Chloroplast DNA Polymorphism in Rice
\textit{(Oryza sativa L.)}

Houxiong Wu\textsuperscript{1*}, Lifang Pu\textsuperscript{2}, Yiji Shu\textsuperscript{1}, Yifeng Li\textsuperscript{3}, Jie Meng\textsuperscript{4}, Hua Yang\textsuperscript{4}, Huan Zhao\textsuperscript{4}

\textsuperscript{1}Hunan Opulent Seed Industry Technology CO., Ltd., Huaihua, China
\textsuperscript{2}Agricultural and Rural Bureau of Zhongfang, Huaihua, China
\textsuperscript{3}Hunan Biological and Electromechanical Polytechnic, Changsha, China
\textsuperscript{4}Agricultural and Rural Bureau of Huaihua, Huaihua, China

Email: *wuhx73@126.com

Abstract
We analyzed the sequence alignment on 25 AA rice and 24 non-AA rice chloroplasts using two length diversity markers (ORF 100 and ORF29-TrnC\textsuperscript{GCA}) and four sequence markers existed in introns of rps16 gene and TrnT\textsubscript{UGU}-TrnLU\textsubscript{AAA} spacer to explore the chloroplast diversity of different types of rice using PCR amplification and sequencing. Results showed that in terms of the length of ORF100 and ORF29-TrnC\textsuperscript{GCA}, chloroplast DNA (cp DNA) of Hainan ordinary wild rice, Dongxiang ordinary wild rice, Hepu ordinary wild rice and three-line cytoplasmic male sterile wild rice were indica-type, indicating ordinary wild rice, Fusui ordinary wild rice, Niwara wild rice, Brazilian upland rice and Lemont were japonica-type among in AA genome. Besides, all non-AA wild rice was japonica-type. There were 4 indica-japonica markers utilizing introns of rps16 gene and TrnT\textsubscript{UGU}-TrnLU\textsubscript{AAA}. We found that all the ordinary wild rice in Chaling and Fusui of AA genome presented as japonica specific sites, while the others owned two indica and japonica specific sites, respectively. There were two indica-japonica sites separately and a 6-base specific fragment in three-line cytoplasmic male sterile materials except Yuetai A, simultaneously, 2-base difference from Hainan wild rice. Moreover, Brazilian upland rice and Lemont were entire japonica specific sites. Result of three markers indicated that the cp DNA of non-AA wild rice was japonica-type and result of one marker showed indica-type. Sequencing results also suggested that wild rice existed many polymorphic base sites, CCDD genome, wart wild rice and malay wild rice had their own specific sites. In conclusion, significant differentiation trend of indica-japonica exhibits in chloroplast of ordinary wild rice, and non-AA wild rice is generally japonica-type. The cytoplasmic polymorphism level of three-line sterile lines is low. It is worth considering whether the cytoplasm of Honglian-type sterile...
line Yuetai A comes from Hainan ordinary wild rice. Furthermore, genetic polymorphisms in wild rice are far more than in cultivar.

Keywords
Rice Chloroplast DNA, Three-Line Sterile Rice, Wild Rice, Polymorphism

1. Introduction

Cytoplasmic DNA is mainly maternal inheritance, which has higher genetic stability and lower mutation frequency than nuclear DNA. Therefore, cytoplasmic DNA is more suitable as a molecular marker for the origin and evolution of species and Chloroplast DNA has more variability than mitochondrial DNA [1]. Especially, analyzing the indica-japonica characteristic of chloroplast DNA (cpDNA) in rice is particularly useful for studying its origin and evolution, which is highly conserved [2]. Japonica-type cpDNA has a 69-bp repeat fragment while deleted in indica-type among in the ORF100 (open reading frame 100), so the band of japonica rice lags behind that of indica rice on the electrophoresis map. Based on this characteristic, Chen et al. discovered that the classification results of cultivated rice according to the 69bp deletion in ORF100 were effectively the same as indica-japonica discriminant function and isozymes [3] [4]. Sun et al. divided 151 cpDNA of ordinary wild rice into indica-japonica taking advantage of ORF100 marker [5]. Since then, it has been widely adopted as a marker for distinguishing the indica-japonica types [6]. Due to one 32-bp insertion between ORF29 and TrnCGCA (the intertranscriptional region ORF29 - TrnCGCA) in typical indica rice but not to exist in japonica rice, Tang suggested that this fragment could be used as a marker for cpDNA indica-japonica typing [7]. In addition, Shaw recommended that the introns of rps16 (ribosomal protein S16) and the TrnTCGU-TrnLUAA (Threonine and Leucine transfer RNA gene) intertranscriptional region were the two hypervariable fragments in plant cpDNA, which were vital for the study of rice cpDNA polymorphism [8].

Oryza is publicly recognized as owing 22 species, including A, B, C, D, E, F, G, H, J, and K [9]. The studied Asian cultivated rice and ordinary wild rice are AA genome type. There are 16 species of wild rice with chromosomes other than AA genome (referred to as non-AA wild rice), however, little research on indica-japonica differentiation. Zhu believed that the type of cpDNA variation was basically divided in accordance with the karyotype level of Oryza after analyzing 138 cpDNA in 14 species of Oryza and 2 species of Leersia, utilizing Restriction fragment length polymorphism (RFLP) [10].

In this paper, distinct 49 genomic types of rice were used as materials. We amplified the four polymorphic fragments of cpDNA including ORF100, ORF29-TrnCGCA intertranscriptional region, introns of rps16 and TrnTCGU-TrnLUAA intertranscriptional region, but also sequenced the last two frag-
ments for comparison and analysis, in order to explore the cp DNA polymorphism and regulation in different genomes of rice.

2. Materials and Methods

2.1. Plant Materials

The test materials include 25 AA genome and 24 non-AA genome of Oryza (Table 1). There are 6 cultivated rice, 11 three-line sterile rice, 5 ordinary wild rice, 1 Nivara wild rice, 1 upland rice and 1 Javanese rice of AA genome, provided by the Plant Physiology Laboratory of Hunan Normal University. Non-AA genome materials cover 12 species, a total of 24 materials, provided by the National Germplasm Nanning Wild Rice Nursery.

2.2. Total DNA Extraction

Total DNA was extracted using CTAB method [11].

2.3. PCR and Electrophoresis

The primers were designed according to the chloroplast genome sequence of Asian cultivated rice 9311 for its’ reliable genome (GenBank accession number AY522329) who was the parent of the first super hybrid rice, using the Primer Premier 5.0 (Table 2). We performed the PCR with a 25 μL reaction system: 1 × PCR Buffer, 2 mmol/L MgCl₂, 0.1 mmol/L dNTPs, 0.2 μmol/L primers, 60 - 100 ng DNA template, 2 U TaqDNA polymerase (Ferments, USA), under 94˚C for 5 min, followed by 32 cycles of 94˚C for 40 s, 50˚C for 40 s and 72˚C for 50 s, ultimately, 72˚C for 10 min. The amplified products were constantly electrophoresed on a 2% agarose gel containing 0.5 µg/mL EB and then imaged by UV.

2.4. DNA Sequencing

We directly sequenced the amplified products after recovered and purified under UV light using Ambio Biotechnology DNA Gel Recovery Kit.

2.5. Data Analysis

Sequencing results were analyzed to compare the differences and similarities between the two polymorphic fragments in various materials by MEGA 3.1.

3. Results

3.1. Comparison of the Length of ORF100 Amplification Fragments

Electrophoresis results of three typical indica rice and three typical japonica rice were in line with the indica-japonica character of ORF100 marker. Four types of three-line sterile materials were indica chloroplast. The bands of Hainan ordinary wild rice, Hepu ordinary wild rice and Dongxiang ordinary wild rice showed that they were consistent with typical indica rice. Fusui ordinary wild rice, Chaling ordinary wild rice, Nivara wild rice, Brazilian upland rice, and
Table 1. The materials and its nuclear genome type.

| NO. | Material Nuclear genome type | NO. | Material Nuclear genome type |
|-----|-------------------------------|-----|-------------------------------|
| 1   | 9311 AA                       | 26  | O. punctata BB                |
| 2   | Nanjing 3 hao AA              | 27  | O. minuta BBCC               |
| 3   | Guanglu ai si hao AA          | 28  | O. minuta BBCC               |
| 4   | Nipponbare AA                 | 29  | O. rhizomatis CC             |
| 5   | Bai ri zao AA                 | 30  | O. rhizomatis CC             |
| 6   | Ai zi nuo AA                 | 31  | O. rhizomatis CC             |
| 7   | Jin 23A1 AA                   | 32  | O. eichingeri CC             |
| 8   | Guangye A1 AA                 | 33  | O. officinalis CC            |
| 9   | V20A1 AA                      | 34  | O. officinalis CC            |
| 10  | Chuanxiang 29A1 AA            | 35  | O. alta CCDD                 |
| 11  | Zhenshan 97A1 AA              | 36  | O. alta CCDD                 |
| 12  | T98A1 AA                      | 37  | O. alta CCDD                 |
| 13  | II-32A2 AA                    | 38  | O. grandiglumis CC          |
| 14  | Zhong 9A1 CC                  | 39  | O. grandiglumis CC          |
| 15  | You 1A2 CC                    | 40  | O. grandiglumis CC          |
| 16  | K17A3 AA                      | 41  | O. latifolia CCDD            |
| 17  | Yuetai A4 CC                  | 42  | O. latifolia CCDD            |
| 18  | Hepu CWR AA                   | 43  | O. australiensis EE          |
| 19  | Dongxiang CWR AA              | 44  | O. eichingeri EE            |
| 20  | Hainan CWR AA                 | 45  | O. australiensis EE          |
| 21  | Chaling CWR AA                | 46  | O. brachyantha FF           |
| 22  | Fusui CWR CC                  | 47  | O. meyeriana GG             |
| 23  | Nivala wild rice AA           | 48  | O. ridleyi HHJJ             |
| 24  | Brazil upland rice AA         | 49  | O. ridleyi HHJJ             |
| 25  | Lemont AA                     |     |                               |

Note: 1 - cytoplasm source from hainan CWR, 2 - cytoplasm source from yinshui 6 hao, 3 - cytoplasm source from K52, 4 - cytoplasm source from hainan red awn CWR, a, b, c - the different species of the material.

Table 2. Primer sequence and target fragment.

| Primer | Forword sequence 5’—3’ | Reverse sequence 5’—3’ | Target fragment |
|--------|------------------------|------------------------|-----------------|
| cp1    | GTGGACCTGACTCCTTGAA    | AGCCGAGGTCTGGTGTA    | ORF100          |
| cp2    | GGAGCCCAGGGAAGGAGACT  | AAGGCTCGGGCATACTG    | ORF29-TrnC_{GCA} |
| cp3    | AGTTGAGCTTACATAACAGAAA | ACCAAAAGCTTCAAATACATCA | rps16 gene intron |
| cp4    | TTTT CCTCTCCATACGGCT  | TAGTCTGTTCATTCCCTGCCC | TrnT_{UGC}-TrnL_{UAA} |

Lemont were the same as typical japonica rice, and all other non-AA genome wild rice behaved similar to japonica rice characteristic (Figure 1).

3.2. Comparison of the Length of ORF29-TrnC_{GCA} Amplification Fragments

Amplification electrophoresis results of cp2 proved the primers cp1. Four types of three-line sterile rice, Hainan ordinary wild rice, Hepu ordinary wild rice and
Dongxiang ordinary wild rice were consistent with typical indica chloroplast. Fusui ordinary wild rice, Chaling ordinary wild rice, Nivara wild rice, Brazilian upland wild rice, Lemont, and all non-AA wild rice were the same as atypical japonica chloroplast (Figure 2).

3.3. Polymorphism Analysis of Bases Sequence in Introns of Rps16 Gene Amplification Fragments

The primers cp3 were used to amplify a 677 - 684 bp fragment. After sequencing and comparison, results indicated that introns of rps16 were dominated by single base polymorphism with rare difference between longer fragments (Table 3). There was an indica-type specific site GTTTATC at 267 - 273 bp, interestingly, the sequences of 10 sporophytic sterile materials in rps16 fragments were completely identical, and a GTTGAG-specific sequence was existed at 220 - 225 bp. However, gametophytic sterile rice Yuetai A as well as others appeared deletion. Such as the base at 595 bp was G in Yuetai A and others were T, Hainan ordinary wild rice was G at 49 bp and others were C. Non-AA genome materials had no the same polymorphic fragments or bases. AA genome was deleted at 133 bp. Except for small grain wild rice was deleted and wart grain wild rice showed AA in non-AA genome, others were A. Apart from Australian wild rice was C at 332 bp and other genomes were T. At 369 bp, Australian wild rice and malay wild rice were T, others deleted. At 512 - 528 bp, wart grain wild rice and malay wild rice were deleted and others were TTATTCGATTCTATA. CCDD genome materials were TCAA at 603 - 606 bp, wart grain wild rice was -AA-, malay wild rice was -AAA, others were TAAAA.

3.4. Polymorphism Analysis of Bases Sequence in TrnTUGU-TrnLUAA Amplification Fragments

An 813 - 824 bp fragment was amplified by the primers cp4 and it found three indica-japonica specific sites, which were 322 - 326 bp, 413 bp and 768 - 779 bp (Table 4). In this fragment, eleven three-line sterile rice had the same sequence structurally, and were the same as indica at three indica-japonica specific sites. Among ordinary wild rice, Fusui and Chaling were consistent with the two specific sites in typical japonica, two bases and one base deletion at japonica specific sites, respectively. Hainan and Hepu had two indica specific sites and 1 japonica specific sites, while Dongxiang had only two indica specific sites. Niwara wild rice had two specific sites for indica and one for japonica. Non-AA genome wild rice did not own indica specific sites but two japonica (Except for Australian wild rice, wart wild rice and malay wild rice which only had one japonica specific site). Simultaneously, there were many polymorphic sites. Such as wart grain wild rice was ------AA, malay wild rice was AAAAAAGAAAA but deletion in
**Figure 2.** The fragments size of ORF29-TrnCGCA. Note: The sequence of materials referring to Table 1.

**Table 3.** Sequence divergence of rps16 gene intron.

| material                  | Base site No. | 49 | 132 - 133 | 220 - 225 | 267 - 273 | 332 | 369 | 512 - 528 | 595 | 603 - 606 |
|---------------------------|---------------|----|-----------|-----------|-----------|-----|-----|-----------|-----|-----------|
| 9311                      | C             | -- | --------- | CTTTATC   | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Nanjing 3 hao             | C             | -- | --------- | CTTTATC   | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Guanglu ai si hao         | C             | -- | --------- | CTTTATC   | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Nipponbare                | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Bai ri zao                | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Ai zi nuo                 | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Jin 23A1                  | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Guangye A1                | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| V20A1                     | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Chuanxiang 29A1           | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Zhenshan 97A1             | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| T98A1                     | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| II-32A2                   | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Zhong 9A2                 | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| You 1A1                   | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| K17A2                     | C             | -- | GTTGAG    | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Yuetai A4                 | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | G   | TAAA      |
| Hepu CWR                  | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Dongxiang CWR             | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Hainan CWR                | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Chaling CWR               | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Fusui CWR                 | G             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Nivala wild rice          | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Brazil upland rice        | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| Lemont                    | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. punctata               | C             | A- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. minutata               | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. minutata               | C             | -- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. rhizomatissi           | C             | A- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. rhizomatis              | C             | A- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
| O. rhizomatis              | C             | A- | --------- | --------- | T         | -   | TATTTTCGATTTCTATA | T   | TAAA      |
Continued

| Species         | Base 1 | Base 2 | Base 3 | Base 4 | Base 5 | Base 6 | Base 7 |
|-----------------|--------|--------|--------|--------|--------|--------|--------|
| O. eichingeri   | C      | A-     | -------| -------| -------| -------| -------|
| O. officinalis  | C      | A-     | -------| -------| -------| -------| -------|
| O. officinalis  | C      | A-     | -------| -------| -------| -------| -------|
| O. alta         | C      | A-     | -------| -------| -------| -------| -------|
| O. alta         | C      | A-     | -------| -------| -------| -------| -------|
| O. alta         | C      | A-     | -------| -------| -------| -------| -------|
| O. grandiglumis | C      | A-     | -------| -------| -------| -------| -------|
| O. grandiglumis | C      | A-     | -------| -------| -------| -------| -------|
| O. grandiglumis | C      | A-     | -------| -------| -------| -------| -------|
| O. latifolia    | C      | A-     | -------| -------| -------| -------| -------|
| O. latifolia    | C      | A-     | -------| -------| -------| -------| -------|
| O. australiensis| C      | A-     | -------| -------| -------| -------| -------|
| O. australiensis| C      | A-     | -------| -------| -------| -------| -------|
| O. brachyantha  | C      | A-     | -------| -------| -------| -------| -------|
| O. meyeriana    | C      | AA     | -------| -------| -------| -------| -------|
| O. ridley       | C      | A-     | -------| -------| -------| -------| -------|
| O. ridley       | C      | A-     | -------| -------| -------| -------| -------|

“-“: the lack base or deletion.

others at 126 - 133 bp, which could be regarded as a specific site between wart and malay. 218 - 222 bp was as well as aspecific site for malay, but deletion in others. 479 bp, 520 bp and 599 bp were the specific sites respectively represented by Guangluai 4, Nanjing 3, and Hundred-day-old. This sequence reflected high genetic polymorphism and verified the view that it was a hypervariable region proposed by Shaw [7].

4. Discussion

Cultivated rice includes indica subspecies and japonica subspecies, previous studies on indica- japonica characteristics of wild rice have been widely recognized by breeders and geneticists. In this research, the length of fragments amplified by primers cp1 and cp2 of Dongxiang ordinary wild rice and Hepu ordinary wild were distinct with others, which were consistent with the typical indica rice and typical japonica rice, respectively. The same was true of cp3 and cp4 sequencing results and relatively supported the two-source origin theory of cultivated rice [12]. There were no differences in the two base sequence polymorphic fragments between introns of rps16 and TrnT^UGU_.TrnL^UAA intertranscriptional region for the tested wild abortive, Yinshui and K-type three-line sterile rice. Adversely, typical indica rice, typicaljaponica rice, ordinary wild rice and non-AA genome wild rice existed differences in various degrees. It demonstrated that the genetic
### Table 4. Sequence divergence of TrnT^{UGU} - TrnL^{UAA} spacer.

| material            | 126 - 133 | 218 - 222 | 322 - 326 | 413 | 479 | 520 | 599 | 768 - 779 |
|---------------------|-----------|-----------|-----------|-----|-----|-----|-----|-----------|
| 9311                | -         | -         | -         | A   | A   | T   |     | AGAAAA--------- |
| Nanjing 3 hao       | -         | -         | -         | A   | G   | T   |     | AGAAAA--------- |
| Guanglu ai si hao   | -         | -         | -         | G   | A   | T   |     | AGAAAA--------- |
| Nipponbare          | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| Bai ri zao          | -         | -         | -         | TATAT | A   | A   | C   | AGAAAAAGAAAA |
| Ai zi nuo           | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| Jin 23A¹             | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Guangye A¹          | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| V20A¹               | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Chuanxiang 29A¹      | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Zhenshan 97A¹       | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| T98A¹               | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| II-32A²             | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Zhong 9A²            | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| You 1A²             | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| K17A³               | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Yuetai A¹           | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Hepu CWR            | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Dongxiang CWR       | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Hainan CWR          | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Chaling CWR         | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| Fusui CWR           | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| Nivala wild rice    | -         | -         | -         | -   | A   | A   | T   | AGAAAAAGAAAA |
| Brazil upland rice  | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| Lemont              | -         | -         | -         | TATAT | A   | A   | T   | AGAAAAAGAAAA |
| O. punctata         | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. minutë           | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. minutëb          | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. rhizomatisë      | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. rhizomatisëb     | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. eichingeri       | -         | -         | -         | T--- | T   | A   | A   | AGAAAAAGAAAA |
| O. officinalisë      | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. officinalisëb    | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
| O. altaë            | -         | -         | -         | A--- | T   | A   | A   | AGAAAAAGAAAA |
Continued

| Plant Name | Primer | Amplification Length (bp) | Polymorphism | Sequence | Polymorphism Type |
|------------|--------|---------------------------|--------------|----------|------------------|
| O. altab | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. altac | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. grandiglumisa | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. grandiglumisb | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. grandiglumisc | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. latifoliaa | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. latifoliab | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. australiensisaa | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. eichingerib | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. australiensisb | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. brachyantha | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. meyeriana | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. ridleya | cp1 | | A--- T A A T | AGAAAAAGAAAA |
| O. ridleyb | cp1 | | A--- T A A T | AGAAAAAGAAAA |

"-": the lack base or deletion.

The background of these three sterile rice chloroplasts was relatively simple, and the level of three-line sterile rice cpDNA polymorphism was lower than that in ordinary wild rice and non-AA genome, indicating the wild rice was the treasure of resources with great value.

Results of non-AA wild rice study showed that the length of fragments amplified by primers cp1 and cp2 was the same as japonica rice. There were three indica-japonica specific sites consistent with japonica of cp3 and cp4, suggesting non-AA genome wild rice was generally biased to japonica. Furthermore, it showed a certain tendency to indica. Ichikawa used molecular markers of the rice chloroplast genome for genetic analysis, and concluded that AA-type was relatively close to CCDD, BB, BBCC, CC, and FF, while EE might evolved from its own Oryza ancestors [13]. In our experiment, results of cp3 and cp4 amplification fragments manifested that Australian wild rice of EE genome did have significant base differences from others and it was relatively close to wart grain wild rice from EE and malay wild rice from HHJJ. At the same time, CCDD genome, wart grain wild rice and malay wild rice had their own specific sites in the two fragments but none in other genomes, which speculated that their kinship might be slightly farther than others.

In the three-line sterile materials, pollen abortion type of wild abortive, Yinshui and K-type was sporophytic sterility, further observed under microscope we defined they were typical, in addition, Honglian-type sterile lines was gametophytic sterility and also described as spherical. Among 11 lines of sterile materials utilized in this study, whether the two base fragments polymorphism in rps16 between Yuetai A and others at 220 - 225 bp and 595 bp could be used as its specific markers to identify the pollen abortion type, that is, the cytoplasmic
genetic characteristics of its cytoplasmic sterility. Especially the GTTGAG insertion of wild aborted materials at 220 - 225 bp in rps16, can it be used as a specific marker, which requires a large number of chloroplast genome sequence alignment results of wild aborted material and is worth further experimental proof. Besides, these two base sequence polymorphisms were also a characteristic marker to discriminate the Honglian-type sterile line Yuetai A from other 10 materials. The cytoplasmic donor of three-line sterile material Honglian-type is Hainan Hongmang wild rice [14], which belongs to the Hainan ordinary wild rice. The amplified electrophoresis images of ORF100 and ORF29-TrnC\textsuperscript{GCA} showed that the chloroplast genome of Hainan ordinary wild rice was japonica-type; Yuetai A was indica-type. Sequencing results of the products amplified by cp3 and cp4 declared that the single base sequence in rps 16 at 49 bp and 595 bp were different between Hainan ordinary wild rice and Yuetai A, revealing that a difference was existed in chloroplast genome between Hainan ordinary rice and Hongmang. According to the study on ORF100 fragment of Chinese ordinary wild rice cp DNA proposed by Sun Chuanqing that most in various regions (provinces) performed indica-japonica differentiation, but no research in Hainan ordinary wild rice [15], merely, our paper certified that there might be indica-japonica differentiation in its cp DNA.

5. Conclusions

Ordinary wild rice chloroplasts have obvious indica-japonica differentiation tendency; non-AA wild rice chloroplast DNA is generally japonica; cytoplasmic polymorphism of three-line sterile lines is low, and genetic polymorphism of wild rice is far more than that of cultivated rice.

Acknowledgements

This research is financially supported by Hunan Province’s strategic emerging industry science and technology research and major scientific and technological achievements transformation projects (2019GK4036) and the National key R & D plan “Seven major crop breeding” projects (2016YFD0101100).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Second, G. and Wang, Z.Y. (1992) Mitochondrial DNA RFLP in Genus Oryza and Cultivated Rice. Genetic Resources and Crop Evolution, 39, 125-140.
[2] Yan, A. and Zhu, D.Y. (2004) The Application of Chloroplast Genome in the Studies of Systematizes and Bioengineering. Chinese Journal of Cell Biology, 26, 153-156.
[3] Chen, W.B., Sato, Y.I., Nakamura, I., et al. (1993) Distribution of Deletion Types in cpDNA of Cultivated and Wild Rice. The Japanese Journal of Genetics, 68, 597-603.
[4] Chen, W.B., Sato, Y.I., Nakamura, I., et al. (1994) Indica-Japonica Differentiation in Chinese Rice Landraces. *Euphytica*, 74, 195-201. https://doi.org/10.1007/BF00040401

[5] Sun, C.Q., Wang, X.K., Atsushi, Y. and Nobuo, I. (1997) Indica-Japonica Differentiation of Chloroplast DNA in *O. rufipogon Griff.* and *O. sativa L.* *Journal of Agricultural Biotechnology*, 11, 319-323.

[6] Kanno, A., Watanabe, N., Nakamura, I., et al. (1993) Variation in Chloroplast DNA from Rice (*Oryza Sativa*): Differences between Deletions Mediated by Short Direct-Repeat Sequences within a Single Species. *Theoretical and Applied Genetics*, 86, 57-584. https://doi.org/10.1007/BF00838712

[7] Tang, J.B., Xia, H.A., Cao, M.L., et al. (2004) A Comparison of Rice Chloroplast Genomes. *Plant Physiology*, 135, 412-420. https://doi.org/10.1104/pp.103.031245

[8] Shaw, J., Lickey, E.B., Beck, J.T., et al. (2005) The Tortoise and the Hare II: Relative Utility of 21 Non-Coding Chloroplast DNA Sequences for Phylogenetic Analysis. *American Journal of Botany*, 92, 142-166. https://doi.org/10.3732/ajb.92.1.142

[9] Zhang, N.Q., Li, Y.X., Zhu, L.L. and He, G.C. (2003) Review of the Research on the Classification of the Genus Oryza. *Chinese Journal of Rice Science*, 17, 393-397.

[10] Zhu, S.H., Wang, M.Q. and Wang, X.M. (1999) Chloroplast DNA Restriction Fragment Length Polymorphism in the Genus Oryza. *Chinese Journal of Rice Science*, 13, 139-142.

[11] Rogers, O.S. and Bendich, A.J. (1988) Extration of DNA Plant Tissue. In: Gelvin, S.B., Schilpe, R.A. and Verna, D.S., Eds., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, Dordrecht. https://doi.org/10.1007/978-94-017-5294-7_6

[12] Chen, W.B. (1999) A Review of Genetic Studies on the Origin and Differentiation of Asian Cultivated Rice (*Oryza sativa L.*). *Acta Agriculturae Shanghai*, 15, 42-48.

[13] Ichikawa, H., Hirai, A. and Katayama, T. (1986) Genetic Analysis of Oryza Species by Molecular Markers for Chloroplast Genomes. *Theoretical and Applied Genetics*, 72, 353-358. https://doi.org/10.1007/BF00288572

[14] Liang, S.H., Li, C.G. and Wu, Y.Y. (1998) Breeding and Main Characteristics of Honglian-Type High-Quality Sterile Line Yuetai A. *Fujian Rice and Wheat Technology*, 16, 1-4.

[15] Sun, C.Q., Wang, X.K., Yoshimura, A., et al. (2002) Genetic Differentiation for Nuclear, Mitochondrial and Chloroplast Genomes in Common Wild Rice (*Oryza rufipogon Griff.*) and Cultivated Rice (*Oryza sativa L.*) *Theoretical and Applied Genetics*, 104, 1335-1345. https://doi.org/10.1007/s00122-002-0878-4