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Utilizing Start Codon Targeted (SCoT) markers for DNA fingerprinting and diversity analysis of Artemisia herba-alba plant species

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Abstract:

Artemisia herba-alba, is a profoundly significant medicinal plant, known for the abundant spectrum of bioactive metabolites. Its potential for therapeutic and medicinal applications is well established, both in modern and traditional medicine. Research was focused towards the identification of the chemical composition for specimens of *A. herba-alba*, but not the genetic diversity present within the same species. In this paper, replicate samples of the plant species were collected from three distinct locations in both Mersa-Matrouh and Saint Catherine (Wadi El Raha). Genomic DNA was extracted from aerial plant materials and amplified via SCoT-PCR to determine their genetic relatedness using Dice coefficient to construct their dendrogram. SCoT-PCR associations indicated genetic variance within the species and close association between (S2 & S3) and (M1 & M2).

Keywords*: SCoT PCR, Artemisia herba-alba, Medicinal plants, Secondary metabolites.*

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1. Introduction:

Artemisia herba-alba is one of the most commonly known medicinal plant belonging to the Asteraceae family that dissipatedly grow in various geographical locations and familiar to tolerate several bioclimatic conditions [1]. This species like all the medicinal plants have gained its potential medicinal effect due to the presence of a large spectrum of bioactive metabolites [2]. After years of extensive researches and data analysis regarding the phytochemistry of Artemisia species including *herba-alba* in particular, it was concluded that this species serve as a huge container of many beneficial compounds in which the terpenoid family is the highlight; the most important within is the sesquiterpene lactone with the greatest structural diversity within the family and other compounds varying between essential oils, phenolic compounds, flavonoids, sterols, acetylenes and coumarins with respect to their subordinates. Over the past two decades, several studies have reported the effectiveness of this plant to cure several human and animal diseases. According to [3], the broad spectrum of its medicinal uses including antispasmodic, antimalarial, anti-diabetic, antioxidant and many other pharmacological properties. DNA fingerprinting is known to be the application of many molecular marker techniques for the identification of cultivars species. These techniques include Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR), Sequence Tagged Microsatellite Loci (STMS), Random Amplified Polymorphic DNA (RAPD).

Start Codon Targeted Polymorphism (SCoT) developed by [4] have been employed to successfully demonstrate a high polymorphic and efficient identification of genetic diversity analysis in rice and peanut. Nowadays, this technique became a widely used technique as a molecular marker for plant fingerprinting. The concept of this technique is relying basically on the presence of short conserved region in plant genes surrounding the translation initiation codon (ATG). DNA markers are produced by using18-mer single primer that is used as forward and reverse at once [5, 6] in the polymerase chain reaction (PCR) that are designed from the short-conserved region flanking the ATG start codon [7] that is conserved for all genes and with 50 ^{0}C as annealing temperature. Markers are then resolved by standard gel electrophoresis; with agarose gels and staining making this technique suitable for the vast majority of plant research labs with standard equipment.

This technique overcomes the drawbacks of RAPD, AFLP and ISSR, it is a gene targeted marker that can be employed without needing the genomic sequence information and can generate more reproducible information correlated with biological traits compared with random DNA markers. This technique has been

demonstrated high polymorphic and efficient when compared to RAPD, AFLP and ISSR, it is a gene targeted marker and can generate more information correlated with biological features compared with random DNA markers [4]. The present investigation was designed to associate and define the genetic variance of Artemisia *herba-alba* using a novel marker system for the plant species SCoT PCR, applied for the first time in fingerprinting studies for Artemisia plants.

2. Materials and Methods

Ethics Statement

There is no particular ethics statement for this field of study as the locations from which the samples were collected are not privately owned or restricted by the government.

Plant Materials

Three soil samples conjugated with their vegetative parts of the species *A. herba-alba* were collected at depth (10-20 cm) in December 2017 from two different locations: Mersa-Matrouh and Saint Catherine (Wadi El Raha). Table 1 demonstrates the exact coordinates of the two sites recorded using the GPS model (Garmin- etrex vista cx)., samples were labeled as M1, M2 and M3 representing the 3 different locations from Mersa-Matrouh province and as S1, S2 and S3 to represent the collected samples from Saint Catherine. The samples were placed in sterile plastic bags and stored at 4 ^{0}C immediately until the delivery, then the vegetative parts were air-dried, and 0.5 mg were separated from each sample to be stored at -20^{0}C for the DNA extraction required in this study.

Table 1: Sample Geographical Coordinates recorded using the GPS model (Garmin- etrex vista cx)

Accession	Location	Latitude (N)	longitude(E)	Altitude (m)
S1	Saint Catherine	28.57507	33.94132	1613
S2	Saint Catherine	28.57558	33.94107	1613
S3	Saint Catherine	28.57562	33.94142	1614
S-control	Saint Catherine	28.57570	33.94083	1609
M1	Mersa-Matrouh	31.23125	27.01582	171
M2	Mersa-Matrouh	31.23107	27.01559	171
M3	Mersa-Matrouh	31.23589	27.01547	173
M-control	Mersa-Matrouh	31.23589	27.02125	176

Abbreviations: N-north; E- east; m-meter

Plant Molecular Analysis

DNA isolation

The collected vegetative samples (three sample from each location) were used to isolate the DNA as a part of developing DNA markers in Artemisia plant and to determine the level of genetic diversity between the two localities. High quality genomic DNA was extracted from young fresh leaves (0.5 mg) of all collected samples using DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) and according to the manufacturer's protocol. The extracted DNA samples were assessed for their quality and quantity through electrophoresis on 1.0 % agarose gel.

Start Codon Targeted Polymorphism Analysis

SCoT-PCR amplification was performed as described by [8]. A set of ten primers with sequences demonstrated in Table 2; were initially screened on the six Artemisia samples. PCR was carried out in 25 µl reaction mixtures containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 µM of each dNTPs, 1 µM of primer, 1U Go-Taq Flexi polymerase (Promega) and 25 ng genomic DNA. A 100 bp plus DNA ladder was used as molecular size standards and the PCR products were visualized on UV light and photographed using a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad®).

Table (2): SCoT Primers sequences used for the SCoT-PCR amplification:

Primer	Sequence
SCoT-1	CAACAATGGCTACCACGA
SCoT-2	CAACAATGGCTACCACGC
SCoT-3	CAACAATGGCTACCACGG
SCoT-4	CAACAATGGCTACCACGT
SCoT-5	CAACAATGGCTACCAGCA
SCoT-6	CAACAATGGCTACCAGCC
SCoT-7	AAGCAATGGCTACCACCA
SCoT-11	ACGACATGGCGACCAACG
SCoT-16	ACGACATGGCGACCATCG
SCoT-20	ACGACATGGCGACCACGC

Data Analysis:

The banding patterns generated by SCoT markers analyses were compared to determine the genetic relatedness of the six Artemisia genotypes. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient [9].

Dice formula: GSij = 2a/(2a+b+c)

Where GSij is the measure of genetic similarity between individuals i and j, (a) is the number of bands shared by i and j, (b) is the number of bands present in i and absent in j, and (c) is the number of bands present in j and absent in i. The similarity matrix was used in the cluster analysis. The cluster analysis was employed to organize the observed data into meaningful structures to develop taxonomic relatedness.

3. Results:

Following the procedure of DNA extraction, quantification and amplification, PCR products were resolved by using 1.5 % agarose gel electrophoresis containing ethidium bromide (0.5ug/ml) in 1X TBE buffer. A 100 bp plus DNA ladder was used as molecular size standards. PCR products were visualized on UV light and photographed using a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad®). The 10 primer sets (sequences previously mentioned in table 2) were used in separate reactions for the six collected plant samples and each gel was photographed separately. The samples arrangement is as follow: 100 bp marker, M1, M2, M3, S1, S2 and S3 respectively. The results for the electrophoresis are exhibited in Fig. 1 and Fig. 2. Results were analyzed to present the genetic similarity based on the dice coefficient, and polymorphism results are exhibited in Table 3 & Table 4. Finally, the similarity matrix was used for cluster analysis to construct a dendrogram based on taxonomic relatedness Fig. 2.

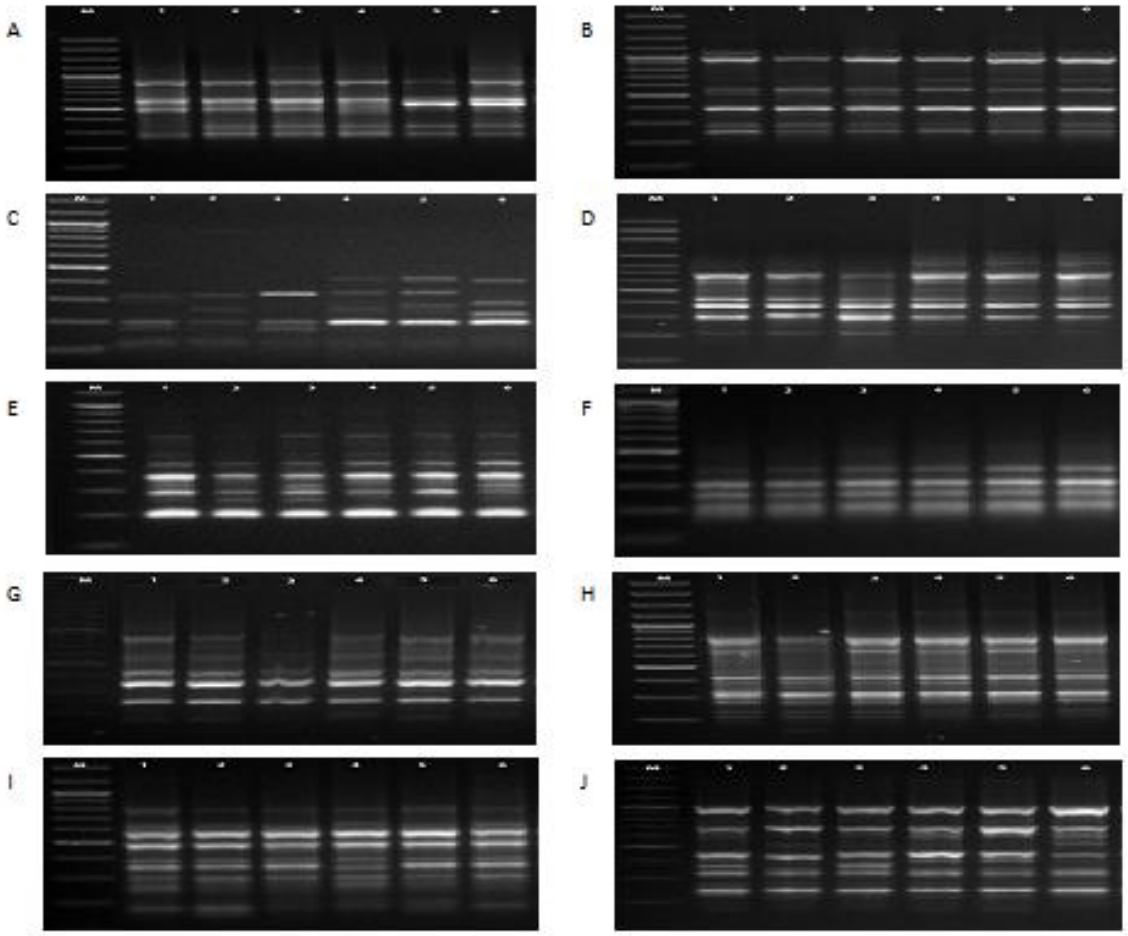
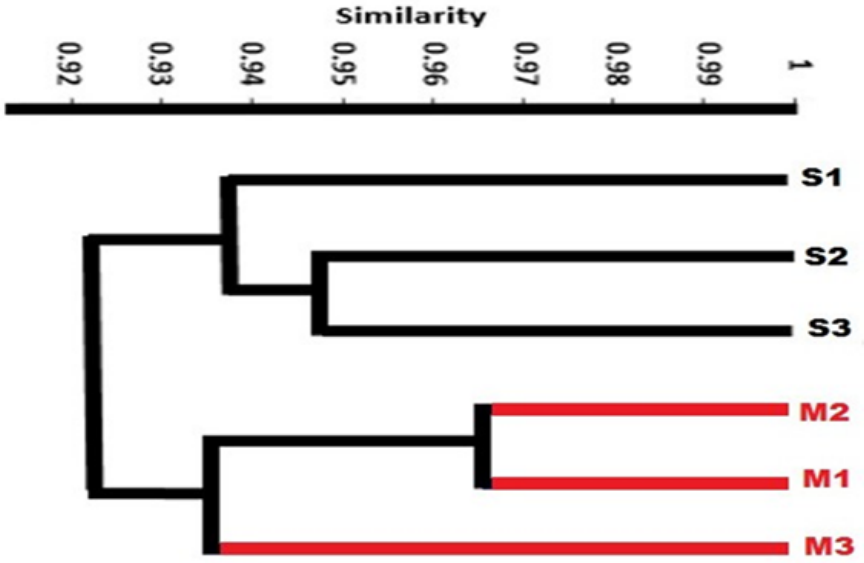


Fig (1): SCoT PCR Gel Electrophoresis; SCoT 2,4,6,7 and 11 showed the same number of bands per lane (4). SCoT 6&7 recorded the same number of polymorphic bands (24), total number of bands (28) and percentage of polymorphic bands (85.7) in all tested samples.



\Fig (2): Dendrogram showing genetic similarity relationship of the six Artemisia samples based on Dice coefficient. The results showed close association between samples (S2 & S3) and (M1 & M2) according to genetic proximity, followed by M3 & S1 association.

Table (3): Average of banding patterns, andpolymorphism for all SCoT primers:

	Average Number of Bands per Lane	NPB	Average NPB	TNB	Average % of PB
SCoT PCR	4.1	246	24.6	339	73.67

Table (4): Detailed banding patterns, number of polymorphic bands, percentage of polymorphism for each primer:

Primer	NPB per Lane	NPB	TNB	% of PB
SCoT 1	3	18	25	72
SCoT 2	4	24	35	68.6
SCoT 3	3	18	27	66.7
SCoT 4	4	24	28	85.7
SCoT 5	5	30	41	73.2
SCoT 6	4	24	28	85.7
SCoT 7	4	24	28	85.7
SCoT 11	4	24	44	54.5
SCoT 16	5	30	42	71.4
SCoT 20	5	30	41	73.2

4. Discussion

All of the assessed primers for *Artemisia herba-alba* successfully produced clear bands in all of the studied samples. The SCoT primers provided in this study provided a total number of 339 bands at an average of 5 bands per primer. The percentage of polymorphism varied where the highest were (85.7%) for SCoT 4, 6, and 7. The banding patterns are shown in Fig. 1 for all the samples. According to [10] scored a total of 79 (83.14%) polymorphic bands detected by 11 SRAP primer pairs in genetic analysis of 29 cultivated individuals of *Artemisia L*. The procedure for identification of the regions (M1-M3; S1-S3) included Genomic DNA extraction of plant materials, and SCoT amplification according to the procedure described by [4]. A set of 10 SCoT primers were initially screened on the six Artemisia samples (Table 1). Understanding of the genetic relationship within populations is essential for founding effective and efficient

conservation for plants [11], and estimation of functional variances. The genetic diversity of *Artemisia herba-alba* growing in Egypt in two distinct regions Mersa-Matrouh and Saint Catherine based on the combined results of SCoT markers ranged from 54.5% in primers 11 to 85.7% in primers 4, 6, and 7 (Table 4). With and average percentage of 73.7 (Table 3), which is consistent in polymorphism patterns observed by [12, 13], for *Artemisia herba-alba* growing in Syria and Tunisia respectively.

Fig. 2 exhibits the genetic similarity between the six samples, where the banding patterns generated by the SCoT markers were compared to determine the genetic relatedness of the samples, the genetic similarity was estimated according to Dice coefficient [14]. The results showed close association between samples (S2 & S3) and (M1 & M2) according to genetic proximity, followed by M3 & S1 association. All samples exhibited some relatedness; however, it is interesting to observe that there are differences within the same plant according to their geographical location. These genetic differences could be due to differences the chemical compositions essential oils from the species as confirmed by [15] who reported similar differences found in the essential oil content and composition of *A. Judaica*, reflects the genetic differences between the studied populations. Additionally, [16] reported that more efficient results are be obtained if all findings correlated with ecogeographical information, which was inclusive in our research. This means that the same species was divided into two main groups, this grouping could be due to altitude and topography between the regions, The findings of grouping work with the findings of [13], who associated the family tree of wormwood in Tunisia and Jordan, where populations were segregated into groups according to the area of growth for the clans, additionally, this coincides with a molecular analysis of *Artemisia capillaris* samples, where they provided variation according to the environments they were in [11]. Genetic variation in the present study, could be attributed to the distinct locations, pollination type, and environmental conditions of the plant [17] confirmed the geographical and environmental effects in and how they contribute to the genetic diversity of the Artemisia plants in Saudi Arabia, similarly [18], observed variance in 3 kinds of wormwood in Jammu and Kashmir. It is worth mentioning that based on the evaluated literature, Artemisia plants were not previously assessed for genetic variation in Egypt, and as far as we have researched never using SCoT PCR, even though it is a powerful identification tool of stable performance in terms of marker associations, the present study demonstrates this. SCoT marker techniques were employed for their targeted techniques, which was classified as simple and reproducible, when resolving on a gel, they are highly reproducible, when compared to RAPD markers which are more arbitrary. Generally, SCoT-PCR was implemented for Chinese sugar cane [19], mangoes [20]), date palm, [8], rice [4], but this is first case of evaluating diversity of Artemisia *herba-alba* using SCoT assay. The relationships of the samples were assessed using similarity indices, where higher indices exhibit closer genetic association, versus lower indices which demonstrate distance in genetic relationships [21]. The similarities of the dendrogram are associated with the results of [11], who estimated the degree between wormwood plants which were 16-82%, closer associations observed within the samples. These assessments though they present interesting and promising results, mandate the utilization of larger number of primer sets, however the present work aimed to estimate the surrounding soil and chemical analysis of the samples via HPLC, thus further work would recommend the use of approximately 25 primer sets for comprehensive assessment, another limitation would be the sample size, where more robust samples (20-100 samples) would be suggested to analyze this phenomenal medicinal plant of significant value in an all-inclusive manner. Which was a portion of the focus presented in this study, and thus justifying the smaller sample size 6 and primer sets 10.

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