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First de novo transcriptome assembly of Iwagaki oyster, *Crassostrea nippona,* and comparative evolutionary analysis of salinity-stress response genes in *Crassostrea* oysters

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ABSTRACT

Crassostrea nippona is a commercially important oyster species in East Asia for it is edible during the summer when the other oyster species are unavailable. Salinity is one of the important limiting factors to the survival and distribution of this stenohaline species. In this study, 535 million reads (74G data) from *C. nippona* were produced and assembled into 66,742 transcripts. The number of 19,253 differentially expressed genes (DEGs) under salinity stress were identified as salinity stress-response genes. Through comparative evolutionary analysis in five *Crassostrea* species from East Asia, salinity stress-response genes were noticed to have higher adaptive evolution rates than other genes. This study presents the first de novo transcriptome of *C. nippona*. Furthermore, comparative evolutionary analysis implies that salinity plays an important role in speciation of *Crassostrea* species.

1. Introduction

Coastal marine systems are the most ecologically and socio-economically vital on the planet (Harley et al., 2006). Seasonal and tidal fluctuations of salinity are characteristics of this area. Aquatic animals that live in the intertidal zone have different adaptations to exist at varying salinity (Drouin et al., 1985). Most invertebrates are known as osmoconformers which adapt the extracellular fluid (ECF) to the ambient environment rather than maintain stable ECF (Bourque, 2008). The salinity stress especially reduced salinity caused by heavy rainfall and river injection influences metabolic and physiological parameters of these organisms (Butt et al., 2006; Gagnaire et al., 2006; Nell and Holliday, 1988; Tirard et al., 1997) and even causes the mass mortality (Gunter, 1955; La Peyre et al., 2013; Soletchnik et al., 2007).

Oysters within the genus *Crassostrea* are important commercial mollusks in East Asia. They have variant biotypes along the coast (Guo et al., 1999; Okutani, 2000) that are hypothesized to reflect differences in their tolerances of salinity (Braby and Somero, 2006). Asia-Pacific is considered as epicenter of extant oyster speciation and most species have originated in estuarine waters such as *Crassostrea gigas*, *C. angulata*, *C. hongkongensis* (Ahmed, 1976; Ren et al., 2010). Among them, *C. nippona* is a benthic species in shallow water along the coast (Okutani, 2000) and diverges early from other Asian *Crassostrea* oysters demonstrated from phylogenetic analysis (Yu and Li, 2012). Other than this, there are limited studies on *C. nippona*. To date, karyotype analysis, shell matrix, taxonomy, and growth and survival of larvae and juvenile have been documented in *C. nippona* (Itoh et al., 2004; Kudo et al., 2010; Samata et al., 2008; Wang et al., 2018; Wang and Li, 2018).

In this study, the Illumina platform was employed to obtain the transcriptome information for *C. nippona*. Furthermore, the adaptive evolution rates of salinity stress-response genes in *Crassostrea* oysters were quantified with an aim to explore the evolutionary history of Asian *Crassostrea* oysters.

2. Data description

2.1. Biological materials

Adult *Crassostrea nippona* were cultured at Rongcheng, Shandong Province, China. Tissues from the mantle (M), visceral mass (V), adductor muscle (A) and gill (G) were collected from healthy oysters and immediately placed into liquid nitrogen to freeze and then stored at −80 °C for constructing *C. nippona* reference transcriptome.

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2.2. Salinity stress

The experimental *C. nippona* were maintained in 70 L tanks containing aerated sand-filtered seawater (30 psu) for one week prior to experimentation, then randomly divided into 3 groups under hyposalinity stress in seawater with salinity of 10 (S1), 20 (S2), and 30 psu (S3) for one week. The low salinity water was prepared by diluting sea water with tap water.

2.3. RNA isolation, library construction and sequencing of C. nippona

Each sample was lysed in 1 ml of TRIzol Reagent (Invitrogen, USA) for total RNA extraction according to the manufacturer's instructions. The purity and integrity of total RNA was determined using a NanoDrop-2000 spectrophotometer (Thermo, USA) and an Agilent 2100 Bio-Analyzer (Agilent Technologies, USA).

The mRNA was enriched by Oligo(dT) beads and then fragmented. The cDNA was synthesized with random hexamers via mRNA fragments as templates. The cDNA fragments were purified and resolved with EB buffer for end repair, single-nucleotide adenine (A) addition and adapter connections. After PCR amplification, the 150 bp library was then sequenced via an Illumina HiSeq™ 4000.

2.4. Transcriptome assembly of C. nippona and reads mapping

Clean reads were obtained by removing "dirty" reads containing adapter sequences, sequences with more than 10% unknown bases ("N"), and low-quality reads containing more than 40% of low quality (Q-value≤10) bases through fastp v0.18.0 (Chen et al., 2018). Reference transcriptome was de novo assembled by Trinity v2.8.4 (Grabherr et al., 2011) and run against metazoan databases with BUCSO v3.0.2 (Waterhouse et al., 2017) to check the completeness. Clean reads were mapped against the reference transcriptome using alignment tool Bowtie2 (Langmead and Salzberg, 2012) with default parameters. RSEM v1.3.1 (Li and Dewey, 2011) was used to quantify the mapped reads. The gene abundances were calculated and normalized to the number of reads per kb per million reads (RPKM) (Mortazavi et al., 2008).

There were 60–300 million raw reads produced from high-throughput sequencing of four *C. nippona* tissues respectively. And then these reads were pooled to assemble a comprehensive reference transcriptome of *C. nippona* (Table 1). As gill is the first line of osmoregulation in oysters, the transcriptome of gill under three different salinities (S1, S2, S3) was sequenced additionally. The RNA was pooled proportionally from three oysters within each experimental group. A total of three samples were used for library construction. The mapping ratio against the *C. nippona* reference transcriptome assembly was 89.09–91.06% (Supplementary Table 1). The RPKM distribution of three samples have similar trends (Supplementary Fig. 1). The Minimum Information about any (x) Sequences (MIxS) information, which is standard description of sequence data, is given in Table 2.

Table 1
Summary of the sequencing and de novo assembly.

	Mantle	Visceral mass	Adductor muscle	Gill
No. raw reads	86,427,524	77,246,768	68,485,094	302,902,344
No. clean reads	86,021,074	76,933,466	68,105,922	299,919,348
Q20% after filter	97.93	98.27	98.4	96.63
No. unigenes	66,353			
GC% of unigenes	38.5793			
N50 length (bp)	2239			
Max length (bp)	30,742			
Average length (bp)	1082			
Min length (bp)	201			
Complete BUSCOs (%)	98			

Table 2
MIxS information for transcriptome assembly of *C. nippona*.

	Value
Investigation type	Eukaryote
Project name	*Crassostrea nippona* transcriptome
Bioproject accession	PRJNA482778
Organism	*Crassostrea nippona*
Collection date	20-Jun-2016
Environment	Sea water
Biome	ENVO: 00000316 (intertidal zone)
Feature	ENVO: 00000016 (sea)
Material	ENVO: 00002149 (sea water)
Geolocation	China:Rongcheng
Development stage	Adult
Sequenced method	Illumina HiSeq TM 4000
Assembly method	Trinity software

2.5. Identification of salinity stress-response genes

The differentially expressed genes (DEGs) under salinity stress were obtained through salinity groups S1, S2 compared with normal salinity group S3. R package edgeR v3.2.4 (Robinson et al., 2010) was used to adjust the read counts and identify DEGs. The threshold for evaluating significance was false discovery rate (FDR) ≤ 0.05 and log2 fold change ratio ≥ 2. DEGs were then subjected to enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al., 2008), taking FDR ≤ 0.05 as a threshold.

Through differential expression analysis, 19,253 unigenes were identified as DEGs, which been taken as salinity stress-response genes. Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing with the whole genome background. KEGG is the major public pathway-related database (Kanehisa et al., 2008). Several osmoregulatory related terms, such as "neuroactive ligand-receptor interaction", "calcium signaling pathway", "ECM-receptor interaction" and "taurine and hypotaurine metabolism" were observed in S2 vs S3 (Supplementary Fig. 2). Those neuroactive ligand-receptors always sense the ambient salinity changes as the gills of oysters are in direct contact with the outside world (Liu et al., 2018). The osmotransduction signaling pathways have well-recognized elements such as an increase in Ca^{2+}, thus the calcium signaling pathway and ECM-receptor interaction have important roles in salinity-stress sensing and signaling transduction (Zhao et al., 2012). Taurine, a non-protein animo acid, is the main organic osmolyte for oysters (Zhao et al., 2017). Apart from those pathways, "apoptosis" was observed to be significantly differentially expressed in S1 vs S3 (Supplementary Fig. 3). Apoptosis is a regulated, physiologic cell death used by organisms to eliminate unwanted cells (Green and Reed, 1998). It is a cellular defense mechanism for osmoconforming mollusks in unwanted conditions (Goedken et al., 2005), which reveals *C. nippona* has little capacity to adapt to 10 psu. And it has been suggested that *C. nippona* is a stenohaline species and has little salinity tolerance capacity (Okutani, 2000).

2.6. Adaptive evolution rates calculation

All translated protein sequences from five species (*C. nippona*, *C. gigas*, *C. angulata*, *C. sikamea* and *C. hongkongensis*) were used to search for single-copy genes by OrthoFinder (Emms and Kelly, 2015). Total of 6559 single-copy genes were shared among five *Crassostrea* species. The number of non-synonymous substitutions per non-synonymous site (Ka) and the number of synonymous substitutions per synonymous site (Ks) against *C. nippona* of another four *Crassostrea* species were calculated by KaKs_Calculator v2.0 (Zhang et al., 2006) and ParaAT v2.0 (Zhang et al., 2012).

The salinity stress-response genes have larger Ka and Ka/Ks values than the non-response genes (Fig. 1, P value $< 10^{-16}$), suggesting that these response genes have more nonsynonymous mutation than non-

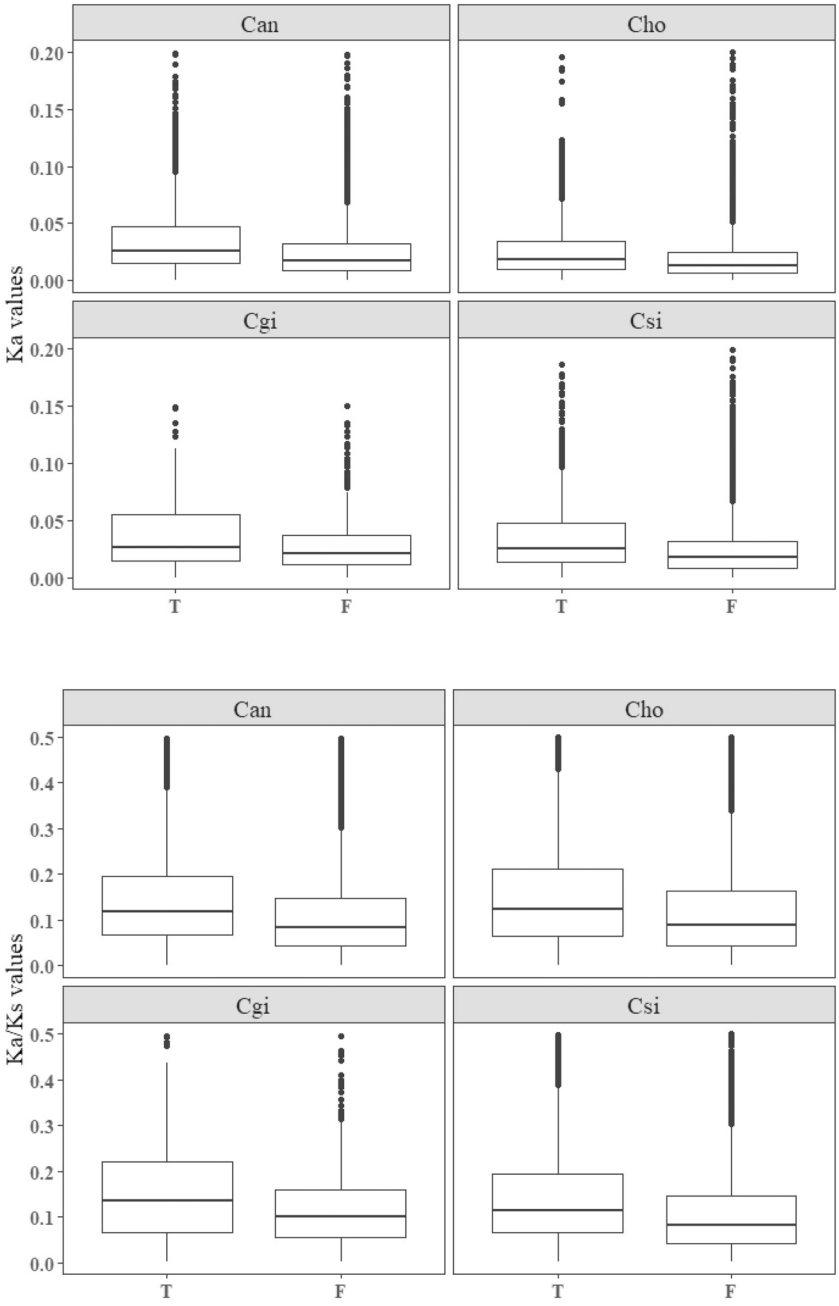

Fig. 1. Boxplot of Ka, Ks and Ka/Ks values for the salinity stress response genes and non-response genes of four *Crassostrea* species against *C. nippona*. Can, *C. angulata*; Cho, *C. hongkongensis*; Cgi, *C. gigas*; Csi, *C. sikemea*; T, salinity stress-response genes; F, non-response genes; Ka, The number of non-synonymous substitutions per non-synonymous site; Ks, The number of synonymous substitutions per synonymous site. The *P* values of Wilcoxon rank test for Ka and Ka/Ks between salinity stress response genes and non-response genes in four species are all less than 10^{-16}.

response genes. This phenomenon is noteworthy, which means there is indeed evidence showing that the intertidal environment with fluctuated physicochemical factors accelerates the speciation of oyster, which was also mentioned in other oyster studies (Guo et al., 2015; Song et al., 2017, 2019).

3. Conclusion

This is the first high-quality transcriptomic resource for *Crassostrea nippona*. Furthermore, the RNA seq data and transcriptome assemblies described in this article provide resources of expressed coding genes in different tissues and condition of adult *C. nippona*. Comparative transcriptome analysis in *Crassostrea* oysters implies that salinity plays an important role in adaptive evolution of oysters, which provides a novel direction for future analysis of evolutionary history of oysters.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.margen.2020.100805.

Data availability

High throughput transcriptomic reads have been deposited in SRA database under SRA ID SRR7646736, SRR10482017, SRR10482018, SRR10482019, SRR10482020, SRR10482021, SRR10482022. This Transcriptome Shotgun Assembly project was been deposited at DDBJ/ENA/GenBank under the accession GGUV00000000. The version described in this paper is the second version, GGUV02000000.

Declaration of Competing Interest

The authors declare that they have no known competing financial

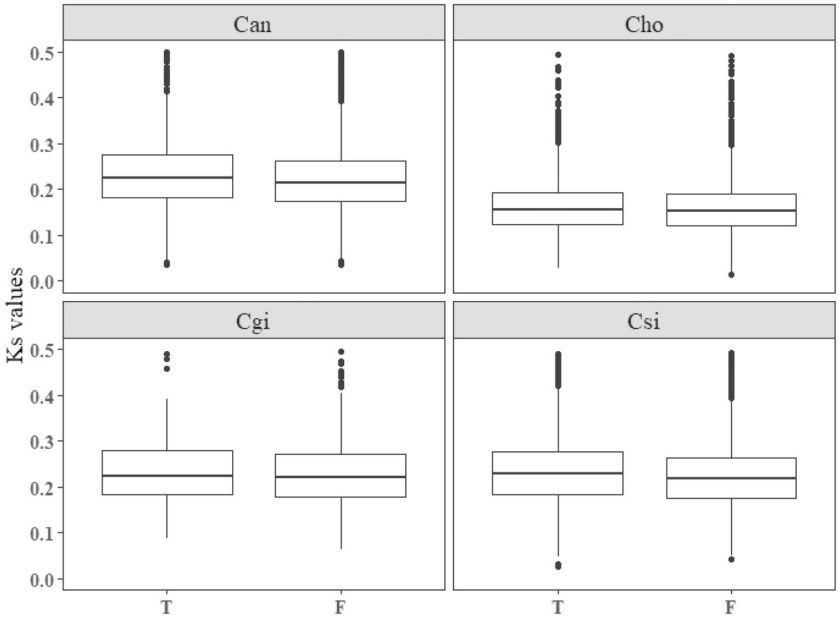

Fig. 1. (*continued*).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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