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Simple sequence repeat (SSR) marker-based DNA fingerprinting of some varieties of rice (*Oryza sativa* L.) released in Nigeria

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Over time, precise, specific and rapid identification of variety have been achieved via fingerprinting with molecular markers. In this research, 13 out of 45 screened simple sequence repeat (SSR) markers were employed to fingerprint 27 rice varieties (22 are commercially released while five are suspected duplicates from the hands of marketers with different names) in Nigeria. The SSR primer pairs (13) were polymorphic and were found to generate 81 allele distinct reproducible bands with an average of 6.233 bands per primer pair. Primer RM400 had the highest allelic frequency of 0.94 resulting from 20 alleles. The polymorphic information content (PIC) values of each primer pair ranged between 0.31 and 0.93 with an average of 0.54. The unweighted pair group method with arithmetic (UPGMA) cluster analysis helped to separate the 27 varieties into 13 major groups indicating wide range of diversity. A large number of the closely related varieties were identified by means of the fingerprinting on the basis of the polymorphic SSR primer pairs. The findings showed a broad genetic variation among the test varieties giving a first-hand insight on how related some of the commercially released varieties are and also disproving the duplicity suspected from the materials with marketers.

Key words: Cultivar, DNA, fingerprinting, rice, simple sequence repeat (SSR) marker, variety verification.

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's seven billion people (Mohanty, 2013). It is an essential food crop globally. It grows in both temperate and tropical regions of the world. Rice belongs to the family Poaceae, tribe *Oryzae* and genus *Oryza*. There are 22 species of rice; of these 22 species, 20 are wild while only two (*O. sativa* and *Oryza glaberrima*) are cultivated. *O. sativa* is the most widely grown worldwide while *O. glaberrima* is grown only in West African countries. Most rice species are diploid (2n=24), although a few are tetraploid (2n=48). Elite cultivars along with rice seeds of high-quality play a vital role in rice production, hence, it is normal for new cultivars to be developed from hybridizations between the members that constitute an elite group of genetically similar parents, and the genetic variability amount among recently developed cultivars will possibly become even smaller (Rahman et al., 2008). This makes it more challenging to clearly distinguish

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cultivars from the others having morphological features and isozyme electrophoresis patterns due to influences by environmental elements.

In rice, there are abundant microsatellites and are distributed evenly across the genome (Akagi et al., 1996; McCouch et al., 1997). Due to their codominance, ability to detect high levels of allelic diversity, and efficient assay via the polymerase chain reaction (PCR) they are valuable as genetic markers (McCouch et al., 1997). Microsatellites exist as PCR-based markers that are cost-effective to use and technically efficient and are available for rice (Chen et al., 1997; Temnykh et al., 2000). Rapid, objective and precise cultivar identification is enabled by means of fingerprinting with molecular markers, and has been certified as an efficient tool for crop germplasm characterization, management and collection. There has been a large deployment of simple sequence repeat (SSR) markers for cultivar identification and genetic analysis due to their co-dominance inheritance, abundance, reproducibility, high polymorphism, and easy to read-off by PCR (Kuleung et al., 2004; Karihaloo et al., 2015; Xie et al., 2011). Past researches on rice have enhanced the development of hundreds of microsatellite markers and a genetic map comprising so many markers for documentation and characterization. In crop species, this approach has recently been used (Ngailo et al., 2016; Rodriguez-Bonilla et al., 2014; Yada et al., 2015; Yang et al., 2015). In Bangladesh, nine soybean cultivars were identified through microsatellite markers, which have provided distinctiveness and will perhaps be protective (Islam et al., 2007). Thirteen maize cultivars were also characterized using microsatellite fingerprinting in conjunction with DUS test (Molla et al., 2007).

Rice is the second major grain crop of Nigeria after maize. Nowadays, one of the hindrances to development of rice production is the duplicity of seeds within germplasm, markets and among farmers within the country. It is therefore, very important, to fingerprint the commercial rice varieties based on molecular markers as a crucial first measure to the ambiguity presently experienced by scientists and farmers and quick identification of similar or closely-related varieties. However, no research on the fingerprinting of the main commercial rice varieties under cultivation at present has been done in Nigeria till date. Therefore, this investigation was to give a firsthand insight on how related some of the commercially released varieties of rice are and some accessions collected within the Niger State region, as a first step to backstopping duplicity of released rice varieties in Nigeria using SSR molecular markers.

MATERIALS AND METHODS

Plant

Twenty seven rice samples, five of which are collected from farmers and markets and 22 of which are from the germplasm of National Cereals Research Institute (Table 1) were used for microsatellite analysis. Seeds were collected and were germinated at aseptic condition and grown in glass house for a period of three weeks.

Genomic DNA isolation

Using the protocol described by Aljanabi and Martinez (1997) with modifications, genomic DNA was isolated from the rice seedlings. Fresh leaf samples (22-days-old) of seedling were employed as the genomic DNA source. Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer (50 mM Tris-HCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 300 mM NaCl and 1% sodium dodecyl sulphate (SDS), pH 8.0). After incubation for 20 min at 65°C with intermittent swirling, the mixture underwent emulsification with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v). DNA was subjected to precipitation using two volumes of absolute alcohol in the presence of 0.3 M sodium acetate and pelleted by centrifugation. The pellets were thereafter washed with 70% ethanol, air-dried and resuspended in a suitable volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). DNA quality was further checked by means of electrophoresis in a minigel and quantification was attained using a spectrophotometer (Spectronic® Genesis™, Spectronic Instruments Inc., USA).

Microsatellite markers and PCR amplification

Thirteen out of 42 screened microsatellite primer pairs were used in the analysis (Table 2). PCR was done in a volume of 10 µl containing 50 ng template DNA, 1 µl 10X PCR buffer containing 15 mM $MgCl_2$, 0.25 mM each of the dNTPs, 0.25 µM of each primer, 1 U ampli Taq DNA polymerase (INQABA Ltd. South Africa) and an appropriate quantity of sterilized deionized water. Amplification was conduct using a thermo cycler (Thermal cycler gradient, Nyxtechnik, USA model A6) with the following program: initial denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final cycle at 72°C for 7 min. PCR products were examined in 2% agarose gel.

Determination of microsatellite allele lengths

PCR products were separated on 6% denatured polyacrylamide gel containing 19:1 Acrylamide:Bis acrylamide and 7 M urea. Electrophoresis was carried out on Sequi Gen GT eletrophoresis cell (Bio-neer Agaro power A7020, Korea). Gels were stained with silver nitrate using the Promega Silver Sequence TM protocol (Gustavo and Grsshoff, 1994).

Data analysis

The most intensely amplified band size (in nucleotides) for each microsatellite marker was calculated on the basis of its migration in relation to the molecular weight (mw) size markers, 100 bp DNA ladder (GENEI Pvt. Ltd. Bangalore, India) using the software DNA frag Ver. 3.03 (Nash, 1991). The data were inputed into a binary matrix as discrete variables; 1 for presence and 0 for absence of the character and this data matrix was subjected to additional analysis. The Excel file containing the binary data was imported into NT Edit of NTSYS-pc 2.02J. Using SIMQUAL subroutine in SIMILARITY routine, the 0/1 matrix was used to estimate similarity as DICE coefficient. Also, to construct dendrograms based on Unweighted Pair Group Method with Arithmetic Means (UPGMA), the resultant similarity matrix was employed to infer genetic relationships and phylogeny while NTSYS-pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software were

Table 1. List of the 27 varieties/accessions used for the fingerprinting.

S/N	Entry	S/N	Entry
1	FARO 34	15	FARO 33
2	FARO 15	16	FARO 37
3	FARO 16	17	FARO 44
4	FARO 17	18	FARO 50
5	FARO 19	19	FARO 51
6	FARO 20	20	FARO 60
7	FARO 21	21	FARO 61
8	FARO 22	22	FARO 62
9	FARO 26	23	ISHAQ
10	FARO 27	24	NDAYIKAKOO
11	FARO 30	25	PETERGII
12	FARO 31	26	WALUYEE
13	FARO 32	27	BERUWA
14	FARO 52	-	-

Table 2. Details of the polymorphic microsatellite markers used for rice genotype identification. SSR Primers from McCouch et al. (2002).

S/N	Primer	Forward	Reverse
1	RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
2	RM228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
3	RM251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
4	RM340	GGTAAATGGACAATCCTATGGC	GACAAATATAAGGGCAGTGTGC
5	RM341	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC
6	RM523	AAGGCATTGCAGCTAGAAGC	GCACTTGGGAGGTTTGCTAG
7	RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
8	RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT
9	RM256	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC
10	RM304	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG
11	RM463	TTCCCCTCCTTTTATGGTGC	TGTTCTCCTCAGTCACTGCG
12	RM400	ACACCAGGCTACCCAAACTC	CGGAGAGATCTGACATGTGG
13	RM244	CCGACTGTTCGTCCTTATCA	CTGCTCTCGGGTGAACGT

used to calculate Jaccard's similarity coefficient.

RESULTS AND DISCUSSION

All 27 cultivars of rice were completely amplified with 13 microsatellite primer pairs. On the basis of past results (Sefc et al., 2000), primer pairs will be known as loci whereas DNA bands as alleles. All 13 microsatellite primer from the 41 screened were found to be polymorphic, showing a total of 81 alleles with an average number of 6.32 alleles per locus among the 27 rice cultivars investigated (Table 3). From the banding patterns produced by the primer pairs, primers RM 288, RM 341 and RM 400 showed that they had the highest frequency of detected alleles of 7, 7 and 20, respectively. For the presence and absence of the SSR bands, all the genotypes were scored. Polymorphic Information Content (PIC) showed the range of the different primer pairs. Out of the 27 observations made for each of the primer pairs, RM400 had the least major allelic frequency of 0.111 which explains its high allelic number and resultant high gene diversity score (0.9410) and PIC number (0.9379). Allele frequency depends on the trait investigated which reflects directly on the allelic number and diversity of a sample as suggested by Fuentes et al. (1999) in their study. However, 13 different clusters were formed from the results of the dendogram.

Genetic diversity assessment is a key component in any breeding programme that is interested in conserving and characterizing its germplasm. Results obtained from this study indicated that more genetic diversity exist

Table 3. Marker Analysis showing gene diversity among 27 Rice varieties.

S/N	Marker	Major allele frequency	Number of obs.	Allele number	Gene diversity	PIC
1	RM225	0.7407	27.0000	4.0000	0.4252	0.3963
2	RM228	0.5926	27.0000	7.0000	0.5926	0.5524
3	RM251	0.3333	27.0000	6.0000	0.7545	0.7155
4	RM340	0.7778	27.0000	5.0000	0.3813	0.3646
5	RM341	0.5926	27.0000	7.0000	0.6173	0.5944
6	RM523	0.4444	27.0000	5.0000	0.6722	0.6162
7	RM224	0.5926	27.0000	5.0000	0.5871	0.5424
8	RM229	0.5185	27.0000	5.0000	0.6584	0.6167
9	RM256	0.5185	27.0000	6.0000	0.6612	0.6213
10	RM304	0.8148	27.0000	5.0000	0.3265	0.3137
11	RM463	0.6667	27.0000	3.0000	0.4829	0.4175
12	RM400	0.1111	27.0000	20.0000	0.9410	0.9379
13	RM244	0.6296	27.0000	3.0000	0.5240	0.4593
	Mean	0.5641	27.0000	6.2308	0.5865	0.5499

among the test entries in this gene pool (Fuentes et al., 1999; Qian et al., 1995). The result shows many cluster groups of closely related genotypes (Figures 1 and 2). However, the frequency of allele shared by the accessions clearly showed the genetic diversity present among the varieties and the higher the frequency of allele shared, the wider the diversity. It is also possible that the rice accessions closely linked to the released variety are similar or same as varieties or they are products of same cross and share parentage which speaks to denote their relative closeness.

Within breeding programs, genetic diversity is affected by classical breeding. Selection raises the alleles frequency or allelic combinations with positive effects at the expense of others which eventually eliminates many essential combinations of alleles (Cao et al., 1998). In this present investigation, SSR markers were used to characterize and assess genetic diversity among 27 rice entries of which 22 are commercially released rice varieties while 5 entries were suspected lines collected from within Niger State.

A total of 13 RM primers were utilized to provide genetic diversity or similarity among 27 commercially released rice variety and all 13 RM primers demonstrated polymorphism between 27 rice cultivars. A total of 351 bands made from 27 observations per SSR marker were scored and no bands were found to be monomorphic. The paper revealed that the primer RM400 had the highest number of alleles with a very low allelic frequency and showing very high gene diversity score. RM251 followed same pattern and similar to a work reported by Akagi et al. (1996). Significantly greater allelic diversity of microsatellite markers has also been reported in a number of studies than other molecular markers (McCouch et al., 2001).

Cluster analysis was employed in grouping the varieties and in constructing a dendogram. The similarity matrix representing the DICE co-efficient was used to cluster the data with the aid of the UPGMA algorithm. The UPGMA based dendogram obtained from the binary data deduced from the DNA profiles of the samples analyzed shows how diverse the test entries were clustering them into different groups. A total of 13 distinct groups resulted out of analysis of pooled SSR marker data (Figures 1 and 2). This dendogram revealed that the genotypes that are derivatives of genetically similar type clustered more together. The Beruwa and FARO 17 clustered in a group showing genetic closeness. Entry ISHAQ, FARO 31 and FARO 62 also clustered together which is indicative of genetic closeness. However, this investigation may not conclude that these clusters means duplicity but maybe said to be genetically close and would need further investigation to authenticate certainty.

In this study, the wide range of similarity values for cultivars revealed by micro satellite markers provides greater confidence for the assessments of genetic diversity and relationships, which can be used in future breeding programs. Principle component analysis was also done to visualize genetic relationships among the elite breeding lines (Figure 2). The results were similar to UPGMA results.

Conclusion

Based on this research, the wide range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of genetic diversity and relationships. The practical approach developed in this study is useful in DNA fingerprinting.

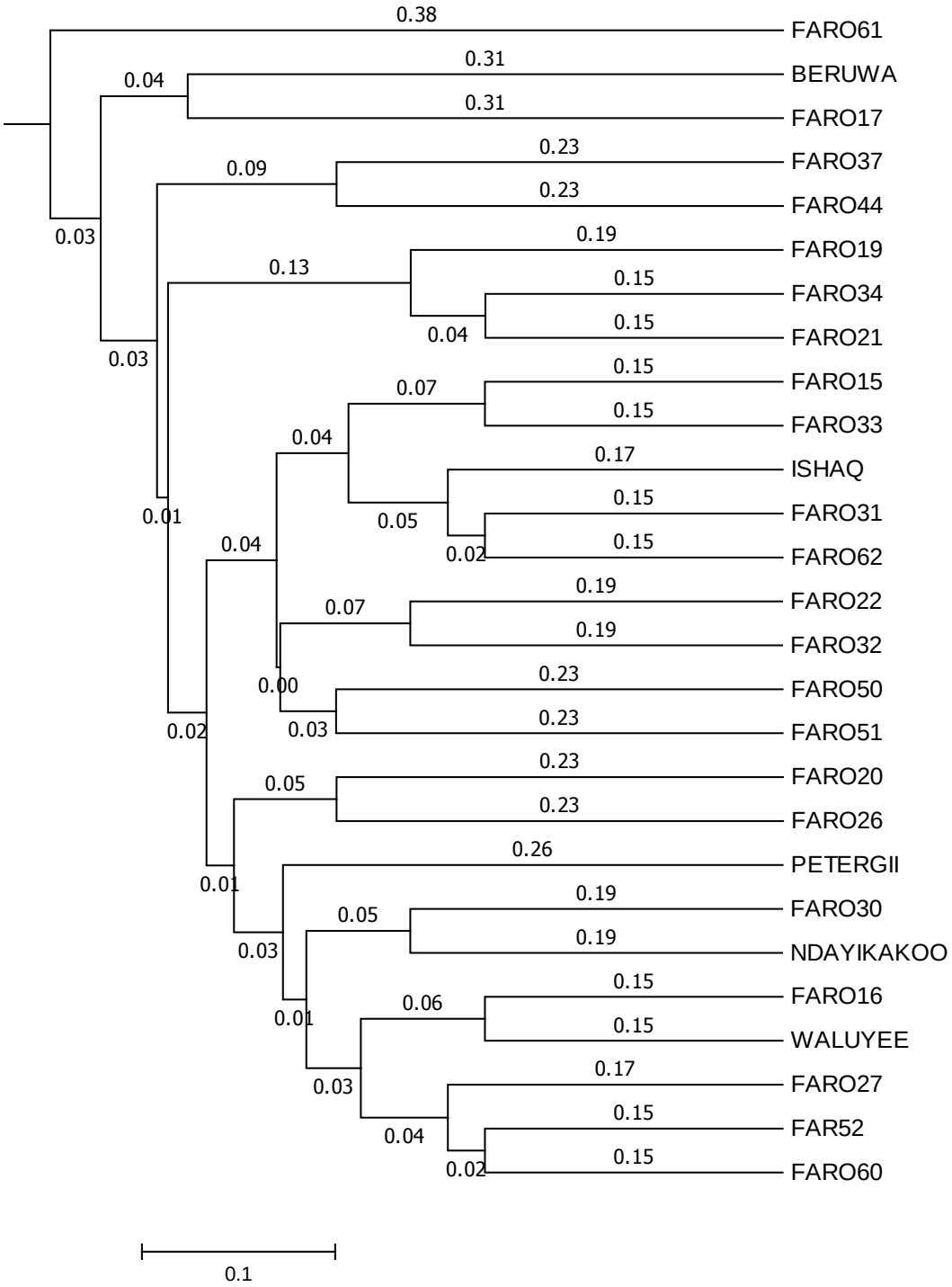

Figure 1. Cluster analysis of 27 accessions of rice showing their similarity.

Among the 45 SSR RM primers studied, 13 primers spread were found to be useful in fingerprinting of 27 commercially released rice varieties. This fingerprinting makes identification and characterization of genotype very easy and will be of greater help in background selections during back cross breeding programs.

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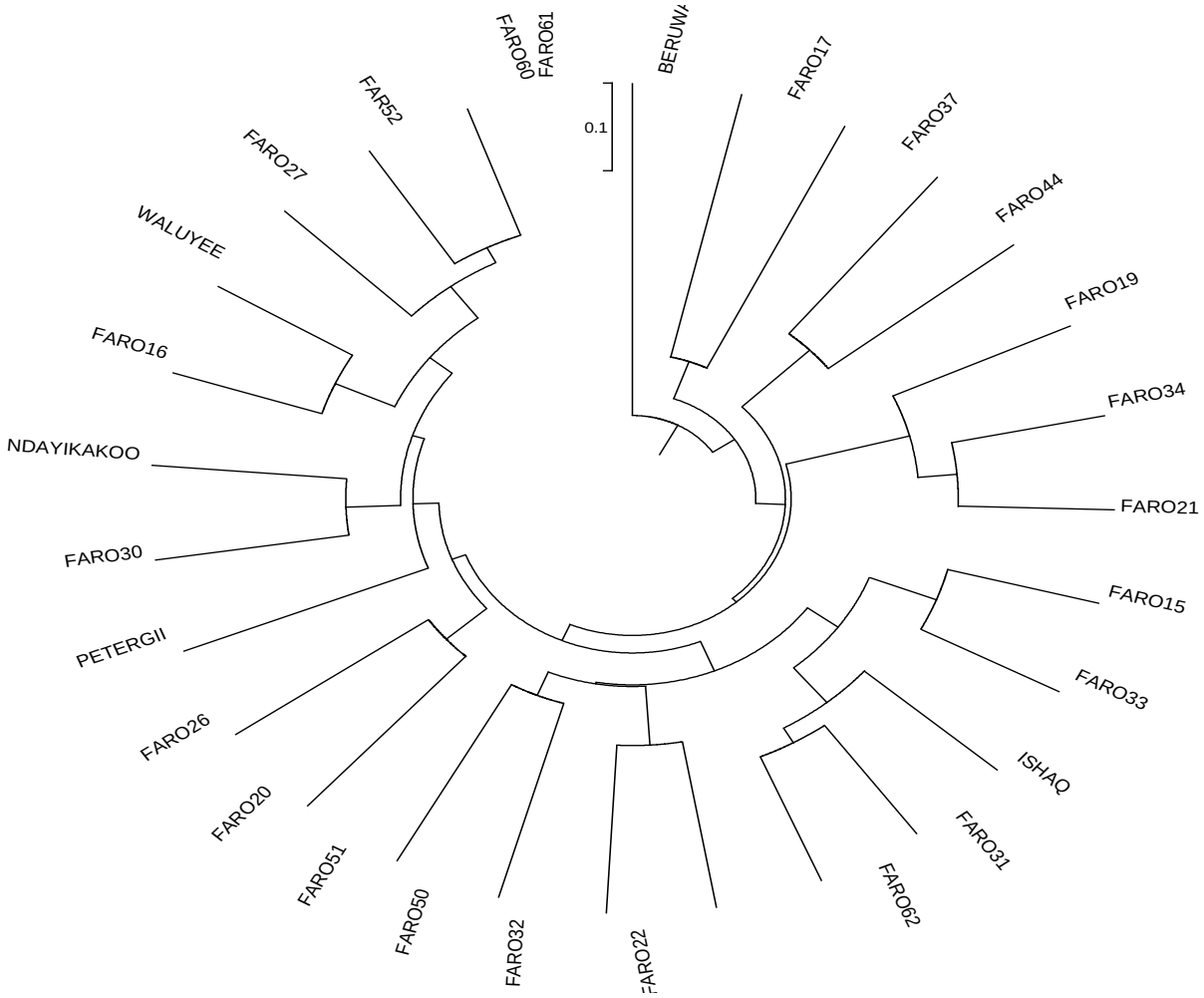

Figure 2. Dendogram showing 27 rice varieties and their relative diversity.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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