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Research article

DNA fingerprinting of foxtail millet (*Setaria italica* L.) variety ATL 1 using SSR and RAPD markers along with morphological descriptors

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Abstract: Foxtail millet (*Setaria italica*) is a cultivated nutritional cereal, which originated in South Asia and is considered one of the oldest cultivated millets in India. DNA fingerprinting is mandatory for registration of newly developed varieties with National Bureau of Plant Genetic Resources (NBPGR) and Protection of Plant Varieties and Farmers' Rights Authority (PPV&FRA). Due to the limited availability of genomic information in foxtail millet, the use of DNA based markers in fingerprinting of crop varieties is also limited. Hence in the present investigation, available RAPD and SSR markers of cereals are used for fingerprinting the foxtail millet varieties. The newly released variety ATL 1 is differentiated from popular variety CO (Te) 7 using SSR and RAPD markers. About 66 maize SSR primers, 16 sorghum SSR primers, and 10 RAPD primers were used in the study. Out of 66 maize SSR markers used for study, one showed polymorphism. The marker umc1704 showed polymorphism between CO (Te) 7 and ATL 1 by the presence of 670 bp allele CO (Te) 7. The RAPD primers OPB4, OPA5, OPA11 and OPB1 also helped for differentiation of the two varieties. The identified makers will help for genetic purity testing of CO (Te) 7 and ATL 1 in the seed chain.

Keywords: DNA fingerprinting - SSR - RAPD primer - Foxtail millet.

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INTRODUCTION

Foxtail millet (*Setaria italica* L.), is one among the cereal annual grass grown for human food (Heuze *et al*. 2015) belongs to the Panicoideae of Poaceae family. Foxtail millet is considered to be a dry farming cereals that forms the oldest cultivated food grain known to humans (Lu *et al.* 2009). It is an important nutritional cereal in China, India and part of Asia because of its drought tolerance and well adapted to arid and semiarid regions. The diploid nature of foxtail millet nowadays is considered as an ideal plant for the genetics of drought tolerance and nutritional research (Andrew *et al.* 1998). Currently, is becoming more popular among the urban population of India because of changing diet habit of the people. Foxtail millet has potential for abiotic stress tolerance and its genetic relatedness to many bioenergy sorts of grasses-like switchgrass, Napier grass and Pearl millet and hence use of genomic information from other cereals will help for detailed study (Muthamilarasan & Prasad 2015). Foxtail millet contains significant levels of protein, fibre, mineral, and phytochemicals. Anti-nutrients like phytic acid and tannin present in this millet are often reduced to negligible levels through modified method of processing. The millet is additionally reported to have hypolipidemic, low-glycemic index, and high antioxidant characteristics. (Sharma & Niranjan 2018). Application of molecular marker techniques to

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identify differences in cultivars is routinely followed in many kinds of cereal except the small millets like foxtail millet. The DNA fingerprints are accurate as it differentiates the individuals based on marker information which is further used for estimating genetic diversity, marker-assisted selection in plant breeding (Weising *et al.* 2005). Finally, DNA fingerprinting plays a vital role in protecting the novelty of a newly evolved plant variety which is submitted to NBPGR and PPV&FRA for notification and its registration. Simple Sequence Repeats are the tandem repeat of around six nucleotides in both the coding as well as non-coding regions. The SSRs have become a marker of choice in genotyping because of their abundance, high level of allelic variation, co-dominant inheritance and analytical simplicity (Miah *et al.* 2013). Moreover, microsatellite markers might be effectively applied to differentiate phylogenetically related species consistent with their conserved sequences, which could be useful to study the genetic constituents of the related species. RAPD marker provides amplicon from one individual and not to the another as it is a dominant marker and tedious to identify the amplified segment is from the heterozygous loci or homozygous loci (Williams *et al.* 1990). RAPD markers are well suited for DNA fingerprinting where the information on genomics about the crops like foxtail millet is limited. RAPD markers can also provide an efficient assay for polymorphism, which would allow rapid identification of DNA fingerprint for varietal identification. Hence the present investigation was focused on the development of varietal-specific fingerprints of popular foxtail millet variety along with the newly released variety for varietal identification and germplasm registration.

MATERIALS AND METHODS

Plant materials

The plant materials used for the study include foxtail millet cultivars *viz*., ATL 1 and CO (Te) 7. The variety CO (Te) 7 was a cross between CO 5 and ISe 248 which was a non-lodging and high yielding variety (Nirmalakumari *et al.* 2005). The tenai (Vernacular name in tamil) culture ATL 1 is a cross derivative of PS 4 x Ise 198 and evolved by Centre of Excellence in Millets, Athiyandal. This variety is going to replace already existing variety, CO (Te) 7 with high yielding ability and resistance to biotic and abiotic stresses for tenai growing regions of Tamil Nadu. The culture ATL 1 having bold grains, high nutrients, good grain qualities for value addition and nutritive and palatable fodder. The two foxtail millet varieties *viz*., ATL 1 and CO (Te) 7 are evaluated at Centre of Excellence in Millets at Athiyanthal for various morphological descriptors and DUS characters during Rabi 2020 season. The DNA extracted from two foxtail millet genotypes were used for fingerprinting with maize and sorghum SSR primers followed by RAPD primers.

DNA extraction and quantification

Total genomic DNA was isolated from foxtail millet cultures by using the modified CTAB method. The leaf sample of 1 g (from 15 days old crop leaf) was ground in CTAB buffer (120mM Tris, pH-8.0, 1.3M NaCl, 25mM EDTA pH-8.0, 0.2% β-mercaptoethanol, pinch of PVP , 2% CTAB), incubated at 65°C for 45 minutes. The extract was purified with 24:1 mixture of chloroform-isoamyl alcohol before precipitation of DNA with a double volume of isopropanol. The precipitate was separated out and dissolved with RNAse then followed by addition of 70% ethanol, sodium acetate and dissolved with TE buffer.

DNA product (2 µl) from each sample was tested for its quality through electrophoresis using 0.8% agarose gel having ethidium bromide in a 1x TAE buffer (40 mMTris, 20 mM acetic acid, and 1 mM EDTA.) at 100 V for about 30 minutes. Finally, the DNA segments on the gel were subjected to a Gel Doc unit and documented. Also, the quantity of the DNA was checked by spectrophotometer. The quantity of DNA was found to be high for all the genotypes. The ratio of 260/280 showed all the DNA samples scored between 1.8 and 2.0 which indicates the absence of other contaminants such as protein and RNAse.

Amplification of foxtail millet cultures was performed using 66 maize SSR primers, 16 sorghum SSR primers and 10 RAPD primers. Maize SSR markers were obtained from the database Maize GDB (https://www.maizegdb.org/datacentre/ssr). RAPD primer sequence obtained from Eurofins genomics India (Table 1).

PCR amplification with SSR and RAPD markers

The polymerase chain reactions (PCR) were performed using Eppendorf, Mastercycler Gradient, Germany. SSR markers in 10 µl reaction volume containing 7 µl of 1x master mix, 0.5 µM of both forward as well as reverse primers, 1 µl of sterilized water and about 200 ng (1 µl) of template DNA. The reaction conditions for maize genomic SSRs were initial denaturation for 7 min at 94ºC and 35 cycles of final denaturation for the 30s

duration at 94ºC, 30 s of annealing temperature at 55ºC, an extension of 45s at 72ºC, with a final extension of 7 min at 72ºC, hold at 10ºC.

Table 1. List of the Primers.

MAIZE SSR			SORGHUM SSR	RAPD PRIMERS
umc 1703	bnlg 2077	umc 1690	xtxp 316	opak 12
umc 1594	umc 1408	umc 2039	xtxp 043	opa 18
umc 1690	umc 1568	umc 1626	xtxp 248	opaw 09
umc 1028	umc 2230	umc 1256	xtxp 075	opa 04
umc 2204	umc 1505	umc 2101	xtxp 006	opb 4
umc 1552	umc 1678	umc 0081	xtxp 145	opb 1
umc 1970	phi 295450	umc 2163	xtxp 331	opa 5
umc 2101	umc 1223	umc 1353	xtxp 024	opb 6
umc 1166	umc 1257	umc 2129	xtxp 297	opa 11
umc 1474	umc 1446	umc 1586	xtxp 027	opaj 18
umc 0381	umc 1137	umc 2208	xtxp 021	
umc 1178	bnlg 198	umc 1525	xtxp 343	
phi 193125	phi 213914	umc 1082	xtxp 042	
bnlg 1209	bnlg 420	umc 0231	xtxp 018	
bnlg 589	bnlg 1396	umc 1703	xtxp 017	
bnlg 371	umc 2214	umc 1014	xtxp 008	
bnlg 198	umc 2324	umc 2257		
bnlg 469	umc 2071	phi 087		
umc 2204	umc 1144	umc 2049		
umc 1552	umc 1060	umc 2321		
umc 1970	umc 1076	umc 1127		
umc 1142	umc 2170	umc 1594		

The polymerase chain reactions (PCR) were performed using RAPD markers in 10 µl reaction volume containing 7 µl of 1x master mix, 1 µM of primer, 1 µl of sterilized water and about 200 ng (1µl) of template DNA. The reaction conditions for RAPDs were initial denaturation for 7 min at 94ºC and 35 cycles of final denaturation at 94ºC for the 30s duration. 30 s of annealing temperature at 37ºC, an extension of 45s at 72ºC, with a final extension of 7 min at 72ºC, hold at 10ºC.

PCR product analysis and documentation

The total volume of the amplified product (10 µl) of each sample was subjected to electrophoresis on 3% agarose gel containing ethidium bromide in 1x TAE buffer at 120 V for 2 h. Finally, the DNA bands were observed on a Gel Doc system and the photographs were captured. The SSR profiles were analysed based on pattern bands. Only clear and unambiguous SSR bands were scored based on base pair (bp) size in each genotype. The amplified product (10 µl) from all the samples were separated through electrophoresis on 1.5% agarose gel having ethidium bromide intact in 1x TAE buffer at 120 V for 1.5 h. Finally, the DNA bands were observed on a Gel Doc system and documented. Scores were done based on the presence or absence of the amplicon.

Morphological descriptors and DUS characters

The crop was raised during Rabi 2020 at Centre of Excellence in Millets, Athiyanthal in the Advanced Yield Trail (ART). The following morphological and the quantitative traits were recorded *viz*., Days to 50% flowering (day), Plant height (cm), Number of basal tillers, Flag leaf length (cm), Flag leaf width (cm), Peduncle length (cm), Panicle length (cm), Panicle exertion, Days to maturity (day), Grain yield per plant (g), Fodder yield per plant (g), Thousand-grain weight (g), Plant pigmentation at flowering, Leaf colour, Blade pubescence, Sheath pubescence, Degree of lodging at maturity, Midrib colour, Inflorescence lobes, Inflorescence bristles, Inflorescence shape, Inflorescence compactness, Fruit colour, Grain shape, Apical sterility in panicle. The mean data is compared between the accessions (Table 2) of the varieties under study are also conducted.

RESULTS AND DISCUSSION

Amplification of Foxtail millet DNA was done using 66 Maize SSR primers, 16 sorghum SSR primers, and 10 RAPD primers. All the primers were initially standardized for the unique amplicon using the gradient PCR. Most of the SSR primers showed unique amplicon between 100 to 300 bp sizes. The RAPD markers showed multiple alleles. The polymorphic primer was always used for the differentiation of cultivars.

SSR marker studies

The SSR primers derived from the maize and sorghum database were used for the amplification of DNA from CO (Te) 7 and ATL 1 varieties. The amplified products having polymorphic nature were scored. Out of 66 maize SSR markers used for the study, only one SSR marker umc1704 showed polymorphism. The other 65 maize SSR markers showed monomorphic bands. The marker umc1704 showed polymorphism for the varieties CO (Te) 7 and ATL 1 by the presence of 670 bp and 680 bp alleles respectively (Fig. 1). The marker umc1704 showed polymorphism for the varieties, CO (Te) 7 and ATL 1 at around 670 bp which was used to differentiate these varieties. The similar results are in accordance with Jia *et al*. (2007), Chandrashekara *et al*. (2007), Panwar *et al*. (2010).

Sixteen sorghum SSR markers were used for polymorphism survey using varieties CO (Te) 7 and ATL 1. But none of the markers showed the polymorphism between the varieties.

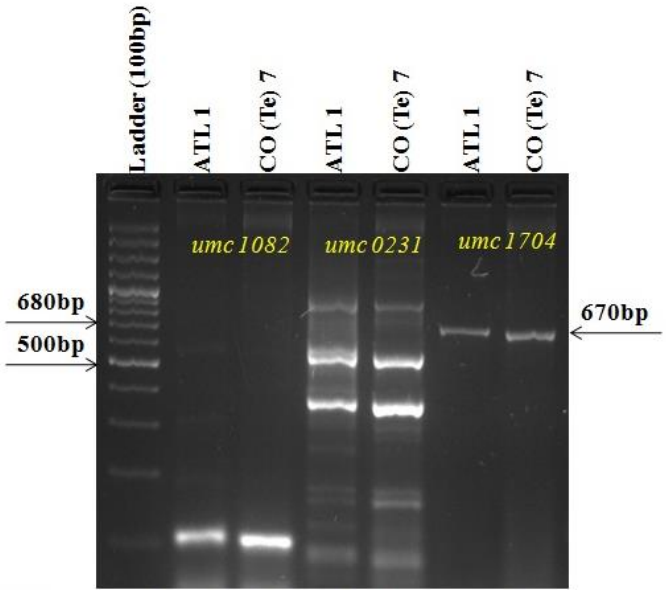

Figure 1. DNA finger printing of foxtail millet varieties using SSR markers.
[Note: Polymorphic primer (umc 1704); Forward primer- TTCACCGGGTAGTCCTTCTTACTG; Reverse primer- AAGTACGCTGTACGCAGGCAG; Annealing temperature- 55ºC; Cycles amplification- 35; Amplicon size- ATL 1 (680 bp); CO (Te) 7 (670 bp)]

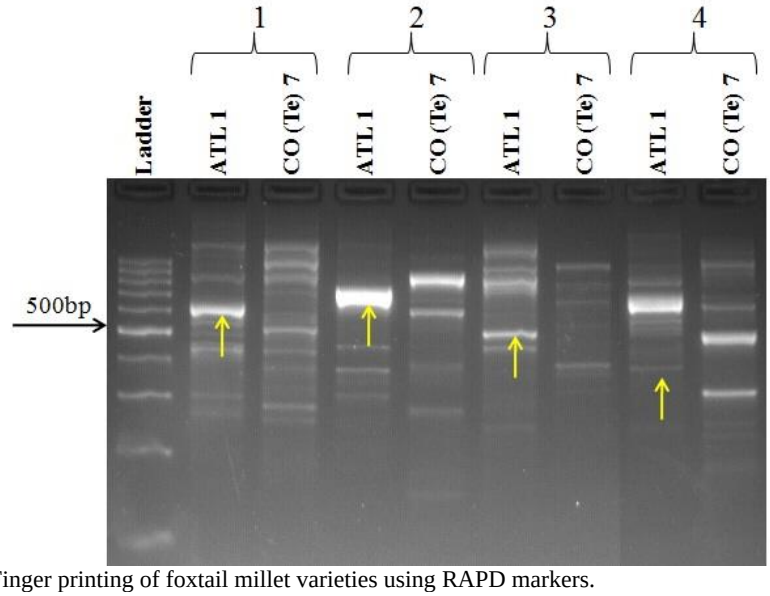

Figure 2. DNA Finger printing of foxtail millet varieties using RAPD markers.
[1- OPB4 (GGACTGGAGT); 2- OPB1(GTTTCGCTCC); 3- OPA5 (AGGGGTCTTG); 4- OPA 11(CAATCGCCGT); Annealing temperature- 37ºC; Cycles amplification- 35]

RAPD marker studies

A panel of RAPD marker survey was followed to identify the polymorphic fragments for the fingerprinting purpose. In this study, the RAPD marker showed clear discrimination between varieties. Ten RAPD primers were used for DNA fingerprinting (Table 1) of foxtail millet varieties. Out of ten primers used, four primers

namely OPB4, OPB1, OPA5 and OPA11 produced the most distinguished and scorable alleles. For the RAPD primers *viz*., OPB4 and OPB1 the alleles are present at 500–600 bp and 600–700 bp in ATL 1 respectively which were absent in CO (Te) 7. Similarly, for other primers like OPA5 and OPA11, the alleles were observed at 400–500 bp and 300–400 bp for ATL 1 (Fig. 2). It was found that there was no amplicon for CO (Te) 7 variety at the same location. The DNA fingerprint generated by each RAPD primer was scored for the presence of specific alleles. RAPD analysis was first developed to detect polymorphism between organisms, despite at the time absence of genomic sequence information in many crops. The RAPD markers also earlier used to develop genetic markers and to construct a genetic map. The different number of bands was observed in PCR for different primers which might be due to the sequence of primer and availability of complementary sequence in the genome or template quality. Similar results were accorded by Sastry *et al*. (1995), Gupta *et al*. (2010), Kumari & Pande (2010).

Morphological traits differentiation between ATL 1 and CO (Te) 7

The morphological traits differentiation between the foxtail millet cultures *viz*., ATL 1 and CO (Te) 7 was discussed in table 2. The pre-release culture flowered within 50 days after sowings which is earlier than the Co (Te) 7 variety which flowered in 55 days after sowing. The height of the crop was found to be 110–120 cm. (Fig. 3) Grain yield per plant of the pre-release culture was 14.5 g which was more than CO (Te) 7 variety which yielded 12.0 g. Per plant grain yield of ATL 1 accounted 20.8% increase over the check variety CO (Te) 7. When comparing the plant height, CO (Te) 7 will be growing taller to 120 cm than the pre-release culture which grows upto 115 cm only. Apart from the height, while comparing the maturity period, the pre-release culture will attain the maturity in 83 days earlier than the CO (Te) 7 which attains maturity in 88 days. In comparison with CO (Te) 7, the variety ATL 1 (3.6 g) has 3.7% increase in thousand-grain weight over CO (Te)

Table 2. Descriptors of foxtail millet culture ATL 1 and CO (Te) 7.

S.N.	Character	ATL 1		CO (Te) 7 *	
		Range	Mean	Range	Mean
1	Days to 50% flowering (day)	48.0–53.0	50.0	53.0–58.0	55.0
2	Plant height (cm)	110.0–120.0	115.0	115.0–130.0	120.0
3	No. of basal tillers	5.0–8.0	6.0	6.0–9.0	7.0
4	Flag leaf length (cm)	32.0–42.5	37.8	30.5–40.6	35.9
5	Flag leaf width (cm)	1.3–2.9	2.4	0.8–2.4	1.6
6	Peduncle length (cm)	15.6–26.2	21.3	13.5–21.2	18.5
7	Panicle length (cm)	28.3–32.8	28.5	25.6–33.9	29.0
8	Panicle exertion		Full exertion		Full exertion
9	Days to maturity (day)	80.0–85.0	83.0	85–90	88.0
10	Grain yield per plant (g)	12.2–19.5	14.5	10.0–16.0	12.0
11	Fodder yield per plant (g)	15.9–23.3	19.3	14.2–20.1	15.6
11	Thousand grain weight (g)	3.1–3.9	3.6	2.8–3.0	3.2
12	Plant pigmentation at flowering		Green		Green to purple
13	Leaf colour		Green		Green to purple
14	Blade pubescence		Intermediate		Intermediate
15	Sheath pubescence		Glabrous		Glabrous
16	Degree of lodging at maturity		Non-lodging		Non-lodging
17	Senescence		Green at maturity		Yellow at maturity
18	Midrib colour		White		Green
19	Inflorescence lobes		Medium		Short primaries
20	Inflorescence bristles		Short		Very short
21	Lobe compactness		Compact		Intermediate
22	Inflorescence shape		Oblong		Cylindrical
23	Inflorescence compactness		Compact		Compact
24	Fruit colour		Brownish Yellow		Yellow
25	Grain shape		Elliptical		Oval
26	Apical sterility in panicle		Absent		Present

7 (3.2 g) (Fig. 4). Fodder yield per plant of pre-release culture is 19.3 g whereas CO (Te) 7 has 3.2 g test weight, which is more than CO (Te) 7 which yields only 15.6 g. During maturity, the pre-release culture will be green in colour and CO (Te) 7 will be in yellow during maturation. This pre-release culture variety can also be used as fodder since it is green in colour even during maturity. The grain shape varies from each other, ATL 1 is elliptical and the check variety is oval. Apical sterility is absent in ATL 1 and present in CO (Te) 7 variety. Fruit colour of ATL 1 is brownish-yellow in colour and CO (Te) 7 variety is yellow. The count of basal tillers was

found to be 5 to 8 per plant. The plant maintains green pigmentation at flowering, and it retains green colour even at senescence stage. The inflorescence lobes were compact and medium, bristles were short and oblong. The apical sterility of the panicle is absent, which was present in CO (Te) 7.

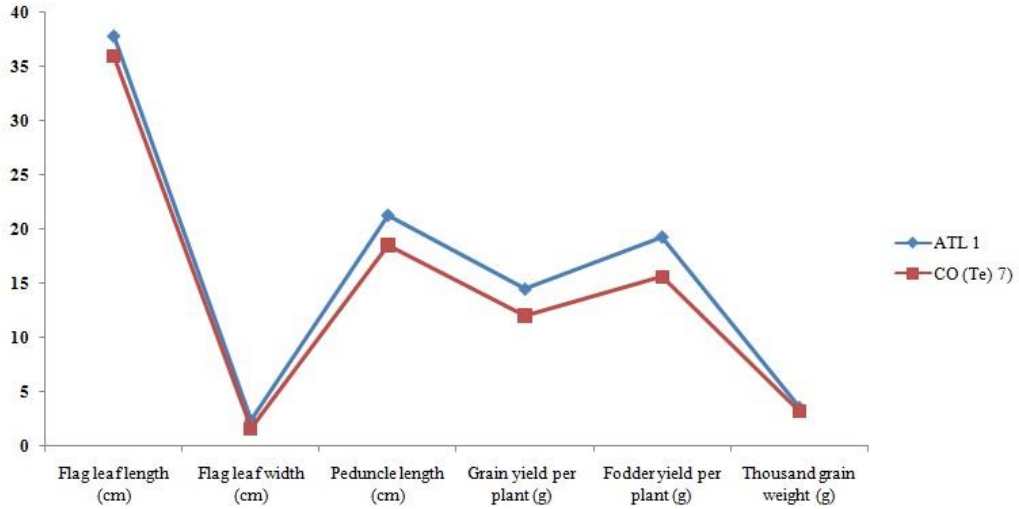

Figure 3. Quantitative characters differentiating the newly released variety (ATL 1) and the existing variety (CO (Te) 7.

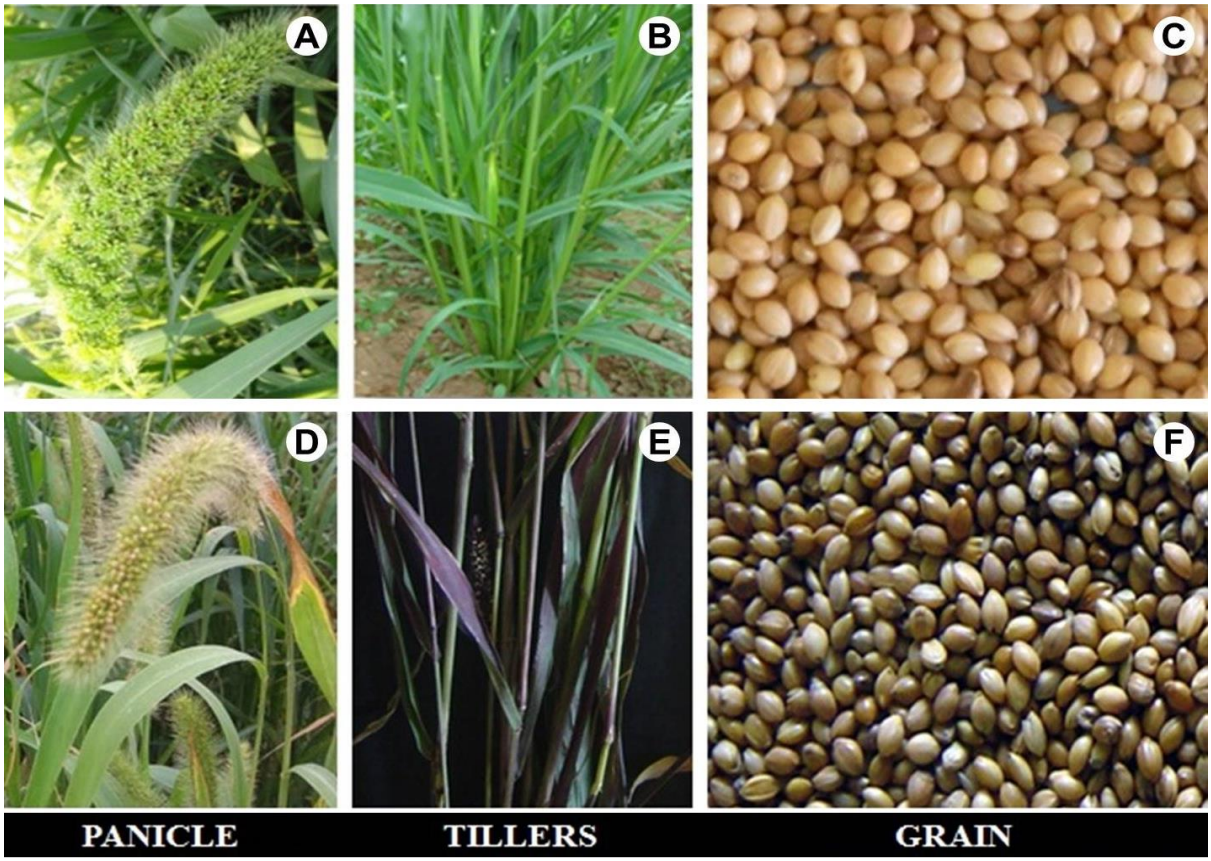

Figure 4. Morphological representation: **A–C**, The newly released variety ATL 1; **D–F**, The existing variety CO (Te) 7.

Earlier report of Foxtail millet says that the plant can be harvested at 75–90 days after planting (DAP) (Cash *et al.* 2002). It can produce good quality hay when gathered into windrows and left until fall/winter grazing (Koch 2002). Plant height is about 120 to 200 cm. It has dense inflorescence and panicle is hairy with 10 to 30 cm long which gives the shape of fox's tail. Its grain diameter is about 2 mm. A grain of foxtail millet is about 2 mm in diameter. Foxtail millet, when grown for hay or silage, takes 65 to 70 days. When grown for the grain, the harvest is after 75 to 90 days (Nirmalakumari *et al.* 2005).

CONCLUSION

The DNA based fingerprinting was generated for the most common cultivated variety CO (Te) 7 and newly released high yielding variety, ATL 1 using SSR and RAPD markers. Hence marker-based cultivar

identification is demonstrated in foxtail millet which will be useful and highly practical, reliable, and efficient. The marker umc1704 showed polymorphism for the varieties, CO (Te) 7 and ATL 1 at around 670 bp. The RAPD studies also helped for developing markers using the primers OPB4, OPA5, OPA11 and OPB1which can be used for screening new cultivars for fingerprinting purpose.

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