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EVALUATION OF GENETIC DIVERSITY AND DNA FINGERPRINTING OF 19 STANDARD REFERENCE RICE VARIETIES USING SSR MARKERS

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SUMMARY

Molecular markers are advanced-tools for identifying new varieties at DNA levels. According to the International Union for the Protection of New Varieties of Plants, new breeded varieties need to be tested for the Distinctness, Uniformity and Stability (DUS), before being recognized as the new ones. Traditional DUS criteria based on 62 - 65 morphological and biochemical characteristics, which evaluated on comparison of new varieties with 19 standard reference varieties for traits of interest. Study on the genotypic polymorphism of 19 standard reference rice varieties provides genotypic information of these varieties for the evaluation of new rice varieties based on genotyping analysis. The reference marker set (30 markers) was used to evaluate the genetic diversity and DNA fingerprinting of 19 standard reference rice varieties. The results showed the similarity coefficient of 19 varieties varied from 0.04 to 0.548. At the genetic similarity coefficient of 0.1, the 19 rice varieties divided into two main groups. Group one included 3 varieties: DH1, DH5, DH13. Group 2 included the remaining 16 varieties. Inside group two, phylogenetic tree divided into two main branches at the genetic similarity coefficient of 0.3. Branch 1 includes 5 varieties including DH2, DH6, DH10, DH11 and DH7. The 11 remaining varieties were in the branch 2. The most closely varieties were DH6 and DH10 with the genetic similarity coefficient of 0.548. This study shows that, the standard reference varieties have high uniformity and high genotypic polymorphism, could used for testing new varieties based on genotyping by DNA fingerprinting combining with phenotype.

Keywords: *DNA, fingerprint, genetic similarity coefficient, marker.*

INTRODUCITON

In the early years of the 21st century, Vietnam was the second rice exporter in the world, and then became the first exporter in 2012. In order to further ensuring food security in the country as well as to hold the position, Vietnam must find ways to improve the rice productivity and quality as well as to enhance the pest resistance and environmental stress tolerance. The new variety being recognized must be passed the DUS tests (Distinctness, Uniformity, Stability) (Deniken, 2005; Michael and Simon, 2006). So far, the DUS test systems in many countries around the world mainly rely on morphological and biochemical traits. Recently, the use of DNA-based assessment methods for the DUS test has been applied in some countries (Michael and Simon, 2006). In Vietnam, to determine the correctness of the new variety as well as to avoid controversy, protect copyright, the construction of DNA fingerprinting set of the rice varieties is necessary. The DNA profile is an important data supporting for DUS test, because it provides an accurate assessment of the identification of a new plant variety. Thus, the data development of DNA fingerprint by modern biotechnology supporting for the DUS test is essential. In 1961, the International Union for the Protection of New Varieties of Plants (UPOV) was established with over 60 member's countries (UPOV, 1991). In 2007, Vietnam officially became the 63rd member of the UPOV. The application of copyright protection of new plant varieties became mandatory when Vietnam joined the WTO. There are five criteria for a new rice variety to be protected: commercial novelty, proper name, distinctness, uniformity and stability. The distinctness, uniformity and stability are technical standards, be determined by the DUS tests. The new varieties

cultivated and compared to existing varieties for the evaluation (Michael and Simon, 2006). In Vietnam, the National Center for Plant Testing has selected and used a set of 19 standard reference rice varieties to evaluate the 62 - 65 morphological and biochemical traits. There are some disadvantages of DUS test if only use morphology and biochemical traits, such as time-consuming (2 years), labor-intensive, less accuracy etc.

Today, with the development of molecular marker technology, overcoming these limitations has become easier. The use of molecular markers to evaluate genetic differences between new rice varieties in the DUS assay could be done quickly and accurately without depending on any external factors. Recently, many researchers have used SSRmarkers for genetics diversity and DNA fingerprinting to identify new rice varieties (Chakravarthi and Naravaneni, 2006; Giarrocco et al., 2007; Kalyan Chakravarthi B and Rambabu Naravaneni, 2006; UPOV, 1991)..

MATERIALS AND METHODS

Materials

Total of 19 standard reference rice varieties from The National Center for Plant Testing used for this study. Their names listed in table 1. Reagents and molecular chemicals used in this study for DNA polymorphisms analysis. The Reference SSR Marker set included 30 markers on table 2 (Tran Long et al., 2018).

Table 1. The list of the standard reference rice varieties set.

List	Rice varieties name	Encode	List	Rice varieties name	Encode
1	KoihikAi kazusa 2go	DH1	11	Khang dan 18	DH11
2	P6 dot bien	DH2	12	NTL 1	DH12
3	DTL2	DH3	13	NV1	DH13
4	Hoa khoi 4	DH4	14	Huong viet 3	DH14
5	Lc 93-1	DH5	15	ST7	DH15
6	Q5	DH6	16	NC5	DH16
7	Nc3	DH7	17	AC5	DH17
8	Q.nam 1	DH8	18	BM 9962	DH18
9	Bac thom so 7	DH9	19	MT131	DH19
10	Tran chau lun	DH10			

Table 2. The list of 30 reference SSR markers.

No.	Marker	Chro.	Allele	PCR Amplification Size (bp)	Forward (F) and Reverse (R) primer sequence
1	RM11	7	5	120-125-132-136-140	F:TCTCCTCTTCCCCCGATC R:ATAGCGGGCGAGGCTTAG
2	RM21	11	6	125-128-132-137-150-156	F:ACAGTATTCCGTAGGCACGG R:GCTCCATGAGGGTGGTAGAG
3	RM163	5	6	130-135-140-153-160-170	F:CGCCTTTATGAGGAGGAGATGG R:AAACTCTTCGACACGCCTTGC
4	RM481	7	12	124-132-139-149-154-158-172-177-182-192-210-224	F:TAGCTAGCCGATTGAATGGC R:CTCCACCTCCTATGTTGTTG
5	RM3412	1	11	148-150-154-155-160-163-165-167-168-182-190	F:TGATGGATCTCTGAGGTGTAAAGAG R:TGCACTAATCTTTCTGCCACAGC
6	RM1	1	6	80-89-92-105-122-125	F: GCGAAAACACAATGCAAAAA R: GCGTTGGTTGGACCTGAC

7	RM5	1	5	105-110-115-118-122	F: TGCAACTTCTAGCTGCTCGA R: GCATCCGATCTTGATGGG
8	RM6	2	4	142-150-156-165	F: GTCCCCTCCACCCAATTC R: TCGTCTACTGTTGGCTGCAC
9	RM17	12	6	150-154-160-175-180-185	F: TGCCCTGTTATTTTCTTCTCTC R: GGTGATCCTTTCCCATTTCA
10	RM25	8	6	128-132-134-140-142-145	F: GGAAAGAATGATCTTTTCATGG R: CTACCATCAAAACCAATGTTC
11	RM206	5	8	125-127-130-135-145-152-160-178	F: ATCGATCCGTATGGGTTCTAGC R: GTCCATGTAGCCAATCTTATGTGG
12	RM215	9	5	96-100-103-106-109	F: CAAAATGGAGCAGCAAGAGC R: TGAGCACCTCCTTCTCTGTAG
13	RM333	10	6	170-175-178-180-189-200	F: GTACGACTACGAGTGTCACCAA R: GTCTTCGCGATCACTCGC
14	RM3252	1	7	162-165-167-170-174-200-205	F: GGTAACTTTGTTCCCATGCC R: GGTCAATCATGCATGCAAGC
15	RM3843	4	4	165-170-175-182	F: ACCCTACTCCCAACAGTCCC R: GGGGTCGTACGCTCATGTC
16	RM7097	3	5	165-167-172-176-179	F: GGGAGGAGGAGAGGAGATTG R: TTAGGCCTGCACTTTTGGAG
17	R4M13	4	4	172-188-200-218	F: TACACGGTAGACATCCAACA R: ATGATTTAACCGTAGATTGG
18	MADS3	6	4	192-222-238-246	F: ATGCGGATAATCAAATAGACTACG R: CTGTGCTGGCCGGAGTGCT
19	SO1160	1	4	170-175-187-210	F: TTGCGATTTATTTGCCAGTG R: CCAGGCATCCAATGCTTATT
20	S11033	11	6	162-165-170-175-178-180	F: TGCCCTAGTCAGTCCCTCTG R: TTTTCGCGTACGGATAGGAT
21	RM19	12	5	190-202-210-225-227	F:CAAAAACAGAGCAGATGAC R:CTCAAGATGGACGCCAAGA
22	RM223	8	5	152-154-160-165-172	F:GAGTGAGCTTGGGCTGAAAC R:GAAGGCAAGTCTTGGCACTG
23	RM341	2	7	156-160-166-166-175-192-206	F:CAAGAAACCTCAATCCGAGC R:CTCCTCCCGATCCCAATC
24	RM3486	5	6	215-221-225-230-250-253	F:TCTCTTTTCCCTCCTTTCCC R:GGCCTGCAAGAGGAGAAAAC
25	RM5758	10	5	96-100-103-106-109	F:GAGGCCCTGATATTCAATGG R:TATGGCTTAGCGTTAGACCG
26	RM10825	1	4	82-87-92-100	F:GGACACAAGTCCATGATCCTATCC R:GTTTCCTTTCCATCCTTGTTGC
27	RM17954	5	7	150-162-167-170-175-180-184-195-200	F:ATTTCAGTACAAGGCACCCATGC R:GTAGACGAGGGAGTACCAACTTGC
28	RM26063	11	6	112-122-130-134-138-143	F:GATCCATATGCCTCTTCGATTGG R:AACTCCAGCAGTGAGAGCGTAGC
29	MADS8	1	3	150-175-200	F:TGCCGTTGCCCTAAGTTGTCTTCT R:AGGCCCTAGGGCTTGCTGTTTCT
30	EST20	11	4	187-196-213-218	F:GACCTGGCTGATCTGGCTTCTTCA R:AACTCCCCCATTTCTCGATGAGCT

Methods

Total genomic DNA extracted and purified by an improved CTAB method (Zheng *et al.,* 1995). PCR reactions were performed at the volume of 15 µl, including 2 µl DNA template (10 – 20 ng/ µl); 1.5 µl PCR buffer; 1 µl dNTPs (2 mM); 0.5 µl forward and reverse primers; 8.5 µl Q-water and 1 µl Taq polymerase (Fermentas, California, USA). Mixed PCR reactions were ran on Thermo cycler (Master cycler Pro S, Germany) type 96 wells. PCR program: 94^0C in 5 minutes, followed by 35 cycles of 94^0C for 1 minute, 55^0C for 1 minute, 72^0C for 1 minute, and the complete step at 72^0C for 5 minutes. PCR products stained with bromo-phenol-blue dye. They used for the electrophoresis on polyacrylamide gel 6 – 8 % at 100 volts. The gel added in SYBR-Safe staining (Invitrogen) to detect DNA bands. The genotypic data analyzed on NTSYS software 2.1. The tree diagram settled according to UPGMA method. Genetic similarities calculated according to the formula of Nei and Li (1972):

$$S_{ij} = 2a_{ij} / (2\ a_{ij} + b + c)$$

In which: S_{ij}: similarity between two samples j and i.

a_{ij}: is the number of DNA bands present in both sample i and j;

b_{ij} is the number of DNA bands available in sample i but not in sample j;

c_{ij} is the number of DNA bands available in sample j but not in sample i.

RESULTS AND DISCUSSION

To evaluate the uniformity of the 19 standard reference rice varieties, 50 seeds of each variety were germinated and used for DNA extraction. The DNA of 10 seeds from one variety mixed with equal molecule to form the combined DNA sample. Doing that way, each variety will have five DNA mixed samples available for uniformity testing. PCR amplification carried out on five SSR primers, including RM481, RM3412, RM163, RM11 and RM21. These primers are the most polymorphic primers in the 30 primers set. The PCR products analyzed on the polyacrylamide gel 6%. If the variety is uniformed, the five DNA mixed samples will show the same band size with one SSR marker on the gel running (figure 1).

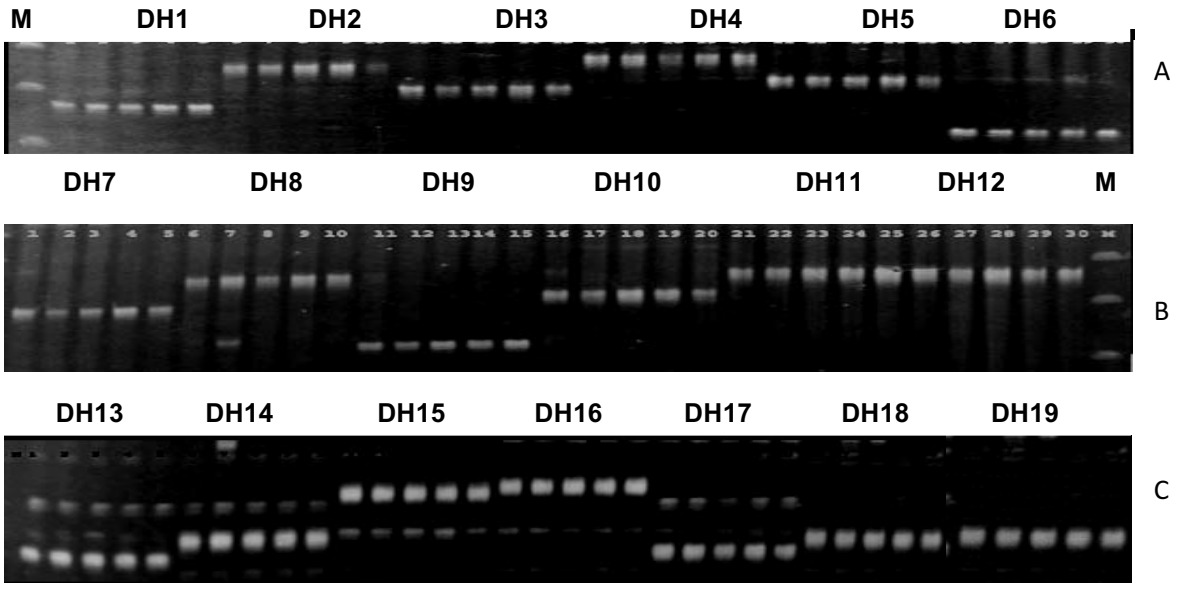

Figure 1. Results of electrophoresis on 6% polyacrylamide gel of PCR products of total DNA from 19 rice varieties using RM21 primer pairs. Figure A: M.50bp ladder; DH1: lane 1-5; DH2: lane 6-10; DH3: lane 11-15; DH4: lane 16-20; DH5: lane 21-25; DH6: lane 26-30. Figure B: DH7: lane 1-5; DH8: lane 6-10; DH9: lane 11-15; DH10: lane 16-20; DH11: lane 21-25; DH12: lane 26-30; M: 50bp ladder. Figure C: DH13: lane 1-5; DH14: lane 6-10; DH15: lane 11-15; DH16: lane 16-20; DH17: lane 21-25; DH18: lane 26-30; DH19: lane 31-35.

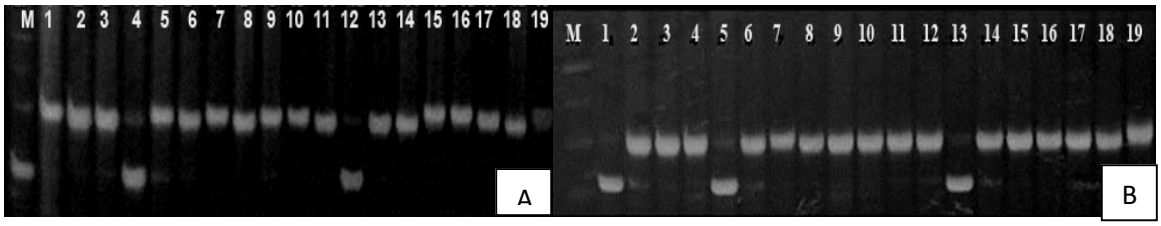

Figure 2: Results of electrophoresis on 6% polyacrylamide gel of PCR products of total DNA from 19 rice varieties using primer RM19 (figure A), MADS8 (figure B). In both figures: M: 50bp ladder; 1.DH1; 2.DH2; 3.DH3; 4.DH4; 5.DH5; 6.DH6; 7.DH7; 8.DH8; 9.DH9; 10.DH10; 11.DH11; 12.DH12; 13.DH13; 14.DH14; 15.DH15; 16.DH16; 17.DH17; 18.DH18; 19.DH19.

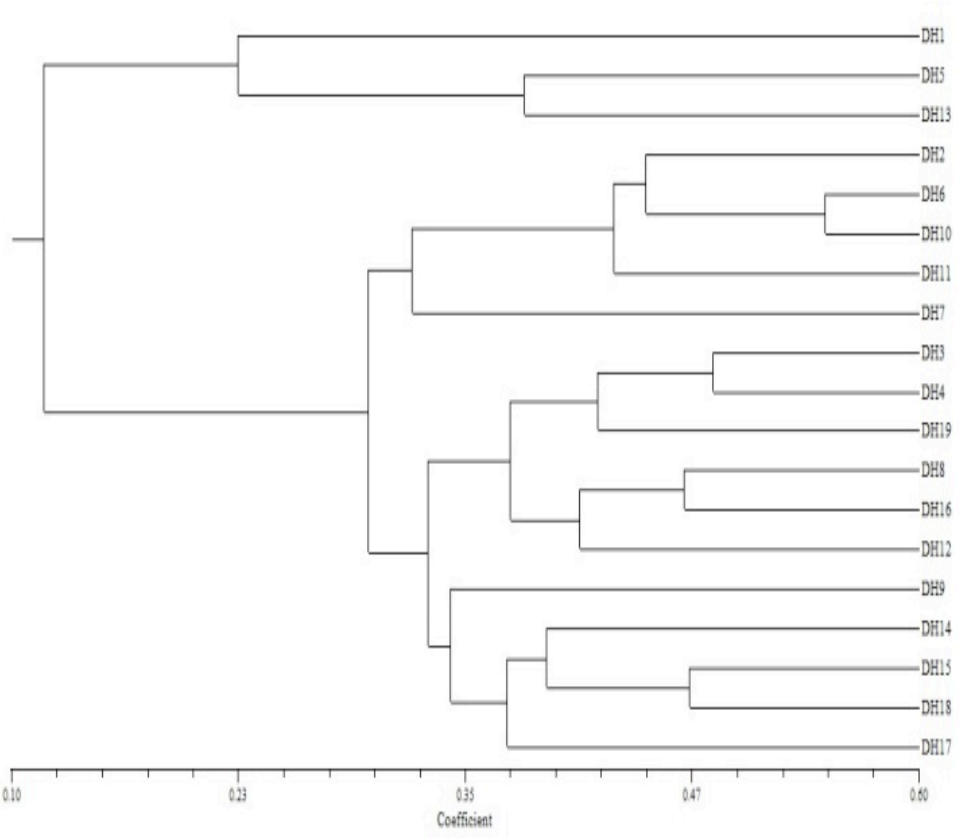

Figure 3. The genetic relationship tree of the 19 standard reference rice varieties when analyzing with NTSYS 2.1.

In figure 1, each of 5 lanes including PCR products from 5 DNA mix samples of one variety. "M" is the 50bp ladder; The order of the varieties is DH1; DH2; DH3; DH4; DH5; DH6; DH7; DH8; DH9; DH10; DH11; DH12; DH13; DH14; DH15; DH16; DH17; DH18; DH19, respectively. From the above results, it had concluded that these 50 seeds of each variety have identical genotypes. After testing 19 varieties with 5 primers, the results showed that all the 19 varieties were uniformity. The stability of 19 standard reference rice varieties was not checked here because they s have been used for many years in The National Center for Plant Testing as shown to be stable.

To calculate the distinctness of 19 varieties in this study, the DNA extracted of 19 standard reference rice varieties used as templates for PCR amplification. Total of 30 SSR primers used for this step. Results showed in table 2.

The genotype data collected from 19 varieties with 30 primers analyzed on NTSYS software 2.1. The genetic relationship tree of the standard reference varieties was constructed. Together with the genetic

relationship tree in figure 3, table 3 is the genetic similarity coefficient of 19 varieties. Based on that, the results showed the similarity coefficient of 19 varieties were 0.04 to 0.548. At the genetic similarity coefficient of 0.1, the 19 rice varieties divided into two main groups. Group 1 is included three varieties: DH1, DH5 and DH13. Group twice included the remaining 16 varieties. Inside group two, genetic relationship tree divided into two main branches at the genetic similarity coefficient of 0.3. Branch 1 included five varieties; they are DH2, DH6, DH10, DH11 and DH7. Branch 2 included 11 remaining varieties including DH3, DH4, DH19, DH8, DH16, DH12, DH9, DH14, DH15, DH18 and DH17. The most closely related varieties were DH6 and DH10, showing the genetic similarity coefficient of 0.548.

The result of this study demonstrated that the 19 standard reference rice varieties were very far away from each other based on the DNA fingerprint. It also approved that, testing new varieties by evaluating phenotype comparisons is time-consuming and labor-intensive. By contrast, the evaluation of new varieties by comparing genotypes will save time, effort and more accuracy.

For that reason, the application of DNA markers should be considered in evaluating new variety trials in the future.

Table 3. Genetic similarity coefficients of the 19 standard reference rice varieties.

DH19	DH18	DH17	DH16	DH15	DH14	DH13	DH12	DH11	DH10	DH9	DH8	DH7	DH6	DH5	DH4	DH3	DH2	DH1	Variety name
0.156	0.106	0.063	0.111	**0.040**	0.043	0.179	0.042	0.065	0.087	0.156	0.067	0.095	0.070	0.270	0.133	0.156	0.111	1.000	**DH1**
0.286	0.286	0.205	0.300	0.286	0.308	0.091	0.333	0.417	0.444	0.350	0.316	0.333	0.455	0.167	0.359	0.350	1.000		**DH2**
0.436	0.333	0.310	0.385	0.302	0.262	0.065	0.385	0.233	0.286	0.302	0.368	0.316	0.282	0.214	0.486	1.000			**DH3**
0.410	0.447	0.256	0.472	0.341	0.300	0.116	0.432	0.268	0.395	0.341	0.417	0.361	0.400	0.250	1.000				**DH4**
0.214	0.186	0.087	0.140	0.133	0.067	0.382	0.136	0.167	0.195	0.133	0.140	0.143	0.175	1.000					**DH5**
0.351	0.351	0.195	0.371	0.190	0.343	0.125	0.324	0.500	**0.548**	0.220	0.412	0.353	1.000						**DH6**
0.282	0.250	0.289	0.371	0.220	0.205	0.093	0.256	0.263	0.333	0.282	0.231	1.000							**DH7**
0.209	0.444	0.275	0.471	0.333	0.289	0.093	0.457	0.429	0.429	0.209	1.000								**DH8**
0.366	0.333	0.310	0.350	0.366	0.359	0.136	0.350	0.205	0.256	1.000									**DH9**
0.200	0.317	0.262	0.300	0.256	0.275	0.143	0.300	0.378	1.000										**DH10**
0.205	0.359	0.238	0.275	0.325	0.351	0.068	0.275	1.000											**DH11**
0.317	0.459	0.262	0.368	0.350	0.308	0.091	1.000												**DH12**
0.111	0.087	0.021	0.068	0.136	0.146	1.000													**DH13**
0.293	0.395	0.333	0.275	0.395	1.000														**DH14**
0.333	0.474	0.375	0.317	1.000															**DH15**
0.385	0.421	0.395	1.000																**DH16**
0.250	0.410	1.000																	**DH17**
0.400	1.000																		**DH18**
1.000																			**DH19**

CONCLUSIONS

The 19 standard reference rice varieties were uniform when evaluating with five SSR markers RM481, RM3412, RM163, RM11 and RM21. The distinctness of 19 varieties showed through the similarity coefficient of them was 0.04 to 0.548, between DH5 and DH19. At the genetic similarity coefficient of 0.1, the 19 rice varieties divided into two main groups. Group one includes three varieties: DH1, DH5 and DH13. Group twice included the remaining 16 varieties. DH6 and DH10 were the

most closed varieties at genetic similarity coefficient of 0.548. In the future, the application of DNA markers should be considered in evaluating DUS to save time, money, effort and increase the accuracy.

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SỬ DỤNG CHỈ THỊ SSR TRONG ĐÁNH GIÁ ĐA DẠNG DI TRUYỀN VÀ VÂN TAY DNA CỦA 19 GIỐNG LÚA CHUẨN

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TÓM TẮT

Chỉ thị phân tử là công cụ tiên tiến để xác định giống mới ở mức độ DNA. Theo Hiệp hội Quốc tế về Bảo hộ giống cây trồng mới, các giống cần được kiểm tra tính khác biệt, tính đồng nhất và tính ổn định (DUS), trước khi được công nhận là giống mới. Các tiêu chí DUS truyền thống dựa trên 62-65 đặc điểm hình thái và sinh hóa, được đánh giá thông qua so sánh các giống mới với 19 giống chuẩn đối với các đặc tính quan tâm. Nghiên cứu đa dạng di truyền của 19 giống lúa chuẩn để cung cấp thông tin kiểu gen của những giống lúa đó, nhằm phục vụ việc đánh giá các giống mới dựa trên phân tích kiểu gen. Bộ chỉ thị chuẩn (gồm 30 chỉ thị) được dùng để đánh giá đa dạng di truyền và vân tay DNA của 19 giống lúa chuẩn. Kết quả cho thấy, độ tương đồng di truyền của 19 giống từ 0,04 to 0,548. Ở mức hệ số tương đồng di truyền là 0,1, các giống lúa được chia thành hai nhóm chính. Nhóm một gồm 3 giống: DH1, DH5 và DH13. Nhóm hai bao gồm 16 giống còn lại. Trong nhóm hai được chia thành hai nhánh chính với hệ số tương đồng di truyền là 0,3. Nhánh một gồm 5 giống là DH2, DH6, DH10, DH11 và DH7. Nhánh hai bao gồm 11 giống còn lại. Hai giống gần nhau nhất là DH6 và DH10, với hệ số tương đồng di truyền đạt 0,548. Kết quả của nghiên cứu đã chỉ ra rằng, các giống lúa chuẩn có độ đồng nhất cao, độ đa dạng di truyền cao, có thể dùng để đánh giá các giống lúa mới dựa trên kiểu gen bằng các vân tay DNA kết hợp với kiểu hình.

Từ khóa: *chỉ thị, DNA, hệ số tương đồng di truyền, vân tay.*