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Linkage Disequilibrium in Wild and Cultured Populations of Pacific Oyster (*Crassostrea gigas*)

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Abstract Linkage disequilibrium (LD) can be applied for mapping the actual genes responsible for variation of economically important traits through association mapping. The feasibility and efficacy of association studies are strongly dependent on the extent of LD which determines the number and density of markers in the studied population, as well as the experimental design for an association analysis. In this study, we first characterized the extent of LD in a wild population and a cultured mass-selected line of Pacific oyster (*Crassostrea gigas*). A total of 88 wild and 96 cultured individuals were selected to assess the level of genome-wide LD with 53 microsatellites, respectively. For syntenic marker pairs, no significant association was observed in the wild population; however, three significant associations occurred in the cultured population, and the significant LD extended up to 12.7 cM, indicating that strong artificial selection is a key force for substantial increase of genome-wide LD in cultured population. The difference of LD between wild and cultured populations showed that association studies in Pacific oyster can be achieved with reasonable marker densities at a relatively low cost by choosing an association mapping population. Furthermore, the frequent occurrence of LD between non-syntenic loci and rare alleles encourages the joint application of linkage analysis and LD mapping when mapping genes in oyster. The information on the linkage disequilibrium in the cultured population is useful for future association mapping in oyster.

Key words *Crassostrea gigas*; linkage disequilibrium; association mapping; microsatellite; mass selection

1 Introduction

Linkage disequilibrium (LD), the non-random association of alleles at different loci in a population (Weir, 2008), has received considerable attention recently because of its usefulness in the fine-scale mapping through association (or LD) mapping in humans (Lander, 2011), crop (Baxter *et al*., 2010; Kump *et al*., 2011), and livestock (Karim *et al*., 2011; Georges, 2007). Conventional linkage analysis is based upon associations in well-characterized pedigrees with large progeny size. Conversely, LD mapping lies in associations within populations of unrelated individuals, and offers the ability to exploit historical and evolutionary recombinants at population level, allowing for increased mapping resolution. Furthermore, LD studies have the power to simultaneously evaluate the varying effects of many alleles (Farnir *et al*., 2000; Lu *et al*., 2010; Nordborg and Tavaré, 2002). Moreover, population-based LD mapping will accelerate the progress of mapping the actual genes responsible for the variation of important traits for species that traditional pedigree-based quantitative trait loci (QTL) mapping approaches are not feasible (Li and Merilä, 2010).

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The feasibility and efficacy of association studies are strongly dependent on the extent of LD which determines the number and density of markers, and experimental design needed to perform an association analysis in study population (Qanbari *et al*., 2009; Flint-Garcia *et al*., 2003). Long-range LD benefits genome-wide LD mapping because a small number of markers are required. In contrast, short-range LD requires much more markers to find associations, but necessitates the fine-scale mapping in association studies (Gray *et al*., 2009; Slate, 2005; Steiner *et al*., 2007). Demographic history (reproductive system, migration, population admixture and bottlenecks), genomic history (recombination and mutation) and selection are all the factors that influence the extent of LD between any two markers within a species (Brazauskas *et al*., 2011). Furthermore, the relationship between LD and physical or genetic distance is highly variable both within and between species (Yu and Buckler, 2006). Accordingly, in order to optimize the strategy for association mapping in the species under study, it is indispensable to investigate the information on possible differences in the extent of LD among different populations (Li and Merilä, 2011). So far, the level of LD has been extensively measured in human, plants, and domestic vertebrates (Conrad *et al*., 2006; Yu and Buckler, 2006; Barnaud *et al*., 2010; Du *et al*., 2007; Farnir *et al*., 2000). However, little is known about the level of LD in the populations of aquaculture

species. To date, only the extent of LD in rainbow trout has been characterized (Rexroad and Vallejo, 2009).

The Pacific oyster (*Crassostrea gigas*) has long received significant attention due to its high commercial value. Several selective breeding programs in oyster have been performed around the world, and major improvements have already been achieved through directional selections on phenotype for complex traits, such as growth rate (Appleyard and Ward, 2006; Li *et al*., 2011), body weight (Evans and Langdon, 2006), and survival (Dégremont *et al*., 2010). In order to speed up the process of genetic improvement of oyster, construction of linkage maps and QTL mapping have been extensively developed (Hubert and Hedgecock, 2004; Li and Guo, 2004; Sauvage *et al*., 2010; Guo *et al*., 2012). Despite the great effort, the dissection of the precise allelic variations underlying phenotypic diversity has not yet been achieved because of the lack of well-characterized pedigrees and numerous molecular markers. Population-based association analysis can obviously benefit the QTL mapping of highly heterozygous species. Association mapping holds promise as a strategy to implement marker-assisted selection of quantitative traits for efficient oyster breeding.

Many studies have showed that selection will lead to an increase in LD (Flint-Garcia *et al*., 2003; Gupta *et al*., 2005). The fact that strong artificial selection is a key force in substantial increase of genome-wide LD in domestic population has been documented in crops and livestock species (Mather *et al*., 2007; Lam *et al*., 2010; Gray *et al*., 2009; Du *et al*., 2007). In order to improve the productivity traits of *C. gigas*, a selective breeding program for faster growth was initiated in China. During the period of 2007–2010, a four successive generation selection for shell height in the base population was carried out to produce the fourth generation selected line (Li *et al*., 2011; Wang *et al*., 2012). So, unlike in the wild population, the level of LD might have changed during mass selection.

In this study, we provide the first characterization on the LD estimation and decay of *C. gigas* with microsatellite markers across the whole genome. The aims of this study were to (1) measure the level of LD in wild and cultured mass selection populations, respectively; (2) examine the impact of selective breeding on the level of LD and possible difference in the extent of LD between wild and cultured populations; and (3) evaluate the upcoming opportunities and conditions for association mapping in oyster. This would benefit the identification and analysis of the genes underlying phenotypic diversity in *C. gigas*.

2 Materials and Methods

2.1 Experimental Populations

One wild and one cultured mass-selected populations of Pacific oyster were used to evaluate the extent of LD between microsatellite markers. A total of 88 wild individuals were caught in Rushan (RS), Shandong Province, China (37.2°N, 122.1°E), while 96 cultured individuals were sampled in 2011 from the fourth generation of mass selection for fast growth of Pacific oyster (MS4). It was produced by four successive generation selection based on a wild population collected from Rushan during 2007–2010. The selection intensity ranged from 1.7 to 1.9 during successive generation selection. In every summer, 80 or 100 individuals were selected from the top end of shell height distribution of the former selection line and served as the parents. 75%–90% of them reached sexual maturity, and the female to male ratio varied between 0.8 and 1.2 during four generation selection. Fertilization was performed by stripping the gonad (Wang *et al*., 2012; Wang *et al*., 2013; Cong and Li, 2014). DNA was extracted from frozen adductor muscle tissue by a modified phenol-chloroform procedure described by Li *et al*. (2006), and stored at −30℃ prior to genetic analysis.

2.2 Genotyping

A total of 53 genomic microsatellites spanning most of Pacific oyster genome were selected from the male genetic map constructed by Hubert and Hedgecock (2004). M13 tailed-primer PCR amplification of microsatellites was performed according to Schuelke (2000) in a 10 μL reaction containing 50 ng of template DNA, 2.0 mmol L^{-1} of dNTP (each), 1×buffer, 1.0–1.5 mmol L^{-1} $MgCl_2$, 0.25 U *Taq* DNA polymerase (Takara), 1.0 μmol L^{-1} of reverse primer and universal fluorescent labeled M13 primer, and 0.25 μmol L^{-1} of M13 sequence tailed forward primer. Amplification was carried out for each microsatellite locus alone in a 96-well DNA thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems). Genotyping of microsatellite loci was performed on ABI 3130 automatic sequencer (Applied Biosystems), following PCR amplification with fluorescent dye-labeled primers. Data were analyzed using software GeneMapper® (Applied Biosystems).

2.3 Statistical Analysis

The total number of alleles, heterozygosity, rare allele with frequency < 1%, and *Fst* values were calculated with MICROSATELLITE ANALYZER software (Dieringer and Schlötterer, 2003). Allelic richness (A_R) as a standardized measure of the sample size was calculated with FSTAT version 2.9.3 (Goudet, 2001) (http://www.unil.ch/dee/page36921_en.html). A nonparametric analysis of variance (Mann-Whitney U test) was performed to test the difference between wild and cultured populations for A_R. Micro-Checker software (Ver. 2.2.3) was used to examine null alleles with the Oosterhout algorithm (Van-Oosterhout *et al*., 2004).

2.4 Linkage Disequilibrium Calculation

The program TASSEL (Bradbury *et al*., 2007) was used to measure the extent of LD as squared allele frequency correlation estimates (r^2), and the significance of r^2. All sites with a frequency < 1% for the rare allele were replaced by missing data to avoid biased estimation of LD. Significance of LD was determined by 1000 permutations

for each pair. LD decay was considered when it is above the threshold of $r^2 = 0.1$. LD extent was estimated separately for unlinked loci and for loci on the same linkage group. The genetic distance (cM) between marker pairs was based on the male map constructed by Hubert and Hedgecock (2004), which was used for calculation of LD decay.

3 Results

3.1 Population Diversity

Observed heterozygosity, expected heterozygosity, total number of alleles, and the number of rare alleles with frequency < 1% for the microsatellites genotyped in the RS and MS4 populations were shown in Table 1. The average number of alleles per microsatellite was 20.7 and 13.5 for the RS and MS4 populations, respectively. Averaged allelic richness was 17.3 and 11.4 for the RS and MS4 populations, respectively. Mann-Whitney U test demonstrated a significant difference between wild and selected populations ($P = 0$). Average observed heterozygosity was 0.53 and 0.55 for the RS and MS4, respectively. Average expected heterozygosity was respectively 0.87 and 0.83 for the RS and MS4. The ratio of rare alleles with frequency < 1% was 16.4% (179/1095) and 10.5% (75/714) for the RS and MS4, respectively. Multilocus *Fst* value was low but significant between the RS and MS4 (*Fst* = 0.0397, $P < 0.001$). Analyses using MICRO-CHECKER suggested the presence of null alleles at 29 out of 54 microsatellites with estimated null allele frequency ranged from 0.105 to 0.305. Nevertheless, simulation studies have shown that the presence of null alleles at microsatellite loci can be safely accommodated in estimating population differentiation (Chapuis and Estoup, 2007).

Table 1 Parameters for microsatellites genotyped in wild and cultured populations of *C. gigas*

Locus	[1]LG (position)	RS population ($n = 88$)					MS4 population ($n = 96$)				
		H_o	H_e	N	A_R	Nr	H_o	H_e	N	A_R	Nr
ucdCg153	1 (6.7)	0.41	0.92	16	14.4	2	0.21	0.84	13	10.5	3
ucdCg018	1 (8.0)	0.56	0.89	22	15.5	6	0.48	0.87	11	9.8	1
ucdCg188	1 (11.7)	0.85	0.92	20	28.3	5	0.92	0.88	15	14.2	0
ucdCg194	1 (11.7)	0.36	0.78	14	12.2	1	0.56	0.87	13	12	0
ucdCg146	1 (39.5)	0.11	0.11	2	2	0	0.3	0.26	3	2.7	0
ucdCg191	2 (0.0)	0.62	0.85	12	10.7	0	0.56	0.82	12	9.5	1
ucdCg006	2 (17.3)	0.69	0.92	20	15.3	5	0.71	0.89	14	12.8	3
ucdCg124	2 (55.0)	0.55	0.63	15	10.7	7	0.72	0.66	8	5.6	3
ucdCg157	2 (65.7)	0.66	0.96	42	30.1	9	0.71	0.93	22	17.3	3
ucdCg145	2 (84.9)	0.27	0.93	16	15.3	0	0.35	0.86	9	8.3	1
ucdCg150	3 (9.9)	0.52	0.94	23	19.4	4	0.36	0.88	13	10.6	1
ucdCg192	3 (31.9)	0.14	0.54	10	8.3	0	0.17	0.61	5	4.6	0
ucdCg195	3 (58.6)	0.82	0.9	14	12.2	2	0.78	0.87	11	10.8	0
ucdCg001	3 (59.8)	0.17	0.92	16	13.9	0	0.23	0.86	14	11.8	3
ucdCg148	3 (67.2)	0.85	0.96	29	23.6	4	0.74	0.91	18	13.6	6
ucdCg199	3 (87.0)	0.59	0.56	5	4.1	1	0.65	0.5	3	2.5	0
ucdCg160	3 (94.4)	0.58	0.87	20	16	3	0.7	0.85	16	12.3	3
ucdCg002	4 (0.0)	0.84	0.94	21	18.5	1	0.81	0.9	15	12.9	2
ucdCg176	4 (36.8)	0.62	0.6	6	4.8	1	0.67	0.68	5	4.7	0
ucdCg198	4 (38.0)	0.38	0.9	15	13.1	1	0.3	0.87	12	10.3	1
ucdCg120	5 (0.0)	0.68	0.75	13	9.4	5	0.65	0.7	7	7.1	0
ucdCg139	5 (30.4)	0.77	0.95	28	22	5	0.84	0.91	16	13.8	2
ucdCg117	5 (31.6)	0.79	0.96	27	23	2	0.95	0.93	22	17.7	2
ucdCg111	5 (43.8)	0.59	0.9	18	14.9	4	0.42	0.85	13	10.6	2
ucdCg119	5 (45.7)	0.53	0.94	21	16.7	5	0.39	0.89	14	12.3	1
ucdCg128	5 (46.0)	0.51	0.95	37	25.6	10	0.44	0.92	18	15.5	1
ucdCg147	5 (52.6)	0.19	0.93	18	16.7	1	0.31	0.9	15	13.9	0
ucdCg164	5 (66.5)	0.46	0.64	9	6	4	0.48	0.68	9	7.1	2
ucdCg014	6 (0.0)	0.68	0.95	27	21.8	6	0.68	0.91	18	14.9	2
ucdCg021	6 (4.2)	0.74	0.96	26	21.7	4	0.57	0.9	16	13.8	2
ucdCg130	6 (47.5)	0.54	0.86	10	19.1	2	0.7	0.86	10	12.4	1
ucdCg141	6 (63.7)	0.39	0.89	14	13	0	0.45	0.77	11	8.4	3
ucdCg133	7 (3.2)	0.61	0.94	20	18	1	0.8	0.9	18	15.4	1
ucdCg131	7 (8.9)	0.64	0.95	25	20.6	3	0.44	0.87	14	11.8	1
ucdCg197	7 (17.0)	0.37	0.96	28	23.8	1	0.42	0.9	18	14.5	3
ucdCg155	7 (18.3)	0.38	0.94	25	18.6	8	0.57	0.92	17	15.3	1
imbCg108	7 (37.3)	0.56	0.94	22	19.9	2	0.35	0.93	22	18.5	1
ucdCg024	8 (10.2)	0.27	0.91	20	22.1	0	0.51	0.88	15	13.1	0
ucdCg175	8 (11.8)	0.78	0.93	28	19.3	7	0.9	0.92	15	14.1	0
cmrCg141	8 (29.8)	0.53	0.95	28	22.4	6	0.44	0.89	17	14.1	2

(to be continued)

(continued)

Locus	[1]LG (position)	RS population (n=88)					MS4 population (n=96)				
		H_o	H_e	N	A_R	Nr	H_o	H_e	N	A_R	Nr
um2CgL16	8 (45.6)	0.24	0.93	17	15.6	1	0.39	0.85	12	10.2	1
cmrCg003	8 (48.6)	0.45	0.95	25	21.6	2	0.41	0.85	13	12.5	2
ucdCg149	8 (49.4)	0.71	0.96	29	23.1	4	0.44	0.8	15	12.1	2
ucdCg022	8 (52.3)	0.25	0.94	25	19	5	0.34	0.86	14	12.1	2
ucdCg166	9 (0.0)	0.46	0.95	27	21.7	4	0.49	0.9	17	13.7	0
ucdCg183	9 (39.6)	0.25	0.96	25	22.3	1	0.52	0.9	20	15.9	4
ucdCg184	9 (44.3)	0.45	0.95	29	21.6	9	0.37	0.91	15	13.2	1
ucdCg171	10 (9.9)	0.85	0.88	13	10.6	3	0.81	0.78	8	7.4	0
ucdCg172	10 (16.2)	0.51	0.56	5	3.8	1	0.59	0.51	3	2.5	0
ucdCg140	10 (24.4)	0.53	0.93	19	18.2	0	0.46	0.86	12	11	0
um2CgL10	10 (29.5)	0.88	0.96	33	33.2	6	0.98	0.92	18	16.1	0
ucdCg189	10 (31.6)	0.44	0.95	33	24.4	6	0.32	0.83	14	10.3	4
Total				1095		179			714		75
Average		0.53	0.87	20.7	17.3	3.4	0.55	0.83	13.5	11.4	1.4

Notes: [1] Linkage groups with the position of microsatellite locus in Kosambi cM as shown in male linkage map constructed by Hubert and Hedgecock (2004); H_o, observed heterozygosity; H_e, expected heterozygosity; N, number of alleles; A_R, allelic richness; Nr, number of rare alleles; n, sample size.

3.2 Linkage Disequilibrium

The extent of LD was estimated for syntenic marker pairs in both RS and MS4 populations. For RS population, the distance of LD decayed rapidly and no significant LD ($r^2 > 0.1$) was observed. For MS4, the observed LD extended to a relatively long distance and three $r^2 > 0.1$ extended to 1.2, 5.7, and 12.7 cM, respectively (Fig.1). In addition, the average r^2 between syntenic marker pairs in RS and MS4 was 0.0097 and 0.021, respectively (Table 2).

The level of LD was measured for nonsyntenic loci in both populations of *C. gigas*. Eleven and 12 LDs were respectively found for RS and MS4 populations. The average r^2 value between nonsyntenic loci for RS and MS4 was 0.0073 and 0.016, respectively (Table 2). Frequency distribution of r^2 with values > 0.01 observed between nonsyntenic marker pairs for both populations of *C. gigas* is shown in Fig.2. By comparison to RS population, r^2 value ($r^2 > 0.01$) in MS4 population was higher in most frequency groups.

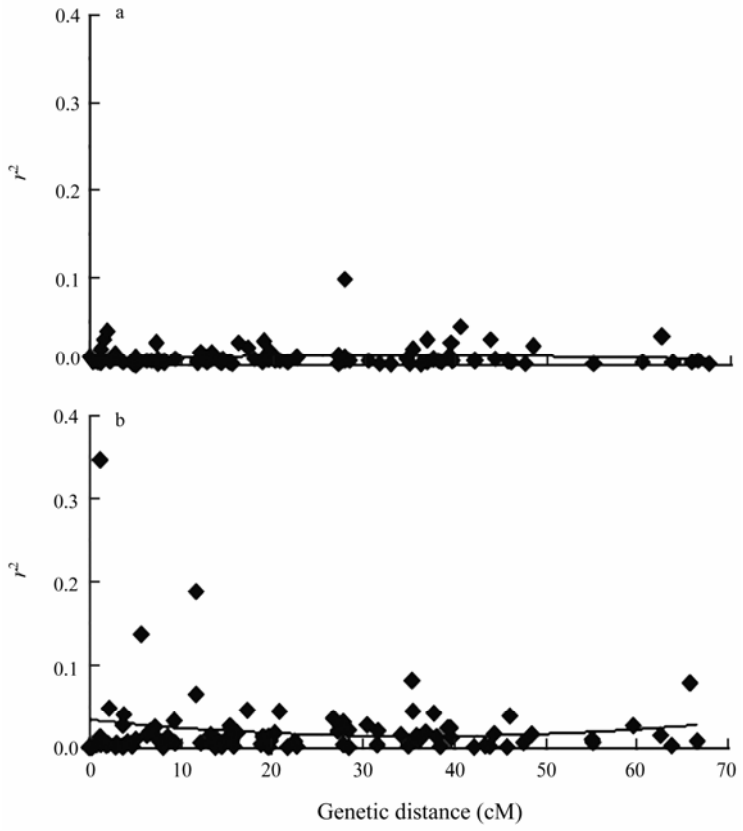

Fig.1 Decline of linkage disequilibrium (r^2) with genetic distance for *C. gigas* in a wild population (RS; n=88) (a), and a cultured population (MS4; n=96) (b). The logarithmic trend line represents the LD decay.

Table 2 Parameters of marker pairs with r^2 values for wild and cultured populations of *C. gigas*

Population	Marker pairs with r^2 values	Total number	Average r^2 value	Number of LD
RS	Marker pairs	88	0.0097	0
	Non-Syntenic marker pairs	1033	0.0073	11
MS4	Syntenic marker pairs	94	0.021	3
	Non-Syntenic marker pairs	984	0.016	12

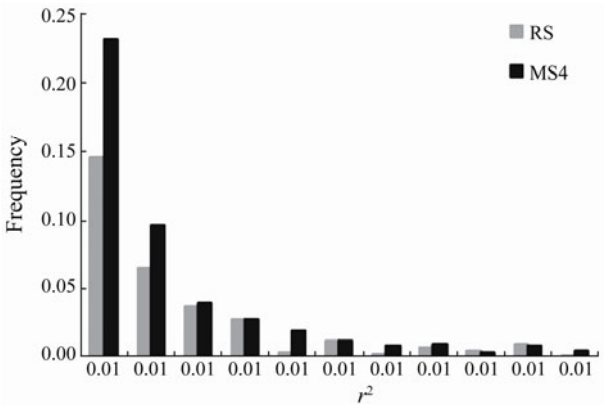

Fig.2 Frequency distribution of r^2 values observed for all nonsyntenic marker pairs in a wild and a cultured population of *C. gigas*.

4 Discussion

Allelic diversity and heterozygosity are both estimated with highly ploymorphic microsatellite markers as measures of genetic variation. Reduced allele diversity was observed for MS4 population relative to RS population. However, no change in heterozygosities was observed between two populations. Such scenario might result from successively artificial selection which led to the loss of low-frequency alleles in selectively bred population. Rare alleles contribute minimal effect to heterozygosity (Allendorf, 1986). These findings are consistent with the reported previously for Pacific oyster (Appleyard and Ward, 2006).

Genome-wide search for LD was performed on wild and mass-selected Pacific oyster populations. The Pacific oyster is an outcrossing heterozygote species with high mutation rate. Sauvage *et al*. (2007) reported an average density of single nucleotide polymorphisms (SNPs) for Pacific oyster, one per 60 bp in coding regions and one per 40 bp in non-coding regions. Both outcrossing and high mutation rate will lead to a decrease in LD (Gupta *et al*., 2005). Therefore, wild oyster population has a low level of LD. However, selection will reduce the number of alleles in selected population, thus enhancing LD of neighboring loci. Moreover, selection may result in LD between unlinked loci (epistasis) despite the fact that the loci are not physically linked (Flint-Garcia *et al*., 2003). With consistently strong artificial selection practiced in this species, the extent of LD has been extensively inflated in MS4 population across the whole genome.

The pattern of LD decay determines the marker density required and the level of resolution that may be obtained in an association study (Brazauskas *et al*., 2011). Wild oyster population has low levels of LD, for which the association approach will require the numerous alleles available but will get closer to the gene of interest. However, in selectively bred population, it might be feasible to identify chromosome segments under selection by identifying regions with higher LD, and testing interaction between chromosomes on the extent of LD with small number of markers (Du *et al*., 2007). This population-specific difference in LD measured in this study could be exploited for genetic mapping by association studies (Rafalski and Morgante, 2004). A two-stage association mapping approach can be applied to *C. gigas* with reasonable marker densities at a relatively low cost by choosing an association mapping population. An initial genome-wide mapping could be performed with a low density molecular markers in a cultured oyter population, following which a high resolution mapping could be conducted with high density molecular markers in a wild oyster population (Rafalski and Morgante, 2004; Georges, 2007).

It should be cautious that LD is relatively extensive between nonsyntenic loci comparing to that between syntenic loci. False-positive results will generate when using association studies as the only mean to identify the genetic basis of complex phenotypes in these populations (Farnir *et al*., 2000). Moreover, a large number of rare alleles and mutation exist in the genome of oyster species (Appleyard and Ward, 2006; Launey and Hedgecock, 2001). Association analysis can discover more phenotype-genotype associations, and provide higher resolution, but QTL mapping can identify critical rare alleles that cannot be detected by association analysis (Famoso *et al*., 2011). Thus the joint application of linkage analysis and LD mapping (Myles *et al*., 2009; Lu *et al*., 2010) may be more effective for mapping genes in oyster species.

In conclusion, this study firstcharacterized the extent of LD in wild and cultured mass selection populations of Pacific oyster. The pattern that LD is greater in cultured population than in wild population was found in this species, indicating that strong artificial selection might increase genome wide LD. Knowledge about the extent and pattern of LD in genome helped to devise the strategy of association mapping to identify the genes underlying phenotypic diversity in oyster. Furthermore, the large number of rare alleles and the frequent occurrence of LD between nonsyntenic loci encouraged the joint application of linkage analysis and LD mapping in *C. gigas* association studies. The information on the linkage disequilibrium obtained in this study is useful for future association mapping in oyster.

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