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Optimization of a High Throughput, Cost Effective, and All-stage DNA Extraction Protocol for Sorghum (*Sorghum bicolor*)

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Abstract: The extraction of DNA for molecular analyses is often a limiting factor in terms of cost, time, and availability of skilled labor. Here the authors describe a simple extraction protocol for obtaining DNA from field and greenhouse grown plants. This method was optimized for sampling mature wild sorghum populations in remote areas of Ethiopia and young greenhouse grown seedlings for genetic studies using microsatellite loci. Initially, the leaf squashes are made on Whatman FTA® plant saver cards or small cards of Whatman® chromatography paper. After several washes, sufficient DNA to run up to 70 PCR amplifications is eluted from a 6 mm disk. Both types of cards were equally effective for collecting genomic DNA from young and mature sorghum plants for PCR-based analyses. The use of this technology for extracting genomic DNA from seedlings and/or mature plants *in situ* is particularly attractive for sampling at sites that are far from the laboratory where samples are ultimately analyzed. Moreover, highly skilled personnel are not required to collect DNA samples using this protocol. The Whatman FTA® card is more expensive than the Whatman® chromatography paper. Therefore, without compromising efficiency, the lower cost chromatography paper can be used for DNA extraction, especially for institutions in developing countries.

Key words: Cost effective, DNA extraction, FTA® card, growth stage, sorghum, PCR, molecular markers.

1. Introduction

Sorghum is the fifth most important grain crop in the world based on tonnage (www.fao.org). Moreover, it is the third most important crop in its center of origin, Ethiopia. Its many uses for food, feed, fiber, and fuel, coupled with its ability to cope up with unfavorable growing conditions, make it the preferred crop in many developing countries where it serves as a staple food. Increased demand for limited fresh water supplies, increasing use of marginal farmland, and global climatic trends all suggest that sorghum will continue to be important in feeding the world's expanding populations [1].

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Molecular tools are now available for sorghum genetic analysis and improvement. The use of gene bank accessions for determining population genetic structure, gene flow, mating systems and genetic diversity is common. However, since the accessions were often collected years ago and many no longer represent sorghum populations at the site of collection, inferences based on these conserved *ex situ* collections may have little relevance for current ecological and evolutionary assessments. Recent, *in situ* collections from sorghum's center of origin are preferred for these genetic analyses. However, facilities and infrastructure are limited in the developing countries in Africa, where the analyses are most needed. It is difficult to collect *in situ,* fresh leaf samples from remote areas; there is a shortage of skilled lab personnel to perform collections

and extractions, and exportation of the seed samples to other countries is often restricted.

A number of protocols have been developed for extraction of DNA from fresh and dry plant leaves [2-9]. Leaves of different species contain varying concentrations of nucleic acids and many plant leaves contain inhibitory secondary compounds. Thus, a single nucleic acid isolation method is not likely to be suitable for all plants [10]. The modified Murry and Thompson [11] DNA extraction protocol described by Saghai-Maroof et al. [12] is commonly used for sorghum. This protocol yields high quality DNA, but requires a relatively large initial amount of freeze dried tissue and must be performed in a well equipped laboratory by trained lab personnel. Thus its use for field collections in the center of origin of sorghum is limited.

Recently, Mbogori et al. [13] optimized the Flinders Technology Associates (FTA®) technology for large scale DNA isolation for use in marker assisted selection in maize. The FTA® card is impregnated with a substance that lyses cell membranes, denatures proteins on contact, and protects nucleic acids from nucleases, oxidation, UV damage and microbial and fungal attack (www.whatman.com). This technology, which results in a dry DNA samples on the card, may be used to safely and efficiently exchange genetic material between countries without the risk of transmitting disease or insect pests, which can be associated with the transfer of seeds. Although the use of the FTA® card technology for *in situ* collections shows promise, the cost of the cards may be prohibitive and the requirement of a separate DNA extraction form each amplification may be unrealistic for researchers in developing countries. The objectives of this study were to compare the effectiveness of the standard FTA® plant card *versus* Whatman chromatography paper for extracting DNA from both seedlings and mature sorghum plants, and to access the practicality of using a single extraction protocol to extract sufficient DNA for multiple PCR analyses.

2. Materials and Methods

2.1 Plant Material

Leaf tissue from 600 mature wild/weedy sorghum plants from 30 populations in five regions in Ethiopia was used in the study. The authors also evaluated the effectiveness of extractions for 1021, 2-3 months old sorghum seedlings. These seedlings, progeny of the mature wild/weedy plants, were germinated in a greenhouse from January through March 2009 at Melkassa Agricultural Research Center, Ethiopia. In addition, samples from volunteers of cultivated sorghum were used in a preliminary experiment carried out at Ohio State University, USA (Table 1).

2.2 DNA Extraction and PCR Run

DNA was collected from leaves of mature wild sorghum plants in the field from October to November 2008 and from seedlings from January to March 2009. Samples were taken from the second or the third leaf, whichever was more succulent and free from disease lesions. Seedlings were uniformly succulent and whole seedlings were used for extractions. Flag leaves of mature plants did not produce adequate staining and thus were not used for the squashes. The extraction procedure consisted of collecting squashes from fresh plant leaves using FTA® cards or cards made from Whatman chromatography paper, allowing the plant squashes to dry, washing the DNA on cards, and eluting DNA into a buffer solution. Whatman chromatography paper cards were prepared by folding 3 cm × 10 cm pieces of Whatman chromatography paper in half. As suggested by the manufacturer [14], leaf squashes were taken onto the cards by pounding with a pestle until the card was stained. Alternately, the authors used a shoe horn to rub the leaves and lyse plant cell so that cards were well stained with squashes. For the FTA card, a piece of the leaf was placed in between the polyethylene bag and the appropriate section of the card with the back of the leaf facing towards the card. The card was placed on a plastic

Table 1 Source of sorghum DNA samples, extraction methods, PCR primers, and visualizations.

Source	Specific area	Age of tissue	Squash source	Extraction protocol	Primer	Visualization
OSU		Mature plant	Frozen tissue	Whatman Card	SB6-36	SFR Agarose gel
OSU		Mature plant	Frozen tissue	FTA Card	SB6-36	SFR Agarose gel
OSU		Seedling	Frozen tissue	Whatman Card	SB6-36	SFR Agarose gel
OSU		Seedling	Frozen tissue	FTA Card	SB6-36	SFR Agarose gel
Ethiopia	Awash	Mature plant	Sampled in field	Whatman Card		ABI Prism 3100 Genetic Analyzer
Ethiopia	Ghibe	Seedling	Fresh leaf from greenhouse	FTA Card		ABI Prism 3100 Genetic Analyzer
Ethiopia	Ghibe	Mature plant	Sampled in field	Whatman Card	Sb5-256 Sb6-84 Sb6-36	ABI Prism 3100 Genetic Analyzer
Ethiopia	Pawe	Mature plant	Sampled in field	FTA Card	Sb5-236 Sb6-84 Sb4-72	ABI Prism 3100 Genetic Analyzer

covered wooden board in the field or on a table in the greenhouse, and the sample was rubbed using a shoe horn and/or pounded with a pestle until it was well soaked from the back of the card. Cards were allowed to air dry for a minimum of 1 hour under shade before storage. Materials (scissors, the polythene sheet covering the crushing board and shoe horn/pestle) were rinsed or wiped with 70% ethanol between each sample to avoid cross-contamination. Moreover, a new polythene bag was used for each extraction.

Extractions were carried out two to twelve months after samples were collected on the cards. A single hole paper punch was used to remove a 6 mm disc from the center of the dried sample area on the FTA® and/or Whatman card. The disc was transferred to a 1.5 mL microfuge tube. In order to ensure no residue was carried over to the next sample, the punch tip was triple rinsed in distilled water, wiped with a tissue paper, and punched onto a blank chromatography paper between samples. After adding 400 μL of FTA® purification reagent (Whatman Inc., USA) to each tube, tubes were vortexed and samples were incubated for 4 minutes at room temperature. Used FTA® purification reagent was discarded using a pipette so that the disc remained in the tube. The FTA® purification reagent wash was repeated once. The disk was then rinsed with 400 μL of a modified TE^{-1} (10 mM Tris, 0.1 mM EDTA) buffer in a similar manner. The TE^{-1} buffer rinse was repeated once. Using tweezers, the disk was transferred to a 0.5 mL microfuge tube and 80 μL of TE (10 mM Tris, 1 mM EDTA) was added. After centrifugation, the disk was incubated in the buffer for 5 minutes at 95 ℃, cooled on ice, and stored at -20 ℃.

In the preliminary OSU experiment, sorghum DNA was amplified as described by Taramino and Tingy [15]. Briefly, 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 50 pmol of each primer, 200 μM of each dNTP, 90 mM Tris-HCl (pH 8.5), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, and 5 ng genomic DNA were used in each 20 μL reaction. DNA was amplified using a Robocycler® (Stratgene, La Jolla, CA) programmed for 3 min at 94 ℃, 35 cycles of 1 min at 94 ℃, 1 min at 58 ℃, 2 min at 72 ℃, and a final 15 min extension at 72 ℃. All OSU samples were amplified using primer SB6-36 [16]. Samples from mature plants and seedlings from Africa were amplified using a QIAGEN® SSR multiplex kit, as described by the manufacturer, and a similar temperature cycling regime, except that initial incubation and final extension times were increased to 15 and 30 min., respectively.

Amplified DNA products were separated on 3% super fine resolution (SFR) agarose gels stained with ethidium bromide and viewed under UV transilluminator. Fluorescently labeled amplified fragments from crop were separated and detected with

an ABI Prism 3100 Genetic Analyzer (Foster City, CA). Fragment sizes of the SSR loci were determined using the GeneMapper 3.7 Software (Applied Biosystems Foster City, CA).

3. Results and Discussion

The manufacturer's recommended extraction protocol was modified to allow multiple amplifications *via* PCR from a single extraction. Accordingly, DNA was eluted from a 6 mm disc into 80 μL TE. This volume was adequate to run more than 70 microsatellite PCR amplifications (1 μL per reaction for PCR). High quality DNA was amplified from extracts eluted form either type of card from both young and mature sorghum leaves (Figs. 1 and 2). Moreover, electropherograms of the fragments from two wild sorghum plant samples amplified by different SSR primer pairs and viewed by ABI 3100 Genetic Analyzer with the associated GeneMapper software. Figs. 3 and 4 showed that the two types of cards work equally well.

Although the protocol suggested by the manufacture is reasonably simple and requires few special tools, the protocol that the authors followed could substitute a 6 mm single hole paper puncher ($1.29) for the 2.0 mm Harris Micro Punch™ (-$109) recommended by the company. The use of this more economical punch also allowed for a larger amount of DNA, sufficient for up to 80 PCR amplifications, to be made from a single extraction. A single FTA® card holds four samples, but extra care must be taken not to contaminate the sample in neighboring quadrants when squashes are made. This kind of cross-contamination risk is not a concern when using a single card prepared from Whatman chromatography card. The opportunity for samples to be inadvertently mislabeled when discs were transferred to microfuge tubes was also greater when four samples were contained on a single card. Although Whatman® doesn't recommend rubbing samples onto the FTA®card, the authors found that using a shoe horn to rub the samples onto the card worked as well as pounding with a pestle. Rubbing with a shoe horn is easier, faster and causes less damage to the FTA® mat than pounding with a pestle or a hammer. Using this method, the authors efficiently collected a large number of samples in a short time. The authors were able to successfully amplify DNA from more than 1,000 samples collected in the field using Whatman chromatography paper. FTA® cards are sold in packs of 100 (designed for 400 samples) for USD $450 (in 2010), whereas, the Whatman chromatography paper is sold in packs of 100, 15 × 20 cm sheets for USD $35. These sheets can be used to make 1000 cards for DNA collections.

As explained in Mbogori et al. [13] the time of the day and stage of the plant when sample squashes collected are critical when using FTA® and Whatman paper cards. It is preferable to collect squashes early in the morning when the stomatal cells in the plant leaves are fully turgid. At noon, plant leaves lose moisture and it is difficult to make squashes and collect DNA samples. In general, humid, cloudy and calm weather is suitable to extract DNA using these cards.

4. Conclusions

Using FTA® technology to extract genomic DNA from mature plants, *in situ*, is particularly attractive for ecological and other field studies. The technology is especially adaptable to sampling at remote sites. Further, this technology may be used to exchange sorghum genetic material across country boundaries since the collection cards are relatively free from the risk of transferring disease and insect pests and pose less risk of unauthorized use (multiplication) of genetic resources, which are common setbacks when using seeds. Perhaps the greatest advantage of using cards and the FTA protocol is the adaptation of the protocol so that unskilled personnel can easily collect plant squash samples.

In the authors' optimization of this technology for extracting sorghum DNA, solution eluted from a 6 mm disc was adequate to run 70 microsatellite multiplex PCR amplifications (1 μL DNA per amplification).

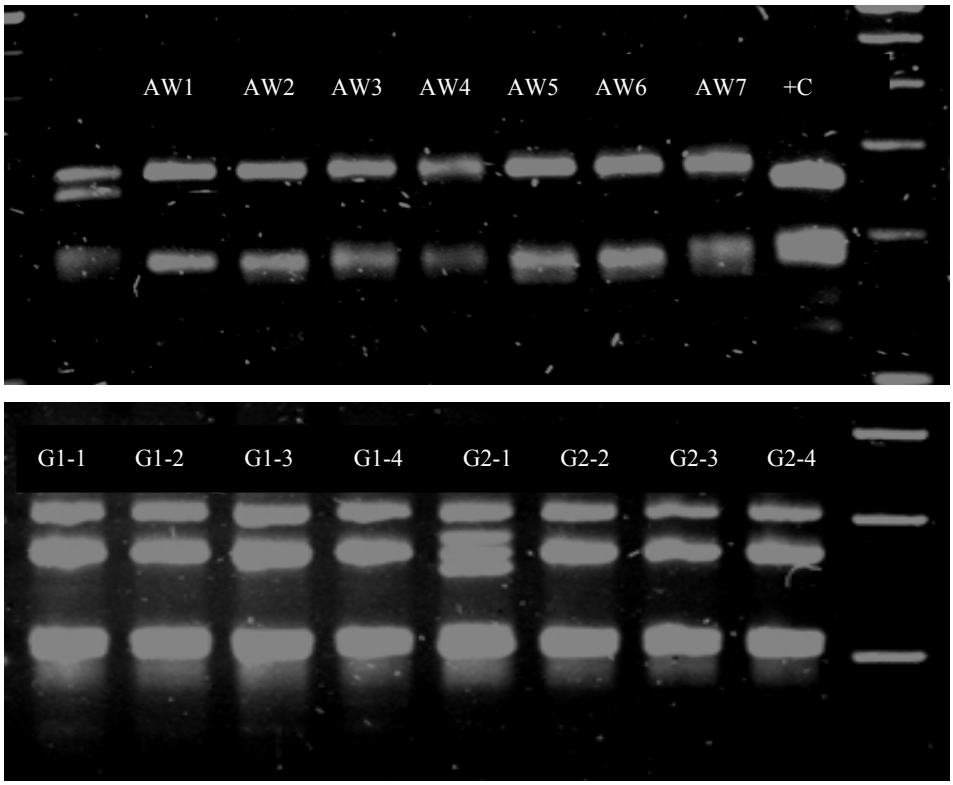


Fig. 1 Amplification results from extractions using ordinary Whatman chromatography paper (top) and standard FTA card (bottom) showing the banding pattern of wild sorghum DNA using SSR markers (Different plant samples were used in each example).

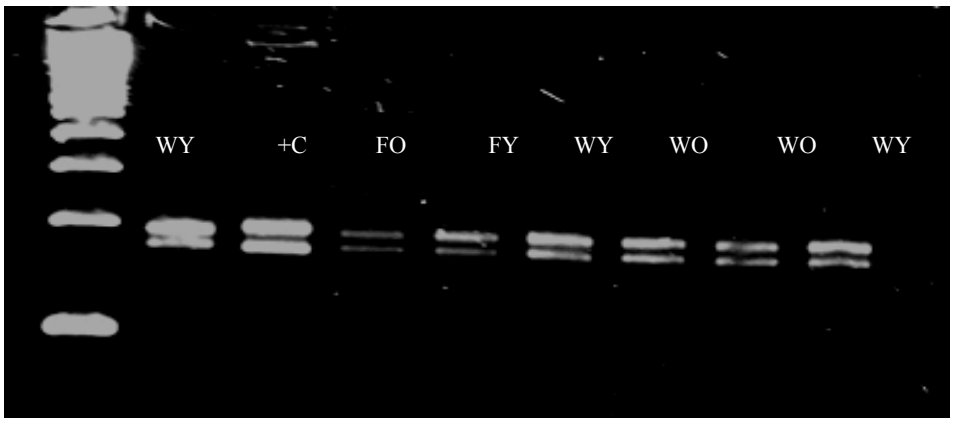


Fig. 2 DNA fragments amplified from volunteer sorghum using primer Sb6-6.
W = Whatman paper, F = FTA® plant DNA card, Y = Young tissue, O = Old/mature tissue, +C = positive control, -C = negative control (No DNA)**.**

This is much more efficient than using a separate extraction and single small disk for each PCR amplification. The authors observed equal efficacy of both types of cards in yielding DNA suitable for SSR amplification. The Whatman FTA® card is expensive and may not be accessible in some parts of the world. However, without compromising efficiency, the lower cost Whatman paper can be used for sorghum DNA extraction. The use of this more readily available and more economical alternative to the FTA cards is especially promising for institutions in developing countries, provided that FTA® purification reagent is accessible. This technology can also be optimized and used for other crop species.

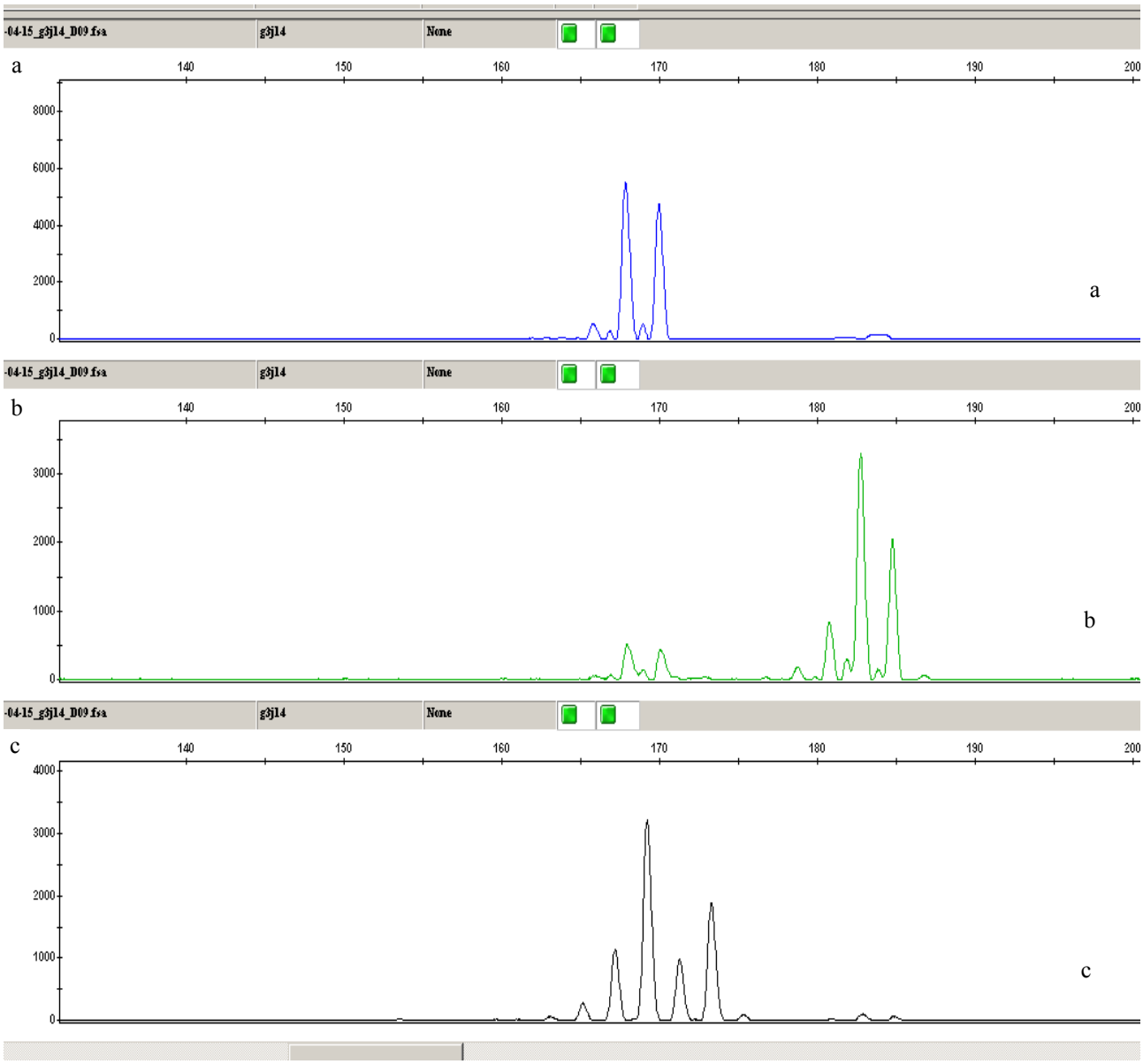
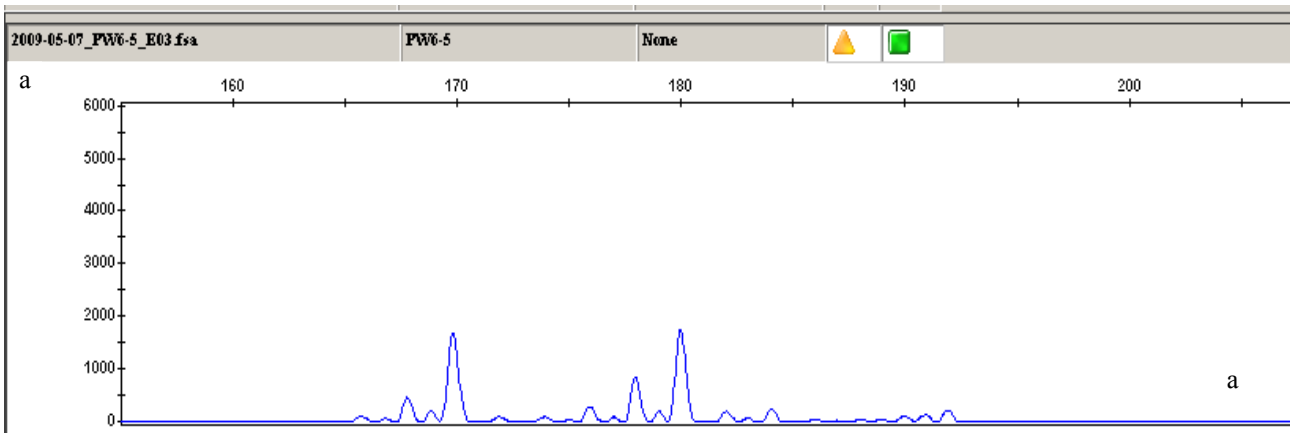


Fig. 3 Electropherogram of DNA from wild sorghum plants collected from Gibe, Ethiopia, using FTA® plant card (from top to bottom, primers Sb5-256 (a), Sb6-84 (b), Sb6-36 (c)).



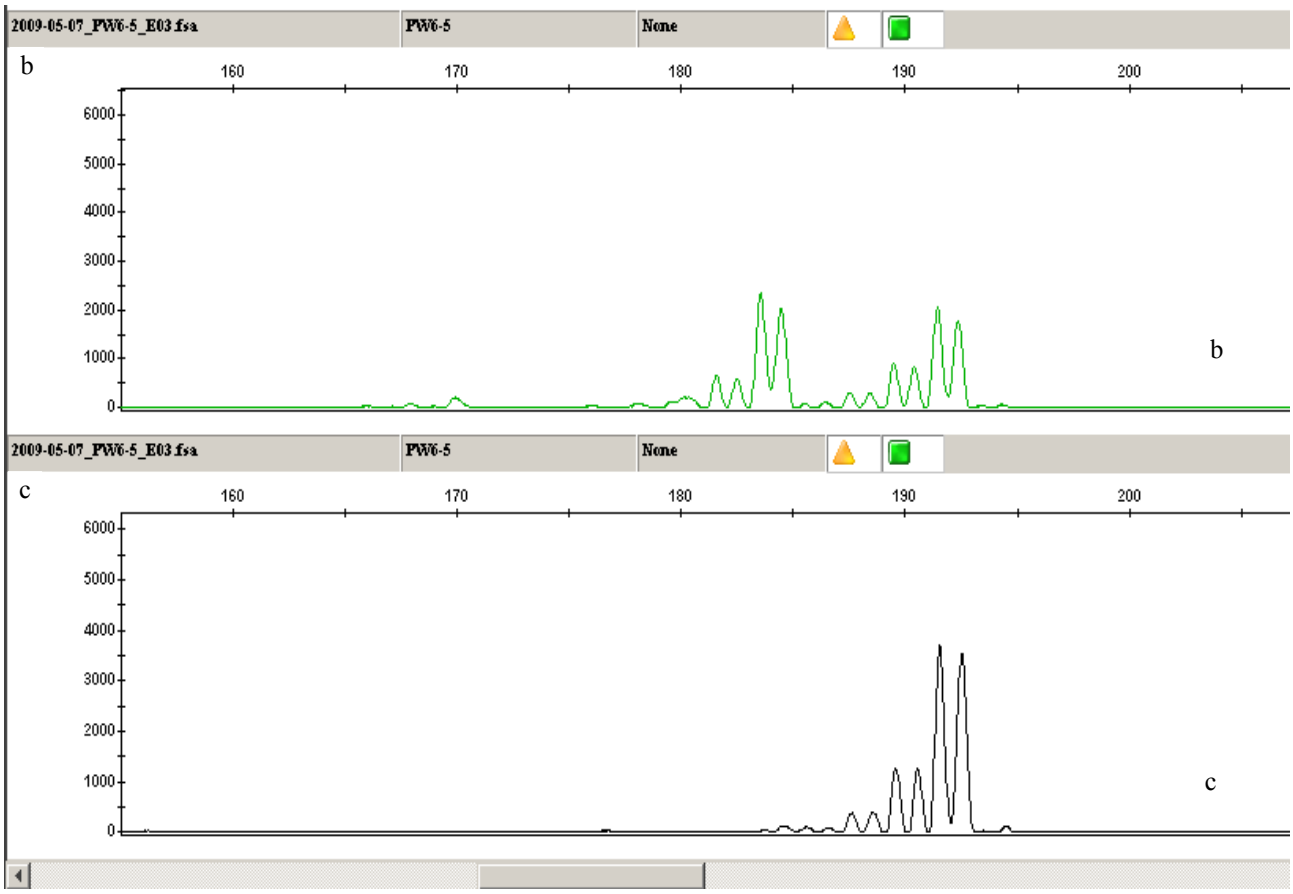


Fig. 4 Electropherogram of DNA from wild sorghum plants collected from Pawe, Ethiopa, using Whatman chromatography paper (from top to bottom, primers Sb5-236 (a), Sb6-84 (b), and Sb6-36 (c)).

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