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Cotton DNA Traceability Technologies

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Introduction

'Cotton traceability' is becoming an important issue as retailers make certain product claims. Every cotton fabric and piece of apparel that we use today is constructed from millions of small fibers. The fabric may be labeled as 100% cotton, or 100% Egyptian cotton, or a cotton-blend, or a manufacturer might claim to have woven fabric from yarn of a specified count, or to have produced a product through a specific process, or in a particular country, or from a certain species or a specific cotton variety. Currently, it is very difficult to objectively verify such claims.

Several identity initiatives operate in the cotton sector, including Organic, Fairtrade, Cotton made in Africa (CmiA), the Better Cotton Initiative (BCI), and E^3 sustainable cotton program. There are also many national sustainability initiatives, including Cotton Leads and myBMP. Given the complexity of the cotton value chain, it is not easy to verify that a cotton fabric is correctly labeled as being of a particular quality or to have been produced in a certain manner in compliance with certain social norms, ethical guidelines, production systems or environmental standards, unless the entire production and processing chain is contained within a knowledge-intensive, trust-worthy system with high levels of accountability and integrity.

To understand the difficulty of verification of fiber, yarn or fabric content, quality or production claims, it is important to understand the complex sequence of processes that connect stake-holders in the cotton value-chain. The sequence starts with the farmer who produces seed cotton, which must be transported from farms to procurement centers to gins and is often co-mingled with seed cotton from other sources in the process. Seed cotton goes through several levels of processing, including ginning, spinning, dyeing, weaving and finally cutting and sewing into a finished product, with each stake-holder performing a role different from the other. In all likelihood, the farmer, ginner, spinner and the weaver do not know each other, though the fiber holds their occupations together.

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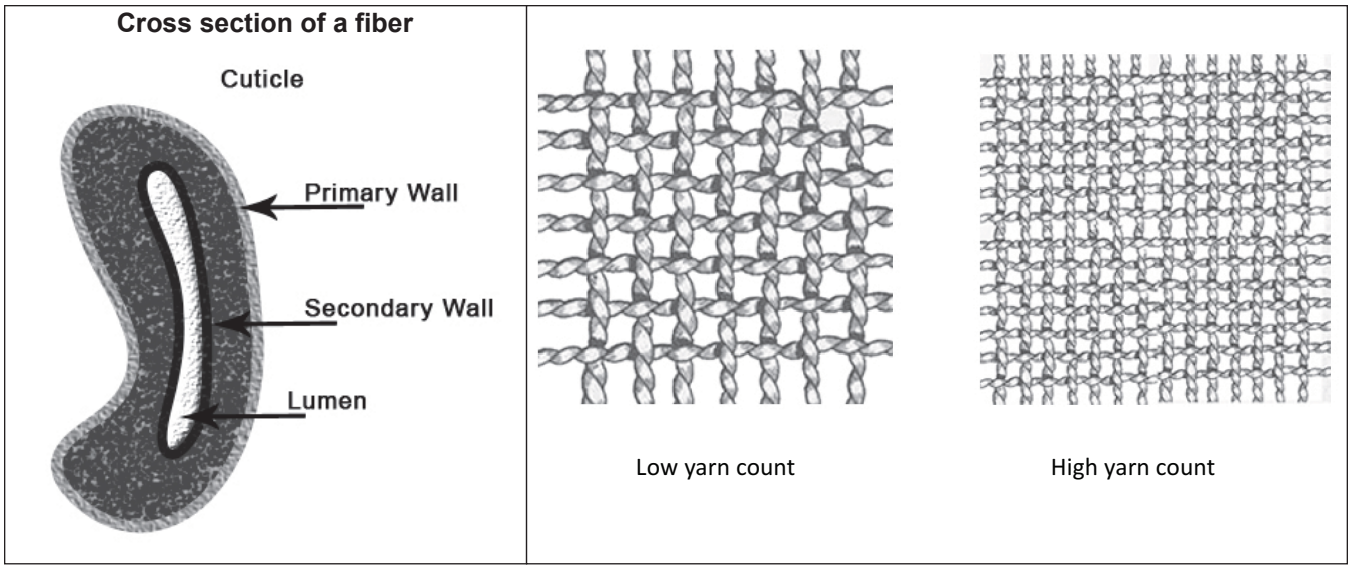
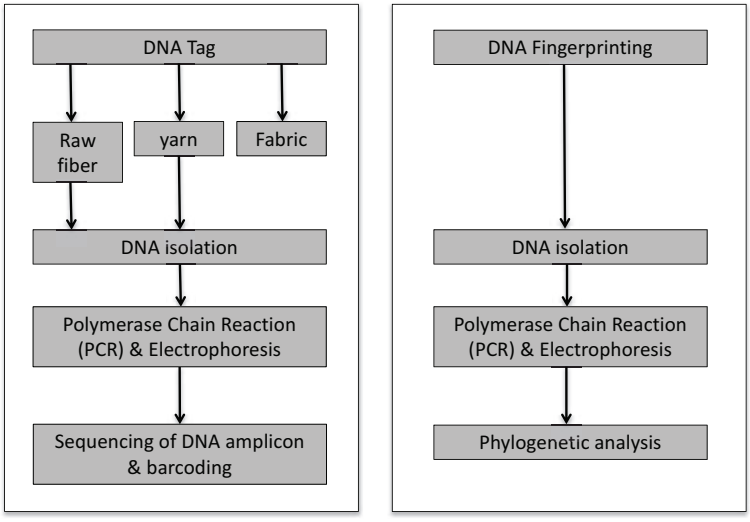


In a disaggregated value chain, labelling often depends on a claim backed by trust. For example, the farmer may claim, and may even produce a certificate to verify, that the cotton he/she produced is in compliance with strict organic-cotton guidelines. Based on this claim, the entire value chain could continue to label the product as 'organic-cotton'. The current identity programs are based at least partially on a 'belief-system' that all claims are trust-worthy. However, there have been recent cases of products labelled as 100% Egyptian cotton that were shown to be produced from upland varieties, and the organic cotton market is rife with rumors. Such incidents raise the need for a robust labelling/genotyping and tracking system to ensure labelling integrity. New Deoxyribo Nucleic Acid (DNA) based technologies provide avenues for a system that can finger-print and detect specific kinds of fiber through genotyping or exogenous DNA tags to fibers, yarn or fabric and detect them to provide traceability.

In an intense competitive environment, companies may fumble in their rigor of ascertaining standards of quality, or any specified kind of product characteristic. A few cases of dubious claims have been causing serious concerns in the market.

One of the most effective ways to identify fibers by origin is through a process called 'matured fiber genotyping'. There are several reports on this process where researchers have developed molecular marker-based tools which have the potential to identify the origin of matured and processed fibers, thus ensuring the genuineness of cotton fiber and textile materials. However, reports also suggest that chances of obtaining intact genetic material from matured fiber are narrow, thus contradicting the robustness of the claimed technological tools which are recommended for fiber genotyping. An alternative method is to tag cotton fibers with a DNA nucleotide-marker at the initial stage of processing. The tag remains always with the fiber in the fabric and can be detected at any point of time. Our review will relook into the scientific basis of several technological tools developed to analyze the genetic material of cotton fiber.



The Cultivated Cotton Species

The genus *Gossypium* comprises more than fifty species, of which only four are adopted for commercial cultivation, including *G. hirsutum* (American), *G. barbadense* (Egyptian), *G. arboreum* (Asiatic/ Indian) and *G. herbaceum* (sub-Saharan African and Arabian) (Wendel and Grover, 2015). All these four species produce unique types of fiber having distinguishable physical properties. For instance, fiber characteristics of the two diploid (two sets of 13 basic chromosomes of A genome) species, *G. arboreum* and *G. herbaceum* are inherently short and coarse compared to the relatively longer and finer fibers of the allotetraploid (two sets each of 13 basic chromosomes of A and D genomes) species, *G. hirsutum* and *G. barbadense*.

American and Egyptian cotton differ remarkably from each other, where the latter yields superior quality fiber typically termed as "extra long staple" (ELS) fiber (Liu *et al.,* 2015). (The ICAC Secretariat prefers the term extra fine to avoid confusion with national labels in Egypt and India that use the ELS designation.) As the name suggests, ELS fibers have naturally longer lint length as compared to that produced by *G. hirsutum* and hence, Egyptian cotton fiber qualifies as a favored raw material for the textile industry. Textile products manufactured out of genuine ELS fiber offer more value to the finished product and attract more vendors and consumers. The quality of ELS fiber from *G. barbadense* varies significantly depending upon the variety, geographical location and crop husbandry during cultivation. Many contentious cases relate to claims of 100% Egyptian ELS cotton that belongs to the species *G. barbadense.*

Endogenous DNA in Cotton Fibers

Cotton fibers are elongated desiccated single cells originating from individual cells of the external epidermal layer of cotton seed (Hernandez-Gomez *et al.,* 2017). Hence, they are also referred to as terminally differentiated unbranched seed trichromes (Tiwari and Wilkins, 1995; Kim and Triplett, 2001). Cotton fiber undergoes four developmental stages to reach maturity: *viz.* initiation, elongation, secondary wall deposition, and maturation. Each epidermal cell that gets transformed into a fiber strand possesses all the organelles similar to non-fiber cells. From the point of traceability, the fate of DNA in nucleus, chloroplast and mitochondria organelles, assumes significance. A sharp increase in the volume and number of nucleus, chloroplast and mitochondria was reported during the fiber initiation and elongation phases (Tatum, 1987). The molecular and physiological basis of initiation, elongation and secondary cell wall synthesis of fiber development is very well studied and documented (Haigler *et al.,* 2009 & 2012). Massive deposition of cellulose and micro-fibrillar rearrangement during secondary cell wall synthesis imparts strength and rigidity to the developing fiber. A difficulty in purification of biomolecules such as proteins and nucleic acids because of the strong cellulosic nature of fiber resulted in poor characterization of the maturation phase of fiber development (Kim and Triplett, 2001).

The fiber initiation and elongation phases are not mutually exclusive developmental events; they rather overlap in synchrony before the cotton fiber attains full maturity (Kim and Triplett, 2001; Haigler *et al.,* 2012). However, the concept of cotton fiber maturity is at times contested with varying opinions. For instance, one of the research groups headed by Hernandez-Gomez *et al.* (2017), referred to the notion of fiber maturity as "misleading" and instead renamed this as fiber desiccation phase. Earlier studies carried out by Berlin and co-workers (1986), had also highlighted desiccation and drying of cotton fiber during the final phase of development.

The cotton fiber development phase is also characterized by pure cellulose deposition during the secondary wall deposition phase, which eventually accounts for more than 95% of dry weight of matured or desiccated cotton fiber (Kim and Triplett, 2001; Stiff and Haigler, 2012). Fiber initiation commences from the day of anthesis arising from specific cells of the outer seed epidermis with a ratio of 1:3.7 among fiber initials and total ovular epidermal cells (Stewart, 1975). Fiber elongates for the next 21-26 days which gradually coincides with secondary cell wall synthesis, which commences at about 16 days post anthesis (DPA), and lasts until approximately 32 to 40 DPA (Meinert and Delmer, 1977; John and Crow, 1992), followed by the desiccation or maturation phase until 45 to 60 DPA (Kim and Triplett, 2001).

Meanwhile, during the course of the fiber development phase over a period of about two months, fiber cells had been hypothesized to undergo programmed cell death (PCD) and die, roughly after 40 DPA (Potikha *et al.,* 1999). PCD is the process of organized and regulated destruction of cells for survival and maintenance of organisms. It is a well documented process in both animals and plants. The process of programmed cell death in tracheary elements differentiation has been extensively studied in plants (Fukuda *et al.,* 2000; Kacprzyk *et al*., 2016; Dauphinee *et al*., 2017). Similarity in the process of tracheary elements (xylem vessels) differentiation and later phases of cotton fiber development led researchers to hypothesize the theory of PCD in cotton fiber development. Coincidence of production of reactive oxygen species such as H_2O_2 in response to initiation of secondary cell wall synthesis convinced Pothika *et al.* (1999) to postulate the theory of PCD in cotton fiber. However, in a subsequent report, Kim and Triplett (2001) contested this theory due to the lack of concrete evidence showing typical disintegration of cellular organelles or any detectable biochemical markers to support PCD in cotton fiber. In the same context, a study was carried out by Roche (2007) exclusively to decipher PCD in cotton fiber.

To examine whether cotton fiber cells succumb to the usual process of PCD, Roche (2007) made keen observations on the fate of the genetic material (i.e. DNA) within cotton fiber cells. DNA content and DNA disintegration were particularly monitored in the study as typical markers for tracing the occurrence and timing of PCD in cotton fiber. DNA was extracted from nuclei isolated from cotton fibers at specific time intervals *viz.* 5, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 DPA. It was observed that DNA could be extracted and visualized from fiber cells until 40 DPA, after which it remained undetected. Notably, there was no DNA laddering, a typical hallmark indicator for PCD linked apoptotic DNA fragmentation (Kressel and Groscurth, 1994; Ryerson and Heath, 1996; Orzaez and Granell, 1997) observed at any point of time. The author therefore, said that her study remained rather inconclusive and was unable to uncover any firm evidence to mark the process of PCD in cotton fiber.

However, the fact that DNA could be traced from cotton fiber

during the course of fiber development has been documented in earlier studies as well (Van't Hof, 1999; Taliercio *et al.,* 2005). Nuclear degradation, vacuolar rupture, organelle destruction are typical hallmark events, along with other important features of PCD that could not be established in cotton fiber. Hence, the fate of DNA after cotton fiber gets fully matured or desiccated and subsequently harvested, is still an enigma to be resolved.

Extraneous DNA Tag on Cotton Fiber

Studies to assess the stability of DNA extraneously introduced or adhered onto various types of fabrics, show that cellulosic fibers such as cotton are good substrates. Such studies are of vital importance in forensic science. For instance, one study included six different fabrics stained with dried blood, including cotton, nylon, rayon, polyester, acrylic and wool. The study revealed that a superior DNA profiling could be derived from the samples of dried blood stained on cotton and nylon as compared to other fabrics tested, even on 14-day old bloodstains (Seah *et al.,* 2004). Thus, cotton fiber acts as a rather good binding substrate for extraneous DNA. Hence, the cotton swab has long been used as a favorable forensic tool for collection and analysis of DNA as a part of evidence in crime scenes (Hansson *et al.,* 2009; Brownlow *et al.,* 2012; Adamowicz *et al.,* 2014).

Apart from forensic applications, the use of extraneous DNA as a marker to tag and authenticate cotton fabrics to combat counterfeiting of branded clothing has been in the news for quite some time. Crypton® and Applied DNA Sciences, Inc. (both are private companies) have developed a unique DNA-marker-based, anti-forging technology which enabled tagging and forensic authentication of textile materials, including cotton fibers in finished products. The technology has been named 'SigNature T DNA platform' which the companies claim provides legitimate proof of the identity and purity of textile goods.

(http://adnas.com/signature_dna/;https://crypton.com/crpt-content/uploads/2017/03/crypton-companies-initiate-forensic-dna-program-with-applied-dna-sciences.pdf).

Stability of DNA During Fiber Development

Several reports suggest that there is considerable evidence for the presence of DNA in matured or harvested cotton fiber. Patent documents claim successful detection and extraction of PCR amplifiable DNA from matured harvested cotton fiber (Liang *et al*., 2014, 2015). As discussed above, the phenomenon of PCD in cotton fiber cells could has not been proven conclusively, due to a lack of conformity with typical PCD associated symptoms. In this case, there arises an obvious question regarding the DNA content and its stability in cotton fiber. Unfortunately, there is no report available that depicts the time line for the decline in integrity of DNA inside fiber cells after 40 DPA. However, based upon earlier studies which relate to storability of genomic DNA of plant samples, some logical conclusions can be drawn in favor of the stability of cotton fiber DNA.

There are reports of the recovery of measurable amount of DNA extracted from herbarium specimens as old as 118 years, and from mummified seeds and embryos ranging, astonishingly, from 500 years to greater than 44,600 years old (Rogers and Bendich, 1985). It is well documented that the nuclear material of biological samples can be preserved for a fairly long duration simply by drying the samples (Doyle and Dickson, 1987). Good quality genomic DNA could be extracted from desiccated plant tissue samples stored for weeks, and even months, at room temperature (Liston *et al.,* 1990; Chase and Hills, 1991; Till *et al.,* 2015). In fact, the DNA extracted from the samples was good enough to carry out restriction site analysis and polymerase chain reaction (PCR) amplification (Chase and Hills, 1991). The final phase of cotton fiber development is marked by a natural process of desiccation of fiber cells (Berlin, 1986; Hernandez-Gomez *et al.,* 2017). In relation to the aforementioned studies, it can be postulated that the genetic material of cotton fiber holds a fair chance of survival for a long period of time. However, a thorough chronological study is needed to define the precise time length for which DNA in cotton fiber may remain intact.

DNA Stability During Fiber Processing

Extraction of DNA from cotton fiber cells from a processed product such as cloth or fabric is a challenging task. The need for traceability has accentuated the demand for methods to extract DNA of a reliable quality that can act as the basic material for detection using DNA-tagging or genotyping to establish the identity of the test cotton fiber. Recovery of good quality DNA from matured cotton fiber, finished fabrics and apparel is the basic prerequisite step to carry out DNA bar-coding for fiber typing and fabric authentication. There is a common belief that, amongst all the currently available methods, technologies based on fiber DNA could provide the most reliable tools for the textile industry as an anti-counterfeiting tool.

Cotton fiber usually undergoes extensive processing before being converted to finished textile products. Moreover, the degree and number of steps of fiber processing vary immensely depending upon the desired end product. Thus, a full cycle of fiber processing in textile mills may impart severe mechanical, thermal and chemical stresses on fiber. For instance, machine harvesting and ginning causes thermal and mechanical damage to cotton fiber, and thermal stress is applied when drying excess moisture from fiber. Chemical treatments during the final phases of spinning and knitting are also common in fiber processing.

In addition, cotton fiber is a unique kind of plant cell with a thick secondary cell wall and is composed of more than 95% pure cellulose. Therefore, there are substantial challenges

in isolating intact and high quality DNA from matured, harvested fiber. Nevertheless, despite the thermal, chemical and mechanical stresses, it has been shown that DNA typing, using either extraneously introduced or naturally occurring DNA, can be carried out reliably from cotton fiber or its processed products which could be further utilized for PCR-based fiber genotyping assays. (http://adnas.com/signature_dna/; Patent EP2318676B1, 2011; Patent US8669079, 2014; Patent US8940485, 2015).

Methods to Isolate DNA Reliably from Raw Fibers, Yarn, Fabric and Apparel

There are a few patent documents available which described protocols for good quality DNA extraction from raw cotton fibers and processed fabrics (Patent EP2318676B1, 2011; Patent US8669079, 2014; Patent US8940485, 2015). The DNA extraction protocols mentioned in these patents are more or less similar to the standard molecular biology protocols (Sambrook *et al.,* 1989) with minor modifications in a few instances. Surprisingly, by following the standard Cetyl-trimethyl-ammonium-bromide (CTAB) method with minor modifications (like enhanced incubation period), it was possible to extract adequate amounts of genomic DNA from matured cotton fibers (Patent EP2318541B1, 2011). Not surprisingly, some earlier reports also suggest that good quality genomic DNA can be isolated from tough (Moncada *et al.,* 2013), mummified (Rogers and Bendich, 1985), dried and old archaeo-botanical plant tissues (Schlumbaum *et al.,* 2008). For example, protocols for DNA extraction from bark cloth, a fabric made from beating fibrous tree barks into sheets, have been elaborated in recent studies (Moncada *et al.,* 2013; Seelenfreund *et al.,* 2016). The DNA of bark cloth was successfully utilized in genetic analysis and characterization of archaeological samples as well. Therefore, these protocols may be explored for the extraction of cotton fiber genomic DNA to ensure its quality and authenticity.

Applied DNA Sciences (ADNAS) Company claims to have discovered that chloroplast DNA has better stability and survival chances compared to nuclear DNA in the fibers. A meticulous study carried out by Roche in 2007 (as discussed earlier), could not comprehend the exact fate of nuclear DNA of cotton fiber due to certain practically translatable experimental limitations. Notably, the author (Roche, 2007) clearly stated in her results that visually detectable nuclear DNA could be isolated from cotton fiber only until 40 DPA, after which (55, 60 and 65 DPA), DNA could not be visualized. It was further found that there was no DNA laddering observed at any point of time. However, there is no clear evidence to ascertain that nuclear genome is more prone to degradation as compared to chloroplast genome. Thus, if ADNAS has validated the survival of chloroplast DNA in mature cotton fibers, there may be an equal possibility of survival of mitochondrial and nuclear DNA as well.

A few patents show that it is very much possible to recover DNA irrespective of nuclear, chloroplast or mitochondrial origin, from matured harvested cotton fibers. A group of inventors, Ming-Hwa Liang and co-workers (2014, 2015) published a patent revealing successful extraction of genomic DNA from mature cotton fibers which can be further utilized for identification of specific cotton cultivar/species and detect genetic variations among cotton species. They have even claimed to have isolated DNA from processed and finished textile materials including anthropological textiles, garments, artwork canvases etc. They have used specific sets of primers targeting sequence polymorphism between distinct cultivars of *Gossypium barbadense*. In addition, they were also able to distinguish between different cotton varieties cultivated at different geographical locations. They could also genotype 25 different ELS cultivars of *G. barbadense* using a combination of merely 5 sets of SSR primers. Using such SSR primers, thousands of different cultivars can be discretely identified, and a genotype profile database can be created to identify specific cultivars of cotton from matured harvested cotton fiber (Liang *et al*., 2015). The patents claim to distinguish between *G. barbadense* and *G. hirsutum*, the two main cultivated cotton species worldwide. In yet another patent Arioli *et al*. (2016) claimed successful extraction of biological macromolecules of DNA, RNA, proteins, peptides etc. from matured or processed cotton fiber. The document has also revealed robust protocols for isolating DNA by incubating lint and processed fibers in specific buffers for a specific time period. Mohamed Negm & Suzan Sanad of Cotton Research Institute, Giza (Egypt) developed specific protocols and fiber-DNA based methods based on DNA melting curves to identify Egyptian varieties and authenticate the purity and presence of Egyptian cotton fibers in textile products (Negm and Sanad., 2015).

Experiments conducted at ICAR-Central Institute for Cotton Research, Nagpur (India) have shown that with a slight modification of standard protocols, good quality PCR amplifiable DNA could be extracted from mature cotton fibers of *G. hirsutum*, *G. barbadense* and *G. arboreum* (Raghavendra et al, unpublished).

Finally, it must be said that so far, research papers have not yet affirmed whether intact nuclear DNA can be isolated from mature fibers, either raw or processed. Interestingly, all such claims of extracting nuclear DNA from mature fibers of fabric or apparel have been made only in patents, which could possibly have been done from a commercial perspective and need to be test-verified for scientific correctness.

Traceability of *Gossypium* Species Using DNA Fingerprinting

DNA fingerprinting is a powerful tool to ascertain the identity of individuals across the animal and plant kingdoms. The DNA fingerprint of a genotype reveals the pattern of allelic variation present in the genome as detected by the molecular

markers. Since its discovery by Jeffreys and co-workers in 1985, many techniques have been developed, optimized, utilized and eventually abandoned when novel and more efficient and/or more reliable methods became available. Of the various molecular marker systems, locus-specific microsatellite analysis is the most popular method for DNA fingerprinting in plants (Nybom *et al.,* 2014) owing to their reproducibility, co-dominant inheritance, genome-wide presence, robustness, higher polymorphism and analytical simplicity. DNA fingerprinting is a relative assessment that is subject to the sample size of the individuals under study and the number of markers employed. It would be practically impossible to genotype an entire population, including the related genotypes within the species and individuals of related species. The credibility of a DNA fingerprint is assessed by using a statistical parameter called 'probability of identical match by chance.' The parameter can be calculated using the formula $(\overline{X}_D)^n$ as described by Ramakishana *et al.* (1994), where '$\overline{X}_D$' is the average similarity index and 'n' is the average number of amplified products per cultivar. The smaller the probability of an identical match, the greater the reliability of the DNA fingerprint.

Table 1: Some example markers for genotyping *Gossypium* sp.

Simple Sequence Repeats (SSR) / Microsatellites	**Reference**
Gossypium hirsutum, G. barbadense, G. darwinii and G. tomentosum.	(Lacape *et al.,* 2007)
378 accessions of *G. hirsutum* and 3 from *G. barbadense*	(Tyagi *et al.,* 2014)
47 upland cotton genotypes	(Rakshit *et al.,* 2010)
157 elite *G. hirsutum* cultivar accessions	(Zhao *et al.,* 2015)
410 *G. barbadense* and 1,523 *G. hirsutum* accessions	(Hinze *et al.,* 2016)
193 *G. hirsutum*	(Fang *et al.,* 2013)
24 *G. hirsutum* accessions with varying degree of drought tolerance	(Abd El-Moghny *et al.,* 2017)
Single nucleotide polymorphism (SNP)	**Reference**
G. hirsutum Texas Marker-1	(Ashrafi *et al.,* 2015)
18 *G. hirsutum* varieties	(Zhu *et al.,* 2014)
363 *G. hirsutum*: 292 cultivated and 71 non-cultivated relatives, 27 from 10 diploid and tetraploid *Gossypium* species which included 6 diploid species (*G. arboreum*, *G. amourianum*, *G. longicalyx, G. raimondii, G. thurberi*, and *G. trilobum*) and four tetraploid species (*G. barbadense, G. ekmanianum, G. mustelinum, and G. tomentosum*).	(Hinze *et al.,* 2017)
Amplified fragment length polymorphism (AFLP) & internal transcribed Spacer (ITS)	**Reference**
41 cultivars of *Gossypium hirsutum, G. barbadense, G. herbaceum and G. arboreum*	(Jena *et al.,* 2011)

DNA fingerprinting in cotton has its own challenges. Cotton is often a self-pollinated, complex, allotetraploid species with a huge genome (2400Mb) and having a high proportion of repetitive sequences. Outcrossing, mediated by insects of varying proportions (5-30%), is reported to happen in cotton. With every outcrossing, the purity of a cotton variety is at risk, and must be maintained through timely selfing and proper roguing. Outcrossing and amplifications emanating from the repetitive sequences of the genome infuses spurious heterozygosity and can reduce the credibility of a DNA fingerprint. However, the number of SSR markers available for research has increased over the years, and their polymorphism in cotton is reported to be low. Nevertheless, techniques used for the separation of PCR amplicons should have a higher resolution and the scoring of gel profiles should be automated to achieve precise estimation of allele size. Studies employing Agarose or Metaphor for amplicon separation and manual scoring of gel profiles can lead to imprecise results. Compared to genomic SSRs, the use of EST-derived SSRs can provide more robust information, as they represent the true variation in the expressed part of a genome connected to trait variation. A common set of markers are to be employed in DNA fingerprinting to compare the genotype profiles across countries and laboratories.

Researchers across the globe have reported several DNA markers that have been developed specifically for genotyping several *Gossypium* species and cultivars. The availability of the genome sequences of diploid progenitors and tetraploid cultivated species of cotton can be explored for the development of robust polymorphic markers for the identification of *Gossypium spp* (Paterson *et al.,* 2012; Wang *et al.,* 2012; Li *et al.,* 2014a; Li *et al.,* 2015; Liu *et al.,* 2015). For instance, utilizing the diversity of chloroplast genome of *G. hirsutum* and *G. barbadense*, Li and co-workers (2014b) identified 50 polymorphic chloroplast simple sequence repeat (cpSSR) markers for diversity analysis and the identification of *Gossypium* species. Similar kinds of markers can be extended to DNA-based tagging and authentication of cotton fiber and textile fabrics. Likewise, several other studies have also revealed many other molecular markers for cotton diversity analysis as depicted in Table 1.

Rakshit *et al.* (2010) developed the DNA fingerprints of 47 upland cotton genotypes using ten identified SSR markers with a moderate probability of an identical match by chance (0.01). Forty-eight of the most popular tetraploid cotton varieties of India were profiled using 68 identified polymorphic SSR markers at the ICAR-Central Institute for Cotton Research,

Nagpur. A robust DNA fingerprint having a low probability of identical match by chance was developed using a selected set of 14 markers (Santhy *et al.,* unpublished). If the probability of identical match by chance is 3.55×10^{-12}, it means one pair in 3.55×10^{12} combinations can have an identical DNA profile by chance. Therefore, to develop a robust DNA fingerprint having a very low probability of identical match by chance, a set of highly polymorphic markers should be utilized for molecular profiling, in combination with a fragment separation system having high resolution power and being amenable to automation. DNA fingerprint profiles can be maintained as databases in the public domain to achieve effective cultivar identification and differentiation across laboratories and countries.

The cultivated tetraploid species, *viz., G. hirsutum* and *G. barbadense,* differ significantly for most fiber quality traits, and many SSR markers tightly linked to these fiber quality characters have been identified through meta-QTL analysis of *G. hirsutum* × *G. barbadense* populations (Said *et al.,* 2015). A dedicated and updatable cotton QTL database (http://www2.cottonqtldb.org:8081/) is being maintained to assist cotton molecular breeding. In order to differentiate lint samples of *G. hirsutum* and *G. barbadense* through DNA fingerprinting, SSR markers, either genic or tightly linked for fiber quality traits sourced from the cotton QTL database can be utilized for greater success.

For over a hundred years, plant breeders have exchanged cotton germplasm lines across continents and used them in varietal improvement programs. For example, varieties from Egypt may have been used by breeders to improve varieties of *Gossypium barbadense* and *Gossypium hirsutum* in other countries. Therefore, a fair amount of the genome from Egyptian cotton varieties would be present in the improved extra-long staple varieties that are commercially cultivated in the major cotton growing countries. Similarly, extra-long staple fibers are produced from inter-specific hybrids of *G. barbadense* × *G. hirsutum* (H×B), which are commonly cultivated in India. It is probable that small-scale farmers may not even have the technical knowledge to differentiate between *Gossypium barbadense* varieties and interspecific hybrids of *G. barbadense* × *G. hirsutum*. Thus, the extra-long staple fibers harvested from H×B hybrids may be traded as *Gossypium barbadense* (Egyptian cotton) fibers mostly out of ignorance and not necessarily with dubious intentions. The quality of extra-long staple fibers of interspecific H×B hybrids may be as good as several *G. barbadense* varieties, but almost half the DNA in the genome of these fibers will come from *G. hirsutum*.

Conclusion & Future Prospects

DNA based 'traceability' methods are being developed as commercial products. With technological advancement, it is possible that very soon techniques will be available that can provide consumers with an absolute assurance of the specified identity, origin or species of fibers within a fabric. However, based on current science, there are still a few challenges that remain to be addressed before a foolproof technology is developed.

The key questions are:

1) Are the DNA methods cost effective and easy to use?

2) Is the DNA tag foolproof?

3) Is DNA fingerprinting / genotyping foolproof for traceability?

The simple answer to the above questions is 'No, not yet'.

Neither the DNA tags nor the DNA fingerprinting and genotyping techniques available today for traceability are foolproof. In addition, whether tagging or fingerprinting techniques are used, DNA methods are very expensive, and the techniques require special labs for DNA isolation, PCR, electrophoresis and interpretation. The DNA testing method itself takes 2-3 days. Tagging fibers with 50 to 100-mer oligos (short single stranded molecules of DNA/RNA oligonucleotides) or small double stranded DNA fragments may not be very expensive, but the detection-testing process can be tedious, time consuming and expensive. For example, each random DNA test to verify a claim could cost about US$50 or more, which could be equal to the cost of the apparel itself.

DNA tags can be misused by dubious operators, and DNA finger printing technology is yet to evolve to the stage where it can provide a credible specific profile of each genotype without any chance of overlap with other genotypes. DNA fingerprinting and the genotyping technology are based on a set of molecular markers that can be used to distinguish genotypes from one another within a population. Single DNA markers that are genotype-specific are very rare. It is possible to develop a multiplex PCR technique using a few reliable markers to obtain robust genotype-specific profiles, but this requires considerable expertise in highly specialized laboratories for testing unknown samples to verify the veracity of claims.

DNA nucleotide fragments are extraneous labels. Molecular tags in the form of small DNA fragments of defined nucleotide sequences are absorbed into fibers during any stage of the textile value chain. The extraneous or exogenous DNA can be incorporated into the fibers at the ginning unit, or in spinning mills or during treatment of the fabric, and they can be tracked at any stage of production. DNA is isolated from the fiber, yarn or fabric and used as a template for PCR amplification of the DNA-tag, separated through electrophoresis to obtain nucleotide amplicons of an expected size, and sequenced to be used as a bar-code for final identification. Some private companies have sensed a commercial opportunity, and they have quickly made claims of having developing reliable technologies of tagging and detection.

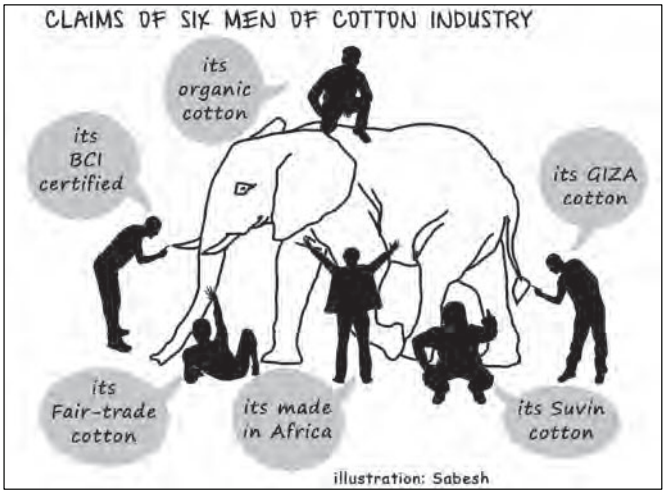
However, one of the main issues with the technology is that, with dubious intensions, any DNA can be tagged with any kind of fiber at the initial stage of processing, and any kind of

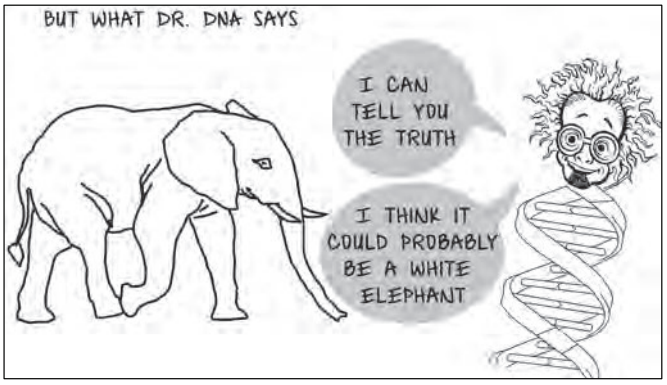
a final claim can be made for the product. In crude terms, a cat's fur can be exogenously tagged with a tiger's DNA and passed off as tiger's fur. It may even pass the legal test, depending on the lawyer's talent! The question once again is of 'integrity'. Therefore, it is possible that DNA tags could be misused by dubious companies as a marketing ploy, or the tags could be used mistakenly by genuine companies to label a product based on their trust in the source of the raw fibers rather than relying on robust scientific methods that confirm the veracity of the claim.

In the absence of traceability technologies, 'trust' plays a major role all through the value chain. Ironically, even if traceability technologies are implemented , products may be labeled with DNA tags based on disclosures made by raw fiber providers, rather than being based on DNA genotyping to confirm the authenticity of a claim of species, variety or geographical area. Thus, the problem of providing an authentic, foolproof traceability system is still a few steps away from being solved through DNA tagging technology. DNA genotyping technology may partly provide traceability information, but this technology is currently not used by any ginners, traders or spinners or any raw material suppliers.

DNA typing or fingerprinting depends on several factors. The technology deploys a set of markers and cannot rely on one or a few markers to distinguish a unique genotype from a population of genotypes. The uniqueness of the DNA fingerprint of a specific genotype is a relative term with reference to the number and type of polymorphic markers used and the relative density of related individual genotypes subjected to the test. The fingerprint of a particular genotype can be considered to be absolutely authentic only if the entire population of its related genotypes / species has been genotyped with a set of markers having high polymorphic resolution power to provide a clearly distinguishable phylogeny profile for all the genotypes within the population. Recent advances in next generation high throughput DNA sequencing techniques such as massively parallel signature sequencing, 454 pyrosequencing, Illumina Sequencing, Polony sequencing, SOLiD sequencing and Single molecule sequencing have not only reduced the cost of DNA sequencing but have also resulted in rapid identification of microsatellites and SNPs in the cotton genome.

The SSR and SNP markers are being either complemented or replaced by new methods of 'genotyping-by-sequencing'. Multiplex-SNP genotyping is also becoming more common. Cotton genome sequence data of *hirsutum* $(AD)_1$, *G. barbadense* $(AD)_2$, *Gossypium arboreum* (AA) and *Gossypium raimondii* (DD) are now available and will provide tremendous help for the identification of novel, highly polymorphic molecular markers that would accelerate future work on DNA fingerprinting. With diligent efforts, it may also be possible to develop species-specific and genotype-specific sequences that can be of immense value in traceability. However, as mentioned above, the development of markers





unique to a specific genotype will depend on sampling density and sequence coverage.

Finally, for the 'traceability-technology' to be reliable and robust, at this point of time, with the given state of the art, it may become necessary to combine an exogenous DNA-tag with at least two or more endogenous DNA markers that represent the species or variety/genotype or geographical indication, etc. Further, it would be important to develop simple and reliable detection methods. Such a set of markers could consist of a combination-tag in consonance for traceability. For example, fibers of Giza-86 could have an endogenous tag of a few SSR markers, combined with an exogenous DNA-tag, to confer high reliability to the technology of traceability. Nevertheless, there is hope that very soon simple and handy 'traceability' tools will be developed and put in place so that consumers get a trustworthy certificate-claim.

While the 'traceability' technology is progressing, some issues still remain. With the given 'state-of-the-art DNA techniques,' fabrics made out of cotton blends either with natural fibers or synthetics will need the more expensive 'Real-time-PCR' to figure out the blending proportion of cotton. Real-time PCR would only reveal the proportion of cotton used in the blend but will not resolve the identity of the components, especially the synthetic fibers used in the blend.

In summary, it would be appropriate to conclude that 'DNA based traceability technology' has evolved commendably in

recent years, but is not foolproof as yet. The major issues with it are as follows:

- DNA or chemical tags are useful but can be easily misused.
- DNA degradation during fiber processing is a serious concern and can affect the isolation and detection of the DNA. The degree of degradation depends on the processing methods employed and the level of stress to which fibers are subjected.
- Varietal-fingerprinting technology is available, but is complicated and difficult to be used for traceability as of now.
- DNA fingerprinting can be very difficult with samples containing physical mixtures of fibers from different varieties.
- DNA fingerprinting tests may suffer false-negatives due to cross-pollinating crops.
- DNA testing cannot reveal proportions and components of a synthetic blend.
- DNA testing can indicate genetic identity, but can be unreliable for geographic identity.
- DNA testing cannot identify or differentiate fibers derived from sustainability initiatives or identity initiatives.
- DNA testing requires considerable infrastructure and technical expertise, apart from being tedious, time consuming, cumbersome and expensive.

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Egyptian Cotton Traceability

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Introduction

"Egyptian Cotton" is characterized by its superior quality. It gives Egypt a competitive advantage on which a comprehensive industry could be developed to make Egypt the main producer of extra fine count yarns which could be processed and exported as the finest and highest quality cottons in the world. Such products need an identity that can be reliably traced and detected. It is now up to Egypt to develop a comprehensive program to establish 'competitive advantage' by developing a distinct brand, increased demand for new products, creating systems to ensure varietal purity associated with robust traceability technologies.

Unlike many other cotton-producing countries, Egypt exclusively produces *Gossypium barbadense*, a type of extra fine cotton endowed with a longer and finer staple than upland cotton. In Egypt, seed for planting has been strictly controlled by the government, which for many years operated as the sole supplier and distributor of cotton planting seed.

This article describes all the traceability initiatives being undertaken in Egypt to ensure seed purity, varietal purity, bale purity and finally to track the fibers from gin to fabric. The DNA testing method developed by us is simple, yet robust enough to detect and distinguish Egyptian varieties. We briefly describe all traceability initiatives, including the DNA techniques used to identify and verify the authenticity of Egyptian cotton.

Traceability of Seed Purity

The Cotton Research Institute, (CRI), of the Ministry of Agriculture and Land Reclamation (MALR), continues to breed high quality cotton varieties. New varieties are developed each year, and the most promising are submitted to a ten to fifteen year path towards the seed's progenies, through Foundation Seeds, Certified Seeds, and Registered Commercial Seeds, grown by 'seed multipliers'. After a few years (generally 2-3 at the Registered Commercial level), new seeds replace the old seeds, and new varieties are promulgated.

Lastly, a revamped cotton extension and marketing system is updated. A new traceability system is being designed in collaboration with the MALR and the cooperatives, including the Central Department for Seed Administration. The structure of the system is briefly described here.

There are three major components of the new traceability strategy that will depend on implementing information technology throughout the process from distribution of seeds to the farmer, follow up on extension programs, agriculture practices, designated cotton production area by variety and yield, Cotton Arbitration & Testing General Organization "CATGO", inspection, and market place bidding.

First, as indicated by the MALR, the cooperatives will play a major role. The MALR initiated measures to prevent genetic deterioration, which was considered one of the principal reasons for a decline in yields. The following strategies were defined: The CRI produces breeder seeds (generation one) and foundation seeds (generation two). The cooperatives will receive the foundation Seeds and distribute them to a set of 'Elite' farmers, who follow the production recommendations developed by the 'extension service', to obtain the highest possible yields of certified seeds (generation three). All seed cotton bags will be delivered to the cooperative warehouse upon harvesting in colored and signed bags. The seed cotton will then proceed to the cotton gins for ginning, and the seeds will be separated into their respective bags, for seed testing, germination, and fiber quality evaluation.

Seeds are selected, graded, labeled as certified seeds and returned to the MALR General Administration. After testing for germination and other characteristics, the MALR will return the seeds back to the cooperatives for the next season's distribution to master lead farmers to act as 'seed multipliers' to produce registered commercial seeds for general distribution. These seeds will have a different colored bag, different markings and labeled as 'registered commercial' seeds.