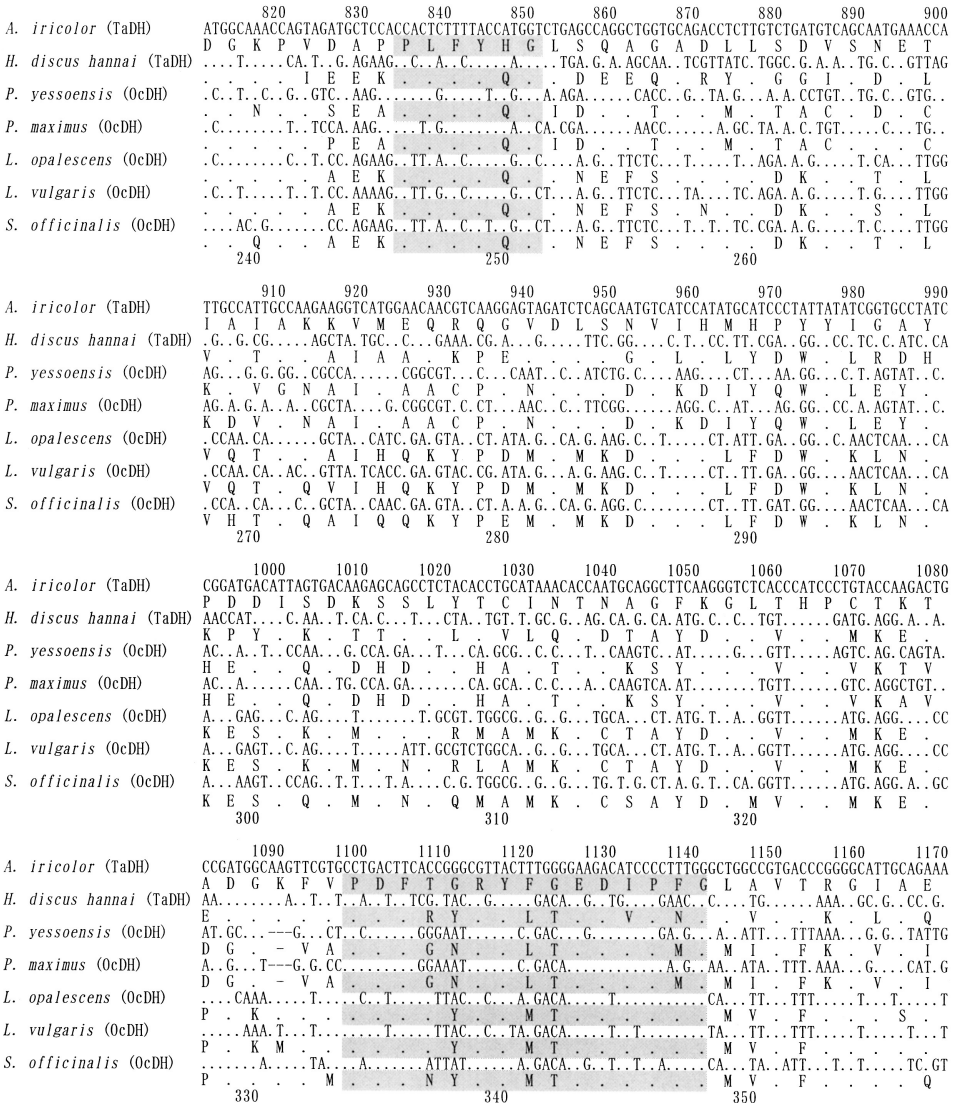

**Figure 1.** Continues.

ments including the flanking regions were obtained by the 5′ and 3′ rapid amplifications of cDNA ends (RACE) methods using the SMART RACE cDNA amplification kit and following the instruction manual (Clontech). The primers used were designed from the internal sequences (TDH-8R, TDH-9F, TDH-11R, and TDH-12F in Table 1).

The primer set of ODH-9F and ODH-11R was constructed from the alignment of nucleotide sequences of the presumed octopine dehydrogenase genes from *Loligo opalescens* (accession number AJ278691), *Loligo vulgaris* (AJ250884), *Sepia officinalis* (AJ250885), and *Pecten maximus* (AJ237916), reported in the DDBJ/EMBL/GenBank database, and from the tauropine dehydrogenase gene of *A. iricolor* (accession number AB081841 and this study). These primers correspond to a conserved domain among all the sequences mentioned above and were used to amplify the gene sequences of opine dehydrogenases from *H. discus hannai*, *P. yessoensis*, *S. sachalinensis*, and *M. lusoria*.

To determine the internal sequences, ODH-9F, ODH-llR, and cDNA from the organisms were used for PCR assays, performed using the following parameters: one cycle of 94°C for 1 minute, 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minute, and a final cycle of 72°C for 5 minutes. Fifty microliters of the reaction mixture included 1 × pfu DNA polymerase buffer, 200 μM dNTPs, 1 μl of template, 50 pmol of each forward primer (ODH-9F) and reverse primer (ODH-11R), and 1.5 U of pfu DNA polymerase. PCR products were subcloned into a Zero Blunt TOPO vector and sequenced after purification by agarose gel electrophoresis.

To determine the complete gene sequences of opine dehydrogenases from *H. discus hannai* and *P. yessoensis*, 5′

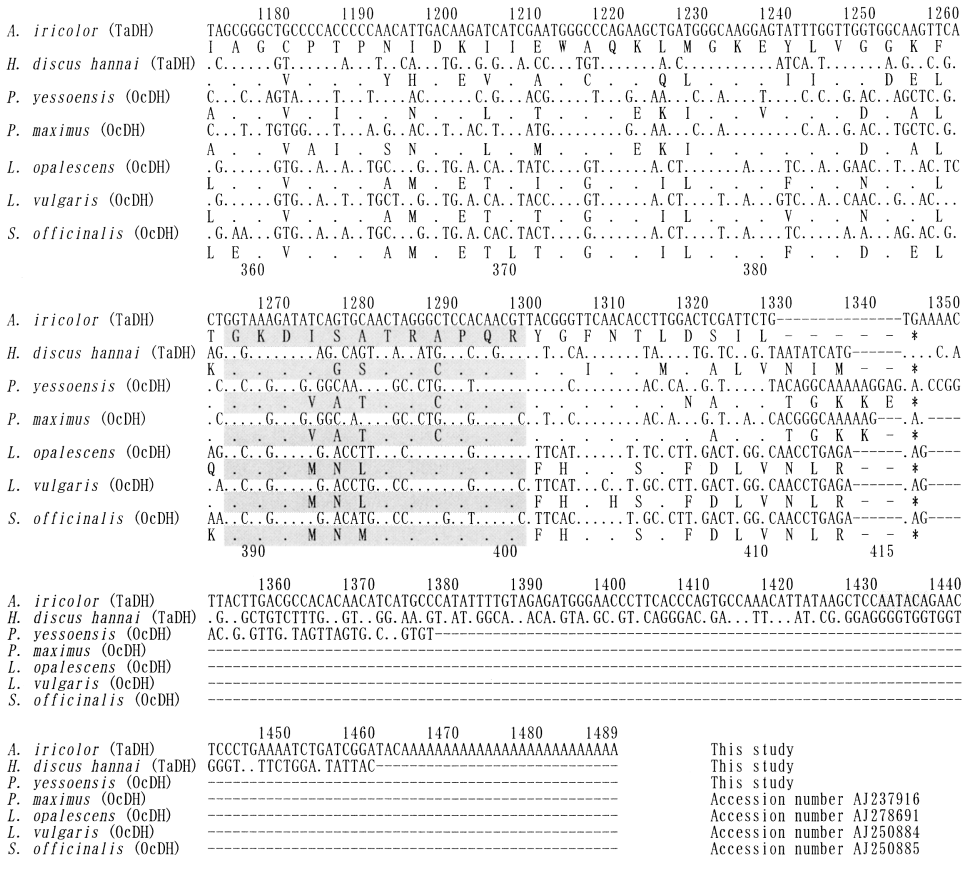


**Figure 1.** Continued.

and 3′ RACE were performed using primers designed from the internal sequences (Hdi-1R, Hdi-2F, and Hdi-3F for *H. discus hannai* opine dehydrogenase, and Pye-2F, Pye-3R, and Pye-6F for *P. yessoensis* opine dehydrogenase; Table 1).

The nucleotide sequence data reported in the present work are available from DDBJ/EMBL/GenBank databases with accession numbers AB081841, AB085183, and AB085184 for cDNA encoding opine dehydrogenases of *A. iricolor*, *P. yessoensis*, and *H. discus hannai*, respectively.

### Sequence Data Analysis

Multiple sequences were aligned using the program CLUSTAL W (Thompson et al., 1994) on the Web site of DDBJ. Phylogenetic trees were constructed using aligned cDNA sequences with the one-parameter method (Jukes and Cantor, 1969). The distance matrix for amino acid substitutions was constructed following the method of Kimura (1969). Trees were constructed by the neighbor-joining algorithm (Saito and Nei, 1987), based on the evolutionary distance matrix. The sequence of *A. iricolor* was used as an outgroup. The degrees of confidence for internal lineages in phylogenetic trees were bootstrapped (Felsenstein, 1985) to compute the distance matrix with 1000 replicates.

## Results and Discussion

The complete nucleotide sequences of cDNA presumably encoding opine dehydrogenases from the marine invertebrates *Arabella iricolor*, *Haliotis discus hannai*, and *Patinopecten yessoensis* were determined. Several colonies were examined, and only a single type of open reading frame was detected in each of the 3 species.

The sequence of the cDNA obtained from the body-wall tissue of *A. iricolor* consists of 1428 nucleotides with an open reading frame of 1191 nucleotides encoding a protein composed of 397 amino acids. No signal sequence was identified, which is consistent with the fact that opine dehydrogenases are not secreted, but are located in the cytoplasm. The stop codon at position 1283 (1344 in Figure 1) was followed by the polyadenylation signal AATACA, starting at position 1370 (1431 in Figure 1).

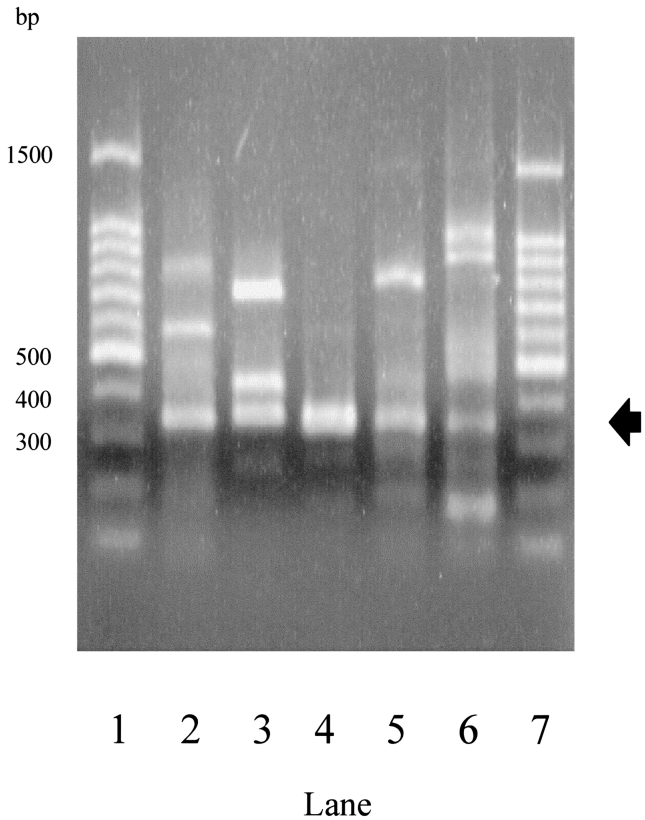


**Figure 2.** Amplification of the internal sequences by PCR with ODH-9F and ODH-11R in various marine invertebrates. Lanes 1 and 7, 100-bp DNA ladder; lane 2, *Arabella iricolor*; lane 3, *Haliotis discus hannai*; lane 4, *Patinopecten yessoensis*; lane 5, *Spisula sachalinensis*; lane 6, *Meretrix lusoria*. The arrow indicates target fragments.

From previous work, it is known that *A. iricolor* possesses high tauropine dehydrogenase activity (116.7 U/g wet weight), which is attributed to the activity of 2 isoenzymes when subjected to isoelectrofocusing (Kanno et al., 1996a, 1996b). Only traces of lactate dehydrogenase and strombine dehydrogenase were detected, and no other opine dehydrogenases were found.

The nucleotide sequence of the isolated cDNA corresponds well with the amino acid sequence of the tauropine dehydrogenase purified from the body-wall tissue of *A. iricolor* (accession number A59226 in protein database), except for the first methionine, as well as isoleucine 52, alanine 328, and glutamic acid 329. The other differences at positions 88 and between 265 and 266 (deletion; Figure 1) are due to mistypings during data transfer to the database. These data are interpreted as the isolated cDNA codes for a tauropine dehydrogenase in the sandworm *A. iricolor*. So far, no cDNA has been obtained for the second isozyme of tauropine dehydrogenase and for the minor activities of lactate dehydrogenase and strombine dehydrogenase (Kanno et al., 1996a, 1996b).

To obtain cDNA clones encoding presumed opine dehydrogenases from the other invertebrate species, several PCR primers were designed from the conserved regions of the aligned nucleotide sequences (data not shown). Among them, only one set of primers, ODH-9F and ODH-11R (Table 1), effectively amplified target fragments encoding presumed opine dehydrogenases in the 5 species *A. iricolor*, *P. yessoensis*, *H. discus hannai*, *S. sachalinensis*, and *M. lusoria* (Figure 2). The expected cDNA fragments, which comprise about 350 bp, correspond to the region between 231 and 580 bp. The fragments were amplified in all species, and their nucleotide sequences were analyzed after subcloning. The complete cDNA sequences presumably encoding opine dehydrogenases from *P. yessoensis* and *H. discus hannai* were finally determined by the RACE method (Figure 1).

The complete cDNA sequence probably encoding an opine dehydrogenase was isolated from *P. yessoensis* (Figure 1). The cDNA contained 1278 nucleotides with an openreading frame of 1200 nucleotides and a stop codon starting at position 1245 (1344 in Figure 1). The sequence can be translated into a protein consisting of 400 amino acid residues. The adductor muscle of *P. yessoensis* harbors 4 opine dehydrogenases: octopine dehydrogenase (27.9 U/g fresh weight); alanopine dehydrogenase (5.1 U/g fresh weight); strombine dehydrogenase (0.6 U/g fresh weight); and tauropine dehydrogenase (0.2 U/g fresh weight) (Sato et al., 1993). Since we could not express the isolated cDNA heterologously, we can only assume that it encodes the octopine dehydrogenase showing the highest activity in the adductor muscle.

The complete cDNA sequence presumably encoding an opine dehydrogenase was obtained from the columella muscle of *H. discus hannai*. The sequence contained 1430 nucleotides with an openreading frame of 1215 nucleotides, corresponding to 405 amino acid residues. A stop codon is found at position 1314 (1344 in Figure 1), and 98 nucleotides at the 5′ and 114 nucleotides at the 3′ end are not translated. The columella muscle tissue of this abalone contains pronounced tauropine dehydrogenase activity (34.7 U/g fresh weight) (Sato et al., 1993), whereas the other opine dehydrogenases showed activities of 0.1 U/g fresh weight or less. Therefore, we tentatively conclude that the obtained cDNA encodes a tauropine dehydrogenase.

Nucleotide sequences of the complete cDNA described for the 3 marine invertebrate species in this study were aligned to one another and to that of *P. maximus*, as well as

**Table 2.** Homologies of Nucleotide (above diagonal) and Amino Acid (Below Diagonal) Sequences

| Taxon | Air | Hdi | Pye | Pma | Sof | Lvu | Lop |
|---|---|---|---|---|---|---|---|
| *Arabella iricolor* (Air) | — | 55 | 56 | 56 | 54 | 56 | 54 |
| *Haliotis discus hannai* (Hdi) | 46 | — | 56 | 57 | 58 | 55 | 58 |
| *Patinopecten yessoensis* (Pye) | 47 | 49 | — | 84 | 55 | 56 | 56 |
| *Pecten maximus* (Pma) | 47 | 49 | 95 | — | 54 | 55 | 55 |
| *Sepia officinalis* (Sof) | 40 | 51 | 49 | 49 | — | 84 | 83 |
| *Loligo vulgaris* (Lvu) | 42 | 52 | 48 | 48 | 89 | — | 91 |
| *Loligo opalescens* (Lop) | 42 | 52 | 48 | 48 | 90 | 93 | — |

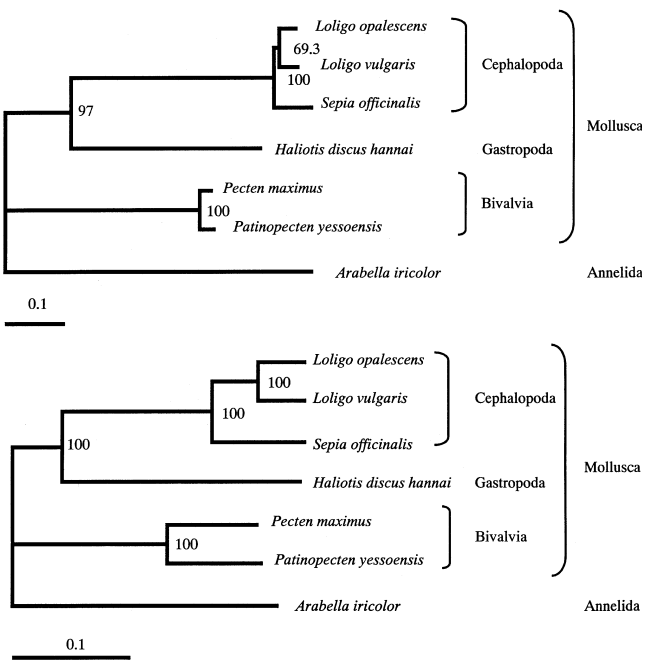


**Figure 3.** Phylogenetic trees derived from amino acid (upper) and nucleotide (lower) sequences of opine dehydrogenases.

that of *L. opalescenss*, *L. vulgaris*, and *S. officinalis*, supposedly possessing octopine dehydrogenase (Table 1). High-level homology is observed between nucleotide sequences presumably encoding octopine dehydrogenase in *P. yessoensis* and *P. maximus* (84%) and between the cepahalopods (83%–91%). Conspicuously lower homologies were observed in the cDNA sequences presumably encoding tauropine dehydrogenase from *A. iricolor* and *H. discus hannai* (55%). However, both sequences align in the same range as the presumable nucleotide sequences of octopine dehydrogenases from the cephalopods and both scallop species (Table 2).

When similar comparisons were made using the amino acid sequences, almost identical homologies resulted. The presumed octopine dehydrogenases were 95% homologous among the scallops and between 89% and 93% homologous among the cephalopods. All other homologies ranged between 42% and 52%, even within the assumed tauropine dehydrogenases from *A. iricolor* and *H. discus hannai* (46%) (Table 2). Obviously, far-ranging homology is conserved for opine dehydrogenases that possess the same activity and occur in closely related orders, such as Pectinidae and Cephalopoda. In contrast, only half of the sequences are homologous within the opine dehydrogenases that presumably have the same activity but occur in species from different orders or phyla. It seems that about half of the protein, which is highly conserved, is required for those sites necessary for substrate and coenzyme binding.

The highly conserved segments CGGGNG/AAH, ADEAERW, TVPAFAH, and FV/ES/TLPWACRI (positions at 17–24, 45–51, 101–107, and 153–162, respectively), were observed in the N-terminal region (Figure 1). The sequence GGGNXA, starting at position 18, corresponds to the consensus sequence of GXGXXG/A, which is involved in the dinucleotide binding site for NAD, FAD, and NADP in dehydrogenases. Unfortunately, no crystal structure is yet available for opine dehydrogenases from invertebrates, but from the analogy of T-DNA-encoded octopine and nopaline dehydrogenase, we propose that this region encodes the 6 β-sheets characteristic of the Rossmann fold (Wierenga et al., 1985; Britton et al., 1998). Another highly conserved region comprises the segments PLFYH/QG, PDFXXRYXXEDI/VPXG, and GKDXXXTRA/CPQR (positions at 246–251, 334–348, and 389–400, respectively), which may be involved in substrate binding.

A phylogenetic branching pattern was derived from the nucleic acid and amino acid sequences (Figure 3). The branching pattern is consistent with the classification of molluscan species from taxonomically derived evolutionary

trees. The configuration showing that Cephalopoda are closer to Gastropoda than Bivalvia is supported by anatomic-organogenetic analyses. Gastropoda and Cephalopoda share several synaptomorphic characters that define them as Visceroconcha (Cyrtosoma) (Salvini-Plawen and Steiner, 1996). Cephalopoda, including *L. vulgaris*, *L. opalescens*, and *S. officinalis*, form a distinct cluster. The bivalves *P. maximus* and *P. yessoensis* also form a cluster. All 5 species synthesize octopine dehydrogenase as the major opine dehydrogenase. However, these 2 clusters are separated by tauropine dehydrogenase from *H. discus hannai*, and tauropine dehydrogenase from *A. iricolor* again branches off from the other opine dehydrogenases.

In conclusion, our data indicate that all opine dehydrogenases analyzed in this study possess a common ancestor. However, it seems that the different amino acid substrate specificities evolved independently after the separation of some subphyla, such as Cyrtosoma (Cephalopoda and Gastropoda in Figure 3) and Diasoma (Bivalvia in Figure 3). In particular, because octopine dehydrogenases from Cephalopoda and Bivalvia are separated by the tauropine dehydrogenase from *H. discus hannai*, one can assume that the substrate specifity for L-arginine had 2 independent evolutionary origins. The same conclusion can be drawn for tauropine dehydrogenases from *A. iricolor* and *H. discus hannai*. Thus cDNA analyses of more opine dehydrogenases from many other species are necessary to provide a comprehensive picture of the evolution of these enzymes.

## Acknowledgments

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