



Article Genetic Diversity Analysis of Different Populations of Lutjanus kasmira Based on SNP Markers

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Abstract: *Lutjanus kasmira* belongs to the family Lutjanidae. Over the past 20 years, the *L. kasmira* population in the South China Sea has been shrinking due to climate change, pressure from human activities, and inadequate food supplies. In this study, single nucleotide polymorphism (SNP) data obtained from restriction site-associated DNA sequencing (RAD-seq) were used to assess the genetic diversity of *L. kasmira* in Zhubi Dao (ZB) and Meiji Dao (MJ). The genome-wide nucleotide diversity (π) of the ZB population and MJ population was 0.02478 and 0.02154, respectively. The inbreeding coefficient (Fis) of the ZB population and MJ population was -0.18729 and 0.03256, respectively. The genetic differentiation (Fst) between the ZB and MJ subpopulations was 0.00255102. The expected heterozygosity (He) of individuals from ZB and MJ was 0.33585 and 0.22098, respectively. The observed heterozygosity (Ho) of individuals from the ZB populations did not have significant genetic differences, the genetic differentiation between them was confirmed using population structure, phylogenetic, and principal component analyses. These results indicated that the genetic differences were small.

Keywords: Lutjanus kasmira; SNP; genetic diversity; genetic differentiation

1. Introduction

Single nucleotide polymorphisms (SNPs) are DNA sequence polymorphisms caused by the transformation or transposition of a single nucleotide at the genomic level [1]. SNP markers are a tool for studying the genetic structure of species [2]. Since any base of genomic DNA can be mutated, SNP molecular markers are ubiquitous in animals and plants [3]. SNPs have the advantages of a large number, a wide distribution, strong representativeness [4], good genetic stability, and convenience for high-throughput and highly automated detection and analysis [5]. The distribution of SNPs in the genome can comprehensively reflect a population's genetic variation [4].

L. kasmira is a typical reef-dwelling fish found throughout the Indo-Pacific region, from Australia in the east to Japan in the north [6]. Individuals of this species are mainly distributed around the South China Sea islands, Taiwan waters, and in the southern portion of the East China Sea [7]. *L. kasmira* has a bright yellow surface and a reddish underside; the side of the body bears four blue longitudinal bands, with an indistinct black spot between



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). the third and fourth blue bands [8]. In recent years, the habitat of this species, i.e., coral reefs and mangroves, has been reduced and degraded due to anthropogenic destruction and environmental pollution [9]. Moreover, *L. kasmira* suffers from overfishing due to its delicious meat, high economic value, and spawning clusters [7]. At present, studies on *L. kasmira* mainly focus on its physiological ecology and molecular systematics [7–9]; there are no reports on the development of SNP markers and the genetic diversity level of *L. kasmira*. In this study, the whole-genome SNPs of *L. kasmira* were first discovered by restriction site-associated DNA sequencing (RAD-seq) technology [10] and then used to analyze the genetic diversity and structure of this fish species, providing a theoretical basis for the rational development and conservation of its germplasm resources.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

L. kasmira samples were collected from Meiji Dao (MJ) and Zhubi Dao (ZB), which are separated by approximately 200 km (Figure 1). A total of 30 *L. kasmira* samples were collected, comprising 14 samples from MJ and 16 samples from ZB (Table 1). Tissue was collected by clipping the fin strip of the experimental samples. The tissue samples were fixed in 75% anhydrous ethanol and stored at room temperature. A Tissue DNA Extraction CZ Kit (Mobio, Guangzhou, China) was used to extract genomic DNA from the pterygiophore of each fish according to the manufacturer's protocol.



Figure 1. Locations of the 30 collected Lutjanus kasmira.

Table 1. San	nple inform	ation for the	e 30 L. k	casmira (collected	in Z	hubi l	Dao and	l Mei	ji Dao
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Sample ID	Sample Location	Length (mm)	Sampling Date	Sample ID	Sample Location	Length (mm)	Sampling Date
ZB_SDDD_1	Zhubi Dao	136	2019	MJ_SDDD_1	Meiji Dao	153	2019
ZB_SDDD_2	Zhubi Dao	146	2019	MJ_SDDD_2	Meiji Dao	179	2019
ZB_SDDD_3	Zhubi Dao	150	2019	MJ_SDDD_3	Meiji Dao	153	2019
ZB_SDDD_4	Zhubi Dao	128	2019	MJ_SDDD_4	Meiji Dao	164	2019
ZB_SDDD_5	Zhubi Dao	133	2019	MJ_SDDD_5	Meiji Dao	123	2019
ZB_SDDD_6	Zhubi Dao	137	2019	MJ_SDDD_6	Meiji Dao	154	2019
ZB_SDDD_7	Zhubi Dao	146	2019	MJ_SDDD_7	Meiji Dao	126	2019
ZB_SDDD_8	Zhubi Dao	148	2019	MJ_SDDD_8	Meiji Dao	137	2019
ZB_SDDD_9	Zhubi Dao	152	2019	MJ_SDDD_9	Meiji Dao	146	2019
ZB_SDDD_10	Zhubi Dao	139	2019	MJ_SDDD_10	Meiji Dao	125	2019
ZB_SDDD_11	Zhubi Dao	120	2019	MJ_SDDD_11	Meiji Dao	143	2019
ZB_SDDD_12	Zhubi Dao	149	2019	MJ_SDDD_12	Meiji Dao	135	2019
ZB_SDDD_13	Zhubi Dao	132	2019	MJ_SDDD_13	Meiji Dao	154	2019
ZB_SDDD_14	Zhubi Dao	125	2019	MJ_SDDD_14	Meiji Dao	144	2019
ZB_SDDD_15	Zhubi Dao	165	2019		-		
ZB_SDDD_16	Zhubi Dao	143	2019				

2.2. RAD-Seq Library Construction and Sequencing

RAD-seq libraries were prepared as described by Yangkun Wang and Yan Hu [11]. First, genomic DNA samples were digested by the restriction endonuclease EcoRI. Then, P1 adapter was added to both ends of the digested genome fragment, which contains a complementary sequence that binds to the primer for PCR amplification and a complementary sequence that binds to the primer for Illumina sequencing. The barcode used for sample tracking and the corresponding restriction enzyme cutting site were also part of the P1 adapter. Next, the sequence of the added P1 adapter was interrupted. Through agarose gel detection, the target band was selected in the range of 400~500 bp. The DNA fragment was then attached to the P2 adapter. The multiplexed libraries were sequenced on the Illumina 1.9 platform with an average sequencing depth of $1.5 \times$ for each individual.

2.3. Sequence Alignment and SNP Genotyping

Stacks (version 2.55, Julian Catchen and Nicolas Rochette, Urbana, IL, USA) software is widely used in RAD-seq data analysis [12]. First, raw reads were processed using the process_radtags program [11]. Through raw data processing, each clean read was assigned to a sample to ensure the quality of the sequencing data for use in subsequent analysis. Ustacks begins by clustering reads from a single sample obtained from the process_radtags program to produce stacks [13]. Ustacks in the Stacks package was applied to cluster the reads for each sample, with the same stack representing one enzyme cutting site (locus). The clustering parameter -m was set to 3 [14], and the loci and locus sequencing depth of each sample were statistically analyzed. Next, we used Cstacks to merge the loci of all samples and allowed up to 2 mismatches between different sample loci to obtain the catalogue consensus sequence of each locus [14]. Sstacks was used next. The minor allele frequency (MAF) was set at 0.05 [15]. Loci in each sample deviating from the catalogue consensus sequence were identified, and then populations were filtered to obtain the SNPs. Only the genotype of the first SNP for each tag and loci shared by more than 75% of individuals were used for further analysis. The loci not in Hardy–Weinberg equilibrium (HWE) were removed [16], and the selected loci were used to analyze genetic diversity and population genetic differentiation.

2.4. Genetic Diversity and Population Structure

The genetic diversity parameters of MJ and ZB were calculated by Stacks-2.55 [15], including observed heterozygosity (Ho), expected heterozygosity (He), genetic differentiation (Fst), the inbreeding coefficient (Fis), and nucleotide diversity (π).

Genetic variation was determined by population structure analysis and genetic differentiation assessment. The R package poppr [17] was used to calculate a distance matrix and draw a heatmap [18]. The R packages igraph and poppr were used to cluster multi-locus genotypes (MLGs) to generate minimum spanning networks (MSNs) [19]. A phylogenetic tree was constructed by PHYLIP (version 3.695, Phylip Team, Washington, DC, USA) software [20] to analyze the relationships between samples. Compared with Structure, Admixture software can be used to estimate the ancestral components of individuals faster [21]. Therefore, ADMIXTURE (version 1.3.0, David H. Alexander et al., Los Angeles, CA, USA) software [22] was used to consider various numbers of putative clusters (ranging from 1 to 10) and compute the ten-fold cross-validation error to select the most suitable number of clusters [23]. The PCA module of GCTA (version 1.93.2, Jian Yang et al., Hangzhou, Zhejiang, China) software [24] was used for principal component analysis (PCA).

3. Results

3.1. Genomic Data Statistics and SNP Discovery

Raw reads were obtained by RAD-seq of 30 individuals of *L. kasmira*. After trimming barcodes and filtering, a total of 286,264,230 high-quality clean reads were obtained from 30 individuals. The number of clean reads per individual ranged from 6,672,063 to 21,514,652, with an average of 9,542,141. In all the libraries, the GC content was sta-

ble between 39% and 40%, and the Phred score was $20 \ge 93.64\%$ (Table 2). A total of 9822 high-quality SNPs were screened out for further analysis after the selection of SNPs with an MAF < 0.05 and in HWE.

Sample ID	Number of Clean Reads	Bases (Q20%)	GC%	Sample ID	Number of Clean Reads	Bases (Q20%)	GC%
ZB_SDDD_1	7,974,070	99.32	39	MJ_SDDD_1	9,265,891	95.32	39
ZB_SDDD_2	6,867,444	96.12	40	MJ_SDDD_2	13,500,657	96.75	39
ZB_SDDD_3	11,342,742	93.64	39	MJ_SDDD_3	9,120,598	99.30	39
ZB_SDDD_4	6,831,672	95.40	40	MJ_SDDD_4	12,363,573	99.64	39
ZB_SDDD_5	10,947,352	96.32	40	MJ_SDDD_5	8,022,139	98.20	39
ZB_SDDD_6	8,673,002	98.89	39	MJ_SDDD_6	10,972,365	97.67	39
ZB_SDDD_7	8,040,810	96.63	39	MJ_SDDD_7	14,935,273	94.03	39
ZB_SDDD_8	7,251,357	97.60	39	MJ_SDDD_8	10,483,102	96.43	39
ZB_SDDD_9	8,440,580	96.30	40	MJ_SDDD_9	9,245,798	94.60	39
ZB_SDDD_10	7,441,155	94.56	39	MJ_SDDD_10	6,732,763	98.75	39
ZB_SDDD_11	8,497,499	93.21	39	MJ_SDDD_11	9,673,994	95.30	39
ZB_SDDD_12	6,672,063	95.63	39	MJ_SDDD_12	9,046,087	98.00	39
ZB_SDDD_13	9,912,874	99.11	39	MJ_SDDD_13	6,814,092	95.19	39
ZB_SDDD_14	7,340,574	98.65	39	MJ_SDDD_14	7,280,377	96.32	39
ZB_SDDD_15	21,514,652	99.30	39				
ZB_SDDD_16	11,059,675	97.21	39				

Table 2. Statistics for the genomic data obtained from 30 L. kasmira individuals.

3.2. Genetic Diversity and Population Structure

The genetic diversity parameters were calculated according to the original SNPs, and the statistical results were obtained. The genome-wide nucleotide diversity (π) of the ZB population and MJ population was 0.02154 and 0.02478, respectively. The inbreeding coefficient (Fis) of the ZB population and MJ population was -0.18729 and 0.03256, respectively. The genetic differentiation (Fst) between the ZB and MJ subpopulations was 0.00255102. The expected heterozygosity (He) of individuals from ZB and MJ was 0.33585 and 0.22098, respectively. The observed heterozygosity (Ho) of individuals from the ZB population and MJ population and MJ population and MJ was 0.33585 and 0.22098, respectively. The observed heterozygosity (Ho) of individuals from the ZB population and MJ population and MJ population and MJ population and MJ was 0.33585 and 0.22098, respectively. The observed heterozygosity (Ho) of individuals from the ZB population and MJ population was 0.46834 and 0.23103, respectively (Table 3).

Table 3. Comparison of population genetic parameters in the ZB population and MJ population.

Population ID	π	Но	He	Average_Fis	Fst
ZB population	0.02478	0.46834	0.33585	-0.18729	0.00255102
MJ population	0.02154	0.23103	0.22098	0.03256	

The heatmap showed positive correlations between the individuals of *L. kasmira*. In general, the correlations between individuals within the ZB population were the strongest, followed by those between the ZB population and MJ population, and the correlations between individuals within the MJ population were the weakest (Figure 2). The POP revealed the genetic distances between the 30 individuals, clustering MLGs based on genetic distance. The distances between individuals from the ZB population and MJ population were within the range of 0.002~0.004, indicating relatively short distances, consistent with other analysis results. Each MLG is a node, and the distance between the nodes represents the genetic distance between individuals (Figure 3). The phylogenetic tree revealed no significant clustering of the 30 *L. kasmira* samples (Figure 4). The population structure of L. kasmira was analyzed by Admixture software. Assuming that the number of groups (K value) is 1–10, the cross-validation error is maximal when K = 7 (Figure 5a,b). Therefore, the 30 samples of *L. kasmira* collected in this study were not from the same population. The genetic backgrounds of the ZB and MJ populations were complex, and there was admixture between the populations, which might be explained by the coexistence of artificial breeding and wild resources. The cross-validation results of the clustering showed that when K = 1

(Figure 5a), the error rate of cross-validation was minimal, indicating that the optimal clustering number was 1. It is speculated that *L. kasmira* individuals in the ZB and MJ populations came from the same primitive ancestor (Figure 5b). The results of PCA showed that the 30 *L. kasmira* individuals were tightly clustered (Figure 6a,b) and the genetic heterogeneity between individuals was small, which was consistent with the phylogenetic tree results.



Figure 2. The distance matrix heatmap of 30 individuals of L. kasmira created using the R package poppr.



Figure 3. The minimum spanning network of 30 L. kasmira individuals.



Figure 4. Phylogenetic tree of 30 *L. kasmira* individuals based on SNP loci created using the neighborjoining method.



Figure 5. The group structure diagrams. (a) The cross-validation error for *L. kasmira* according to the admixture value K; (b) results of Bayesian cluster analysis of *L. kasmira* based on SNP loci using ADMIXTURE software for K = 1-10 clusters.



Figure 6. Population relationships of the collected *L. kasmira* individuals. (**a**) Principal component analysis (PCA) plot of 30 *L. kasmira* individuals based on all SNP loci between the ZB and MJ populations. A and B represent the MJ population and ZB population, respectively; (**b**) three–dimensional PCA clustering map of 30 *L. kasmira* individuals based on all SNP loci between the ZB and MJ populations. A and B represent the MJ population and ZB population, respectively; (**b**) three–dimensional PCA clustering map of 30 *L. kasmira* individuals based on all SNP loci between the ZB and MJ populations. A and B represent the MJ population and ZB population, respectively.

4. Discussion

RAD-seq is a simplified genome sequencing technology based on whole-genome restriction sites developed on the basis of second-generation sequencing [11]. The number of RAD markers developed by this method is 10 times higher than that of traditional molecular marker development technology [11], with high accuracy and high data utilization [25]. RAD-seq shortens the marker development cycle compared to that of traditional markers and reduces experimental costs [26]. The technology can also screen for SNPs on a large scale in species without a reference genome [27]. SNPs are a new type of DNA molecular marker with broad application prospects [28]. SNPs occur at a high frequency and have a high marker density in most genomes. Compared with simple sequence repeat (SSR) markers, SNPs have higher genetic stability and are easier to automate [29]. RAD-seq technology can also be used to construct linkage maps [30]. For example, Palaiokostas et al. used RAD-seq technology to construct the first linkage map of Dicentrarchus labrax based on high-density SNPs [31].

In previous studies, genetic diversity was studied mainly by SSR analysis and D-loop sequence analysis [32], but these techniques have inherent difficulties in obtaining high-quality DNA from wild individuals [33]. By referring to the study of 29 Rhinopithecus roxellana by Zhang et al. [34], we used RAD-seq to detect SNP markers in 30 *L. kasmira* individuals in this study and further analyzed the genetic diversity levels of different populations of this species. Based on a study of *L. kasmira* in the South China Sea by microsatellite analysis [32], further exploration was performed. A comparison of the results of this study with those of other studies shows that the degree of variation of *L. kasmira* in the ZB and MJ areas is low. This reflects its p ability to adapt to environmental change and respond to natural selection. Therefore, it is necessary to investigate the wild population as soon as possible and determine how to improve its genetic diversity. A larger sample size would produce more statistically robust results. More samples should be collected in different regions for more comprehensive genetic diversity analysis in the future.

In the phylogenetic tree based on RAD-seq data, the 30 samples of *L. kasmira* did not form obvious groups, and the population structure diagram also showed that they came from the same ancestor. Similar results were obtained with PCA, which were mutually confirmed with the results of the phylogenetic tree. The genome-wide nucleotide diversity (π) of the ZB population and MJ population was 0.02478 and 0.02154, respectively, indi-

cating high nucleotide diversity. The expected heterozygosity (He) of individuals from ZB and MJ was 0.33585 and 0.22098, respectively. However, the observed heterozygosity (Ho) of individuals from the ZB population and MJ population was 0.46834 and 0.23103, respectively. The He of the two populations was lower than the Ho, indicating a low degree of variation for *L. kasmira* in the two areas and a certain degree of heterozygote deficiency. The genetic differentiation (Fst) between the MJ and ZB subpopulations was 0.00255102. The Fst value was consistent with the results of the heatmap and MSN. The distance between individuals was short, and differentiation was not obvious. Although π was high, He and Ho were low. The overall analysis showed that the population structure of *L. kasmira* was simple and had a low degree of variation, which might lead to poor adaptability to the environment. Furthermore, the genetic diversity of the MJ population was lower than that of the ZB population.

These results may be due to overfishing and global warming, which have reduced the genetic diversity of wild populations. Therefore, there is an urgent need to protect the population genetic resources of *L. kasmira*, and we can improve its genetic diversity through fishing restriction protection measures and artificial breeding of superior varieties. It is suggested that effective population size analysis and population history tracing should be performed for *L. kasmira*.

5. Conclusions

In this study, SNP data obtained by RAD-seq technology were used to analyze the genetic diversity in two populations of *L. kasmira*, providing a new approach for genetic diversity evaluation. The results showed that the genetic diversity of the two populations was relatively low at the genome level. To ensure adequate survival of the species, it is necessary to protect existing diversity and take measures to improve genetic diversity. To promote the healthy and sustainable development of germplasm resources of *L. kasmira*, much attention should be given to improving and maintaining its population genetic diversity, to implementing methods to increase gene flow, and to breeding excellent varieties by applying molecular breeding techniques.

Finally, our results indicate that the RAD-seq technique can detect SNP markers and apply them to the research of genetic diversity, with the benefits of a huge number of markers, low costs, and simple automation. This study is useful for the conservation of aquatic germplasm resources as well as for the development and utilization of high-quality resources.

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