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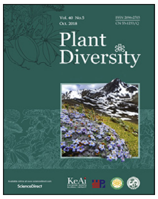

Generation and characterization of expressed sequence tags (ESTs) from coralloid root cDNA library of *Cycas debaoensis*

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ABSTRACT

A normalized full-length cDNA library was constructed from the coralloid roots of *Cycas debaoensis* by the DSN (duplex-specific nuclease) normalization method combined with the SMART (Switching Mechanism At 5′ end of the RNA Transcript) technique. The titer of the original cDNA library was about 1.5×10^6 cfu·mL^{-1} and the average insertion size was about 1 kb with a high recombination rate (97%). The 5011 high-quality expressed sequence tags (ESTs) were obtained from 5393 randomly picked cDNA clones. Clustering and assembly of ESTs resulted in 2984 unique sequences, consisting of 618 contigs and 2366 singlets. EST sequence annotation revealed that 2333 and 1901 unigenes were functionally annotated in the NCBI non-redundant database and Swiss-Prot protein database, respectively. Functional analysis demonstrated that 1495 (50.1%) unigenes were associated with 4082 Gene Ontology (GO) terms. A total of 847 unigenes were grouped into 22 Cluster of Orthologous Groups (COG) functional categories. Based on the EST dataset, 22 ESTs that encoded putative receptor-like protein kinase (RLK) genes were screened. Furthermore, a total of 94 simple sequence repeats (SSRs) were discovered, of which 20 loci were successfully amplified in *C. debaoensis*. This study is the first EST analysis for the coralloid roots of *C. debaoensis* and provides a valuable genomic resource for novel gene discovery, gene expression and comparative genomics, conservation and management studies as well as applications in *C. debaoensis* and related cycad species.

1. Introduction

The Cycadales (cycads), whose origin can be dated to the Late Paleozoic (~265–290 Ma), are the most primitive living seed plants. The earliest Cycad fossil in the world was found in China and dates to Lower Permian, whereas peak abundance and diversity of cycads date to the Mesozoic (Martínez et al., 2012; Gao and Barry, 1989). Extant cycads are distributed in tropical and subtropical regions of Africa, Asia, Oceania, and America. Ten genera and ~300 species are currently accepted (Hill et al., 2004; Norstog and Nicholls, 1997). Cycads, which originated from seed ferns, have many of same characteristics as ferns. Such characteristics include pinnately compound leaves, circinate vernation, unique girdling leaf traces, no axillary buds, dichotomous branching (versus axillary branching in higher plants). However, cycads have recognizable intermediate morphological traits between angiosperms and gymnosperms, and have therefore been classified as gymnosperms. Of the four order that comprise the gymnosperms—Ginkgoales, Gnetales, Coniferales, and Cycadales—Cycadales are considered the most ancestral (Nixon et al., 1994; Soltis et al., 2002). Their pollen tubes possess multiciliate sperm, and their ovules are borne on the margins of leaf-like megasporophylls (Loconte and Stevenson, 1990). Clearly, cycads represent a key node in the phylogeny of seed plants.

Previous research has also indicated the importance of understanding cycad biological nitrogen fixation (BNF). Cycads have a particular root type, referred to as coralloid roots due to their 'coral-like' appearance. Nitrogen-fixing cyanobacteria invade coralloid roots, where BNF processes occur (Vessey et al., 2005; Lindblad and Costa, 2002). To date, no symbiosis-related genes in cycads have been identified (or searched) and the molecular mechanisms of symbiotic nitrogen fixation in these plants are still largely unclear (Rai et al., 2000).

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In non-model species with a large genome size, EST (expressed sequence tag) sequencing and annotation is a means for gene discovery and a way to understand the transcription and expression patterns of specific genes. Normalization techniques reduce the frequency of highly expressed genes and increase the rate of rare gene discovery (Soares et al., 1994). Cycads have a large genome size (~20–30 Gbp) and are evolutionarily and horticulturally important, long-lived plants (Zonneveld, 2012). Cycads are also the only early seed plants that have evolved a specialized coralloid root to host endophytic bacteria that fix nitrogen for the plant.

Microsatellites, also called SSRs (simple sequence repeats) or STRs (short tandem repeats), are highly polymorphic genetic markers. They have been extensively used for plant population genetic studies because of their co-dominant inheritance, relative abundance, multi-allelic nature, high reproducibility, and ease of detection (Powell et al., 1996). The development of SSR markers has improved the characterization and use of genetic variation in cycads (Ju et al., 2011; Yang et al., 2008). SSRs have been adopted to evaluate genetic diversity and to reconstruct the population structure, allowing researchers to design reasonable conservation and management protocols.

Cycas is at the basal node of the Cycadales (Treutlein and Wink, 2002). *Cycas debaoensis*, endemic to southern China, is a rare and endangered plant species (Chen and Zhong, 1997; Ma et al., 2003). In this study, we constructed a normalized full-length cDNA library using RNA derived from the coralloid roots of *C. debaoensis*. We assembled ESTs into contigs and singletons, and subsequently performed comparative protein annotations. Using this database, we identified a total of 22 ESTs that encode putative receptor-like protein kinase (RLK) genes, which play a variety of important defensive and symbiotic roles in plant–microbe interactions (Shiu and Bleecker, 2003; Shiu et al., 2004). Finally we used EST data to detect SSRs.

2. Materials and methods

2.1. Plants and total RNA extraction

Coralloid roots of *C. debaoensis* were collected from National Cycad Germplasm Conservation Center, Shenzhen Fairylake Botanical Garden. Tissue was obtained from the topsoil, washed with sterile water, and then 70% ethanol, wrapped with aluminum foil bags, frozen in liquid nitrogen for more than 2 h, and stored at −80 °C. Total RNA was extracted from pulverized, frozen tissue using trizol reagent (Invitrogen). The integrity of the total RNA was confirmed by examining the ratio of 28S and 18S ribosomal RNA with 1% agarose gel electrophoresis. Quality and quantity of the isolated RNA were determined using a spectrophotometer. The extracted RNA was found to be of high quality (OD260/OD280 = 2.02).

2.2. Construction of full-length normalized cDNA library

The cDNA library was constructed using Creator SMART cDNA library construction kit (Clontech, USA). Double stranded cDNA was synthesized according to the manufacturer's protocol. DSN normalization was applied according to the instructions of Trimmer-Director kit (Evrogen, Cat. No. NK002). The cDNA inserts were directionally cloned in pDNR-LIB vector and transformed using DH10B electrocompetent cells of *Escherichia coli*. The recombinants were selected in LB agar plates supplemented with ampicillin. The quality and quantity of the isolated plasmid DNA was confirmed on 0.8% agarose gels before sequencing.

2.3. EST sequencing and assembly

Plasmid DNA was sequenced by 5′ end single-pass sequencing on automated DNA capillary sequencer ABI 3730XL (Applied Biosystems) according to the manufacturer's instructions using T7 primer (5′TAATACGACTCACTATAGG3′). All EST sequences were deposited in the GenBank database under the accession numbers dbEST JZ917721–JZ922731. Sequence analysis was performed at Beijing Luhe Huada Gene Sci-Tech Company. ESTs were scanned and trimmed for vector sequences using NCBI's VecScreen tool. Low quality and short (<100 bp) sequences were also removed. The processed sequences were assembled into contigs and singletons using Contig Assembly Program CAP3. Then EST and contig redundancy was calculated.

2.4. Annotation and classification of singlets

All contigs and singletons were compared against the NCBI non-redundant protein (Nr) and Swiss-Prot database (Boeckmann et al., 2003). Based on the Nr annotations, GO annotations of the unigenes were obtained using the Blast2GO program. GO terms were assigned to each unigene and classified into three functional annotation categories: biological process, cellular component, and molecular function. All unigenes were also compared to the proteins in the Cluster of Orthologous Groups (COG) databases (Tatusov et al., 2000) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2008).

2.5. EST-SSR search and primer design

SSR loci of unigenes were identified and analyzed by MISA software (http://pgrc.ipk-gatersleben.de/misa/). Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA) was employed to design PCR primers for the conserved flanking regions of the SSRs. The minimum repeats of sequences with di-, tri-, tetra-, penta- and hexanucleotide motifs was set as 6, 5, 5, 5 and 5, respectively. The range of PCR product size was set between 100 and 300 bp.

2.6. Analysis of sequences

Databases used to perform BLASTN analyses included *Cycas rumphii* PUT (10,901), *Zamia vazquezii* PUT (7,657), *Gnetum gnemon* PUT (6,193), and *Ginkgo biloba* PUT (10,210). All databases as described above were downloaded from plantGDB (http://www.plantgdb.org). All BLAST searches were subject to an expect value < 1e-5.

3. Results and discussion

3.1. EST sequence quality, contigs and singlets

A total of 5393 ESTs were generated from this cDNA library; removing vector and low quality sequences resulted in 5011 high-quality ESTs. The EST length was distributed from 100 to 500 bp (19.04%), 501 to 800 bp (80.42%) and 801 to 840 bp (0.54%). The cluster analyses of ESTs generated 2366 singlets and 618 contigs. The average lengths of singlets and contigs were respectively 538.87 bp and 720.66 bp. The redundancy of the library was calculated as 40.45% ((1 − Number of Unigenes/Number of ESTs) × 100%) (Tatusov et al., 2000). This is lower than that reported in other gymnosperms studies like *C. rumphii* (Brenner et al., 2003), *Ginkgo biloba* (Brenner et al., 2005), and *Picea glauca* (Birol et al., 2013). The number of ESTs in a contig ranged from 2 to 150. About 96.28% of the contigs had less than 10 ESTs with 338 having

Table 1
Summary of EST sequencing and assembly results.

EST sequences and contigs	Number
Number of EST sequences	5011
Number of Contigs	618
Number of singletons	2366
Average assembled EST length	600.59
Average number of sequences per contig	4.23
Number of contigs containing:	
2 ESTs	338
3 ESTs	119
4~5 ESTs	89
6~10 ESTs	49
11~20 ESTs	17
21~50 ESTs	1
51~100 ESTs	3
>100 ESTs	2

only 2 sequences. EST sequencing and assembly results are shown in Table 1 and the distribution of ESTs in contigs is shown (Fig. 1).

3.2. Functional gene annotation and gene discovery in unique sequences

All unique sequences (including 2366 singlets and 618 contigs) were subjected to BLASTX searches against the NCBI Nr (non-redundant) protein database. A total of 2333 unigenes had significant hits. Among the 2333 unique sequences, 577 matched proteins of known function and 1756 matched predicted proteins of unknown function. These unique sequences were also compared to proteins in Swiss-port, KEGG, and COG databases, which revealed there were 1,901, 2255 and 951 unigenes that showed high similarity, respectively. Of these, 938 sequences had annotations in all four databases, 2343 sequences in at least one of the four database, and 641 sequences had no annotation in any database (Fig. 2).

3.3. GO and COG categories

Gene Ontology (GO) is a standard system defining gene classes (Ashburner et al., 2000). Fifty percent of 2984 unique sequences were successfully annotated with 4082 GO terms (Fig. 3). The unigenes were thus functionally classified with one or more ontologies: 954 sequences (63.8%) were assigned GO terms associated with biological processes, 1329 sequences (88.9%) were involved in molecular functions, and 415 sequences (27.8%) were cellular components. Under the category “biological process”, 51.2% sequences were associated with “physiological process”, 39.4% with “cellular process”, 5.7% with “regulation of biological process”, and 3.5% with “response to stimulus”. Under the category “molecular functions”, sequences associated with “binding” (805), “catalytic activity” (739), and “transporter activity” (111) were respectively 35.8%, 32.8%, and 4.9%. Under the category “cellular component”, 57.3% sequences were associated with “cell”, 23.7% with “organelle”, and 17.8% with “protein complex”.

The COG protein database is an attempt to classify orthologous gene products (Tatusov et al., 2000). All unigenes were compared to proteins in the COG database. The results showed that a total of 847 sequences were assigned to 22 COG categories (Fig. 4). The cluster for “general function prediction only” (154, 18.18%) was the largest group, followed by “posttranslational modification, protein turnover, chaperones” (132, 15.58%), “translation, ribosomal structure and biogenesis” (90, 10.63%), “carbohydrate transport and metabolism” (73, 8.62%), “energy production and conversion” (61,7.2%), and “amino acid transport and metabolism” (56, 6.61%). In addition, the clusters for “signal transduction” (29, 3.42%) and “defense mechanisms” (5, 0.59%) were found.

3.4. Gene expression and highly abundant transcripts

The expression levels of corresponding genes can be estimated preliminarily according to the distribution frequency of transcribed ESTs in the cDNA library (Mann et al., 2013). Among the annotated sequences, 22 contained at least 10 supporting ESTs (Table 2). A family of metallothionein-like proteins was the most abundant transcript, with 470 ESTs detected. These are small metal ion binding proteins. By binding heavy metals, metallothionein-like proteins can effectively reduce the toxicity of heavy metals to the body (Zhou et al., 2005; Liu et al., 2002). Other highly abundant transcripts were genes associated with DJ-1 protein, antimicrobial proteins, germin-like proteins, aquaporin proteins, ERD (early responsive to dehydration) proteins, WAT1-related proteins, and membrane steroid-binding proteins. High abundance of genes involved in stress and defence responses is quite expected because the cDNA library was constructed from coralloid roots of *C. debaoensis* under natural conditions. The genes identified in our study provide a valuable transcriptomic resource for structural and functional genomics studies in *C. debaoensis*.

3.5. Sequence similarity and evolutionary relations

The BLASTX comparisons of unique *C. debaoensis* sequences were conducted against the published PUT (putative unique transcripts) of four major gymnosperms (*C. rumphii*, *Z. vazquezii*, *Ginkgo*

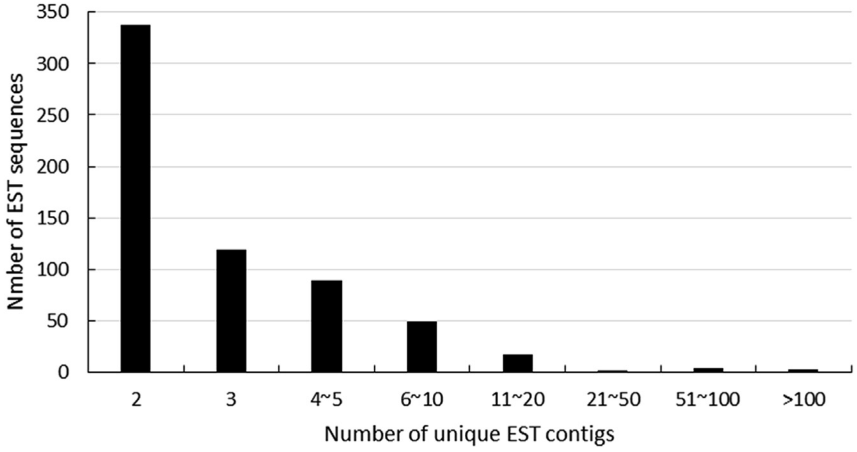

Fig. 1. Distribution of individual EST sequences among the clustered contigs.

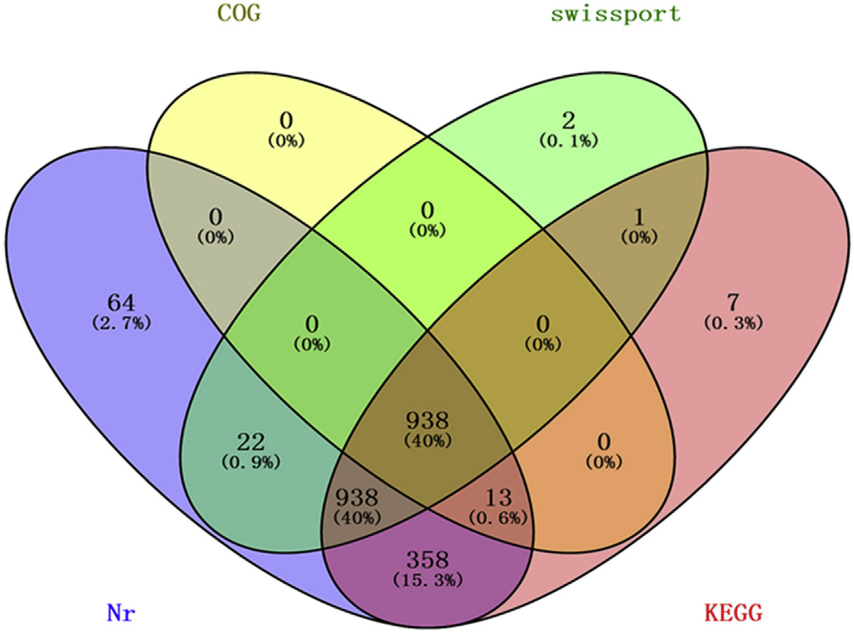

Fig. 2. Venn diagram of annotation results against Nr, Swiss-Prot, COG, and KEGG databases. The numbering each color block indicates the number of unigenes that is annotated by single or multiple databases.

biloba, and *Gnetum gnemon*). Phylogenetially, *C. debaoensis* is closest to *C. rumphii*, next to *Z. vazquezii*, *Ginkgo biloba*, and last to *Gnetum gnemon*.

Accordingly, *C. rumphii* and *C. debaoensis* had the highest matches in 1354 sequences (45.4%). The similarity results indicate that 45.4% of *C. debaoensis* genes discovered are homologues (orthologs) of *C. rumphii* genes and may have originated from a common ancestor. *Ginkgo biloba* followed, with 436 (14.6%) unique sequence similarities. The number of unique *C. debaoensis* sequences that were similar to *Z. vazquezii* PUTs was 429 (14.4%). *Ginkgo biloba* had higher similarity than *Z. vazquezii* since the EST resource generated for *Ginkgo biloba* (10,210 PUTs) is much larger than that of *Z. vazquezii* (7,657). Only 50 (1.7%) sequences had statistically significant similarity between *C. rumphii* and *Gnetum gnemon* (Fig. 5).

3.6. Simple sequence repeats

A total of 94 different 2–6 nucleotide repeats were developed from unigenes obtained in this experiment (Table 3). Trinucleotide repeats were the most frequent (46, 49%), followed by dinucleotide repeats (38, 40%), tetranucleotide repeats (8, 8.5%), pentanucleotide repeats (1, 1.1%), and hexanucleotide repeats (1, 1.1%). Among the various SSRs, AG/CT repeats were the most abundant (23, 24.5%), followed by AAG/CTT (10, 10.6%), and ATC/ATG (9, 9.6%). Our results are in agreement with previous studies that have shown that

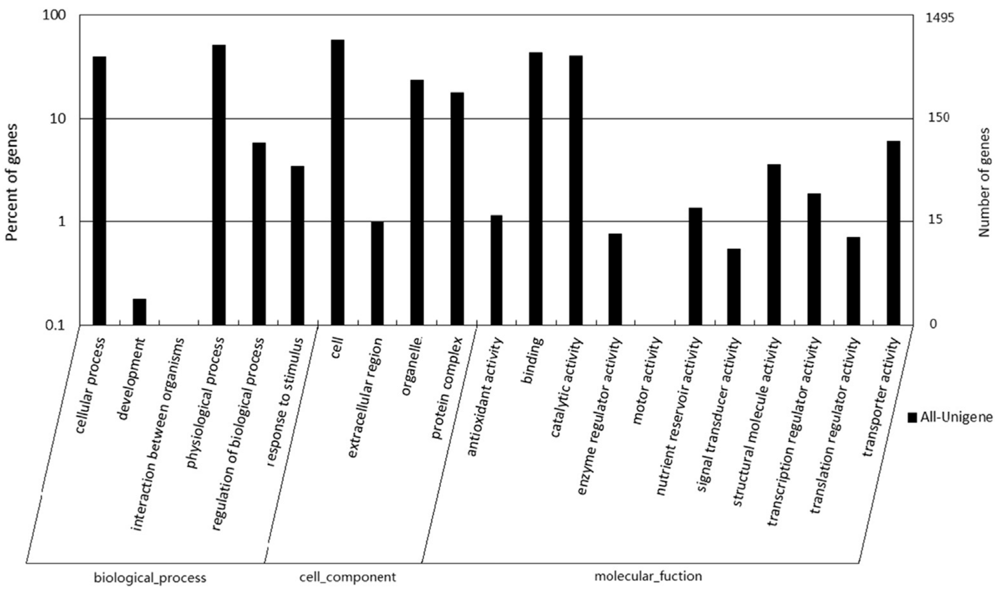

Fig. 3. GO analysis and functional classification of the *C. debaoensis* unigenes.

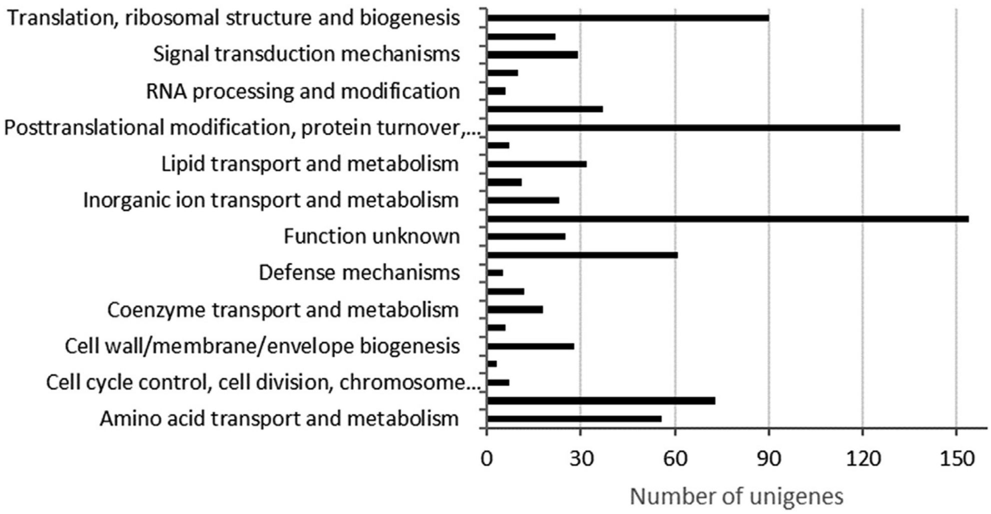

Fig. 4. COG functional classification of the *C. debaoensis* unigenes.

Table 2
Estimation of gene expression: unique EST sequences with >10 ESTs.

Putative protein identification	Number of ESTs	Number of unique ESTs
Metallothionein-like protein EMB30	470	90
Protein DJ-1 homolog D	101	26
Antifungal protein ginkbilobin-2	55	17
Germin-like protein 9-3	50	19
Ubiquitin-conjugating enzyme E2 28	42	10
Glycine cleavage system H protein, mitochondrial	38	6
WAT1-related protein At5g07050	23	4
Glucoamylase	23	5
Protein early responsive to dehydration 15	17	1
Chitotriosidase-1	15	7
High mobility group B protein 1	14	2
Clavaminate synthase-like protein At3g21360	14	6
Subtilisin-like protease SDD1	13	4
Retrovirus-related Pol polyprotein from transposon TNT 1-94	13	13
Probable aquaporin PIP2-8	12	7
Flavanone 3-dioxygenase	11	3
Small nuclear ribonucleoprotein E	10	1
Probable aquaporin PIP1-5	10	1
Non-functional NADPH-dependent codeinone reductase 2	10	4
Membrane steroid-binding protein 2	10	1
EndochitinaseA2	10	2
EC protein homolog 1	10	3

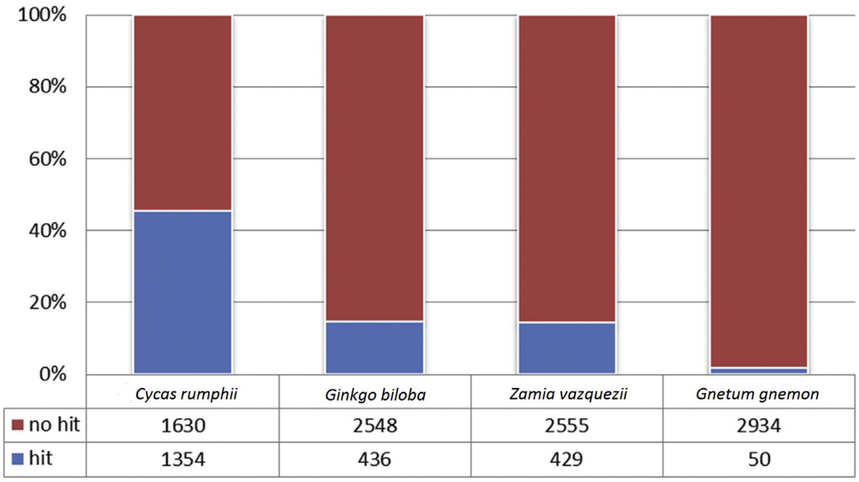

Fig. 5. Conservation between PUT sequences of *C. debaoensis* and other gymnosperms.

Table 3
Type and number of nucleotide repeats in SSRs.

Repeats motif	Number of repeats							total
	5	6	7	8	9	10	>10	
AC/GT	–	2	3	1		1	–	7
AG/CT	–	14	4	–	1	2	2	23
AT/AT	–	5	2	–	1	–	–	8
AAC/GTT	3	1	–	–		–	–	4
AAG/CTT	6	2	1	–	1	–	–	10
AAT/ATT	4	3	–	–	–	–	–	7
ACG/CGT	2	–	–	–	–	–	–	2
AGC/CTG	4	2	1	–	–	–	–	7
AGG/CCT	3	3	1	–	–	–	–	7
ATC/ATG	5	1	1	1	1	–	–	9
AAAT/ATTT	6	–	–	–	–	–	–	6
AATT/AATT	1	–	–	–	–	–	–	1
ACAT/ATGT	1	–	–	–	–	–	–	1
AAAAT/ATTTT	–	1	–	–	–	–	–	1
ACAGCC/CTGTGG	1	–	–	–	–	–	–	1
Total	36	34	13	2	4	3	1	94

angiosperms and gymnosperms have a higher abundance of AG/CT and AAG/CTT motifs. These sequences are also known methylation targets in plants (Ranade et al., 2014). In gymnosperms, some studies report the AG/CT motif as the most abundant in *Cycas* (von Stackelberg et al., 2006) and *Gnetum* (Victoria et al., 2011). In other studies, the AAG/CTT motif has been shown to be the most abundant trimer in *Picea* (Rungis et al., 2004) and *Cycas* (von Stackelberg et al., 2006). Finally, 60 pairs of primers were designed for PCR amplification. Of these primer pairs, 20 successfully amplified the target regions using genomic DNA of *C. debaoensis* (Table 4). As newly developed molecular markers, they provide valuable resources for the population and conservation genetics of *C. debaoensis* and other cycads.

3.7. Potential candidate RLK genes involved in signaling in symbiosis and defense

The receptor-like protein kinase (RLKs) is the main receptor for plant extracellular signals (Shiu et al., 2004; Shiu and Bleecker, 2003). Plant RLKs function in diverse signaling pathways, including the responses to microbial signals in symbiosis and defense (Antolínllovera et al., 2012). Because the cDNA library was constructed from coralloid root tissues, genes involved in plant–microbe interactions were expected to be expressed. In this study, we focus on RLKs involved in plant–microbe interactions. Three types of the RLKs highlighted here include the leucine-rich repeat (LRR) type, lysin-motif (LysM) type, and lectin (Lec) domain type. The LRR-RLKs contain a tandemly repeated (9–26) Leu-rich motif, which plays an important role in plant development, defense symbiosis, and other biological processes (Liu et al., 2017). RLKs with lysin-motif (LysM) ectodomains confer recognitional specificity toward N-acetylglucosamine-containing signaling molecules, such as chitin, peptidoglycan (PGN) and rhizobial nodulation factor (NF), which induce immune or symbiotic

Table 4
Characteristics of 20 SSR loci designed from an EST library of *C. debaoensis*.

Locus	Primer sequence (5′–3′)	Repeat motif	product size (bp)	Ta (° C)	GenBank Accession No.
Cdb01	F:CGCCCCATTTTAGATCTCTC R:AAACGATGTGAGCCAAAACC	(TC)6	155	55	JZ918061
Cdb02	F:CAATGCCAACGCTGTGTCTA R:CCCTCAACCTGCAATTTCTC	(CAT)9	222	57	JZ918389
Cdb04	F:TTGCACCTGCCATTAGTCAA R:TGATCGGTCTCAACAGGTAATG	(AATA)5	196	55	JZ918792
Cdb05	F:TTGCACCTGCCATTAGTCAA R:TGATCGGTCTCAACAGGTAATG	(AATA)5	196	55	JZ919036
Cdb07	F:ATCCAAGCTAAAGGGTTCGG R:TGAACTGCTGCTGCTATAAAAA	(TGA)5	141	55	JZ919105
Cdb08	F:CGACTGATCTCGTCCCAAAT R:AGACATAATCCGCCACGAAG	(GA)6	221	57	JZ920586
Cdb09	F:AAATCCAAGCCAAAGGGTTC R:CCCCCAACAACAACTGAACT	(TGA)5	157	55	JZ921520
Cdb11	F:TTGCACCTGCCATTAGTCAA R:TGATCGGTCTCAACAGGTAATG	(AATA)5	196	55	JZ921918
Cdb12	F:CCTGTACCAGGGACGAAGAA R:CCCTCAACCTGCAATTTCTC	(CAT)8	273	57	JZ922529
Cdb13	F:CGGACCCTCAATGTGTCTTT R:CAGCAGCCAAATGAGCACTA	(CT)6	163	57	JZ920486
Cdb18	F:ATTGTATATGCAGCAGCCCC R:CAAGACCACGCGTTGAGATA	(GCA)6	265	57	JZ920178
Cdb19	F:ATTGTATATGCAGCAGCCCC R:CAAGACCACGCGTTGAGATA	(CCT)7	265	57	JZ920178
Cdb33	F:AAGTTCCGTGCCAACCATAA R:GATCTGCTGCCTTCACCTTC	(ATA)5	164	55	JZ918520
Cdb45	F:TGGATTCATGAGCATTGGAA R:TAATGCAAACAGGGCAATGA	(CAT)5	148	53	JZ920472
Cdb48	F:AAGCCAAAAAGGGCAAGATT R:CTTCTACTTCGCCCCTCCTT	(CAA)5	186	53	JZ921258
Cdb50	F:TACTTACAGCAGGGGGAAGG R:CACATGACAGAGGTCTAGTGGG	(TATG)5	263	59	JZ921416
Cdb53	F:TCTGTAGCGAGTTTGGGGTT R:CCGCTAAGATTGCCACATTT	(TAT)6	255	55	JZ921726
Cdb54	F:TACATCAGGCAATGGCAAAA R:TGCAAACTCCAATAATTCAAGAGA	(AT)7	259	53	JZ922036
Cdb55	F:CCTCCGAGGAACACAAACAT R:ATATCGCCCTCGCTCCTAAT	(AAG)7	241	57	JZ922127
Cdb56	F:ATCGGTCTCAACTTGGATGC R:CGTCGTTCTCCCGAGTTTTA	(TC)10	261	57	JZ922158

Table 5
Identification of ESTs encoding putative RLK (LRR-RLKs,LysM-RLKs,LecRLK) in coralloid roots of *C. debaoensis*.

<table>
<tr><th>GenBank_Accn</th><th>Annotated sequence identifier</th><th>Annotation description</th></tr>
<tr><td>JZ919236</td><td>sp|C0LGQ5|GSO1_ARATH</td><td>LRR receptor-like serine/threonine-protein kinase GSO1</td></tr>
<tr><td>JZ922644</td><td>sp|C0LGP4|Y3475_ARATH</td><td>Probable LRR receptor-like serine/threonine-protein kinase At3g47570</td></tr>
<tr><td>JZ920215</td><td>sp|Q9XID3|Y1343_ARATH</td><td>G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300</td></tr>
<tr><td>JZ920265</td><td>sp|O64780|Y1614_ARATH</td><td>G-type lectin S-receptor-like serine/threonine-protein kinase At1g61400</td></tr>
<tr><td>JZ917839</td><td>sp|Q9LFG1|Y3359_ARATH</td><td>Putative leucine-rich repeat receptor-like serine/threonine-protein kinase At3g53590</td></tr>
<tr><td>JZ921696</td><td>sp|C0LGS2|Y4361_ARATH</td><td>Probable LRR receptor-like serine/threonine-protein kinase At4g36180</td></tr>
<tr><td>JZ920273</td><td>sp|C0LGP4|Y3475_ARATH</td><td>Probable LRR receptor-like serine/threonine-protein kinase At3g47570</td></tr>
<tr><td>JZ921196</td><td>sp|C0LGP4|Y3475_ARATH</td><td>Probable LRR receptor-like serine/threonine-protein kinase At3g47570</td></tr>
<tr><td>JZ922387</td><td>sp|C0LGH3|Y5614_ARATH</td><td>Probable LRR receptor-like serine/threonine-protein kinase At1g56140</td></tr>
<tr><td>JZ917721</td><td>sp|O64825|LYK4_ARATH</td><td>LysM domain receptor-like kinase 4</td></tr>
<tr><td>JZ919870\JZ920485\JZ922291</td><td>sp|Q9M2S4|LRKS4_ARATH</td><td>L-type lectin-domain containing receptor kinase S.4</td></tr>
<tr><td>JZ918074\JZ921455\JZ918973\JZ917791\JZ919688\JZ920169</td><td>sp|Q9LYX1|LRK82_ARATH</td><td>L-type lectin-domain containing receptor kinase VIII.2</td></tr>
<tr><td>JZ918026</td><td>sp|O49445|LRK72_ARATH</td><td>Probable L-type lectin-domain containing receptor kinase VII.2</td></tr>
<tr><td>JZ919127</td><td>sp|Q9LT96|Y5977_ARATH</td><td>Probable leucine-rich repeat receptor-like protein kinase At5g49770</td></tr>
<tr><td>JZ919147</td><td>sp|O22938|Y2182_ARATH</td><td>Leucine-rich repeat receptor-like tyrosine-protein kinase At2g41820</td></tr>
</table>

responses (Antolínllovera et al., 2012). The lectin receptor-like kinases (LecRLKs) possess a characteristic extracellular carbohydrate-binding lectin domain. LecRLKs play important roles in plant development and innate immunity (Vaid et al., 2013; Prashant and Laurent, 2013). Using this database, we identified a total of 22 ESTs that encoded putative RLK genes (LRR-RLKs, LysM-RLKs, and LecRLKs), which play a variety of important defensive and symbiotic roles in plant–microbe interaction (Table 5). Further study of these genes will help us to understand the signaling pathways leading to symbiosis and defense.

4. Conclusions

This study is the first to successfully construct high quality cDNA library using RNA derived from coralloid roots of *C. debaoensis*. We have obtained 2984 unigenes, including 641 sequences with no nucleotide similarity with public databases. These sequences are an important addition to existing databases. We have identified highly expressed genes (mainly stress-responsive genes and antimicrobial genes). A total number of 94 SSR loci were detected, of which 20 loci were successfully amplified in *C. debaoensis*. These SSR markers can be used for various population and conservation genetics studies of *C. debaoensis* and other cycads. The cDNA library also provides an excellent resource for discovering genes involved in signaling in symbiosis and defense. A total of 22 ESTs that encoded putative receptor-like protein kinase (RLK) genes were identified.

Author contributions

Conceived and designed the experiments: Yunhua Wang, Nan Li, Ting Chen; Performed the experiments: Yunhua Wang, Ting Chen; Analyzed the data: Yunhua Wang, Yiqing Gong; Wrote the paper: Yunhua Wang.

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