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Research Article

Molecular Cloning and Characterization of *Dmc1* from the Chinese Mitten Crab (*Eriocheir sinensis*)

Abstract

Dmc1, a member of the RecA/Rad51 superfamily, is essential for meiotic recombination. In this study, a *Dmc1* gene (*EsDmc1*) was identified from screening the larval transcriptomes of Chinese mitten crab *Eriocheir sinensis*. The full-length cDNA of *EsDmc1* was 1478 bp long and contained a 1026 bp open-reading frame encoding 341 amino acids. The genomic fragment of *EsDmc1* contained two exons separated by one intron. Several tandem repeats were found in intron. The deduced EsDmc1 protein contained motifs conserved in the RacA/Rad51 superfamily, including the Walker A and B motifs, and L1 and L2 loops. EsDmcl shared 87.3%, 86.5% and 77.4% identity with its homologues in *Litopenaeus vannamei*, *Penaeus monodon* and *Ixodes scapularis*, respectively. Phylogenetic analysis revealed that EsDmc1 had a closer relationship with Dmc1s from arthropods than vertebrates. The *EsDmcl* transcripts could be detected in all examined larval stages with the highest expression level in the fifth zoeal stage. These results suggest that *EsDmc1* could be expressed before reproductive maturity and might have complex functions in crab reproduction.

Introduction

The Chinese mitten crab *Eriocheir sinensis* (Henri Milne Edwards 1854) is a commercially important species in Southeast Asia and has been widely farmed in ponds, reservoirs and lakes of China since 1990s [1]. Like other crabs, the mitten crabs exhibit bimodal growth patterns in which males exhibit higher growth rate and larger size than females [2-4]. However, female mitten crabs have greater economic value than males. In addition, sexual precocity has occurred in cultured *E. sinensis* populations [5] and caused catastrophic economic losses to crab aquaculture. Understanding the mechanisms involved in reproduction or sexual development and characterization of the related genes in *E. sinensis* would be helpful for the aquaculture industry.

Dmc1 (disrupted meiotic cDNA), a member of the RecA/Rad51 superfamily that encodes a DNA recombinase, plays a central role in meiotic recombination [6,7]. The *Dmc1* gene was initially identified in budding yeast *Saccharomyces cerevisiae* as a meiosis-specific gene [8]. Afterwards, *Dmc1* genes have been isolated from higher eukaryotes, such as human and mouse [9]. In mammals, *Dmc1* was also a specifically expressed gene in testicular germ cells and embryonic ovaries in the period of meiosis. Further studies showed that *Dmc1* mutations cause meiotic arrest at the zygotene stage without homologue synapsis [10] or with an occasional synapsis between non-homologues [11]. All of these suggest the crucial role of *Dmc1* during meiosis appears to be conserved from yeast to mammals.

Recently, *Dmc1* genes have been identified and characterized from several aquatic animals, such as Japanese eel [12], cyprinid fishes [13], and only two crustacean species, giant tiger shrimp *Penaeus monodon* [14,15] and whiteleg shrimp *Litopenaeus vannamei* [16]. Despite the finding of *Dmc1* from testis transcriptome [17], no study has described the sequence characterization and expression pattern of *Dmc1* in *E. sinensis*. Here, a *Dmc1* gene (designated as *EsDmc1*) showing a predicted amino acid sequence similarity to *L. vannamei* was found from screening the transcriptomes of larval *E. sinensis*. The main objectives of the present study are to clone this gene from *E. sinensis*, detect genomic organization and investigate the expression pattern during larval developmental stages based on transcriptome data.

Materials and Methods

cDNA library construction and gene annotation

Four libraries were conducted from whole bodies of *E. sinensis* at four larval developmental stages, including the fifth zoeal stage (Z5), the megalopa stages before and after desalination (MB and MA), and the first juvenile instar (J1). Transcriptome sequencing was carried on an Illumina HiSeq™ 2000 platform. A total of 23.48 Gb clean data were generated from larval transcriptomes [18-20]. BLASTx analysis revealed that a unigene (comp19040_c0_seq1) was homologous to *Dmc1* in *L. vannamei* (ADM45305).

Full-length cDNA sequence determination

Gene specific primer (P1), nested primer (P2) and oligo (dT)-adaptor (Table 1) were used to clone the 3' end of *EsDmc1*cDNA by 3' RACE technique. Initial amplification was carried out with P1 and oligo (dT)-adaptor, followed by nested PCR using P2 and oligo (dT)-adaptor. The amplification was performed in a 25 μl reaction volume containing 17.3 μl sterile distilled H_2O, 2.5 μl of 10×PCR buffer, 1.5

μl of $MgCl_2$ (25 mM), 0.5 μl of dNTP (10 mM), 1 μl of each primer (5 μM), 0.2 μl (1 U) of Taq polymerase (TaKaRa), and 1 μl of template. The PCR reactions were performed on TaKaRa PCR Thermal Cycler Dice Model TP600 (Takara Bio Inc.) with an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 50 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were gel-purified and cloned into pMD18-T simple vector (TaKaRa). After being transformed into the competent cells of *Escherichia coli* DH5α, the positive recombinants were identified through anti-Amp selection and PCR screening with M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced to verify the full-length cDNA of *EsDmc1*.

Genomic DNA amplification

Genomic DNA was extracted from the muscle tissue by standard phenol-chloroform method [21]. To detect the genomic structure of *EsDmc1*, two gene-specific primers (P3 and P4, see Table 1) were designed according to the obtained cDNA sequence. The PCR was performed in a 25 μl reaction volume containing 17.3 μl sterile distilled H_2O, 2.5 μl of 10×PCR buffer, 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTP (10 mM), 1 μl of each primer (5 μM), 0.2 μl (1 U) of Taq polymerase (TaKaRa), and 1 μl of DNA template (approximately 30 ng). The PCR temperature profile was 94°C for 3 min, followed by 34 cycles of 94°C for 30 s, 55°C for 50 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were cloned and sequenced according to the method described above.

Sequence and phylogenetic analysis

Blast algorithm at National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast/) was used to search the homology of nucleotide and protein sequences. The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). SignalP 3.0 program was utilized to predict the presence and location of signal peptide, and the cleavage sites in amino acid sequences (http://www.cbs.dtu.dk/services/SignalP).

Multiple amino acid sequence alignment was performed using the Clustal X with the default settings [22]. A neighbor-joining (NJ) tree with bootstrap values was constructed for phylogenetic analysis using MEGA 4.0 [23]. All the reference sequences for phylogenetic analysis were derived from GenBank.

Expression analyses based on transcriptome data

The reads for *EsDmc1* were counted by mapping reads to assembled unigene sequences in the four larval transcriptomes. The expression of *EsDmc1* was calculated using the reads per kb per million reads (RPKM) method [24]. The fold change values > 2 and false discovery rates (FDR) adjusted significance values < 0.05 (-log10 (0.05) = 1.3) were considered as the threshold to judge the significance of expression.

Results and Discussion

cDNA cloning and sequence analysis of *EsDmc1*

The full-length cDNA sequence of *EsDmc1* was obtained by overlapping the corresponding unigene with the amplified fragments.

Table 1: Primers used in this study.

Name	Sequence (5′ to 3′)	PCR objective
P1	CAGGGACTTACACTGGAGGGA	3′ RACE and cDNA cloning
P2	CGAAGATTTACGACAGTCCGGAG	3′ RACE and cDNA cloning
oligo (dT)-adaptor	$GGCCACGCGTCGACTAGTACT_{17}$	3′ RACE
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing
P3	AGGTGGCAGCTAAGGTGAGC	Genomic cloning
P4	GGTCGGCGGTCATCTGGTTAGT	Genomic cloning

It was 1478 bp, and consisted of a 5′-UTR of 213 bp, 3′-UTR of 239 bp and an open-reading frame (ORF) of 1026 bp. A canonical polyadenylation signal-sequence (AATAAA) and poly (A) tail was detected in *EsDmc1*. The ORF encoded 341 amino acids and no signal peptide was identified (Figure 1). The estimated molecular weight of *EsDmc1* was 37.49 kDa and its theoretical isoelectric point was 5.41. The sequence of *EsDmc1* was deposited in GenBank under the accession number KP876487.

Genomic organization of *EsDmc1*

Until now, no genomic sequence of *Dmc1* has been identified in crustaceans. The amplified genomic DNA fragment of *EsDmc1* was 856 bp and deposited in GenBank under accession number KP876488. By aligning with the corresponding cDNA sequence, the exon-intron boundaries of *EsDmc1* were determined (Figure 2). The *EsDmc1* genomic sequence consisted of two exons (248 and 355 bp) separated by one intron (253 bp). All splice sites in *EsDmc1* followed the canonical GT/AG splicing recognition rule. Several mononucleotide repeats $(T)_{4-7}$ and one pure dinucleotide repeat $(GC)_3$ were found in the intron (Figure 2). Intronic microsatellites found in *EsDmc1* will be helpful in understanding the genetic structure of *Dmc1*s and studying the possible roles of microsatellites.

Homologous and phylogenetic analysis of *EsDmc1*

BLAST analysis indicated that the deduced amino acid sequence of EsDmc1 shared significant identity with other reported Dmc1s. EsDmc1 displayed 87.3% identity with published Dmc1 of *L. vannamei* (LvDmc1), 86.5% with *P. monodon* (PmDmc1), 77.4% with *Ixodes scapularis* (IsDmc1), 75.9% with *Anguilla japonica* (AjDmc1) and 74.8% with *Homo sapiens* (HsDmc1). As reported in LvDmc1 [16], multiple alignments revealed that EsDmc1 contained motifs conserved in the RecA/Rad51 superfamily, including L1 and L2 loops, which are single-strand DNA binding motifs [25,26], and the Walker A and B motifs of the ATP binding site [26,27] (Figure 3). Moreover, the helix-hairpin-helix (HhH) motif, a double-strand DNA binding site that is commonly present in Rad51 and Dmc1, but not in RecA [26,28,29], was also detected in EsDmc1.

Furthermore, a phylogenetic tree was constructed based on 14 amino acid sequences of Dmc1 members by NJ method (Figure 4). The Clustal X alignment consisted of 346 characters, 149 being constant, 195 variable but parsimony-uninformative and 103 informative. The phylogenetic tree showed that the Dmc1s formed two major clades:

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CAGAAACAATGTGCTGTGGACCCACAACCCATTGTTTTTATTCCGTATTCAACTTATAACATTTAAAAACAACAGATATT 80
ACGTGATTTTTCTGGATCCGCTCCCTGGAAATAGAAAAAAAAAGTCCCATTACGAACTCTATAGCGTCAGTTCGCGTCAC 160
CATTGGGTACGAGGAAACCATTTTTGCGTCGCTCCTCCAAAAATCGCCTCAAAATGCAAGACCAAGCGCTGGACGTCGAG 240
                                                    M  Q  D  Q  A  L  D  V  E   9
GTGGAAGACGGGGAAGAAGACGTAAGCTTTTTCACGGACATAGATGAATTACAAGCCCATGGAATTAACGCAGCGGACCT 320
 V  E  D  G  E  E  D  V  S  F  F  T  D  I  D  E  L  Q  A  H  G  I  N  A  A  D  L  36
CAAGAAGCTGAAGACGGCCGGGATTTGCACTGTCCGCGGCGTGCAAATGACGACGCGGAGGAAAATGTGCAATATCAAAG 400
  K  K  L  K  T  A  G  I  C  T  V  R  G  V  Q  M  T  T  R  R  K  M  C  N  I  K   62
GTCTTTCCGAAGCCAAGGTGGATAAGATTAAAGAGGTGGCAGCTAAGGTGAGCGGCGGTGACGGCTTCGTGACAGCTCTG 480
G  L  S  E  A  K  V  D  K  I  K  E  V  A  A  K  V  S  G  G  D  G  F  V  T  A  L  89
GTGGTGCTTGAGCGTCGGAGGCACATCTTCAGGGTCAGCACGGGGTCAGGGGAGCTGGACAGCCTGCTCGGGGGCGGCAT 560
 V  V  L  E  R  R  R  H  I  F  R  V  S  T  G  S  G  E  L  D  S  L  L  G  G  G  I 116
CGAGAGCATGGCCATCACGGAGGTCTTCGGCGAGTTCCGCACGGGCAAGACGCAGTTAGCGCACACCCTCTGCGTCACGG 640
  E  S  M  A  I  T  E  V  F  G  E  F  R  T  G  K  T  Q  L  A  H  T  L  C  V  T  142
CACAGATCCCCAACGATGCAGGGACTTACACTGGAGGGAAGGTCATTTTCGTCGACACGGAGAACACGTTTCGCCCAGAC 720
A  Q  I  P  N  D  A  G  T  Y  T  G  G  K  V  I  F  V  D  T  E  N  T  F  R  P  D  169
CGGCTGCGTGGTATCGCGGATCGTTTCAACCTGGAGCAGGAGGCGGTGCTGGAGAACGTGCTGTACACCCGCGCCTTCAC 800
 R  L  R  G  I  A  D  R  F  N  L  E  Q  E  A  V  L  E  N  V  L  Y  T  R  A  F  T 196
TTCGGAGCACCAGTTCGAGATCCTGGACCATGTCGCGGCACAGTTCCACGAGGAGCCAGGCATCTTCAAGCTCCTCATCA 880
  S  E  H  Q  F  E  I  L  D  H  V  A  A  Q  F  H  E  E  P  G  I  F  K  L  L  I   222
TCGACTCCATCATGGCACTATTCCGCGTGGACTTCAGCGGCCGCGGGGAGCTTGCAGACCGACAGCAGCGCCTCGCCCAG 960
I  D  S  I  M  A  L  F  R  V  D  F  S  G  R  G  E  L  A  D  R  Q  Q  R  L  A  Q  249
TACTTGTCCCGCCTGCAGAAGATCAGCGAGGAGTACAATGTGTCCGTCTACATCACTAACCAGATGACCGCCGACCCAGG 1040
 Y  L  S  R  L  Q  K  I  S  E  E  Y  N  V  S  V  Y  I  T  N  Q  M  T  A  D  P  G 276
GGCTGCCATGTCCTTTCAGGCTGACCCCAAGAAGCCCATCGGCGGACACATCCTCGCTCACGCCTCCACCACCCGTGTCT 1120
  A  A  M  S  F  Q  A  D  P  K  K  P  I  G  G  H  I  L  A  H  A  S  T  T  R  V   302
CCCTGCGCAAAGGCCGCGGCGAGACACGCATCGCGAAGATTTACGACAGTCCGGAGCTGCCCGAGAACGAAGCCACTTTT 1200
S  L  R  K  G  R  G  E  T  R  I  A  K  I  Y  D  S  P  E  L  P  E  N  E  A  T  F  329
GCTATCACTGCAGGCGGTGTGGCAGATGCAAAGGAGTGAGGACGCTCTAGTACTCCTTAGCCCCTAGCCTATAACTTTAT 1280
 A  I  T  A  G  G  V  A  D  A  K  E  *                                          341
AATTTTGCCTGGACTCTCAGTTTTTTAATCAGGGTATAGTTTGTGTTCTGACAAGTGCGGGAGGACACAGAACCACCCAC 1360
CCGCCATAATATAGGAATTGTAGTATGTTCTTTTTTTGTCTTTAATATTTCAATGCAGCACGTATTCATACTTCAGTGAA 1440
ATAAACACAAATTACATCAAAAAAAAAAAAAAAAAAAA                                          1478
```

Figure 1: Nucleotide and deduced amino acid sequences of *EsDmc1* from *Eriocheir sinensis*. Numbers on the right of the sequence give the positions of the last nucleotide and amino acid on each line, respectively. The start and stop codons are boxed and the classical polyadenylation signal in the 3′-UTR is underlined.

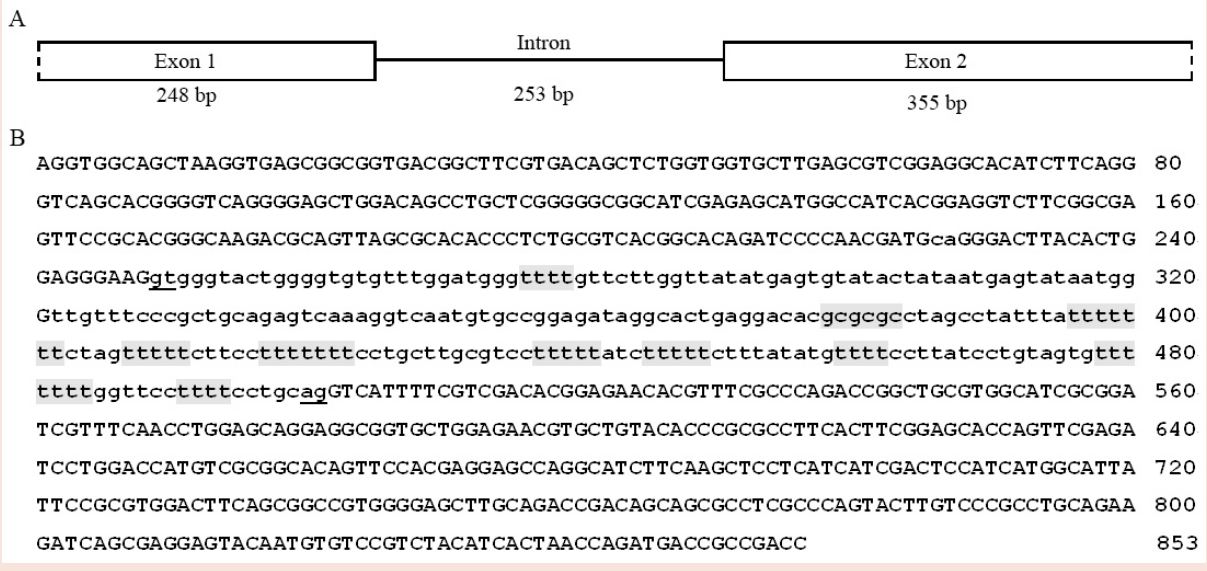

Figure 2: Genomic organization (A) and nucleotide sequence (B) of *EsDmc1*. The exon and intron sequences are illustrated with capital and lowercase letters, respectively. The tandem repeats in intron are shadowed. Intron dinucleotide acceptor and donor sites (gt/ag) for RNA splicing are underlined.

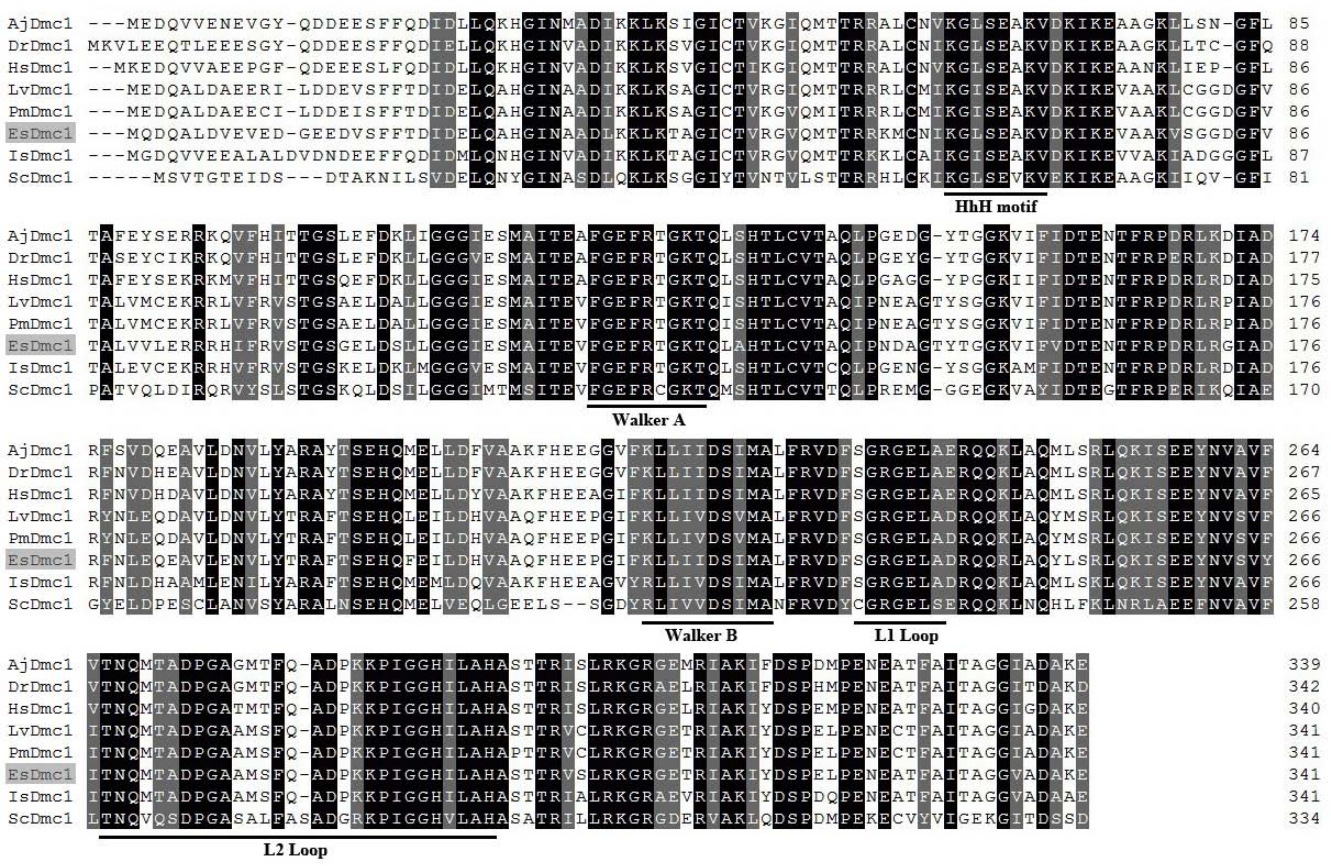

Figure 3: Multiple alignments of EsDmc1 with other known Dmc1s. Amino acid residues with 100% conservation are shaded in dark, and similar amino acids are shaded in grey. Numbers on the right indicate the amino acid position of the different sequences. Each domain conserved in the RacA/Rad51 superfamily is underlined. The species and the GenBank accession numbers are as follow: *Anguilla japonica* AjDmc1 (BAD98459), *Danio rerio* DrDmc1 (BAD98462), *Homo sapiens* HsDmc1 (NP_008999), *Litopenaeus vannamei* LvDmc1 (ADM45305), *Penaeus monodon* PmDmc1 (ACC62173), *Ixodes scapularis* IsDmc1 (XP_002436011) and the budding yeast *Saccharomyces cerevisiae* ScDmc1 (NP_011106).

one with four arthropods and the other containing two subclades from the vertebrates. EsDmc1 was clustered together with Dmc1s from the whiteleg shrimp *L. vannamei* and the giant tiger shrimp *P. monodon*, and then had a closer relationship with blacklegged tick *I. scapularis*. Both homology and phylogenetic analysis together showed that *EsDmc1* is a clearly member of *Dmc1* homologous belonging to RecA/Rad51 superfamily.

Expression of *EsDmc1* in larval developmental stages

Most studies on expression profiles of *Dmc1* transcripts have focused on the gonads and somatic tissues of adults or subadults [12,14-16]. However, little is known about the expression pattern of *Dmc1* in larval developmental stages. In the present study, the transcripts of *EsDmc1* could be detected in all examined larval stages (Figure 5). *EsDmc1* had the highest expression level in Z5, moderate expression in MB and MA, while only a week expression level in JI. Moreover, the expression of *EsDmc1* in Z5 was significantly higher than that in MA and JI. This expression pattern would suggest *EsDmc1* could be expressed at earlier developmental stages before reproductive maturity in *E. sinensis*. Contrary to the results from *L. vannamei*, *P. monodon Dmc1* did not show gonad-specific expression [14-16], indicating the complex roles of *Dmc1* in crustaceans. Further investigations regarding crab *Dmc1*, particularly functional studies, are needed to determine the biological roles of this gene in crustaceans.

Conclusion

This study reported a *Dmc1* gene in the Chinese mitten crab, *E. sinensis*, encoding amino acid sequences characteristic of the RecA/Rad51 superfamily. Multiple alignment and phylogenetic analysis showed *EsDmc1* was a member of *Dmc1* homologues. Different from the previous studies in other species, *EsDmc1* transcripts were found to be expressed at earlier larval stages, from Z5 to JI. Our study provides more information about crustacean *Dmc1*, and will facilitate further investigations of the molecular mechanisms involved in crab reproduction.

Acknowledgement

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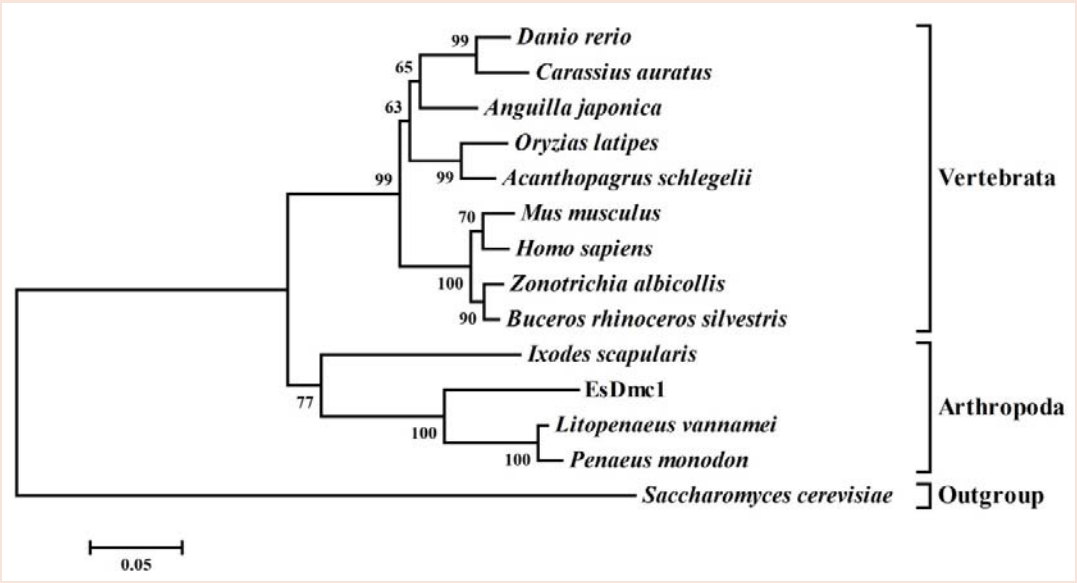

Figure 4: A bootstrapped NJ tree inferred from amino acid sequences of EsDmc1 and other reported Dmc1s. The species and the GenBank accession numbers are as follow: *Danio rerio* DrDmc1 (BAD98462), *Carassius auratus* (ABQ23182), *Anguilla japonica* AjDmc1 (BAD98459), *Oryzias latipes* (ACH91672), *Acanthopagrus schlegelii* (AFV31617), *Mus musculus* (NP_034189), *Homo sapiens* HsDmc1 (NP_008999), *Zonotrichia albicollis* (XP_005488680), *Buceros rhinoceros silvestris* (XP_010136730), *Ixodes scapularis* IsDmc1 (XP_002436011), *Litopenaeus vannamei* LvDmc1 (ADM45305), and *Penaeus monodon* PmDmc1 (ACC62173). The budding yeast *Saccharomyces cerevisiae* ScDmc1 (NP_011106) serves as outgroups. Numbers in each branch indicate bootstrap values.

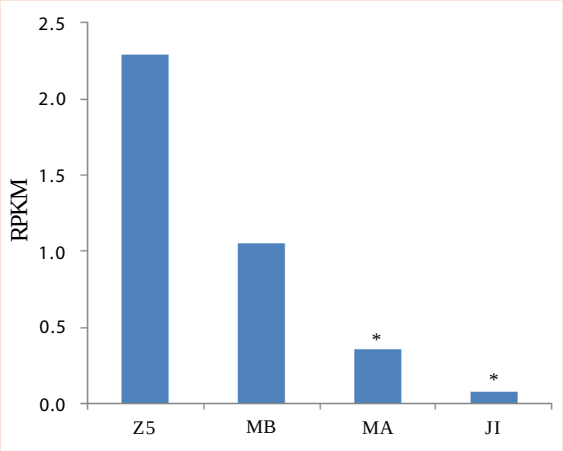

Figure 5: Expression levels of *EmDmc1* transcripts in four larval transcriptomes of *Eriocheir sinensis*. Significant differences across Z5 are indicated with an asterisk.

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