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Molecular mechanism of inbreeding effects based on RNA-Seq analysis of the adductor muscle of bay scallop (*Argopecten irradians*)

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Abstract

Understanding the effects of inbreeding is critical for the long-term viability of shellfish breeding and for understanding inbreeding depression (ID) of the offspring of selfed individuals and full-sib crosses. The bay scallop (*Argopecten irradians*) is a simultaneous hermaphroditic marine bivalve with distinct male and female gonads and is a species with external fertilization. It is a canonical model invertebrate for analysis of the genetics of ID and evolution of outcrossing mating system. ID in bivalves is well documented with regard to the phenotype and molecular markers aspects, but little is known on how inbreeding affects genome-wide gene expression. Here, we compare the gene expression profiles of adductor muscle of *A. irradians* lines inbred to F=0.5 with those of its counterpart outbred lines which are originated from the same paternal individual. Inbred and outbred individuals have some differences in growth traits. The shell length and the total weight of inbred scallops are lower than the counterpart of outbred lines. We found that 1 175 genes were up-regulated while 1 390 were down-regulated in inbred lines. Regarding components, differentially expressed genes (DEGs) were concentrated on sarcomeres, contractile filaments, and cell membranes. Among others, whereas regarding their function, the Gene Ontology (GO) was concentrated on transferase, phosphotransferase, peptidase, and others. The DEGs-enriched metabolic pathways mainly belonged to the oxidative phosphorylation pathway and apoptosis pathway. We used another two families to verify our bioinformatic results. The selfed and the outbred family originated from different ancestors, but the selfed family showed obvious ID. By using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), we verified that DEGs with inbreeding are involved in the oxidative phosphorylation and cell apoptosis pathways, suggesting that inbreeding may affect energy metabolism and homeostasis.

Key words: *Argopecten irradians*, inbreeding depression, differentially expressed genes, transcriptome

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1 Introduction

The bay scallop (*Argopecten irradians*) was initially introduced to China in 1982 from the USA and has since become one of the most important mariculture mollusks in China. Male and female gonads develop simultaneously, and external fertilization is usually exerted as one of three breeding strategies: outbreeding, inbreeding, and selfing. This species is a compelling model to study inbreeding depression, as several problems in the bay scallop breeding industry have emerged, including inbreeding depression and decline of commercial traits, probably caused by a founder effect, self-fertilization, or breeding mismanagement.

Inbreeding depression (ID), which manifests as reduced survival and fertility of offspring of related individuals, is also pervasive in natural populations. It is characterized by an increase in homozygosity of expressed deleterious alleles or a reduction in heterozygosity (Charlesworth and Hughes, 1999). Theoretical and empirical studies suggest that the level of ID is specific to environments and breeding lines (Bijlsma et al., 1999; Fowler and Whitlock, 1999a, b; Reed et al., 2002; Kristensen et al., 2003; Vermeulen and Bijlsma, 2004a, b) and depends on the inbreeding coefficients. Molecular and biochemical alterations caused by inbreeding may be due to specific changes in single proteins or

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pathways and regulatory systems (Birchler et al., 2005). Empirical studies on the effects of fixation of deleterious mutations on protein instability and disturbance of processes related to the protein quality control system have the potential to reveal causative explanations for biochemical changes induced by inbreeding (Sangster et al., 2004; DePristo et al., 2005).

The effects of inbreeding have become more apparent with the attempts of shellfish breeders to develop selected lines from hatcheries with small effective population sizes (Newkirk and Haley, 1983; Hedgecock and Sly, 1990; Saavedra, 1997). However, studies of ID in shellfish are scarce and are somewhat inconsistent regarding their conclusions on dioecism (Longwell and Stiles, 1973; Mallet and Haley, 1983); thus, *A. irradians* is an intriguing model species to study the effects of ID.

Here, we provide a new approach to unveil genes that are differentially expressed between inbred and outbred lines. We selected significant differentially expressed genes (DEGs) and classified them in order to investigate several critical pathways and to subsequently verify differential gene expression by using a qRT-PCR approach in large inbred families. On the basis of the obtained results we conclude that inbreeding leads to differential expression of a considerable number of genes involved in oxidative phosphorylation and cell apoptosis.

2 Materials and methods

2.1 *Study animals and tissue collection*

Adult outbred and inbred bay scallop families were Zhongkehong bay scallop groups, obtained from the Jiaonan Breeding Base, Qingdao, and originated from the same paternal individual. Fifty scallops were assigned to each group. The average length of outbred family scallops is (61.08±1.49) mm, the average weight is (39.62±1.45) g, whereas the average shell length of the inbred family scallops is (55.73±2.45) mm, with an average total weight of (35.12±2.23) g. Before the experiment, the animals were kept in an indoor pool at (18±8)°C for one week to allow for adaption to the laboratory environment. Seawater was changed daily. For tissue sampling, the adductor muscle of three scallops of each family was collected. The respective muscle samples from each family were mixed and subsequently stored in liquid nitrogen until RNA extraction.

2.2 *cDNA library preparation and Illumina sequencing*

The muscle samples of the inbred and outbred families were delivered to the Beijing Genomics Institute (BGI). Total RNA was extracted (Invitrogen), and RNA was purified using Oligo(dT) magnetic beads to remove rRNA. The remaining mRNA was sheared to 200 bp fragments, and transcribed to cDNA. After this, sequencing adaptors were ligated to the fragments. The target fragments were isolated by agarose gel electrophoresis and enriched in a PCR amplification. The library products were sequenced using an Illumina HiSeq$^{\text{TM}}$ 2000 kit, and resulting sequences were and aligned to the transcriptome of bay scallops (unpublished data).

2.3 *Bioinformatic data processing and analysis*

Raw data were quality-filtered before data analysis, as raw sequencing data typically includes reads with adaptors with unknown bases to more than 10%, and low quality reads. The bay scallop transcriptome was used as an alignment reference. The bioinformatic analysis pipeline of the RNA-Seq procedure is presented in Fig. 1.

2.4 *Data quality assessment*

The assembled sequences included reads with high similarity such as alternative splicing isoforms, which caused redundancy or over-representation of the same gene. Cluster database at high identity with tolerance (CD-HIT) was used for clustering the assembly with a minimum similarity cut-off of 95%. The TIGR gene indices clustering tools (TGICL) and the Contig Assembly Program (CAP3) (Huang and Madan, 1999) were used for further clustering and removal of redundant reads, based on terminal region matching for at least 40 bp and 94% identity. A recently released de-novo assembler, Trinity, was evaluated with a k-mer size of 25. Two methods were used to examine the integrity and assembly accuracy of the contigs. First, 14 560 *C. virginica* ex-

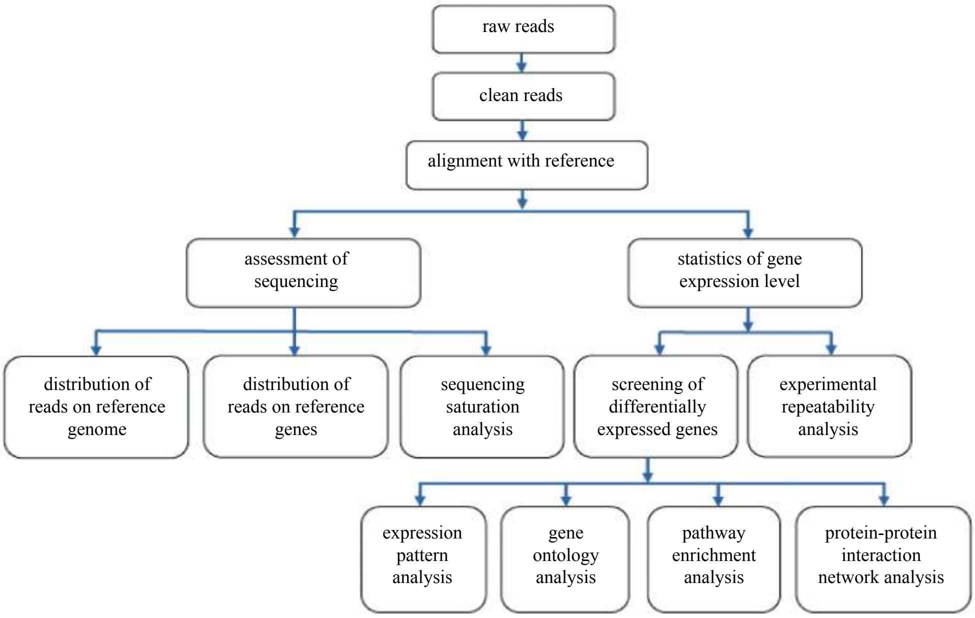


Fig. 1. The pipeline of bioinformatics analysis.

pressed sequence tags (ESTs) were downloaded from the NCBI database and aligned against our assembly using the package BLAT with default parameters. Second, raw reads were mapped against the *A. irradians* transcriptome.

2.5 *Identification and annotation of DEGs*

The gene expression level was calculated using the Reads per kb per Million reads (RPKM) method, according to the following formula:

$$\mathrm{RFKM}\,(\mathrm{A}) = \frac{10^9 C}{NL},$$

where RPKM(A) is the expression of gene A, C is the number of reads that uniquely align to gene A, N is the total number of reads that uniquely align to all genes, and L is the number of bases in gene A. The RPKM method is able to eliminate the influence of different gene lengths and sequencing discrepancy for the calculation of gene expression. Therefore, the calculated gene expression can directly be used for comparing differences of gene expression among samples. If more than one transcript existed for any one gene, the longest transcript was used to calculate the expression level and coverage. We identified the most DEGs ($\mathrm{FDR} \leqslant 0.001$, $\mathrm{RPKM} \geqslant 5$ and $\log_2(\text{expression fold change}) \geqslant 1$) and grouped and annotated them based on the biological process ontology according to the GO database.

2.6 *Gene Ontology (GO) analysis*

Gene Ontology is an international standardized gene function classification system that offers a dynamically updated and controlled vocabulary, and a strictly defined concept to comprehensively describe properties of genes and their products in any organism. GO has three ontologies: molecular function, cellular component, and biological process. Firstly, it maps all DEGs to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for every term, and then used hypergeometric tests to find significantly enriched GO terms in DEGs compared to the genome background.

2.7 *Primers designed for real-time PCR*

We used a primer design software for primers for almost all the genes in the two pathways for real-time PCR, using β-actin as an internal control to identify expression levels. Primer sequences, respective melting temperatures and expected product sizes are shown in Table 1.

2.8 *Verification of DEGs by qRT-PCR*

We used another two families to verify our bioinformatic results. The selfed and the outbred family originated from different ancestors, but the selfed family showed obvious ID. The average length of the outbred family is (60.99±3.21) mm, the average weight is (37.8±2.11) g, whereas the average length of inbred family is (53.73±1.51) mm with weight of (33.2±1.09) g. These scallops were also cultured for a week to allow for adaption to the environment. Three adductor muscle samples from each family were mixed and stored at –80°C until extraction of total RNA using a commercially available RNA Extraction Kit (Feijie, Shanghai). RNA was transcribed to cDNA using the PrimerScript RT reagent kit (Takara, Japan). We amplified housekeeping gene β-actin to assess cDNA quality. Primers were designed using Primer Premier 5 software. SYBR Green I was used as a fluorescence dye, and PCRs were performed according to the $Premix^{EX}$ Taq^{TM} protocol (Takara, Japan). Details on the selected DEGs are shown in Table 1. Again, β-actin was used as an internal control. The ΔCt for each sample was subtracted from the ΔCt of the calibrator, and the respective expression levels of the target genes were calculated by $2^{-\Delta\mathrm{Ct}}$. The magnitude of differences between the selfed and the outbred family was determined by $2^{-\Delta\mathrm{Ct(selfed)}}/2^{-\Delta\mathrm{Ct(outbred)}}$.

3 Results

3.1 *Alignment results*

The reads of two samples were mapped to the transcriptome (Table 2), showing more total mapped reads in the outbred sample than in the inbred sample. The percentages of clean reads reflecting the sequencing quality were all above 99% in each of the two samples.

3.2 *DEGs*

Compared to the outbred sample, 1 175 genes were up-regulated, and 1 390 genes down-regulated in all the DEGs ($\mathrm{FDR} \leqslant 0.001$ and $\log_2(\text{expression fold change}) \geqslant 1$) in inbred samples.

3.3 *GO*

Regarding components, DEGs were concentrated on sarcomeres, contractile filaments, and cell membranes. Among others, whereas regarding their function, the GO was concentrated on transferase, phosphotransferase, peptidase, and others.

3.4 *Pathway enrichment analysis of DEGs*

While ID significantly up-regulated all genes in the apoptosis pathway and down-regulated all genes in the oxidative phosphorylation pathway (for detailed information on these genes see Table 3).

More stringent criteria with smaller FDR and a greater value for the magnitude of change can be used to identify DEGs. The most DEGs (FDR=0 or RPKM=0) belonged to four categories: muscle-related, cell apoptosis, oxidative phosphorylation, and protein metabolism.

The DEGs-enriched metabolic pathways mainly belonged to the oxidative phosphorylation pathway, apoptosis pathway. Among them, the genes of the oxidative phosphorylation pathway were down-regulated in the inbred family. These genes include NADH oxidordeuctase, iron-sulfur protein, coenzyme Q, cytochrome oxidordeuctase, and ATP synthetase. The expression of apoptosis pathway-related genes was up-regulated in the inbred family, including the following genes: tumor necrosis factor, cytochrome C, IKK protein, complement C3, IAP, caspase 9, caspase-recruited protein, NF-kappa-B, HSP70, HSP90, TNFR, TRAF, P53, bak, and bcl-2. Environmental stress-related genes were up-regulated, include metastasis associated protein (MTA), cathepsin, Ras family GTP-binding protein, HSP70, non-selenium glutathione peroxidase, glutathione synthetase, Δ9 desaturase, D-LDH, citroyl synthetase, and eIF-2B (Table 3).

3.5 *DEGs related to ID*

We selected DEGs with FDR expression level from outbred and selfed transcriptome indicated that most DEGs were mainly in genes related to immune defense, detoxification, oxidative phosphorylation, and metabolism (Fig. 2).

We used real-time PCR to verify most of the DEGs. The results showed that DEGs in the oxidative phosphorylation pathway were down-regulated in the inbred family compared to the outbred family, whereas DEGs in the apoptosis pathway were up-

Table 1. Primers designed for DEGs

Gene ID	Sense primer	Temperature /°C	Anti-sense primer	Temperature /°C	Product length/bp
OE12_c42585	GGGCGGCCTTTATGTCTTGAGAGT	61.2	ATAACCTGAGCTAGAGCACGAGCC	60.6	120
OE12_c619	CCGAGGACCTGACTACCACCAT	59.2	GCGATTTGCCTGCGAGCCTAA	59.9	171
OE12_c717	AGTCTGTCCCAGTGCCACTTGT	59.8	TGGACGGACCGAAGATGAACGA	59.7	121
OE12_c51310	ATTCGGTTGGTGGTGAGGGTGT	60.1	ACGAACTTGCCTAGCCGCTAAA	58.7	118
OE12_lrc7882	TCCGATGACCACAAGCCAATCC	59.0	TTCCCGAGGGCAAGAGCACAA	60.1	170
OE12_c4853	CACGGATCACTGAGGCCAAAGC	60.1	TGCAGTCCAGCGACTTCCTGAA	60.2	161
OE12_c88938	TGGTGCGTTGGTCTACGTTGG	59.1	ACCTCAGAGCTAGGAAGCCACA	58.8	167
OE12_c73786	GTGGCAGCATCTTCAAACAGTGGT	60.6	ATCAAGGCAGAGATGGCTGACGAA	60.7	162
OE12_c60975	TGCCCAAACTCCACAACGAAAGC	60.8	GCGAGCACGAATGGACTAAGAAGG	60.3	167
OE12_c903	TGCATCATGCCAGCAAATCCAAAG	59.4	TGCCTGCCTGTATCTACATGGGAG	60.3	178
OE12_c13385	CAGGGCTTACACACGGCAATGGT	61.9	TCGGCGTTGGTTCTTCTGACATCT	60.9	170
OE12_c36933	ATTCTGCTAAGTGGCTCCTGACCT	59.9	GTACTGTAGGGATCGCATCATCGT	58.6	140
OE12_c12896	AGCTGCATACCGGCGTATAGAT	57.5	GCAGTGGTATCAACGCAGAGTG	58.0	122
OE12_c41307	GCACCTGAGCCACCAGCTACTA	60.1	CACTTCCCGACGCTTCCATTCC	59.9	210
OE12_c19514	CGATCCCGTGGCGTTACAAACA	59.8	CATAGCATTGCGTCTCCTACCTGT	59.0	176
OE12_c8292	ACAGCGACAATAGTCACCGTTTCC	59.9	GCCAAGAGCACAGAGCAGAAAGT	59.8	119
OE12_c20835	AGTTTCTGGCTCTCTTCCTCTGGT	59.5	AACCGTCGAGGCCATCAATAAGG	59.2	102
OE12_lrc1678	ACATCCCTCTCCTTCTCCACTTGG	59.9	CGAAGCGGTGACGGTTCATTCAA	60.4	164
OE12_c28339	ATCCTGCTCCCACTCATCTCGTT	59.6	GGTCCACCAACACAAGAACCAAGA	59.7	173
OE12_c62971	GCCTCGCTTCATTCACTGCTGA	59.6	TGGTTGTTGACGACTGGCTGTT	59.0	119
OE12_c6464	AAGCATTCGGTCCACGTTGAGG	59.5	GCCGACGAACGCATCTTCACA	59.8	115
OE12_c15870	AAGCAGTGGTATCAACGCAGAGTG	59.9	TAACCTTGAGCCCGACGACATGA	60.1	130
OE12_c17838	CTGTTCAGCAGCCTTCCTGAGTTG	60.4	CTTATGCCATTGACCGTCGCTCTC	60.4	156
OE12_c41346	TCCCTCAGATGTTTCTCCATGTGG	58.6	CCAGGTTGTAGTTGGACTGATGCT	59.1	131
OE12_c5732	TCCTGCACATACGTTGGCATCTCT	60.7	ACGCAAGACCTCTGTGACACCTTC	61.3	140
OE12_c39693	AGCAGAATGGTTGGGAACTGGTGA	60.8	GGCTGGAACTCCGATGGGAAATCT	61.0	158
OE12_c16299	TCCTCTTCCTTGATGTCTGCCTGT	59.8	CGAGAGCCAGCAACGAAGTGTT	59.8	127
OE12_c24505	CGATGTGCTGGACACGAGTTGGTA	61.4	TCGGCTTCTCAGTGCTGGACTAAC	60.9	125
OE12_c42694	AAGCAGTGGTATCAACGCAGAGTG	59.9	GCTGCTGTGGTATTGCCGTATCT	59.5	177
OE12_c4763	CTTGCCGATGCTCCTTGAAGTGTC	60.7	GAGTTGGGATGACGCAGTTGGTTG	60.9	116
OE12_c21514	AGAAAGGAAATGCTGAGCCGATGG	60.0	GCTGTAGTGGTGCTGGATGTTGTC	60.4	177
OE12_c45274	ATGACTCGCTTGCCTGAAGTTGTT	59.8	GTAAATAGTTGCCCAGCAGCCGAT	60.0	158
OE12_c41794	TGGCGTACAAGGCATCCTCTCT	59.5	AGTGTCCTGTGGGCATGGGATT	59.9	200
OE12_c16794	GCTCACAGACGGATTTGCGACAT	60.2	TACATGCGTGCTCGCTGTACAAG	60.0	121
OE12_c23581	GGAAGCAATGAACCCTGGTGATGT	59.9	TTCTGCTGGTCTGCCTGTGTCT	59.9	114
OE12_c20393	AAGCAGATCAGGTGAAGCGATTGG	60.0	TCCTGCGTTGCGTTCTGACATT	59.4	189
OE12_c20598	GCGAGTCCCTTGCTTTGGAAGT	59.4	GGATGGAGGTGGAAGGTCCGAT	59.5	119
OE12_c33179	TTGCAGCCATTGCCGCTTTAGG	60.6	AGCAGTGGTTCAACGCAGAGTG	59.7	207
OE12_lrc743	CCTGCTACCACCTGAGACCTGT	59.4	AGCGGGAAGATGGCGATGGT	59.7	161
OE12_lrc780	GGTGATTCAGGTTACCAGCAGTTC	58.2	AGGCTACATCAAGATGGCAAGGA	57.9	104
OE12_c49950	GTCCGTCACTGGAGCCATTCATTC	60.3	GCGTCAACAACATGAGCAGGGT	60.0	165
OE12_c26486	ATGGCGAGCAGTGCTGAATCCT	60.6	CGTGCAGTTGTTGTAGCTGAGTGT	60.3	181
OE12_c11964	ACGAGTCTGCGTGGAAATGTAACC	59.9	ACAGTCGGGCAAGGATGGCTAA	60.0	190
OE12_c46996	AAGGTGTTGGCCCGGTATTTAGAC	59.4	CAGTGCTGGACCCGTCAAAGTT	59.4	172
OE12_c18444	CAAGGCTGGAATCGAGACAACAGT	59.6	CAGGGCAAGAGCATGACTACCATT	59.4	145
OE12_lrc45825	GACAGGAGTCAAGGCGGTAGTAGT	59.9	GCGGTGAAAGGCAGACGATTCTT	60.1	195
OE12_c2323	AGAGCTGTAATGCAGTGCAGGAAG	59.7	ACGCTGGATGTCGGACTTGTCT	60.0	125
OE12_c4685	AAGCAGTGGTATCAACGCAGAGTG	59.9	ACCAAGACAGGTTCCAGCATGAC	59.2	162
OE12-c9897	TGCCAACCGTTAGCTTGAAGACTC	59.9	ATGTCTTTGGGCTGGTCATGGAGT	60.6	171
β-actin	CCCTCTATGCCTCTGGTCGT	64.0	TTCTCTCTCGGCTGTGGTTG	62.0	189

regulated in the inbred family compared to the outbred family (Figs 3 and 4). The qRT-PCR results were consistent with the profile data of differential gene expression.

4 Discussion

Inbreeding depression was first documented in plants (Darwin, 1876) and has become a major concern for evolution, ecology, and conservation biology. The mechanisms of ID are very complex and cannot be generalized. For this study, we chose to investigate gene expression profiles of a substantial trait in an economically important bivalve, the adductor muscle of the bay scallop in order to identify DEGs between selfed and outbred

Table 2. Alignment statistics of outbred and inbred samples

Map to gene	Outbred sample		Inbred sample	
	Number of reads	Percentage/%	Number of reads	Percentage/%
Total reads	12 265 303	100.00	12 534 881	100.00
Total base pairs	600 999 847	100.00	614 209 169	100.00
Total mapped reads	8 333 226	67.94	7 872 480	62.80
Perfect match	6 794 460	55.40	6 440 841	51.38
⩽2 bp mismatch	1 538 766	12.55	1 431 639	11.42
Unique match	2 390 541	19.49	2 685 246	21.42
Multi-position match	5 942 685	48.45	5 187 234	41.38
Total unmapped reads	3 932 077	32.06	4 662 401	37.20

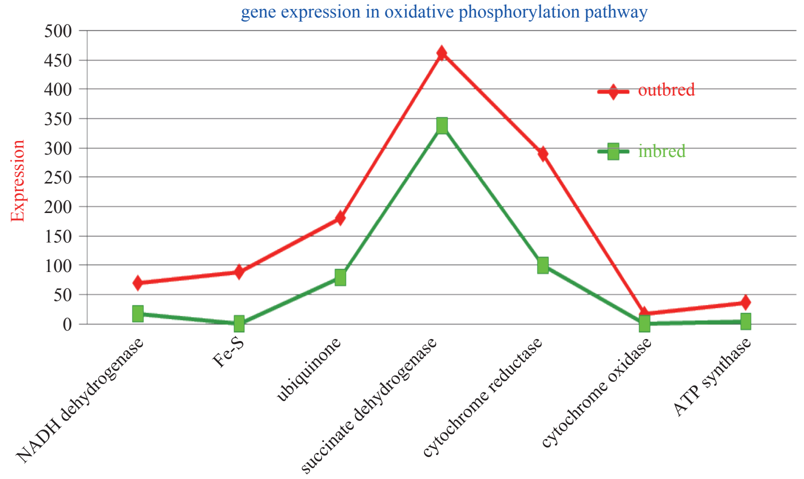
Table 3. Map of differentially expressed genes between inbred and outbred families

Gene name	Gene ID	Gene length/bp	Expression		RPKM		$\log_2$(inbred /outbred)	Inbred /outbred	*P* value	FDR
			Outbred	Inbred	Outbred	Inbred				
NADH dehydrogenase	OE12_c42585	680	18	2	11.07	1.095	–3.337 642 1	down	7.86×10^{-5}	0.000 98
Ubiquinone	OE12_c619	619	69	17	46.63	10.23	–2.188 778 7	down	0.213 54	1.03×10^{-8}
	OE12_c717	1 091	180	79	69.02	26.97	–1.355 789 5	down	0.336 54	1.74×10^{-11}
	OE12_c51310	364	157	41	180.4	41.95	–2.104 785 9	down	0.385 71	1.50×10^{-18}
Iron-sulfur protein	OE12_lrc7882	899	88	0	40.95	0.001	–15.321 486	down	0	1.94×10^{-27}
Cytochrome reductase	OE12_c4853	554	16	0	12.08	0.001	–13.560 489	down	0	9.17×10^{-5}
Cytochrome oxidase	OE12_c88938	463	289	99	261.1	79.63	–1.713 286 2	down	0.425 83	6.51×10^{-26}
ATP synthase	OE12_c73786	680	24	0	14.76	0.001	–13.849 803	down	0	3.52×10^{-7}
	OE12_c60975	708	36	4	21.27	2.104	–3.337 642 1	down	0.045 26	3.7×10^{-7}
Succinate dehydrogenase	OE12_c903	1 026	461	338	188	122.7	–0.615 460 6	down	0.443 62	5.84×10^{-8}
Tumor necrosis factor	OE12_c13385	1 100	4	35	1.521 1	11.849	2.961 565 9	up	0.686 95	0.000 5
	OE12_c36933	505	57	139	47.216	102.5	1.118 333 95	up	0.920 36	6.29×10^{-6}
Caspase recruitment domain	OE12_c12896	622	0	5	0.001	2.993 6	11.547 671 6	up	0.540 43	0.171 73
	OE12_c41307	1 068	0	19	0.001	6.625 2	12.693 745 9	up	0.580 85	9.74×10^{-5}
IAP	OE12_c19514	797	31	87	16.271	40.652	1.321 030 07	up	0.893 21	6.83×10^{-5}
	OE12_c8292	648	63	151	40.67	86.78	1.093 407 7	up	0.912 74	3.41×10^{-6}
HSP70	OE12_c20835	825	173	445	87.719	200.87	1.195 316 19	up	0.964 56	2.00×10^{-11}
HSP90	OE12_lrc1678	1 104	13	0	4.925 8	0.001	–12.266 146 7	down	5.28×10^{-5}	0.000 68
P53	OE12_c28339	562	38	87	28.285	57.65	1.027 298 87	up	0.912 74	0.001 78
NF-kappa-B	OE12_c62971	535	9	54	7.037 1	37.589	2.417 245 39	up	0.891 28	3.23×10^{-5}
Caspase 9	OE12_c6464	862	33	59	16.014	25.489	0.670 531 82	up	0.856 87	0.133 94
Caspase 3 like protein	OE12_c15870	453	30	43	27.703	35.35	0.351 657 05	up	0.891 28	0.593 48
JNK	OE12_c17838	656	29	113	18.493	64.149	1.794 480 86	up	0.912 74	1.71×10^{-9}
TNFR associate factor	OE12_c41346	756	10	30	5.533 3	14.778	1.417 245 39	up	0.727 05	0.031 78
	OE12_c5732	1 061	157	412	61.9	144.61	1.224 162 67	up	0.942 83	2.16×10^{-12}
Fas	OE12_c39693	490	35	92	29.88	69.921	1.226 561 83	up	0.912 74	0.000 12
	OE12_c16299	1 789	318	559	74.357	116.36	0.646 104 41	up	0.935 25	3.22×10^{-9}
	OE12_c24505	875	13	38	6.215	16.173	1.379 770 68	up	0.760 55	0.014 17
	OE12_c42694	503	8	23	6.653 1	17.028	1.355 844 84	up	0.775 16	0.087 88
I-kappa-B kinase (IKK)	OE12_c4763	1 595	125	214	32.783	49.965	0.607 965 59	up	0.912 74	0.001 71
	OE12_c21514	918	125	436	56.96	176.87	1.634 682 93	up	0.959 18	4.49×10^{-12}
Apoptotic	OE12_c45274	595	7	16	4.921 4	10.014	1.024 927 97	up	0.627 97	0.335 05
	OE12_c41794	813	0	6	0.001	2.748 4	11.424 365 3	up	0.540 43	0.106 58
TNFR	OE12_c16794	841	11	65	5.471 4	28.783	2.395 219 08	up	0.864 59	9.46×10^{-6}
	OE12_c23581	624	106	277	71.06	165.31	1.218 104 6	up	0.951 68	0
TRAF	OE12_c20393	509	48	78	39.448	57.068	0.532 722 61	up	0.912 74	0.171 81
Complement C3	OE12_c20598	1 298	19	108	6.123 3	30.986	2.339 242 88	up	0.873 36	5.73×10^{-8}
Metastasis-associated protein	OE12_c33179	676	51	80	31.559	44.072	0.481 785 64	up	0.061 85	0.217
Cystatin A1	OE12_lrc743	1 770	231	355	54.594	74.691	0.452 209 06	up	0.912 74	0.002 06
Cathepsin L	OE12_lrc780	1 420	0	174	0.001	45.633	15.477 782 6	up	0	0
	OE12_c49950	500	30	215	25.099	160.13	2.673 585 14	up	1.14×10^{-12}	4.95×10^{-11}

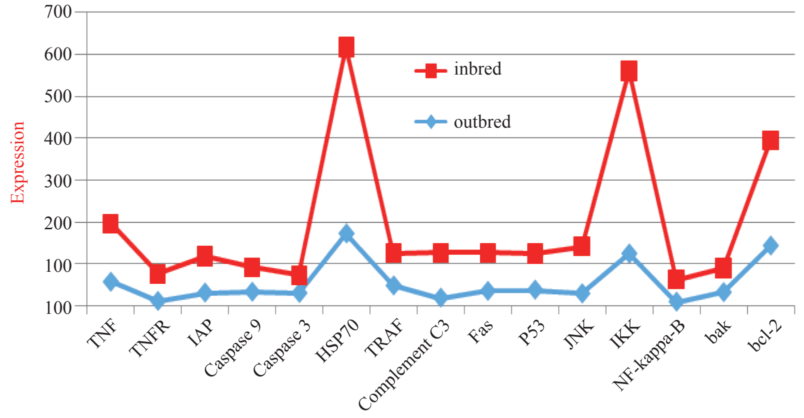
to be continued

Continued from Table 3

Gene name	Gene ID	Gene length/bp	Expression		RPKM		$\log_2$(inbred /outbred)	Inbred /outbred	*P* value	FDR
			Outbred	Inbred	Outbred	Inbred				
Non-selenium glutathione peroxidase	OE12_c26486	993	532	686	224.11	257.27	0.199 065 22	up	0.016 8	0.084 41
D-lactate dehydrogenase	OE12_c11964	1 184	235	410	83.027	128.96	0.635 246 04	up	4.73×10^{-8}	1.15×10^{-6}
Glutamine synthetase	OE12_c46996	568	8	31	5.891 8	20.325	1.786 479 2	up	0.807 89	0.006 47
Factor eIF-2B subunit beta	OE12_c18444	614	55	108	37.471	65.505	0.805 810 68	up	0.000 59	0.005 5
Mannosidase	OE12_lrc45825	962	0	27	0.001	10.452	13.351 508 7	up	0.628 53	8.94×10^{-7}
	OE12_c2323	1 054	18	91	7.143 9	32.153	2.170 152 53	up	0.873 36	1.10×10^{-7}
Proteasome	OE12_c4685	1 277	23	3	7.534 3	0.874 9	–3.106 316 57	down	0.011 2	0.000 21
Endonuclease	OE12_c44952	705	12	68	7.120 3	35.92	2.334 783 23	up	0.891 28	5.2×10^{-6}
Bcl-2 like protein 1	OE12_c4923	1 416	144	251	42.541	66.013	0.633 901 44	up	0.912 74	0.000 3
P63	OE12-c42012	569	28	65	20.585	42.542	1.047 295 78	up	0.900 31	0.008 18
Bak	OE12-c9897	924	32	58	14.5	23.38	0.690 263 9	up	0.842 54	0.123 98



DEGs in oxidative phosphorylation parthway



DEGs in apoptosis pathway

Fig. 2. Relative expression level of DEGs in two pathways. Genes on the oxidative phosphorylation pathway were down-regulated, and those of the apoptosis pathway were up-regulated in the selfed sample compared to the outbred sample.

lines to explain how inbreeding influences genotype and phenotype.

The number of DEGs found in this study is considerably large (for a comprehensive list of DEGs see Supplementary information Table S1), which indicates that inbreeding depression affects various processes of growth and development. Here, we present gene groups categorized by the most crucial pathways.

A serial of protein synthesis-related genes such as ribosomal protein, eIF3e, and HSP90 were down-regulated, and protein ubiquitination genes were up-regulated. The obvious result is that inbreeding manifested in lower protein levels for offspring survival and growth. The molecular chaperone HSP70 is an important indicator of environmental stress, and was also up-regulated in the selfed lines, which partly supports that aging, in-

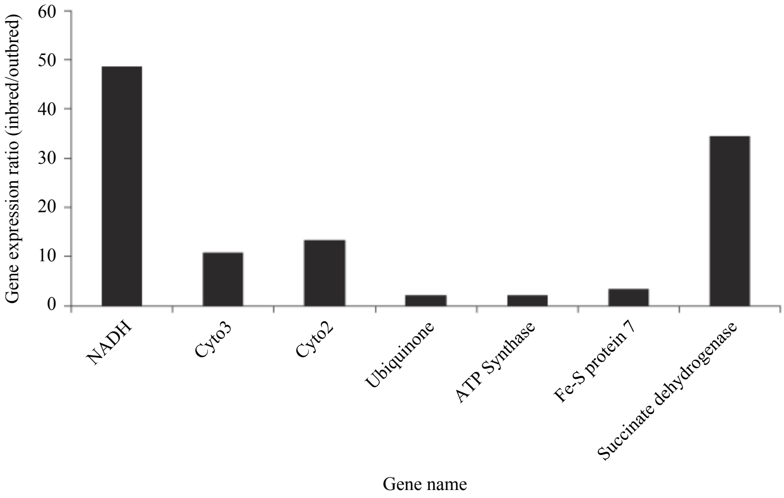


Fig. 3. The chart of the DEGs ratios (outbred/selfed) in the oxidative phosphorylation pathway. Vertical bars represent the mean±SD (*N*=3). β-actin gene expression is used as an internal control, the vertical bar indicate the gene expression ratio (inbred/outbred).

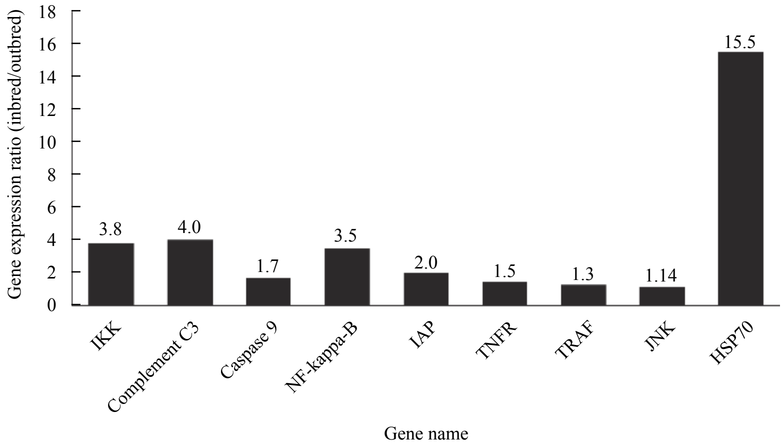


Fig. 4. The chart of the DEGs ratios (selfed/outbred) in the apoptosis pathway. Vertical bars represent the mean expression±SD (*N*=3). β-actin gene expression is used as an internal control, the vertical bar indicate the gene expression ratio (outbred/inbred).

breeding, and environmental stress may induce similar cellular responses (Kristensen et al., 2002, 2005; Pletcher et al., 2002).

The oxidative phosphorylation pathway is known as energy networking in mitochondria that produces ATP and NADH. The core components of this are five enzyme complexes and ATP synthetase. Apoptosis is a cellular response to a variety of stimuli in order to maintain organismic homeostasis. The process of apoptosis is controlled by a multitude of cell signals, which can be both extrinsic or intrinsic.

Internal stimuli such as oxidant- and ceramide-induced mitochondrial signals elicit the release of cytochrome c to bind to Apaf-1, ATP, and pro-caspase-9 to create an apoptosome that activates the effector caspase-3. Extrinsic signal transduction pathways include the TNF-induced and the Fas-Fas ligand-mediated signaling pathway. Our qRT-PCR and RNA-Seq results strongly indicate that inbreeding affects the network of chemical reactions for the formation of ATP, NADH, and building blocks for bio-synthesis. Thus, the inbred lines seem to maintain lower metabolic efficiency that may result in lower productivity and resistance.

Higher expression levels of genes of the apoptosis pathways indicate that inbred lines might be more susceptible to environmental stressors, thus inbred lines presumably activate cell death pathways more often in order to maintain homeostasis.

Inbreeding is a widespread phenomenon in nature, particularly in small populations. Self-compatibility and inbreeding depression raise various intriguing scientific questions. Therefore, investigations on the mechanisms of ID may contribute to the conservation of rare and endangered species and may help to support the development of healthy and resistant pure-bred lineages.

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Supplementary information:

Table S1. A comprehensive list of differentially expressed genes (DEGs).

The supplementary information is available online at https://doi.org/10.1007/s13131-019-1351-4 and www.hyxb.org.cn/aosen/ch/index.aspx. The supplementary information is published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.