

Gene discovery in *Carcinus maenas* and *Homarus americanus* via expressed sequence tags

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Synopsis Expressed sequence tags (ESTs) were produced for normalized cDNA libraries prepared from several tissues of 2 marine crustaceans, the green shore crab *Carcinus maenas* and the American lobster *Homarus americanus*. Tissues represented in the *Carcinus* library were anterior and posterior gills, hypodermis, heart, hepatopancreas, antennal gland, brain, testis, and skeletal muscle, obtained from animals acclimated to 35 and 10‰ salinity. Tissues represented in the *Homarus* library were gill, epipodite, branchiostegite, heart, ovary, testis, antennal gland, skeletal muscle, hepatopancreas, and brain, obtained from intermolt and post-molt animals. Directional libraries from oligo-dT-primed cDNA were constructed in the pCMVSPORT6.1 vector and normalized by self-subtraction at 2 different Cot values. Randomly picked clones were single-pass sequenced from the 5' end. Raw sequence data were trimmed and prepared for submission to dbEST using phred, cross-match, and blastx protocols embedded within trace2dbest software. A total of 4604 *Homarus* and 12 401 *Carcinus* ESTs produced 540 and 2651 clusters, respectively, as determined by TIGR Gene Index Clustering software. Gene Ontology analysis with reference to a *Drosophila melanogaster* database using GOBlet revealed 187 positive hits (35%) with the *Homarus* clusters and 1037 positive hits (39%) with the *Carcinus* clusters. Combining the number of assembled sequences with the number of singlets obtained after cluster analysis suggested that transcripts representing as much as 25% of the total number of genes in *Carcinus* have been isolated.

Introduction

The green shore crab *Carcinus maenas* is a highly invasive portunid crab, originating in coastal regions of northern Europe and now occupying marine and estuarine habitats around the globe (Carlton and Cohen 2003). Nominated as 1 of the "World's 100 Worst Invaders" by the IUCN Species Survival Commission Invasive Species Specialist Group, the green shore crab is capable of disrupting coastal ecosystems as a result of its aggressive behavior as a predator (Grosholz and Ruiz 1996). For example, recent studies have suggested that *C. maenas* may compete with juvenile and sub-adult lobsters for limited food supplies, negatively impacting lobster growth and survival (Rossong and others 2006; Williams and others 2006). A sister species, *Carcinus aestuarii*, with its origin in the Mediterranean and Adriatic Seas, has also invaded distant ecosystems (Geller and others 1997).

Clues regarding the adaptability of *Carcinus* species have come from physiological studies that detail their capacity to tolerate wide ranges of salinity (Siebers and others 1982; Lucu 1990; Henry and others 2003), oxygen concentration (Truchot 1975; Johnson and Rees 1988; Zinebi and others 1990; Lucu and Pavicic 1995),

temperature (Aagaard 1996; Queiroga and others 1997), and ammonia levels (Weihrauch and others 1998; Weihrauch and others 2002).

The American lobster *Homarus americanus*, in addition to providing an important commercial fishery, is the subject of many studies in neurobiology, developmental biology, behavioral biology, and physiology (Factor 1995). The lobster fishery in the United States was valued dockside at ~\$375 million in 2004, with 76% of the catch obtained by Maine lobstermen (Diaby 2004). In southern New England, the lobster fishery has declined over the last decade, possibly in part due to increasing problems with disease (Floreto and others 2000) and pesticide toxicity (De Guise and others 2004).

Prior to the expressed sequence tag (EST) study described here, <70 nucleotide sequences from *C. maenas* and 100 sequences from *H. americanus* were known, making gene-based studies of their physiology and development difficult and time-consuming. The goal of the EST project described here was to alleviate, at least in part, this paucity of nucleotide sequence data, facilitating gene discovery and gene expression analysis in these 2 genera.

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cDNA library construction and normalization

Source tissues were selected to provide a diverse collection of transcripts representing a broad range of tissue function and physiological state. To prepare material for the *Carcinus* library, crabs were either maintained in 35‰ seawater or transferred to 10‰ for 1 week. Anterior and posterior gills were sampled from both groups, in addition to hypodermis, heart, hepatopancreas, antennal gland, brain, testis, and skeletal muscle. Tissues were stored overnight in RNeasy lysis buffer (Qiagen) at 4°C then kept at -20°C for a maximum of 2 weeks prior to RNA extraction.

To prepare material for the *Homarus* library, 1 male and 1 female intermolt lobster were transferred from 35‰ seawater to 20‰ 18 h prior to tissue removal to induce salinity-sensitive transcripts. To include molt-related and calcifying transcripts, tissues from 1 male and 1 female post-molt (soft-shell) lobster were sampled as well. From each animal, 0.12 g of individual tissues were obtained, including gill, epipodite, branchiostegite, heart, ovary or testis, antennal gland, skeletal muscle, hepatopancreas, and brain.

Total RNA extracts were prepared according to Chomczynski and Sacchi (1987), using materials supplied in the RNeasy Total RNA Isolation System (Qiagen). Total RNA samples were analyzed by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer. Electropherograms of total RNA from *Carcinus* tissues revealed 3 sharp peaks of ribosomal RNA, the 28S rRNA appearing as 2 discrete fragments typical of crustacean rRNA (Skinner 1968) (Fig. 1A). In electropherograms of total RNA from *Homarus* tissues, 1 of the 28S fragments apparently overlaps the 18S peak, giving 2 sharp peaks (Fig. 1B). All tissue RNA preparations used for library construction showed no indication of degradation. For the *Carcinus* library, 100 µg of each tissue RNA preparation were combined into one pool, and for the *Homarus* library, 175 µg.

The *Carcinus* and *Homarus* multiple-tissue RNA preparations were submitted to Invitrogen where reverse transcription of mRNA transcripts was carried out by oligo-dT priming. Normalization of the cDNA libraries was accomplished by subtractive hybridization at 2 different Cot values (15 and 7.5) using Invitrogen's proprietary technology. Normalization reduced the abundance of a highly expressed gene by 400-fold for the *Carcinus* library and 53-fold for the *Homarus* library (Table 1), thus enriching the libraries in rare transcripts. The cDNAs remaining after normalization

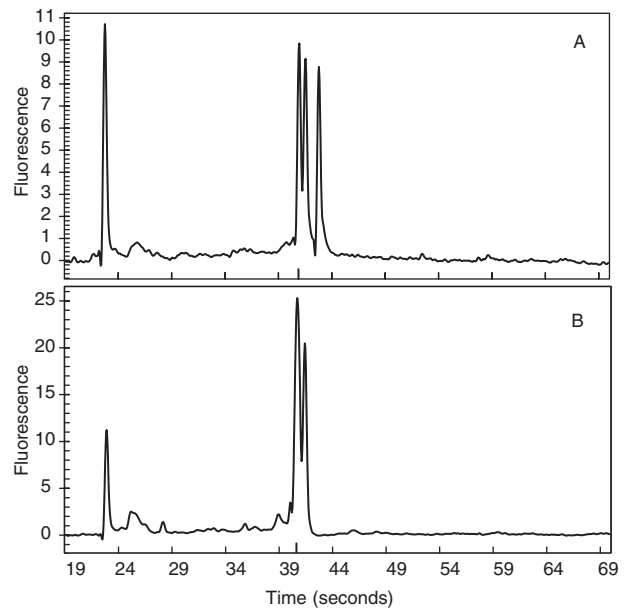


Fig. 1 Electropherograms of total RNA preparations from tissues of *Carcinus maenas* (A) and *Homarus americanus* (B), produced by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Chip System. The left-most peak in each trace represents a marker included in the electrophoresis buffer for standardization. The 18S rRNA peak identified by instrument software is marked on the electropherogram with an upward tick at ~41 s elution time. The 28S rRNA exists as 2 smaller fragments in arthropods, 1 or both of which may overlap the 18S peak.

were directionally ligated into the pCMV Sport 6.1 vector and electroporated into competent DH10B *Escherichia coli*, yielding 6.6×10^7 colony forming units (cfu) for the *Carcinus* library and 2.5×10^6 cfu for the *Homarus* library (Table 1).

Sequencing and EST submission

An aliquot of each library was diluted with 2× LB broth, spread on LB agar plus carbenicillin (100 mg l^{-1}), and incubated at 37°C overnight. Using a QPix2 robot (Genetix), colonies were picked at random into 96-well plates containing 100 µl 2× LB broth, 8% glycerol, and carbenicillin (100 mg l^{-1}). After overnight incubation at 35°C, deep-well plates containing 1 ml 2× LB broth and carbenicillin (100 mg l^{-1}) were inoculated from the original plates that were then stored at -80°C. Following a 16-h incubation at 37°C, plasmids were isolated from the 1-ml cultures by alkaline lysis using a BioMek 2000 robot (Beckman-Coulter) and Millipore miniprep reagents. Inserts were then single-pass sequenced from the 5' end using SP6 (5'-ATTAGGTGACACTATAG-3') as primer on an ABI 3100 16-capillary sequencer (Applied Biosystems).

Table 1 Normalization and properties of multiple-tissue cDNA libraries from green crab *Carcinus maenas* and American lobster *Homarus americanus*

Species	Normalization probe	Positive % in non-normalized library	Positive % in normalized library	Fold reduction	Total colony forming units	% With insert	Average insert size (kb)
<i>C. maenas</i>	β -Actin	4.00	0.01	400	6.6×10^7	100	2.10
<i>H. americanus</i>	Arginine kinase	2.47	0.047	53	2.5×10^6	96	2.41

Sequence traces were processed for submission to dbEST using the trace2dbest component of partigene software from the University of Edinburgh (Parkinson and others 2004). This Linux-based software performs base-calling with the phred protocol, removes vector and low quality sequences with cross-match, performs local blastx analysis for putative identification by homology, and prepares records for direct submission to dbEST at NCBI. As of January 23, 2006, 14 880 clones from the *Carcinus* library have been processed, yielding 12 401 acceptable ESTs (83.3% of the clones sequenced). For the *Homarus* library, 5568 clones have been sequenced, producing 4604 acceptable ESTs (82.7% of the original total). All of the ESTs are searchable in dbEST, available at <http://www.ncbi.nlm.nih.gov>, to facilitate discovery of sequences of specific interest to individual investigators. Samples of the bacterial clones underlying these ESTs are available from the authors upon request.

Clustering and preliminary blast analysis

The *Carcinus* and *Homarus* ESTs were clustered using TIGR Gene Indices Clustering software (Pertea and others 2003). The 12 401 *Carcinus* ESTs yielded 2651 contigs with greater than 1 member plus 1145 singlets. The 4604 *Homarus* ESTs yielded 540 contigs plus 3289 singlets (Table 2). Sequence clusters containing more than 40 ESTs were rare in both libraries while, as expected, clusters of 2 ESTs were the most frequent.

The most populated cluster in both libraries (45 members in *Homarus* and 133 in *Carcinus*) produced no hit upon searching NCBI's non-redundant protein-sequence database with the blastx algorithm (Altschul and others 1997). However, searching the nucleotide database with the blastn algorithm produced high-scoring matches between the most populated *Homarus* and *Carcinus* cluster and mitochondrial 16S rRNA genes (Schubart and others 2000; Giribet and others 2001). Blastx analysis showed that the second-most populated cluster in the *Homarus* ESTs (16 members) matched the sequence for *Homarus* cytochrome oxidase subunit 1 (Giribet and others

Table 2 Assembly of ESTs derived from multiple-tissue cDNA libraries of *C. maenas* and *H. americanus* using the TIGR Gene Indices Clustering program (<http://www.tigr.org/tdb/tgi/software/>).

	<i>C. maenas</i> library	<i>H. americanus</i> library
Total ESTs analyzed	12 401	4604
Number of ESTs in contigs	11 256	1315
Number of contigs	2651	540
Number of singlets	1145	3289
Number of putative unique transcripts	3796	3829
Number of contigs containing:		
2 ESTs	1116	413
3–4 ESTs	839	115
5–10 ESTs	535	9
11–20 ESTs	134	2
21–40 ESTs	24	0
>40 ESTs	3	1

2001) and in *Carcinus* (67 members) matched the nuclear transcript encoding myosin 3 light chain of insects (Veiga and others 2005) (Table 3).

Functional annotation of crab and lobster ESTs

All of the resulting contigs and singlets were submitted to blastx analysis using the Tera-BlastP algorithm (translated nucleotide sequence versus protein sequence) on a DeCypher server (TimeLogic), with reference to the non-redundant protein sequence database from NCBI. Positive hits with an *E*-value <0.0001 were obtained for 44.7% of the *Carcinus* contigs and 33.9% of the *Homarus* contigs, and for 47.4% of the *Carcinus* singlets and 39.3% of the *Homarus* singlets (Table 4). These results will be made available on a publicly available server at the Mount Desert Island Biological Laboratory (<http://www.mdibl.org/~dtowle/DNA/DNAFacility.htm>).

The frequency of positive blastx hits in the range of 30–50% agrees with EST projects for other non-model arthropods. A study of 7210 ESTs from the water flea *Daphnia magna* produced 2958 clusters representing

Table 3 Putative identification of the 12 most populated contigs in the assembled EST database for *C. maenas* and *H. americanus*, determined by the blastn or blastx mode of Tera-BlastP software on a DeCypher processor

Contig ID	No. ESTs	Blast type	Putative functional identification	E-value	Target acc. no.
<i>Carcinus</i>					
Cm1	133	n	Mitochondrial 16S rRNA	1.4e-047	AJ130811
Cm2	67	x	Myosin 3 light chain	1.1e-044	AAW22542
Cm3	53	x	Cytochrome c oxidase polypeptide IV	1.2e-044	AAV66918
Cm4	35	x	ATP synthase subunit B	5.0e-062	AAN39695
Cm5	32	n	28S Ribosomal RNA	4.2e-234	AY210842
Cm6	30	x	Eukaryotic translation initiation factor 3, subunit 6 interacting protein	6.1e-155	AAH61351
Cm7	29	x	Peritrophic membrane chitin binding protein	2.8e-063	AAV46199
Cm8	28	x	Cytochrome c-1	2.4e-111	AAH45127
Cm9	27	x	ATP synthase 1, delta subunit precursor	5.8e-024	XP_625060
Cm10	27	x, n	No hits		
Cm11	27	x	Ribosomal protein L3	1.5e-103	AAX62422
Cm12	25	x	Aldehyde dehydrogenase	2.5e-118	XP_520432
<i>Homarus</i>					
Ha1	45	n	Mitochondrial 16S rRNA	0.0	AF370876
Ha2	16	x	Cytochrome oxidase subunit I	2.0e-110	AAF63420
Ha3	11	x	RNA-binding protein	3.0e-014	XP_706449
Ha4	10	x	Ovary development related protein	3.0e-003	AA073305
Ha5	6	x, n	No hits		
Ha6	5	x	Growth hormone inducible transmembrane protein	9.0e-092	XP_976122
Ha7	5	x, n	No hits		
Ha8	5	x	Glycerol-3-phosphate dehydrogenase	9.0e-083	AAR13229
Ha9	5	x	Cystein proteinase 3 precursor	0.0	P25784
Ha10	5	n	Mandibular HMG-CoA reductase	1.2e-103	AY292877
Ha11	5	x	Translocase of inner mitochondrial membrane	2.9e-49	NP_001026197
Ha12	5	x	Actin-related protein 2/3 complex	5.6e-61	NP_005710

The significance threshold was $E < 0.0001$. The 10 top hits for each contig were inspected for the most specific functional identification and a representative E-value and its corresponding target accession number are given.

unique, non-redundant sequences of which 41% showed significant similarity to known nucleotide or protein sequences (Watanabe and others 2005). Analysis of 14 642 high-quality ESTs from the brain of honeybee *Apis mellifera* revealed 8912 unique sequences, of which 39% were similar to known sequences in the non-redundant protein database (Whitfield and others 2002). A recent study of 11 761 high-quality ESTs from hypodermis and gill of blue crab *Callinectes sapidus* produced 2176 unique sequences, of which 55% could be identified by significant homology to known sequences (Coblentz and others 2006).

Gene Ontology (GO) annotation of the assembled sequences was accomplished using GOblast (<http://goblet.molgen.mpg.de/>) (Hennig and others 2003), employing the highly annotated genome of the arthropod *Drosophila melanogaster* as the reference (Adams

and others 2000). Positive GO hits with an E-value $< 1e-10$ were obtained for 39.1% of the *Carcinus* contigs and 34.6% of the *Homarus* contigs, roughly corresponding to the returns for blastx analysis. Assignment of GO terms within the "molecular function" category revealed representatives within each of 12 categories (Table 4), with the most frequent annotations occurring in 3 categories: binding activity (25%), catalytic activity (36–38%), and transporter activity (9–10%).

Because of the long-standing interest of our laboratory in osmoregulatory and transport physiology in marine crustaceans, we examined the distribution of GO terms within the transporter activity category more closely. Among the *Carcinus* assembled sequences, representatives of 15 transporter categories were identified, including 1 contig tentatively identified as encoding an aquaporin. The smaller number of contigs

Table 4 Blastx and Gene Ontology (GO) analysis of contigs and singlets derived from *Carcinus* and *Homarus* ESTs

	<i>C. maenas</i> library	<i>H. americanus</i> library
Contigs with blastx hits (% of total)	1185 (44.7%)	183 (33.9%)
Singlets with blastx hits (% of total)	543 (47.4%)	1292 (39.3%)
Contigs with GO hits versus <i>Drosophila</i> database (% of total)	1037 (39.1%)	187 (34.6%)
Number of contigs assigned a GO term within "Molecular Function":		
Antioxidant activity	12	2
Binding activity	395	73
Catalytic activity	578	108
Chaperone activity	35	7
Enzyme regulatory activity	55	7
Motor activity	16	2
Nutrient reservoir activity	5	1
Signal transduction activity	89	11
Structure activity	141	22
Transcription regulatory activity	91	22
Translation regulatory activity	30	4
Transporter activity	148	28

Positive blastx hits with an *E*-value <0.0001 were obtained using Tera-BlastP (translated nucleotide sequence versus the non-redundant protein database) on a DeCypher processor. GO classification was accomplished using GOblet (<http://goblet.molgen.mpg.de/>) with reference to GO annotations in the Berkeley *Drosophila* Genome Project database (threshold *E*-value <1 e⁻¹⁰). Contigs were putatively identified by assigning GO terms within the molecular function category. Note that contigs may be assigned to more than 1 term.

in the *Homarus* library resulted in a lower diversity of transporters, with 11 sub-categories represented. The 3 most abundant sub-categories of transporter activity identified in both libraries were the GO terms carrier activity (37–39%), electron transporter activity (9–10%), and ion transporter activity (24–26%) (Fig. 2). Among the latter were several cation transporters that we had identified in earlier experiments using degenerate primers with reverse transcription and the polymerase chain reaction (Towle and others 1997, 2001; Weihrauch and others 2001).

Of particular interest for future studies of osmoregulatory and transport physiology are a number of newly identified sequences that were made possible by the EST projects at Mount Desert Island Biological Laboratory. These include ATPases, ion channels, and transporters that may play important roles in osmoregulatory adjustments to changing salinity (Table 5). The availability of species-specific sequence data for these transcripts will facilitate detailed studies of gene expression and its control.

Implications for future genome-based studies

The haploid genome size of *C. maenas* is estimated at 1.07 pg, the smallest known genome among the decapod crustaceans (Gregory 2001). Thus *C. maenas* may serve as a suitable target for whole-genome shotgun sequencing if the necessary informatics resources become available. Among these resources should be an extensive, well-annotated EST library. The total of 3796 putative unique transcripts (assembled sequences plus singlets) presented in this study likely represents 25% or less of the total

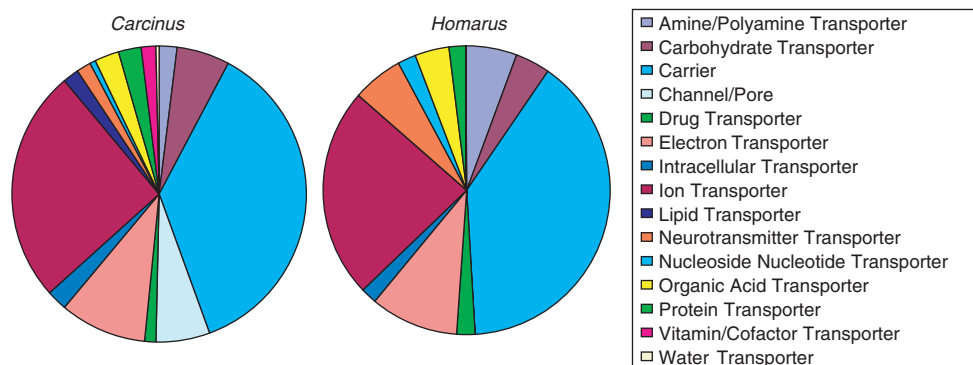


Fig. 2 Expansion of Gene Ontology (GO) terms within the transporter category of molecular function, with reference to cDNAs and GO annotations in the Berkeley *Drosophila* Genome Project database, as identified by GOblet (<http://goblet.molgen.mpg.de/>) for contigs derived from *C. maenas* and *H. americanus* EST libraries. Interpreting the pie chart is facilitated by beginning at "12 o'clock" and reading clockwise to match colors with transporter sub-category.

Table 5 ESTs in the *Carcinus* library putatively encoding proteins of interest in transport physiology and osmoregulation, identified by blastx analysis

Accession no. of EST	Putative identification	E-value
DN635124	Zinc transporter	6e-18
DN738771	Calcium-activated chloride channel	5e-28
DN739449	Vacuolar ATPase Subunit M9	5e-20
DN796463	Potassium channel tetramerisation domain	3e-71
DV111820	Phospholipid-transporting ATPase	3e-96
DV642652	Voltage-gated calcium channel	9e-18
DV944903	Organic anion transporter	1e-30
DW251129	Amino acid transporter	3e-28
DW584526	Epithelial chloride channel protein	2e-30

number of structural genes in the *Carcinus* genome. Thus, a more complete EST collection will require additional sequencing and analysis using cDNA libraries that may be more complex in their composition.

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