INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

INTER SIMPLE SEQUENCE REPEAT (ISSR) AND START CODON TARGETED POLYMORPHISM (SCoT) AS DISCRIMINATION TECHNIQUES BETWEEN CERTAIN APPLE AND PEAR CULTIVARS.

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Article Received on: 05/08/18 Approved for publication: 18/09/18

DOI: 10.7897/2230-8407.099192

ABSTRACT

Rosaceae is a large family in the plant regality, includes 3200 species in 115 genera such as *Malus* and *Pyrus* to which belong *Malus domestica* Borkh. fruits (apples) and *Pyrus communis* L. fruits (pears). Apples and pears have a wide variety of active constituents like anthocyanins, flavonoids, sterols, and tannins. *P. communis* and *M. domestica* fruits exhibit good antioxidant, antihyperglycemic, and chemopreventive activities. Botanists prefer to keep apple and pear under genus *Pyrus* but recently, American authors keep them distinct, apple under genus *Malus* and pear under genus *Pyrus*. Hybrids were developed to produce cultivars adapted to Egypt warm weather, so there is a large similarity between the different pear and apple cultivars which requires accurate and rapid techniques for their differentiation. The genetic discrimination between apple cultivars, Anna (1), Volus (2), Dorset golden (3) and pear cultivars, Le-Conte (4), MKM (5) and Flordahome (6) were carried out using start codon targeted and inter simple sequence repeat techniques with ten decamer primers, five for each technique. All primers gave bands with all cultivars with total 49 bands, 26 with ISSR and 23 with SCoT techniques, respectively with 27, 19 and 3 monomorphic, polymorphic and unique bands, respectively. According to combined dendrogram and similarity matrix, apple and pear cultivars grouped into two main groups, one contains the apple cultivars and the other contains pear cultivars with similarity coefficient 0.76-0.92. As a result, we can use ISSR and SCoT techniques for differentiation between apple and pear cultivars which have large morphological similarity.

Keywords: Rosaceae, *Malus domestica*, *Pyrus communis*, SCoT, ISSR, DNA primers.

INTRODUCTION

Precise identification of closely related species of medicinal herbs, moreover the cultivars in the same species is an important issue in quality control of these herbs and thus in their medicinal utility. Authentication of the plant origin is essential for accurate and correct use of closely related Plants in different medicinal remedies and products. The use of popular methods in plant identification such as morphological [1], anatomical [2] and chemical [3, 4] methods is difficult between the closely related plants and furthermore, require professional taxonomist's specialty. Genetic analysis is a preferred tool for resolving taxonomists' differences in discrimination between close species and cultivars resulting in the use of useful genotypes which increase the quality and efficiency of drug standard formulations [5]. DNA based authentication is a useful method in quality control assurance and safety monitoring of medicinal herbal products. It can also raise the medicinal potential of herbal products [6]. Botanists prefer to keep apple and pear under genus *Pyrus* but recently, American authors keep them distinct, pear under genus *Pyrus* and apple under genus *Malus* [7] due to the presence of slight botanical distinctions between the two genera which is difficult to clear by unprofessional students. Apple and pear fruits require cold weather for blooming and production of important antioxidants as anthocyanins [8], so hybridization for developing of cultivars being adapted to warm weather as apple cultivar Anna and pear cultivar Le-Conte was carried out. Thus, new cultivars will differ more or less in their active constituents' composition and quantity in reference to other apple and pear cultivars. Therefore, accurate and rapid authentication methods of medicinal plants will ensure quality and efficiency of herbal formulations [9]. Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. PCR amplification of these regions using a single primer yields multiple amplification products that can be used as a dominant multilocus marker system for the study of genetic variation. ISSR markers are easy to use, low-cost, and less demanding compared to other dominant markers, the generation of ISSR markers makes use of microsatellite sequences that are highly variable and absolutely distributed across the genome, at the same time ISSR achieves higher reproducibility than Random amplified polymorphism (RAPD)[10]. ISSR technique was widely used in genetic diversity analysis of medicinal plant species, such as *Memecylon* species [11] and *Ocimum* species [12]. Start Codon Targeted Polymorphism (SCoT) is a simple and novel DNA marker technique, uses 18-mer single primer in PCR and an annealing of 50 ºC. PCR products are resolved using standard agarose gel electrophoresis. SCoT based on the short conserved region flanking the start codon (ATG) in plant genes. This technique is more polymorphic, efficient and reproducible than RAPD and successfully utilized in rice for cultivar identification and genetic diversity analysis [13], moreover, SCoT polymorphism is correlated to functional genes and their corresponding traits such as different metabolites in closely related species. SCoT polymorphism used also in genetic diversity of Jojoba genotypes [14]. Combined ISSR and SCoT techniques were used in discrimination between closely related cultivars, such as Mango cultivars [15] and Indian Jujube cultivars [16]. *Malus* genus is reported to consist of 25–35 species [17]. *Pyrus* recorded species were listed into two categories, one including 38 species and the other including 47 taxa of

hybrids and ferals [18]. *P. communis* L. and *M. domestica* Borkh., contain many chemical constituents, as proanthocyanidin, flavonoids, caffeoylquinic acid derivatives [19, 20], sterols [21, 22] and pectin [23]. *P. communis* L. have antibacterial, antiseptic, antipyretic, aphrodisiac, astringent, diuretic, laxative and antihyperglycemic activities [24], also *M. domestica* Borkh. reported to possess chemopreventive in liver cancer [25], furthermore, apples and pears have antioxidant and antihyperlipidemic activity [26]. Our main purpose of this study, therefore, was to develop accurate, rapid and reproducible DNA-based marker (ISSR) and (SCoT) techniques for discrimination of different apple and pear cultivars, as there is no report of usage of ISSR and SCoT combination for study of genetic diversity between apple and pear cultivars in Egypt.

MATERIAL AND METHODS

Plant Material

The leaves of the six cultivars, apple cultivar Anna (1), Volus (2), Dorset golden (3), pear cultivar Le Conte (4), MKM (5) and Flordahome (6), were collected in March 2017 from Horticulture Research Institute, Giza, Egypt. The plants were authenticated by Dr. Galal Eliwa professor in the institute and by Dr. Abdel Halim Abdel Mogaly, taxonomist in the Agricultural Museum, Giza, Egypt. Voucher specimens no. 12.03.2017.01 and 12.03.2017.02 were deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt. Samples of leaves were stored at -70 ^{o}C and then, were ground using liquid nitrogen to a fine powder before DNA isolation. DNA analysis was carried out in Horticulture Research Institute, Agricultural Research center, Ministry of Agriculture and Land Reclamation, Giza, Egypt in 2017.

DNA Mapping Material

Buffers: Extraction buffers: Lysis Buffer AP1: 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 0.1M NaCl, 1X PVP, 10mM DTT, RNase (add fresh prior to use). Protein Precipitation buffer AP2: 60 ml of 5M potassium acetate (98.14 g in 200 ml d H20), 11.5 ml of glacial acetic acid in 28.5 ml d H20. Binding Buffer AP3: 1M Guanidine Hydrochloride (4.78 g GH in 50 ml 100% ethanol). Wash buffer AW: 70% ethanol. 5X Tris-borate (TBE) (pH 8), Tris-base 5.4 g, Boric acid 2.75g, 500 mM EDTA (pH 8) 0.29 g and deionized water (d.w) up to 100 ml. Reaction buffer: 10X TBE, 25mM $MgCl_2$, 2.5 mM dNTPs, 10 pmol primer, Taq DNA polymerase, Template DNA and (d.w) H_2O. Loading dye buffer 5X: 2 ml Na-EDTA (pH 8), 5 ml Glycerol (100%), 0.75 ml Bromophenol blue and 1.5 ml (d.w.) H_2O.

Primers: ten primers were purchased from Metabion international AG (Germany), five primers were used for ISSR analysis and their sequences are as follows:

14A; [CTC TCT CTC TCT CTC TTG], 44B;[CTC TCT CTC TCT CTC TGC], HB-12;[CAC CAC CAC GC], HB-14; [CTC CTC CTC GC], HB-15;[GTG GTG TGG C].

Five primers for SCoT analysis with the following sequences.

SCoT 2;[ACC ATG GCT ACC ACC GGC], SCoT 3;[ACG ACA TGG CGA CCC ACA], SCoT 6;[CAA TGG CTA CCA CTA CAG], SCoT 8;[ACA ATG GCT ACC ACT GAG], SCoT 11;[ACA ATG GCT ACC ACT ACC].

Molecular weight marker: DNA ladder 100 bp, Bio-Rad laboratories.

Apparatus: The DNA amplifications were performed in an automated thermocycler (model Techno 512), agarose gel electrophoresis was carried out by (mini-submarine gel Bio-Rad) tool. Visualization of ISSR and SCoT fragments was carried out by UV Polaroid camera**.**

Methods for Molecular Examination

DNA extraction*:* DNA extraction was performed using DNeasy Plant Mini Kit (QIAGEN) by DNeasy-like protocol [27].

Amplification of ISSR and SCoT markers: PCR was performed with 25 ng DNA template according to [28] following thermal cyclic profile.

Thermal cyclic profile: The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one denaturation cycle at 94º C for 4 min followed by 45 cycles each of 1 min at 94º C, an annealing cycle for 1 min at 57º C, and an elongation cycle for 2 min at 72º C. Finally, the primer extension section was prolonged to 10 min at 72º C. The amplified products were determined by electrophoresis using 1.5% agarose gel containing (0.5µg/ml) ethidium bromide in 1X TBE buffer for 30 min at 80 V in mini submarine gel Bio-Rad.

ISSR and SCoT Analysis

DNA extraction was performed using DNeasy Plant Mini Kit (QIAGEN), PCR amplifications were performed in an automated thermal cycle (model Techno 512). Amplification products were determined by electrophoresis, visually studied and recorded for the presence (1) or absence (0) of DNA bands. Cluster analysis of data using unweighted pair group method with arithmetic average (UPGMA) was used for obtaining of the similarity matrix. Each of DNA bands was treated as a unit character for assessment of genetic diversity between the tested samples [28]. The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among genotypes as revealed by dendrograms were done using SPSS windows (Version 24) program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among cultivars [29].

RESULTS

Six different cultivars, three apple and three pear cultivars were selected from the local market to examine the accuracy and precision of quality control and genetic discrimination by using ISSR and SCoT techniques. The fresh leaves are more or less like to each other in their morphological characters despite being apple or pear cultivars (Fig.1). ISSR and SCoT techniques were carried out using ten decamer primers, five for each technique. The banding pattern produced by ISSR primers, 14A, 44B, HB-12, HB-14 and HB-15 and those produced by SCoT primers, SCoT 2, SCoT 3, SCoT 6, SCoT 8 and SCoT 11 were all represented in (Table. 1). ISSR and SCoT bands were considered as present or absent regardless of their percentage. The ISSR and SCoT-PCR produced loci indicated that all primers were produced. Each DNA locus is considered as a unit character. The total number of resulted bands was 49 bands, 26 by ISSR technique and 23 by SCoT technique, mainly produced by 7 primers of the 10 used primers, 7 bands by primer HB-14 ranging from 140 to 6700 bp, 6 bands by primer SCoT 2 ranging from 360 to1280 bp and 5 bands by each of these primers, 44B primer ranging from145 to 1960 bp, HB-12 primer ranging from130 to 2950 bp, HB-15 primer ranging from 135 to 1830 bp , SCoT 3 primer ranging from 220 to 625 bp and SCoT 11 primer ranging from 315 to 980 bp, as a result, selection of primers HB-14, 44B, HB-12 and HB-15 for ISSR based analysis and primers SCoT 2, SCoT 3 and SCoT 11 for SCoT based analysis is proper for discrimination of apple and pear cultivars from each other. ISSR based analysis produced 26 bands divided into 14 and 12, monomorphic and polymorphic bands, respectively. SCoT based analysis resulted in 23 bands divided into 13, 7 and 3

monomorphic, polymorphic and unique bands, respectively. All used apple and pear cultivars are differentiated by presence or absence of unique bands in ISSR or SCoT profiles. The total number of different band types resulted with each primer is represented in (Table. 1) and the common and unique bands produced by ISSR and SCoT PCR amplification are provided in (Table. 1). Dendrograms are computational diagrams used for clustering of samples and genes. They have been widely used in clustering of closely related pear cultivars [30] and in the differentiation of jojoba male and female genotypes 14 and used in the evaluation of genetic diversity of wild *Pistacia* Species [31]. Use of dendrograms based on unweighted pair group method with arithmetic average (UPGMA) analysis results in clustering of the six cultivars as follows, according to ISSR data (Fig. 3 A) they were grouped into three main groups with Dice similarity coefficient ranging from 0.77 to 0.96 (Table. 2), the first group includes two apple cultivars, Volus and Dorset golden and the second group includes, pear cultivar Le-Conte, MKM and Flordahome and finally, the third group include, apple cultivar Anna alone. Dendrogram based on SCoT data (Fig. 3 B) grouped the six cultivars into two main groups with Dice similarity coefficient ranging from 0.74 to 0.92 (Table. 3), the first include the three apple cultivars and the second includes the three pear cultivars with further division of the first group to two sub groups, one contains cultivars Anna and Volus and the other contains only cultivar Dorset golden, also, the second main group is divided into two subgroups, one includes pear cultivars MKM and Florda home and the other has only cultivar Le-Conte. According to the dendrogram based on combined ISSR and SCoT data (Fig. 3 C), the six cultivars were clustered into two main groups with Dice similarity coefficient ranging from 0.76 to 0.92 (Table. 4), the first included apple cultivars with two subgroups, one for cultivar Anna alone and the other contains cultivars Volus and Dorset golden. The second main group contains pear cultivars with also, two subgroups, one contains only cultivar Le-Conte and the other contains cultivars, MKM and Florda home.

DISCUSSION

Herbal drug adulteration with cheaper or less active adulterants is widely spread whereas, it is intended or due to unawareness, despite the reason, herbal drugs adulteration can cause harmful effects on human health [32]. Assurance of traditional medicines safety, quality and efficacy is one of the WHO guidelines that are required firstly in the evaluation and research methodology of traditional medicines which requires necessarily, the correct identification. DNA-based discrimination of different plants is undoubted, a superior technique that provides accurate and reproducible identification based on unique genetic structure and furthermore, it is not affected by age, physiological or environmental factors. Moreover, DNA-markers are not tissue specific and thus can be used in the identification of the plants in any developmental stage [6]. In comparison with morphological and chemical authentications, DNA based technology provides precise, efficient and fast means of differentiation for hundreds of samples because it can be operated automatically. DNA based authentication is utilized in differentiation between species, individuals and populations as a widely distributed technique in many fields so, DNA based tools of discrimination used now as an important pharmacognostic measure in medicinal plants quality control and quality assurance. The great importance of DNA based discrimination in quality control of commercial medicinal plants which are adulterated is due to the utility of this technology in both processed and unprocessed forms of the plants[33]. Apples and pears are well known edible fruits with high nutritional value but also, they are a great source of different biologically active compounds whereas in fruits or leaves such as, phenolic compounds with their different classes as flavanoids, phenolic acids and procyanidins [19, 20] beside the presence of high amounts of fiber and pectin [23]. Scientific studies suggested that apples and its products had a wide range of biological activities that include antioxidant, antiproliferative, anti-diabetic, anti-inflammatory, lipid oxidation inhibition, and cholesterol-lowering activities which may contribute to health beneficial effects against cardiovascular disease, asthma and pulmonary dysfunction, diabetes, obesity, and cancer [34]. It was reported also, that pears have several biological activities such as antioxidant, antiulcer and antibacterial activities [35-37]. Polyphenols concentration in apple is influenced by the plant variety as well as environmental factors, including geographic region, growing season, and storage [38] that is assuring the importance of using DNA-based technology for evaluation of genetic diversity of apple and pear cultivars. Recent studies were carried out for evaluation of genetic diversity between Chinese wild apple species and apple cultivars [39] and between northeastern Spain local apple cultivars [40] by using simple sequence repeats (SSR) markers. Genetic diversity of some pear cultivars and genotypes by use of (SSR) Markers was done [41]. In this study, we preferred to use both ISSR and SCoT techniques due to their advantages comparable to RAPD technique and for obtaining more accurate, reproducible and higher levels of polymorphism. In this study, the six apple and pear cultivars are widely cultivated in Egypt, whether for eating as apple cultivar Anna and pear cultivar Le Conte and Flordahome or for using as rootstock as apple cultivar Dorset golden and Volus. The six cultivars including apple cultivars, Anna (1), Volus (2) and Dorset golden (3) beside pear cultivars, Le Conte (4), MKM (5) and Flordahome (6) were chosen for genetic discrimination by ISSR markers 14A, 44B, HB-12, HB-14 and HB-15 and by SCoT markers SCoT 2 SCoT 3, SCoT 6, SCoT 8 and SCoT 11. Based on ISSR markers, the higher percentage of polymorphism was exhibited by two decamer primers 14A, 75% polymorphism and 44B, 60% polymorphism. According to SCoT markers, the higher polymorphism exhibited by decamer primers, SCoT 8 and SCoT 11 with 67% and 60% polymorphism, respectively. All ISSR primers produced polymorphic bands. Concerning SCoT primers, three unique bands were produced as follows, two bands produced by sample (3) with primers SCoT 2 and SCoT 6 at 1280 bp and 540 bp, respectively and one produced by sample (2) with primer SCoT 11 at 880 bp. Primer SCoT 3 gave totally monomorphic bands which means that is indicative of a functional gene present in all tested cultivars. Based on the percentage of polymorphism, primers 14A and 44B are preferred to use in ISSR based authentication, while for SCoT based discrimination, it is preferred to use primers SCoT 8 and SCoT 11. Cluster analysis of the six cultivars showed slight differences based on the technique used in the evaluation of genetic diversity, which indicate the close relationship between apple and pear cultivars thus, gathering of more than one DNA based techniques is recommended for more accurate and reliable results. According to the combined ISSR and SCoT based dendrogram, the six cultivars were grouped into two main groups, one includes the apple cultivars with further separation into two subgroups one contains Apple cultivar Anna alone and the other contains cultivars Volus and Dorset golden and the second main group includes the pear cultivars which also subdivided to two subgroups, one contains cultivar Le Conte alone and the other includes cultivars MKM and Flordahome.

Table 1: Polymorphic pattern and degree of polymorphism for the genetic relationship in apple and pear cultivars

Primer name	Sequence	Gel polymorphism					
		Monomorphic bands	Polymorphic (without unique)	Unique bands	Polymorphic (with unique)	TNB	% P
14A	CTC TCT CTC TCT CTC TTG	1	3	0	3	4	75%
44B	CTC TCT CTC TCT CTC TGC	2	3	0	3	5	60%
HB-12	CAC CAC CAC GC	4	1	0	1	5	20%
HB-14	CTC CTC CTC GC	4	3	0	3	7	43%
HB-15	GTG GTG GTG GC	3	2	0	2	5	40%
SCoT 2	ACC ATG GCT ACC ACC GGC	3	2	1	3	6	50%
SCoT 3	ACG ACA TGG CGA CCC ACA	5	0	0	0	5	0%
SCoT 6	CAA TGG CTA CCA CTA CAG	2	1	1	2	4	50%
SCoT 8	ACA ATG GCT ACC ACT GAG	1	2	0	2	3	67%
SCoT 11	ACA ATG GCT ACC ACT ACC	2	2	1	3	5	60%

Table 2: Genetic similarity matrix based on ISSR data among six of *P. communis* L. and *M. domestica* Borkh. cultivars estimated according to dice method

	1	2	3	4	5	6
1	1.00					
2	0.87	1.00				
3	0.82	0.96	1.00			
4	0.81	0.78	0.77	1.00		
5	0.93	0.89	0.84	0.84	1.00	
6	0.85	0.86	0.86	0.91	0.93	1.00

Table 3: Genetic similarity matrix based on SCoT data among six of *P. communis* L. and *M. domestica* Borkh. cultivars estimated according to dice method

	1	2	3	4	5	6
1	1.00					
2	0.92	1.00				
3	0.9	0.88	1.00			
4	0.84	0.81	0.74	1.00		
5	0.82	0.86	0.84	0.9	1.00	
6	0.86	0.89	0.87	0.87	0.91	1.00

Table 4: Genetic similarity matrix based on combined ISSR and SCoT data among six of *M. domestica* Borkh. and *P. communis* L. cultivars estimated by dice method

	1	2	3	4	5	6
1	1.00					
2	0.89	1.00				
3	0.86	0.92	1.00			
4	0.82	0.8	0.76	1.00		
5	0.88	0.88	0.84	0.87	1.00	
6	0.85	0.88	0.86	0.89	0.92	1.00

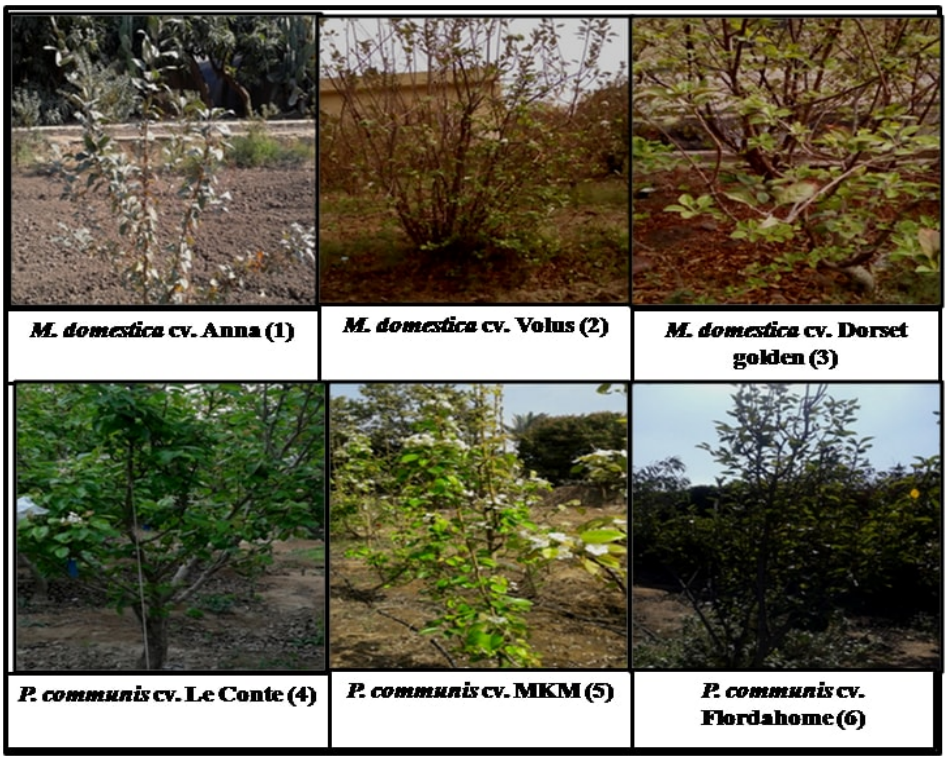

Fig. 1: Photos of the three apple and three pear cultivars' trees

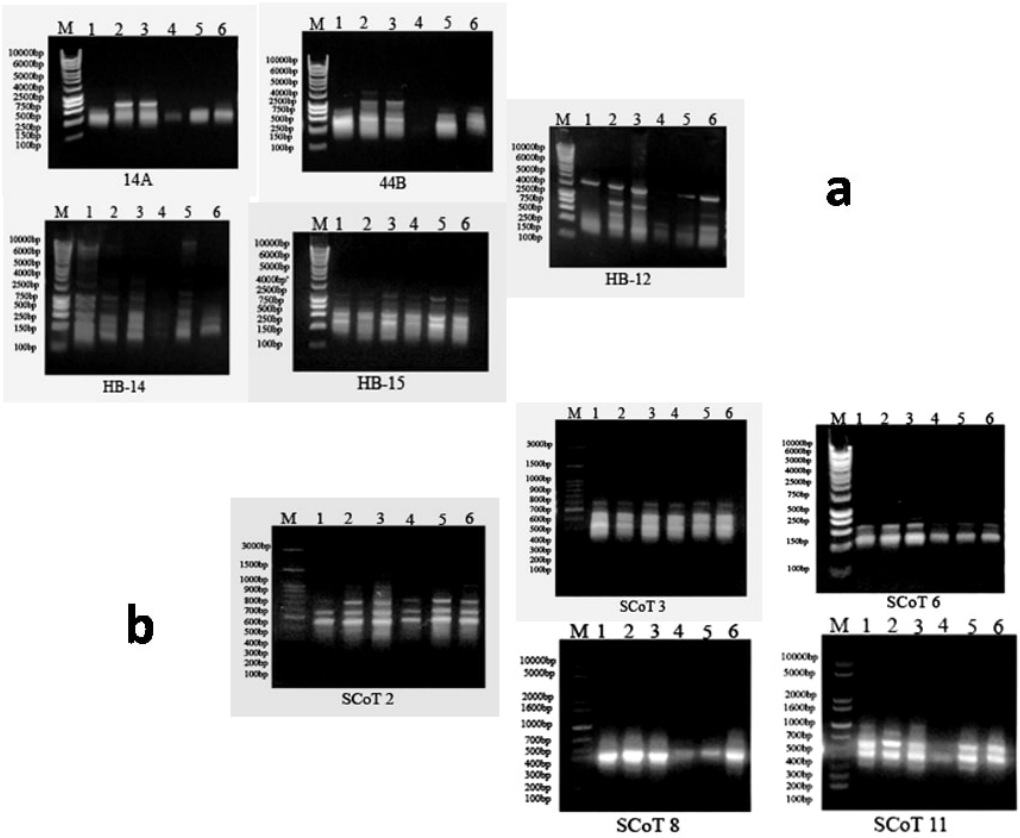

Fig. 2: The obtained PCR bands of *Malus domestica* borkh. cvs. Anna (1), Volus (2), Dorset golden (3) and P. *communis* l.cvs Le-conte (4), MKM (5) and Flordahome (6) with (a) ISSR primers (14A, 44 B, HB-12, HB-14 and HB-15) and (b) SCoT primers (SCoT2, SCoT 3, SCoT 6, SCoT 8 and SCoT 11).

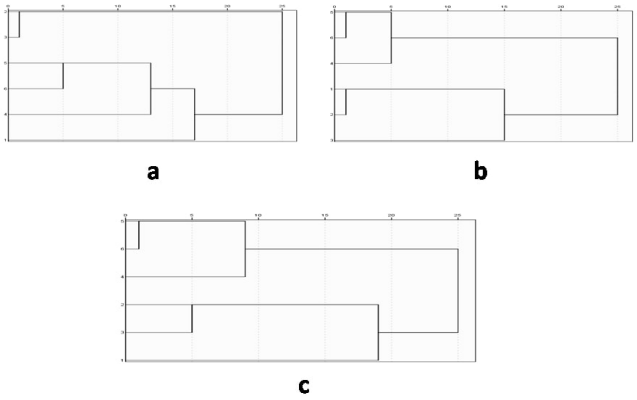

Fig. 3: UPGMA dendrograms showing the clustering of the six apple and pear cultivars based on (a) ISSR data, (b) SCoT data and (c) combined ISSR and SCoT data.

CONCLUSION

Use of different DNA based analysis techniques whether alone or preferably in combination as we used in this study can provide large, accurate and reference data base can be used for fast and precise authentication of closely related medicinal plants from their adulterants.

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Cite this article as:

Seham S. El-Hawary *et al*. Inter simple sequence repeat (ISSR) and start codon targeted polymorphism (SCoT) as discrimination techniques between certain apple and pear cultivars. Int. Res. J. Pharm. 2018;9(9):80-86 http://dx.doi.org/10.7897/2230-8407.099192

Source of support: Nil, Conflict of interest: None Declared