# Sequence Variation and Phylogenetic Relationships Among Ten Iraqi Rice Varieties Using RM171 Marker

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**Abstract**

In the present study rice microsatellite marker (RM 171) was used to evaluate the genetic diversity and determining cultivar identity among ten rice varieties (*oryza sativa* L.) (Seven local and three commercial varieties). PCR technique was performed using two specific primers. The result showed presence of a band (305bp) DNA sequencing was done to PCR product to detect sequence variation between the ten rice varieties. In order to detect the relationship among all varieties, alignment of RM171 marker sequence was carried out for each variety. Amber and Daawat varieties showed the highest similarity with 98% identity, while the difference (2%) consists of two gaps and two transition mutations (T/C) and (C/T). Furthermore, Amber was aligned with mashkhab-1; 6% variation was noticed includes 5% gaps of 16 nucleotides which are not found in Amber that distributed in four different locations. In addition to the gaps, two transversion mutations were identified (G/C) and (G/T). Phylogenetic relationships among varieties were achieved, which showed that genetic distances were ranged from 0.029 to 1.999 among rice varieties. Cluster analyses grouped the ten varieties into five main clusters depending on their geographic origin, their ancestor and their aroma characteristics and this revealed relatedness between aromatic and non -aromatic with few of independent varieties. The result of this study could be helpful in the future for rice breeding programs.

**Key Words:** *Oryza sativa*, RM171 Marker, DNA Sequence Alignment, Phylogenetic Tree.

# العلاقات التطوريه والتغاير التسلسلي لعشرة اصناف من الرز العراقي باستعمال مؤشر الرز الوراثي (RM171)

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**الخلاصه:**

استخدمت في الدراسه الحاليه مؤشرات وراثيه لتقييم التنوع الوراثي وتحديد التماثل بين الاصناف العشرة تحت الدراسه (سبعه محليه وثلاثه تجاريه ). تم تضخيم ناتج تفاعل البوليمريز التسلسلي (305)bp باستخدام زوج من البوادى المتخصصه ، بعدها تم اجراء تحديد تتابع الأحماض النووية للناتج وذلك لمعرفة التباين في التسلسلات بين اصناف الرز. اجريت المحاذاة بين تسلسلات RM171 لكل صنف وذلك للكشف عن العلاقه بينهما. اظهرت النتائج ان اعلى نسبة تشابه كانت بين صنفي العنبر والدعوات (98%) حيث ان الفرق (2%) يتضمن فجوتين وطفرتين انتقاليه (T/C ) و (C/T)، وعند محاذاة صنف العنبر مع المشخاب 1 لوحظ وجود نسبة تغاير (6%) والذي يتضمن (5%) فجوات ل 16 نيوكليوتيده غير موجوده في صنف العنبر والتي وزعت في اربعة مواقع مختلفه .بالاضافه الى الفجوات تم تحديد طفرتين تباديليه(G/Cو G/T ). اتضح من خلال علاقات النشوء والتطور ان البعد الوراثي يتراوح بين (0.029_1.999). قسمت اصناف الرز العشره الى خمسة مجاميع رئيسه اعتمادا على الاصل الجغرافي و الاسلاف المنحدره منها وصفاتها العطريه . لوحظ وجود علاقه بين الاصناف العطريه ،غير العطريه والاصناف المستقله .يمكن استخدام النتائج المستحصله من هذه الدراسه في برامج تربية و تحسين الرز المستقبليه.

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## Introduction

Rice (Oryza Sativa L.) is a Global Grain that cultivated widely in the world feeding millions of mankind. As the humankind faces nutrient deficient in cultivated crops, rice supplemented with micronutrients is an important substitute to overcome such malnutrition. Rice is cultivated in a wide range of ecological environments worldwide [1]. In Iraq, rice is one of the staple foods of the greater majority of the Iraqi population. It cultivate as a summer crop in Iraq especially in the southern machinery as well as in the valleys of northern Iraq [2]. A number of traditional varieties and improved cultivars have been cultivated in different regions of Iraq since the early 20th century. The most important variety of rice in Iraq is Amber which cultivate particularly in the southern region [3]. The wild abortive cytoplasmic male sterility (CMS-WA) system, an ideal type of sporophytic CMS in indica rice, is used for the large-scale commercial production of hybrid rice. Searching for restorer genes is a good approach when phenotyping is very time-consuming and requires the determination of spikelet sterility in test cross progeny [4].

To establish more precisely the genetically and physical maps of fertility restorer (R*f*) gene, high-resolution mapping of this locus was carried out using simple sequence repeat (SSR) markers and newly designed markers in a F2 population. The genetic linkage analysis indicated that two SSR (simple sequence repeats) markers, RM171 and RM228, were linked to RF gene located on the long arm of chromosome 10 [5].

Microsatellites (SSRs) are motifs of 1-6 bp in length that randomly repeated in DNA. They have been detected ubiquitously in the genomes of all organisms analyzed so far. It was found that eukaryotic genomes, generally contain much more microsatellite than Prokaryotes [6, 7]. Microsatellite has been used widely as a useful and efficient DNA marker for multiple purposes in plants, including genome mapping, QTL tagging, association mapping, marker assisted selection (MAS), genetic diversity, cultivar identification, population genetics, and taxonomic/phylogenetic analysis. This is largely due to salient properties of microsatellite, such as genome-wide coverage, hyper variability, condominant inheritance, reproducibility, chromosome-specifically and amenability to high through put genotyping [8].

The objective of this study was to compare the effectiveness of PCR-based molecular marker (RM 171) to determine the genetic relationships among several Iraqi rice genotypes. Also to investigate the suitability of this unique rice molecular marker characterizing the ten rice varieties, and develop unique fingerprint for each variety.

## Materials and Methods:

### *Samples Collection and DNA Extraction*

Seeds of seven rice varieties, cultivated in Iraqi, were obtained from the State Board for Agricultural Research (SBAR) and AL-Mashkhab Rice Research Station (MRRS) and 3 commercial rice varieties were obtained from local market (table 1). DNA was isolated by wizard genomic (DNA purification kit, Promega, USA) according to the isolation of genomic DNA from plant tissue protocol. DNA extracted from 60 mg of seeds after grinded of each sample. The volume of the extracted DNA solution was usually 100 µl were stored at -20°C.

**Table 1-** Local and improved rice varieties used in the study

| Varieties' name | Pedigree | Varietal group | Breeding Institute |
|---|---|---|---|
| 1.*Yasmin | Introduced from (Vietnam) in 1998 | Aromatic | AMRR & SBAR |
| 2.*Brnamge -4 | Introduced from IRRI (Philippines) in 2001 | Non- Aromatic | AMRR & SBAR |
| 3.*Amber | Local (Iraqi) | Aromatic | AMRR & SBAR |
| 4.*Furat | Introduced from (Vietnam) in 1996 | Aromatic | AMRR & SBAR |
| 5.*Mashkhab | Introduced from IRRI (Philippines) in 1987 | Non- Aromatic | AMRR & SBAR |
| 6.*Mashkhab | Introduced from IRRI (Philippines) in 1978 | Non- Aromatic | AMRR & SBAR |
| 7.**AL-abasia | Radiation grain of Mashkhab -1- by Gamma ray | Non- Aromatic | SBSTC |
| 8. Daawat | Commercial | Aromatic | Market |
| 9. Al-aila | Commercia | Aromatic | Market |

*SBAR: State Board for Agriculture Research, and AMRRS: Al-Mashkhab Rice Research Station
**SBSTC: State Board of Seeds Testing and certification

***Polymerase Chain Reaction (PCR) (detection of SSR marker):***

- *Primer selection*

The primers that selected in this study were amplified the SSR marker (RM171). The primers and their sequences used in the PCR reaction were selected from previous studies [9, 10], and supplied by Alpha DNA company, (Canada). A forward primer (RM171 F: 5'-ACGAGATACGTACGCCTTTG-3') and a reverse primer (RM171 R: 5'-AACGCGAGGACACGTACTTAC-3') were produced about 305-331 bp fragment.

- *PCR reaction*

The PCR reaction was performed with final volume of 50 µl and contained: 25 µl of Go Taq green master mix 2X (Promega, USA), 4µl DNA (conc. 50 ng/µl), 0.66 µl of each primer (10 pmol/µL) and up to 50 µl with nucleases free water.

- *PCR program*

The thermal cycling (MultigeneTM Gradient Thermal Cycler, Labnet International, Korea) was programmed as follows: initial denaturation of 5 min at 94°C and then 33 cycles of the following three steps: 94 °C for 1min, 60°C for 1 min and 72 °C for 1min and final incubation at 72 °C for 7 min [10].

***Determine the Molecular Size of Amplicons :***

The PCR products were separated on 2% agarose gel electrophoresis in the presence of 100 bp DNA ladder marker (Promega, USA) and visualized under the ultraviolet light (302nm) after staining with ethidium bromide [11].

***Sequencing, Sequence Alignment and phylogenetic tree:***

Sequencing of PCR product was carried out by Scigenom Company (India) for sequencing through using individual up and downstream primer which was used in each sequencing reactions. Sequence Alignment searches were conducted between the sequencing results of PCR products and the sequence of standard gene by BLAST program (Basic Local Alignment Search Tool (BLAST)-*Oryza sativa* (rice) which is available at the national center biotechnology information (NCBI) online at (http://www.ncbi.nlm.nih.gov). Phylogenetic tree were generated using Mafft, a multiple sequence alignment based on fast Fourier tool program, which is an online tool available at (http://mafft.cbrc.jp/alignment/server/).

**Results and Discussion:**

Unlike morphological and biochemical markers SSR markers are unlimited in numbers and are not affected by environmental factors and / or the developmental stages of the plant [12, 13]. The genetic markers arise from different classes of DNA mutations such as substitution mutation, rearrangements errors in replication of tandem by repeated DNA.

In the PCR technique, the primers were used to amplify the RM171 marker. This marker linked to fertility restorer gene (***Rf4***) on chromosome 10 [14]. These primers produced fragments about 305-331 bp in size (Figure 1). In this context, many studies showed similar results [13,14].

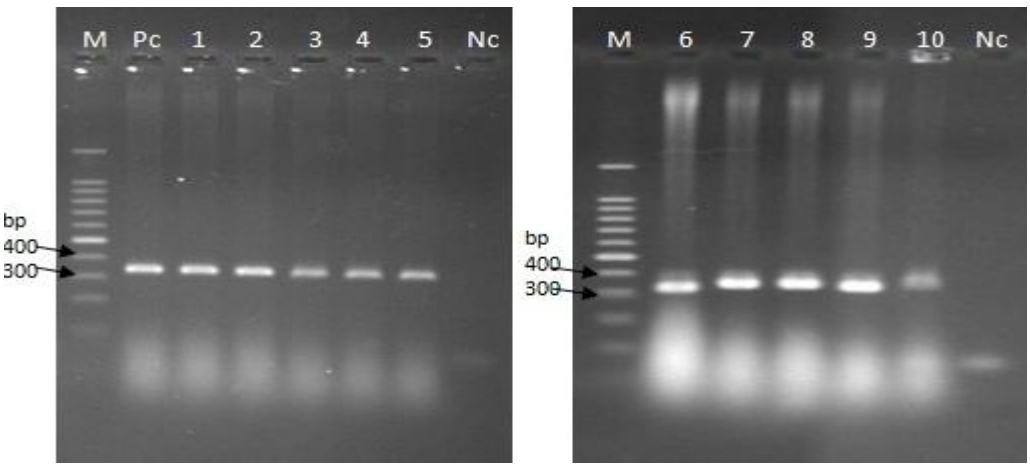


**Figure 1-** Agarose gel electrophoresis of PCR products of RM171 marker across 10 rice varieties. Lanes: 1-Amber, 2-Yasmine, 3-Furat, 4-Mashkhab-1-, 5-Mashkhab-2-, 6-Brnamge-4-, 7-AL-abasia, 8-Daawat, 9-AL-aila, 10-Karman. M: 100bp

ladder, Pc: positive control, Nc: negative control. Bands were separated by 2% agarose gel electrophoresis (2hr, 5V/cm, 0.5XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide.

The PCR products for all samples were sequenced and then published on the national center biotechnology information (NCBI). Alignment of the sequence of RM171 marker was achieved for all varieties with each other to detect the relationship among them.

Amber and Daawat varieties showed the highest similarity in the RM171 marker sequence which is 98% identity, while the difference (2%) consists of two gaps and two transition mutations (T/C) and (C/T) (Figure 2A). Amber was also aligned with Mashkhab-1, 5% variation was noticed between the two DNA sequences that means there is about 94% similarity between each other. The differences in the genetic material include 5% gaps of 16 nucleotides, 12 of them were not present in Mashkhab, these gaps were mainly distributed into two locations, and 4 were absent in Amber, that distributed with four different locations. In addition to the gaps, two transversion mutations were identified (G/C) and (G/T) (Figure 2B).

(A)

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 497 bits(269) | 1e-145 | 280/285(98%) | 2/285(0%) | Plus/Plus |

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Amber   32  GTCCTCGATGCTAGCTGGCTGCCTGCCTTCGTGCGTGCGTGCGAGAGGAGGCCAGTTCGG  91
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Daawat  33  GTCCTCGATGCTAGCTGGCTGCCTGCCTTCGTGCGTGCGTGCGAGAGGAGGCCAGTTCGG  92

Amber   92  TGAGCCGCCCAatccatccatccatccatccatccatcGTGTGTACCGTGTACGTGTGTA  151
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Daawat  93  TGAGCCGCCCAACCCATCCATCCATCCATCCATCCATCGTGTGTACCGTGTACGTGTGTA  152

Amber   152 GGTGTCATGCATATGCAATCTAGtt-tttttCGAACACGTAAAAGAATTACACGTTAAT  210
            |||||||||||||||||||||||| ||||| ||||||||||||||||||||||||||||
Daawat  153 GGTGTCATGCATATGCAATCTAGTTATTTTTACGAACACGTAAAAGAATTACACGTTAAT  212

Amber   211 TGATCACGCATACGCTATCATCGGCCGAATTTGCAAAGTGCAGTTGCTGATCGCCTGATC  270
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Daawat  213 TGATCACGCATACGCTATCATCGGCCGAATTTGCAAAGTGCAGTTGCTGATCGCCTGATC  272

Amber   271 GCCTTAATATTTGGAGTGCGTAAGTACTGCCCCTC-GCGTTAAAA  314
            |||||||||||||||||||||||||||| ||||| |||||||||
Daawat  273 GCCTTAATATTTGGAGTGCGTAAGTACTGTCCCTCCGCGTTAAAA  317
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(B)

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 438 bits(237) | 7e-128 | 283/302(94%) | 16/302(5%) | Plus/Plus |

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Amber       17  GCTGGCTCGGACGTAAGTCCTCGATGCTAGCTGGCTGCCTGCCTTCGTGCGTGCGTGCGA  76
                ||||||||  | ||||||||||||||||||||||||||||||||||||||||||||||
Mashkhab-1  17  GCTGGCTCCG-CGTAAGTCCTCGATGCTAGCTGGCTGCCTGCCTTCGTGCGTGCGTGCGA  75

Amber       77  GAGGAGGCCAGTTCGGTGAGCCGCCCA----atccatccatccatccatccatcGTGTGT  132
                |||||||||||||||||||||||||||    ||||||||||||||||||||||||||||
Mashkhab-1  76  GAGGAGGCCAGTTCGGTGAGCCGCCCAATCCATCCATCCATCCATCCATCCATCGTGTGT  135

Amber       133 ACCGTGTACGTGTGTAGGTGTCATGCATATGCAATCTAGTTATTTTTGCGAACACGTAAA  192
                |||||||||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||
Mashkhab-1  136 ACCGTGTACGTGTGTAGGTGTCATGCATATGCAATCTAGTT-TTTTTTCGAACACGTAAA  194

Amber       193 AGAATTACACGTTAATTGATCACGCATACGCTATCATCGGCCGAATTTGCAAAGTGCAGT  252
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Mashkhab-1  195 AGAATTACACGTTAATTGATCACGCATACGCTATCATCGGCCGAATTTGCAAAGTGCAGT  254

Amber       253 TG--------CTGATCGCCTTAATATTTGGAGTGCGTAAGTACGTGTCCCTCGCAGTTAA  304
                ||         |||||||||||||||||||||||||||||||| || ||||||| |||||
Mashkhab-1  255 TGCTGATCGCCTGATCGCCTTAATATTTGGAGTGCGTAAGTAC-TGCCCCTCGC-GTTAA  312

Amber       305 AA  306
                ||
Mashkhab-1  313 AA  314
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**Figure 2-** Alignment of RM171 marker sequence for (A) Amber against Daawat (B) Amber against Mashkhab-1 variety as obtained from NCBI.

Figure 3- shows the polygenic tree of RM171 microsatellites for 10 rice varieties based on the sequencing results. These varieties were distributed into 5 groups .The First group includes Mashkhab-1 and Mashkhab-2 attached to Brnamge-4 and Al-abasia respectively, to form two sub-cluster (Mashkhab-1, Brnamge-4) and (Mashkhab-2, Al-abasia) which has 98% sequence similarity. The 2% dissimilarity of the last sub-cluster was resulted from the radiation of Mashkhab-1 to introduce Al-abasia. The second group in the phylogenic tree consists of Daawat, Al-aila and Amber. Both of Daawat and Al-aila have a very high genetic identity which is 99%, while Amber is 98% similar to both of them. The last three varieties were distributed to three separated clusters Yasmin, Furat and Karman which has less similarity to any variety. Yasmin and Furat were similar to each other with 82% identity; both of them are originally from Vietnam and aromatic. Finally, the phylogenic tree shows that aromatic varieties were arranged together and distinct from the non-aromatic varieties.

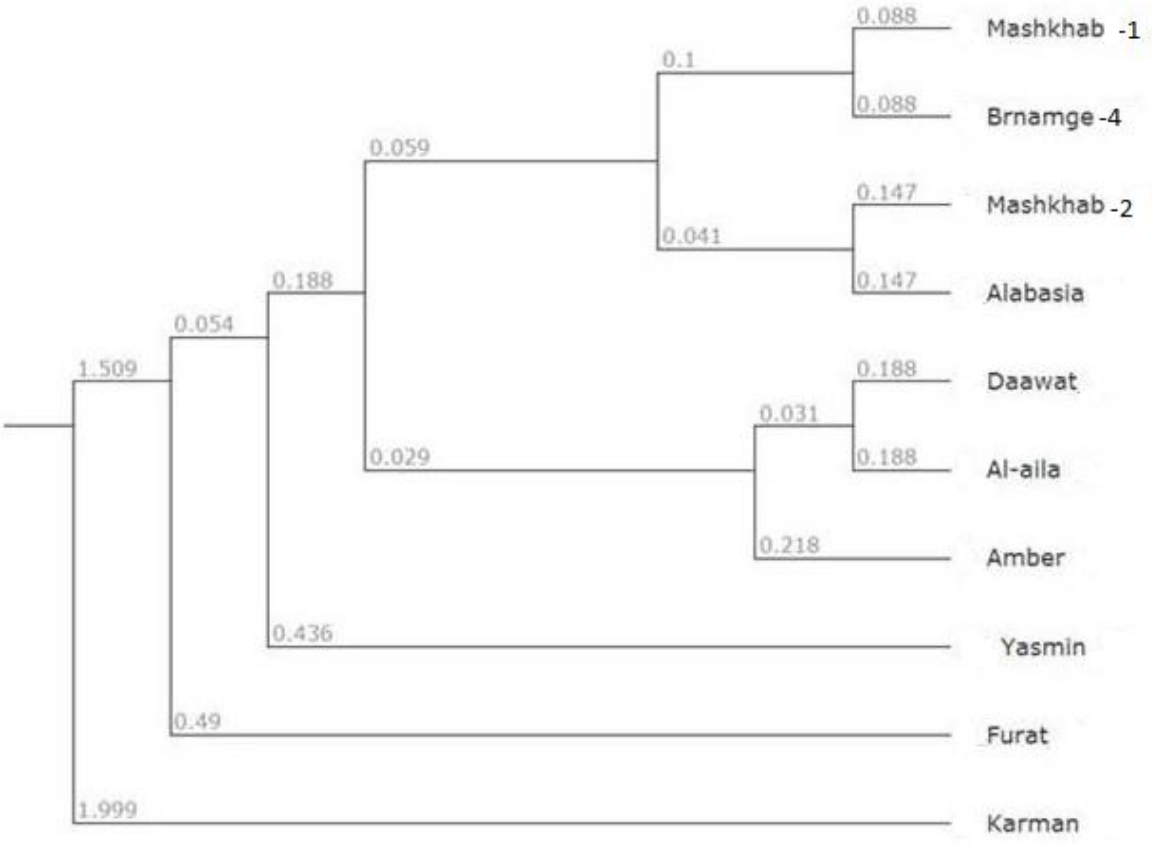


**Figure 3-** Phylogenic tree of RM171 marker (linked to ***Rf4*** genes) of rice varieties using MAFFT program

These results confirm the conclusion of Al-Judy [15] and Younan *et al*. [10], who mentioned that Amber variety may come from Indian origin. As shown in this study, there was 98% identity between Amber and both of Daawat and Al-aila varieties which formed one group, while there was just 94% identity between Amber and Mashkhab varieties, that means Amber variety tends to be comes from Indian origin as Daawat and Al-aila varieties.

The present study represent one of the first attempts to find out a small set of microsatellite markers to discriminant Iraqi rice cultivars providing meaning full data that can be enlarged by additional rice cultivars and new microsatellite markers.

In conclusion, the microsatellite rice markers (RM171) were used here to provide a positive assessment to the ability of SSR marker to produce unique DNA profile of rice genotypes. The data obtained can be used for variety survey of the construction of a data base of all rice varieties grown in Iraq, providing also additional genetic information of the agronomic and quality characteristics of rice variety.

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