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Wagutu GK, Njeri HK, Fan XR, Chen YY. Development and transferability analysis of SSR primers in wild rice *Zizania latifolia* (Poaceae)[J]. *Plant Science Journal*, 2020, 38(1): 105-111

# 菰核基因组 SSR 引物的开发及其在稻族植物中的通用性检测

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**摘 要**: 利用数据库中已有的部分菰(*Zizania latifolia* Turcz.)核基因组序列, 采用 *in silico* 方法开发其 SSR 引物, 并选取我国不同纬度的 5 个菰野生种群, 对合成的 64 对引物进行筛选。结果显示: 64 对引物中有 15 对至少在一个种群中表现出多态性; 共发现 84 个等位基因, 每个位点平均有 5.6 个等位基因。在 5 个种群中, 观察杂合度为 0.000 ~ 0.941, 预期杂合度为 0.072 ~ 0.625。种群间的基因流($N_m$ = 0.576)水平较低导致了种群间表现出较高的遗传分化($F_{ST}$ = 0.432)。进一步对稻族其他物种的通用性检测发现, 15 个多态位点中, 有 8 个位点在亚洲栽培稻(*Oryza sativa* L.)中得到扩增, 有 9 个位点在普通野生稻(*O. rufipogon* Griff.)中得到扩增。

**关键词**: 微卫星标记; 参照基因组; 简单重复序列(SSR); 野生稻; 菰

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# Development and transferability analysis of SSR primers in wild rice *Zizania latifolia* (Poaceae)

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**Abstract**: *Zizania latifolia* Turcz., also known as Manchurian wild rice, is a member of the tribe Oryzeae, and a major wild ecological and genetic resource. In this study, nuclear SSR primers were developed *in silico* based on part of the existing genome sequences of *Z. latifolia*. Five wild populations of the species from different regions across China were selected to screen 64 developed primers. Results showed that 15 primer pairs were polymorphic in at least one population. In addition, we identified a total of 84 alleles, with an average of 5.6 alleles per locus. For the different populations, the level of observed and expected heterozygosity ranged from 0.000 to 0.941 and 0.072 to 0.625, respectively. Relatively high genetic differentiation between populations ($F_{ST}$ = 0.432) was found, as

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evidenced by low levels of gene flow ($N_m$ = 0.576) among populations. These newly developed markers will facilitate further study of the level and pattern of genetic diversity, and the development of germplasm resource conservation strategies for natural extant *Z. latifolia* populations. In the cross-species transferability test, eight and nine of the 15 loci were successfully amplified in *Oryza sativa* L. and *O. rufipogon* Griff., respectively.

**Key words**: Microsatellite markers; Reference genome; Simple sequence repeats (SSR); Wild rice; *Zizania latifolia*

The aquatic/wetland wild rice genus *Zizania* is a member of the tribe Oryzeae. *Z. latifolia* Turcz., also known as Manchurian wild rice, is a perennial emergent aquatic plant growing along the littoral zones of freshwater marshes and streams[1]. It shares the genus *Zizania* with *Z. aquatica* L., *Z. palustris* L., and *Z. texana* Hitchc.[2, 3] and possesses important ecological functions. For example, it can help consolidate dykes and purify wastewater due to its high clonal reproduction and nutrient uptake capacities[4, 5]. It also serves as an important aquatic crop for its seeds and young shoots, which is a popular vegetable in China[6]. Moreover, some *Z. latifolia* traits are used for rice breeding due to its close relationship with *Oryza sativa* L.[1].

Wetland systems have become globally threatened and degraded from climate change and human activity, thus posing a risk to the genetic variability of wild rice and its adaptive potential. Wild populations of *Z. latifolia* in China are distributed along a wide stretch of latitudinal zones (21° – 50°N) that differ greatly in climate. However, most previous genetic studies on wild populations of *Z. latifolia* have been conducted independently based on different markers and samples from different areas[5, 7–10], resulting in discordant findings on its genetic structure. These complex results are not beneficial for the future management and conservation of the plant. Thus, further genetic investigations based on extensive sample collection and consistent molecular markers are needed to gain insight into the genetic basis for the high degree of adaptability in *Z. latifolia*. Such results will help identify potential new germplasm resources for the domestication of this aquatic crop.

Quan *et al.*[11] developed 16 polymorphic SSR markers for *Z. latifolia* using the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) method. However, only seven primers produced clear polymorphic bands when the primers were tested on natural *Z. latifolia* populations, which therefore necessitates the development of more effective SSR markers for the species. Here, 15 polymorphic marker primers were discovered and characterized *in silico* based on the *Z. latifolia* genome sequence[12]. Marker validation tests were conducted on five *Z. latifolia* populations spread across different latitudinal zones, with transferability tests also performed on two *Oryza* species: i.e., *O. sativa* L. and *O. rufipogon* Griff.

## 1 Materials and Methods

### 1.1 SSR identification and primer design

The reference genome of *Z. latifolia* was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/)[1]. Microsatellite motifs were identified using SSRHunter software with search criteria of more than five repeat units for di-, tri-, tetra-, and penta-nucleotides[13]. This initial screening yielded a total of 232 microsatellite-containing fragments, which were used to design primers based on Primer Premier 5.0 software. Primers flanking the sequences of each unique SSR were designed based on an optimum length of 20 bp,

optimum temperature of 50℃ – 60℃, and product size range from 100 – 300 bp. Following highly stringent filtering for dimers, hairpins, and false priming, 64 primer pairs were obtained. The primers were further tested on the *Z. latifolia* genome *in silico* using FastPCR software[14]. The number of mismatches allowed in the 3′ end of the template was set to one nucleotide, whereas the length range of the PCR product was set to 50 – 350 bp. All primers produced at least one amplicon. Amplification efficiency and polymorphism of the developed microsatellites were assessed using 89 accessions from five natural populations of *Z. latifolia*. Cross-transferability tests were performed on 20 accessions, 10 samples each for *O. sativa* and *O. rufipogon* (Table 1).

### 1.2 SSR validation and cross-transferability test

Total DNA was extracted from 0.3 g of young leaves following a modified CTAB protocol[15]. Primers were screened using five individuals from each population of *Z. latifolia*. The PCR amplifications were performed in a final volume of 20 μL containing 2 μL of template genomic DNA, 2 μL of *Taq* buffer, 1.6 μL of $Mg^{2+}$, 0.4 μL of dNTPs, 2 μL of each forward and reverse primer, 0.2 μL of 5 U *Taq* polymerase, and 9.8 μL of $ddH_2O$. The amplification procedure was performed as described by Quan *et al.*[11]. The PCR products were separated in 6% denaturing PAGE gel and visualized through silver staining. A 25 bp ladder (Promega, Madison, WI, USA) was used to identify alleles.

### 1.3 Data analysis

Fifteen primer pairs were selected based on their high polymorphism and clear banding patterns (Table 2). Using BLASTn, the microsatellite-containing fragments were aligned against the SSR markers developed by Quan *et al.*[11]. No similarity was identified, thus indicating the novelty of the 15 microsatellites. Genetic diversity was estimated by the total number of alleles ($A$), observed allele number ($N_a$), effective number of alleles ($N_e$), observed heterozygosity ($H_o$), and expected heterozygosity ($H_e$) using GenAlEx 6.501[16]. Using the same software, deviation from the Hardy-Weinberg equilibrium was also calculated.

To investigate the efficiency of the markers in the estimation of population differentiation, fixation index ($F_{ST}$) values with 1000 permutations were calculated in FSTAT[17]. Based on the $F_{ST}$ values, the average level of gene flow ($N_m$) was estimated using the formula: [$N_m = (1 - F_{ST}) / 4F_{ST}$]. The genetic relationships among populations were assessed by the neighbor-joining method in MEGA 7.0.26[18] using the genetic distances calculated in GenAlEx.

## 2 Results

A total of 84 alleles from the 15 polymorphic primers were identified, ranging from two to 14, with an average of 5.6 alleles per locus. Observed and expected heterozygosity ranged from

Table 1　Geographical information for samples used in this study

| Species | Population | No. | Locality | Geographic location | Voucher no. |
|---|---|---|---|---|---|
| *Zizania latifolia* Turcz. | LQQ | 15 | Heihe, Heilongjiang | 49°54′20.00″N, 127°29′38.20″E | HIB-Zlf-005 |
| | JH | 15 | Liuhe, Liaoning | 42°23′15.50″N, 125°46′04.90″E | HIB-Zlf-008 |
| | DP | 20 | Dongping, Shandong | 35°58′50.05″N, 116°15′28.05″E | HIB-Zlf-014 |
| | SJ | 21 | Shengjin, Anhui | 30°24′07.50″N, 117°02′51.50″E | HIB-Zlf-022 |
| | WC | 18 | Wuchuan, Guangdong | 21°20′58.90″N, 110°38′10.00″E | HIB-Zlt-027 |
| *Oryza sativa* L. | OS | 10 | Wuhan, Hubei | 30°34′55.13″N, 114°16′05.04″E | HIB-Osv-001 |
| *Oryza rufipogon* Griff. | OR | 10 | Wuhan, Hubei | 30°34′55.13″N, 114°16′05.04″E | HIB-Orf-001 |

Table 2 Characteristics of 15 polymorphic loci

| Locus | WGS accession no. | Primer sequence (5′–3′) | Repeat motif | Allele size range (bp) | *N* | *A* | $H_o$ | $H_e$ | $F_{st}$ | $N_m$ |
|---|---|---|---|---|---|---|---|---|---|---|
| ZL1 | ASSH01048101.1 | F: GCTGCTACAATCTTTCTAACTACTA<br>R: TTCCAGGCTGCGTTTT | (TA)11 | 200–308 | 89 | 13 | 0.941 | 0.625* | 0.250 | 0.751 |
| ZL3 | ASSH01048101.1 | F: TTTGATGCTGCTACAATCTTTC<br>R: TTCCAGGCTGCGTTTT | (TA)11 | 170–248 | 89 | 11 | 0.111 | 0.464* | 0.450 | 0.305 |
| ZL4 | ASSH01048101.1 | F: GCTGCTACAATCTTTCTAACTACTA<br>R: TTCCAGGCTGCGTTTT | (TA)11 | 188–240 | 89 | 12 | 0.180 | 0.465* | 0.471 | 0.281 |
| ZL5 | ASSH01048101.1 | F: GTTCTTTGATGCTGCTACAAT<br>R: TTCCAGGCTGCGTTTT | (TA)11 | 184–256 | 89 | 14 | 0.146 | 0.461* | 0.467 | 0.286 |
| ZL9 | ASSH01048102.1 | F: CATTGCCACTAGACATACA<br>R: TTGAGGATTCGACGATA | (AT)8 | 246–258 | 89 | 5 | 0.059 | 0.236* | 0.483 | 0.268 |
| ZL10 | ASSH01048102.1 | F: ATCATTGCCACTAGACATAC<br>R: CTTGATGACAAAGGATAGAA | (AT)8 | 130–146 | 89 | 6 | 0.057 | 0.269* | 0.591 | 0.173 |
| ZL31 | ASSH01000014.1 | F: TTGAGGATCAGGTGGCAGTC<br>R: TGACCATTCAGCTTCTTGGA | (CT)5 | 238–256 | 89 | 4 | 0.260 | 0.263* | 0.094 | 2.397 |
| ZL32 | ASSH01020883.1 | F: TTGAGGATCAGGTGGCAGTC<br>R: ACCATTCAGCTTCTTGGAGA | (CT)5 | 262–282 | 89 | 4 | 0.216 | 0.219 | 0.098 | 2.302 |
| ZL36 | ASSH01026238.1 | F: CATGCTTCTCATCGGTAGAG<br>R: GTGACACCAAACAATGTCAA | (AT)5 | 194–204 | 89 | 2 | 0.093 | 0.072 | 0.724 | 0.095 |
| ZL42 | ASSH01000025.1 | F: TTGTTACGAGGACTTTATGAG<br>R: ATTCTGTACGTTTGAGGTTGT | (AT)5 | 334–342 | 89 | 3 | 0.000 | 0.198* | 0.315 | 0.543 |
| ZL43 | ASSH01000025.1 | F: TTGTTACGAGGACTTTATGAG<br>R: TGATTCTGTACGTTTGAGGTT | (AT)5 | 334–352 | 89 | 2 | 0.000 | 0.133* | 0.406 | 0.365 |
| ZL55 | ASSH01000043.1 | F: GTTTGAGACGGCTGTTTTG<br>R: CAGGAGGCATGAGGAAGG | (TC)5 | 158–164 | 89 | 2 | 0.253 | 0.168 | 0.532 | 0.220 |
| ZL56 | ASSH01000043.1 | F: GTTTGAGACGGCTGTTTTG<br>R: AGCAGGAGGCATGAGGAA | (TC)5 | 162–168 | 89 | 2 | 0.253 | 0.168 | 0.532 | 0.220 |
| ZL57 | ASSH01000043.1 | F: GTTTGAGACGGCTGTTTTG<br>R: AGAGCAGGAGGCATGAGG | (TC)5 | 170–176 | 89 | 2 | 0.253 | 0.168 | 0.532 | 0.220 |
| ZL58 | ASSH01000043.1 | F: CTCTAGGACGGTTTGAGACG<br>R: CAGGAGGCATGAGGAAGG | (TC)5 | 174–180 | 89 | 2 | 0.253 | 0.168 | 0.532 | 0.220 |

Notes: *A*, Number of alleles; $H_e$, Expected heterozygosity; $H_o$, Observed heterozygosity; *N*, Number of individuals genotyped; $F_{ST}$, Fixation index; $N_m$, Gene flow. *, Significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$). Same below.

0.000 to 0.941 and 0.072 to 0.625, respectively. Significant deviation from the Hardy-Weinberg equilibrium was observed in nine loci ($P < 0.001$) (Table 2). At the population level, the number of alleles ranged from 1.4 to 3.4 (mean = 2.133) and the observed and expected heterozygosity ranged from 0.074 to 0.382 (mean = 0.205) and 0.139 to 0.354 (mean = 0.272), respectively (Table 3). In cross-genus transferability tests, eight and nine loci were successfully amplified in *O. sativa* and *O. rufipogon*, respectively (Table 4).

Table 3 Summary measures of genetic diversity for each population of *Zizania latifolia*

| Population | *N* | $N_a$ | $N_e$ | $H_o$ | $H_e$ |
|---|---|---|---|---|---|
| LQQ | 15.000 | 1.800 | 1.435 | 0.293 | 0.264 |
| JH | 15.000 | 2.133 | 1.651 | 0.382 | 0.305 |
| DP | 20.000 | 1.933 | 1.627 | 0.130 | 0.297 |
| SJ | 21.000 | 3.400 | 1.915 | 0.146 | 0.354 |
| WC | 18.000 | 1.400 | 1.296 | 0.074 | 0.139 |
| Mean | 17.800 | 2.133 | 1.585 | 0.205 | 0.272 |

Notes: *N*, Number of individual plants; $N_a$, Observed alleles number; $N_e$, Effective allele number.

Table 4　Transferability results for 15 *Zizania latifolia* SSR markers

| Locus | Allele size (bp) | |
|---|---|---|
| | *Oryza rufipogon* | *Oryza sativa* |
| ZL1 | – | – |
| ZL3 | – | – |
| ZL4 | – | – |
| ZL5 | – | – |
| ZL9 | 272 | 272 |
| ZL10 | 104 | 104 |
| ZL31 | – | – |
| ZL32 | 102 | 102 |
| ZL36 | 294–296 | – |
| ZL42 | 348 | – |
| ZL43 | 350 | 354 |
| ZL55 | 212 | 212–216 |
| ZL56 | 300 | 298 |
| ZL57 | – | 244 |
| ZL58 | 200 | 200–204 |

Note: "–", no amplification.

The genetic differentiation across all populations was high ($F_{ST}$ = 0.432) and gene flow was low ($N_m$ = 0.576) (Table 2). Pairwise comparisons of $F_{ST}$ were significant for genetic differentiation between populations ($P$ = 0.05). Levels of differentiation were moderate; $F_{ST}$ = 0.094 to 0.591. The microsatellites were able to distinguish genotypes from different locations, as evidenced by the clustering of the 89 individuals into four groups using the neighbor-joining tree (Fig.1).

## 3　Discussion

In this study, 15 polymorphic microsatellite markers were developed and evaluated to estimate genetic diversity in *Z. latifolia*. A total of 84 alleles were identified, ranging from two to 14, with an average of 5.6 alleles per locus. The observed and expected heterozygosity ranged from 0.000 to 0.941 (mean = 0.205) and 0.072 to 0.625 (mean = 0.272), respectively.

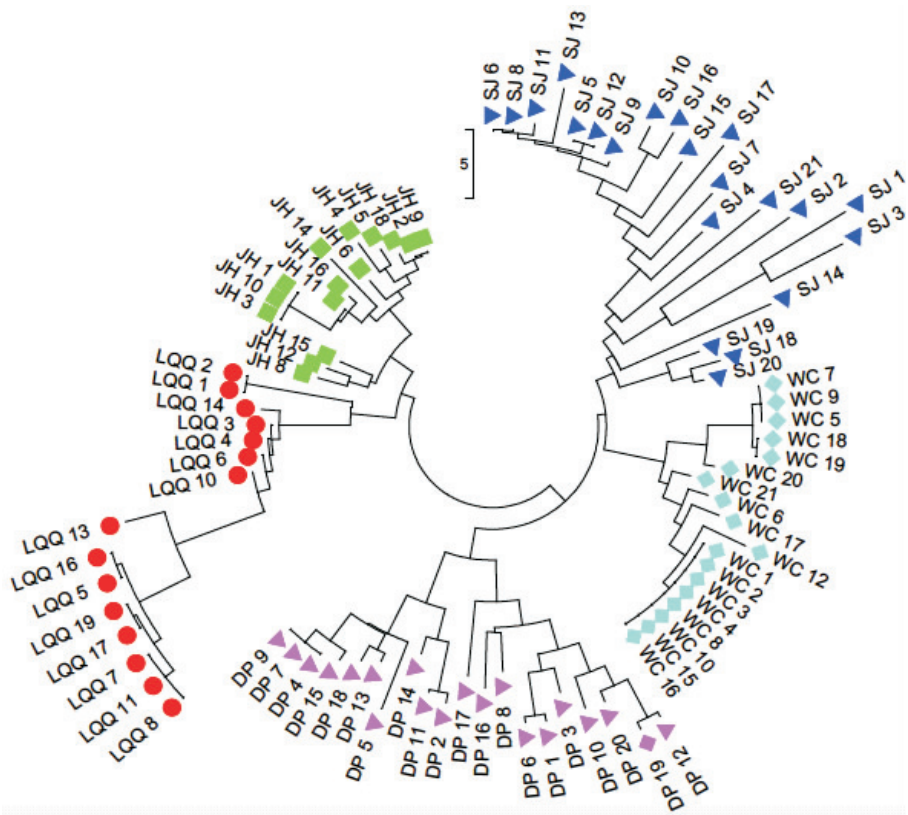


Fig. 1　Neighbor-joining tree based on 15 microsatellite markers for 89 samples of *Zizania latifolia* across China (Samples are color coded based on collection location. Population names correspond with those in Table 3.)

These measures of genetic diversity were comparable to previously developed markers; allele range of two to 14, with an average of 5.6 alleles per locus, and mean observed heterozygosity of 0.241[11]. However, the developed markers differed in the range of observed and expected heterozygosity, with existing markers ranging from 0.071 to 0.690 and 0.174 to 0.812, respectively[11]. The average expected heterozygosity of the previous markers (0.483) was also higher than for the newly developed markers. These dissimilarities and the huge difference between $H_o$ and $H_e$ for the existing markers compared to the new SSRs could be attributed to differences in the geographical range of genotyped individuals, number of samples used, and method of marker development.

The mean $H_e$ estimated using the 15 primers showed that *Z. latifolia* had relatively low genetic diversity (0.272). Similar levels of genetic diversity have been reported in natural populations from northeast wetlands ($H_e$ = 0.328)[5] and lower-middle Yangtze wetlands ($H_e$ = 0.271)[8] using SSR markers. Moreover, our results aligned to the expected average genetic diversity for Poaceae at $H_e$ = 0.201 based on the family characteristics inherent to gene flow[19]. Nine loci deviated significantly from the Hardy-Weinberg equilibrium. This could be attributed to the asexual reproduction in *Z. latifolia*, which is expected to yield heterozygote deficiency.

We also found a high level of genetic differentiation among populations using the developed markers ($F_{ST}$ = 0.432). Similar levels of genetic divergence have been reported recently for *Z. latifolia* (i.e., $F_{ST}$ = 0.405[5]; $F_{ST}$ = 0.481[20]). The observed high genetic divergence could be attributed to decreased gene flow between populations ($N_m$ = 0.576). According to Wright, $N_m$ can be interpreted as the effective number of migrants exchanged between demes per generation[21]. Therefore, an $N_m$ value > 1 suggests little divergence, whereas low migration results in increased divergence. Conventional fragmentation of wetlands into islands within the expansive terrestrial habitat and induced fragmentation[5] may explain the low gene flow and hence high genetic divergence.

The 16 previously reported markers were developed from cultivated *Z. latifolia* from a research station in Wuhan, Hubei[11]. In our study, we developed 15 markers from natural populations across five latitudinal zones in China. These novel microsatellites will supplement the already available markers for the species. This current total of 31 markers will enable research that requires the higher statistical power provided by a larger marker set.

In conclusion, the 15 polymorphic microsatellite markers will be useful for examining the levels and patterns of genetic diversity in extant natural populations of *Z. latifolia*. The baseline genetic information should be beneficial for collecting and conserving economically important germplasm resources. The transferability efficiency within the tribe Oryzeae could also be useful for further research on molecular breeding.

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