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DNA profiling of Egyptian *Astragalus spinosus* (Forssk.) Muschl. species using AFLP markers

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ABSTRACT

Background: Species of Astragalus have been used medicinally for more than a thousand years. *Astragalus spinosus* (Forssk.) Muschl. extract has been used in the treatment of various diseases. Despite its numerous and varied uses, studies on the genetic makeup of Egyptian *A. spinosus* are lacking. Therefore, the current study was conducted to generate a molecular fingerprint for this species in two successive growth periods which will be used to recognize, record, and act as a molecular reference for future research.
Method: *Astragalus spinosus* samples were collected from El-Hawala region of Marsa-Matrouh, Egypt's North Coast and subjected to molecular analyses using the amplified fragment length polymorphism technique (AFLP) with four primer combinations.
Results: Our results revealed minute variations among *A. spinosus* samples regardless of different collecting periods.
Conclusions: The current work confirmed the accuracy and reliability of the AFLP technique in constructing an accurate genomic picture of the investigated plant.

1. Background

The genus Astragalus includes more than 2,500 species grouped in 100 subgenera and is the largest in the Fabaceae family. It is regarded as one of the most varied genera. Egypt has about 37 species that have been identified [1].

Astragalus spinosus (Forssk.) Muschl. is a commonly terrestrial shrub species from the Leguminosae family. It is locally known as Kaddad. It develops as a self-supporting growth type in an arid environment. As a result, *A. spinosus* is a drought-tolerant plant. It is a little perennial shrub with a height of about 60 cm and a semi-circular width.

In the spring, the plant renewed its growth. It distinguishes with a white flower in January, besides its fruits which look like chickpeas that are in April [2,3]. The plant produces powerful, cylindric, 15 cm long, sharp thorns which protect the plants from the grazing animals. Nevertheless, during the early phases of growth the branches are flexible allowing animals to graze. Moreover, *A. spinosus* fixes nitrogen and functions as a photoautotroph (able to convert light into chemical energy). Also, it is crucial to plan for regenerating deteriorated habitats [2], Fig 1. Species of Astragalus have been used medicinally for more than a thousand years [3].

Astragalus spinosus extract has been used to treat leukaemia, allergic reactions, wound healing, scorpion bite, and other inflammatory conditions. In addition, the plant extract has antifungal, antibacterial, anti-anxiety, antidepressant, and immunostimulant effects. Furthermore, *Astragalus spinosus* is used in the treatment of renal, hepatic, and cardiac toxicities, the modulation of neurotoxicity, and DNA damage [4].

Fig. 1 *Astragalus spinosus*

Noteworthy to mention that, traditional organism identification was based on many shared morphological characteristics, mainly collecting the entire plant at its vegetative, blooming, and fruiting stages during the same study season. Even though, these techniques are considered time-consuming due to the great influence of environmental or developmental factors on the features of the plant during its growth. They may not be able to distinguish these organisms at the species level. Furthermore, if the taxa have the same phenotyping or were gathered when they were still juveniles, even an experienced taxonomist could make a mistake [5].

To accurately identify the investigated species, geneticists use specific molecular markers in conjunction with morphological characteristics. DNA fingerprinting, also known as DNA typing or profiling, is a method for differentiating between members of the same and distinct species. Therefore, displaying a collection of DNA fragments from a particular DNA sample constitutes DNA fingerprinting. These DNA fragments that show variations or mutations can be used to identify polymorphism in a population or gene pool between various genotypes.

Prokaryotic and eukaryotic organisms can be identified and typed at the DNA level using various techniques. These techniques include those with a potentially broad application, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), Restriction fragment length polymorphism (RFLP), microsatellites or simple sequence repeats (SSR), and single nucleotide polymorphisms (SNP) that differ in their standardization, ease of result interpretation, taxonomic range, reproducibility, and discriminatory power.

The best genotyping technique yields results that are constant at different labs enabling specific comparative analysis and the creation of trustworthy databases [6]**.** The amplified fragment length polymorphism (AFLP) is considered one of several molecular biology techniques based on Polymerase chain reaction (PCR) amplification for identifying genomic restriction fragments. It may be applied to DNAs of any complexity or origin and is very helpful in identifying polymorphism across genotypes that are closely related. AFLP has many advantages over other DNA fingerprinting methods [6,7].

For convenience, prior knowledge of the genome sequence is not required for the AFLP technique. It has been successfully used in numerous fields, including breeding, taxonomy, microbiology, ecology, population genetics, and evolutionary biology. Additionally, AFLP-based marker-assisted analysis provides accurate and reliable data that may be contrasted with information from other analytical techniques. AFLP provides much information to identify strains or variants with the highest level of specificity. It is possible to create genotyping databases that several laboratories can utilize for different purposes. AFLP is regarded as one of the best tools for detecting and comparing genetic relationships between and within species [8].

Moreover, AFLP can also be used to research species with sequenced genomes. For instance, AFLP has been applied to paternity tests and criminal investigations to analyze human DNA samples [9]**.** it has been used to track infectious outbreaks in hospitals to determine whether an outbreak is related to the transmission of only one strain or several different strains [10] . The pathogenic organism is isolated from infected patients and put through AFLP analysis in this investigation. Researchers can quickly ascertain if the epidemic is caused by a single strain or a variety of strains by comparing AFLP data obtained from various patients.

The significance of AFLP markers and their applications has been demonstrated in the literature over many years. AFLPs were used in sweet potato, wheat, sorghum, and mango as an effective tool to observe genetic diversity, tagging important agronomic traits, fiber-quality traits, and fingerprinting studies in cotton, rice, and soybean [11].

Additionally, in maize, AFLP techniques have been applied to DNA fingerprinting, genome mapping**,** genetic diversity studies, and hybrid performance prediction [12] . Furthermore, Molecular diversity and diagnostic fragments for *Cymbopogon* cultivars were developed through AFLP markers which provided detailed cataloguing of *Cymbopogon* cultivars for genetic conservation purpose [13].

Although, the two dominant markers (RAPD and AFLP) successfully measured the genetic variation in the 16 Zea Mexicana genotypes. AFLPs have the advantage of having a higher marker index than RAPD and high reproducibility [14]. Due to the lack of studies on the genetic makeup of *A. spinosus* plant, the current work was carried out to create a molecular fingerprint for *A. spinosus* plant species that would be utilised to recognise, record, and serve as a molecular reference for future research.

2. Materials

2.1. Astragalus spinosus samples

The *Astragalus spinosus* plant samples were collected from El-Hawala region, Marsa-Matrouh, North Coast, Egypt, In April 2018 (sample A) and 2019 (sample B). The young fresh leaves were taken and kept frozen at -80º C until they were used for DNA fingerprinting research.

3. Methods

3.1. Preparation of A. spinosus samples for Amplified Fragment Length Polymorphisms (AFLP)

Young leaves from the plants were ground using liquid nitrogen, and the complete genomic DNA extraction was done using a DNeasy Plant Mini Kit (Qiagen, Santa Clarita, CA). The AFLP analysis was performed using the AFLP® Analysis System II (Invitrogen, USA) following the manufacturer's instructions (Cat. No. 10483-022) at Agricultural Genetic Engineering Research Institute (AGERI), Giza, Egypt. From each sample, 400 ng of DNA were simultaneously digested with EcoRI and MseI restriction enzymes during an overnight incubation at 37ºC.

The restriction endonucleases were inactivated by incubating the digested samples at 70 °C for 15 min. to produce template DNA for amplification, EcoRI and MseI adapters were used to ligate the digested DNA samples [15, 16]. Pre-amplification is carried out using EcoRI and MseI primers, each having a single chosen nucleotide at the 3'position. Four primer combinations (E-ACT/M-CAA, E-ACG/M-CAC, E-ACT/M-CTT, and E-ACG/M-CAA) were utilized to amplify restriction fragments selectively Table 1.

3.2. Calculation of the polymorphism information content (PIC)

Polymorphism information content (PIC) was calculated according to Smith *et al.,* [17] using the formula PIC=1-fi2, where fi2 is the allele frequency. The PIC values range from 0 (monomorphic) to 1 (very high discriminative with several alleles in equal frequencies).

4. Results

The current study employed the AFLP approach to detect the molecular fingerprint of the Egyptian *A. spinosus* plant species using 4 primer combinations (E-ACT/M-CAA, E-ACG/M-CAC, E-ACT/M-CTT, and E-ACG/M-CAA). Based on the utilised primer pair, the results demonstrated little differences in the total number of bands observed in the banding profile of *A. spinosus* species Fig. 2. The findings will be discussed hereafter.

4.1. Combination of E-ACT/M-CAA (lanes 1&5)

This primer combination resulted in 34 bands ranging between 112 and 1838 bp, which was considered the smallest number of bands compared to the other primer combinations. Twenty-nine monomorphic bands were produced with asimilarity percentage of 85%, while the remainder is polymorphic ones at 186, 373, 537, 547, and 1838 bp, the percentage of polymorphism is 14.7%, Table 2.

4.2. Combination of E-ACG/M-CAC (lanes 2&6)

This primer combination exhibited 39 bands ranging from 100 to 1743 bp. Four amplified fragments are polymorphic with sizes 537, 547, 691, and 1535 bp, with a polymorphism percentage of 10.3%. Thirty-five monomorphic bands were obtained with a similarity percentage of 89.7%.

4.3. Combination of E-ACT/M-CTT (lanes 3&7)

It produced 40 bands with size ranging from 112 bp to 1743 bp, out of which 39 bands are monomorphic (common) while one band is polymorphic with a size of 547 bp Table 2. Across primer combinations, this primer pair is thought to have the greatest number of amplified fragments. The similarity percentage obtained using this primer combination is 97.5%, while the polymorphism percentage is 2.5%

4.4. Combination of E-ACG/M-CAA (lane4&8)

Additionally, this primer combination revealed 35 bands ranging from 106 to 1743 bp. Other than these, only 4 bands are polymorphic at 373, 537, 547, and 1535 bp, with a polymorphism percentage of 11.4%. Thirty-one monomorphic bands were obtained with a similarity percentage of 88.5%.

Additionally, the genetic similarity among the two collected samples of *A. spinosus* was performed using the SPSS computer program (version 28.0.1.1). For each primer pair, the estimated similarity between *A. spinosus* samples was 0.92, 0.95, 0.98, and 0.94 for the primer pairs E-ACT/M-CAA, E-ACG/M-CAC, E-ACT/M-CTT, and E-ACG/M-CAA, respectively.

Table 1. Sequence of AFLP adapters and primers

Primer adapters	**Sequence**
EcoRI forward adapter	5'- CTCGTAGACTGCGTACC-3'
EcoRI reverse adapter	5'- AATTGGTACGCAGTCTAC-3'
MseI forward adapter	5'-GACGTGAGTCCTGAG-3'
MseI reverse adapter	5'-TACTCAGGACTCAT-3'
Primer core region	
EcoRI (E)	5'-GACTGCGTACCAATTC-3'
MseI (M)	5'-GATGAGTCCTGAGTAA-3'
Pre-selective amplification primers	
EcoRI (E+1)	5'-GACTGCGTACCAATTCA-3'
MseI (M+1)	5'-GATGAGTCCTGAGTAAC-3'
Selective amplification primers (E+3)/(M+3)	
E-ACT/M-CAA	5'-GACTGCGTACCAATTCACT- 3' 5'-GATGAGTCCTGAGTAACAA-3'
E-ACG/M-CAC	5'-GACTGCGTACCAATTCACG-3' 5'-GATGAGTCCTGAGTAACAC-3'
E-ACT/M-CTT	5'-GACTGCGTACCAATTCACT-3' 5'-GATGAGTCCTGAGTAACTT-3'
E-ACG/M-CAA	5'-GACTGCGTACCAATTCACG-3' 5'-GATGAGTCCTGAGTAACAA-3'

While the computed similarity based on the outcomes of all primer combinations was 0.95, This demonstrates that *A. spinosus* plant samples, regardless of the time of collection, are highly similar to one another and exhibit minimal variances, as seen in Table 3.

The PIC values were used to investigate the efficacy of the utilized primer combinations and the accuracy of the AFLP method. The resulting PIC values are very high, ranging from 0.97 to 0.98, as depicted in Table 4. The maximum PIC value was 0.98 for primer pair E-ACG/M-CAC followed by 0.97 for primer pair E-ACT/M-CAA, demonstrating its effectiveness in exhibiting the minute variations (polymorphisms) between *A. spinosus* samples.

5. Discussion

The utility of AFLP data to resolve various genetic questions has been stated. This method can be extremely helpful for addressing issues with organisms, such as various plants, fungi, and bacteria, whose genome sequence has not yet been identified.

A further application of AFLP is to determine whether two organisms belong to the same species. The genetic diversity of a species or between closely related species can also be determined using this method. The taxonomic classification of species based on AFLP-based genetic markers has been significantly improved by AFLP technique. It also became extremely popular for phylogenetic analysis adding new dimensions to the evolutionary theories in plant and animal research. Moreover, as a tool for improvement of species by marker assisted selection [18] .

In the current study AFLP was used to detect variation in the DNA sequence in *A. spinosus* plant within two collection periods, April 2018 and 2019. Our results revealed that the primer pairs E-ACT/M-CTT and E-ACG/M-CAC successfully provided a good genomic picture of *A. spinosus* by exhibiting the greatest common bands unique to that species which are considered species specific markers for *A. spinosus* species. Therefore, one can recommend these reliable primer pairs for future studies on *A. spinosus*. In addition, our data based on polymorphism information content values (PIC) indicated that the primer combination E-ACG/M-CAC was more successful in revealing the variations (polymorphisms) between the studied samples.

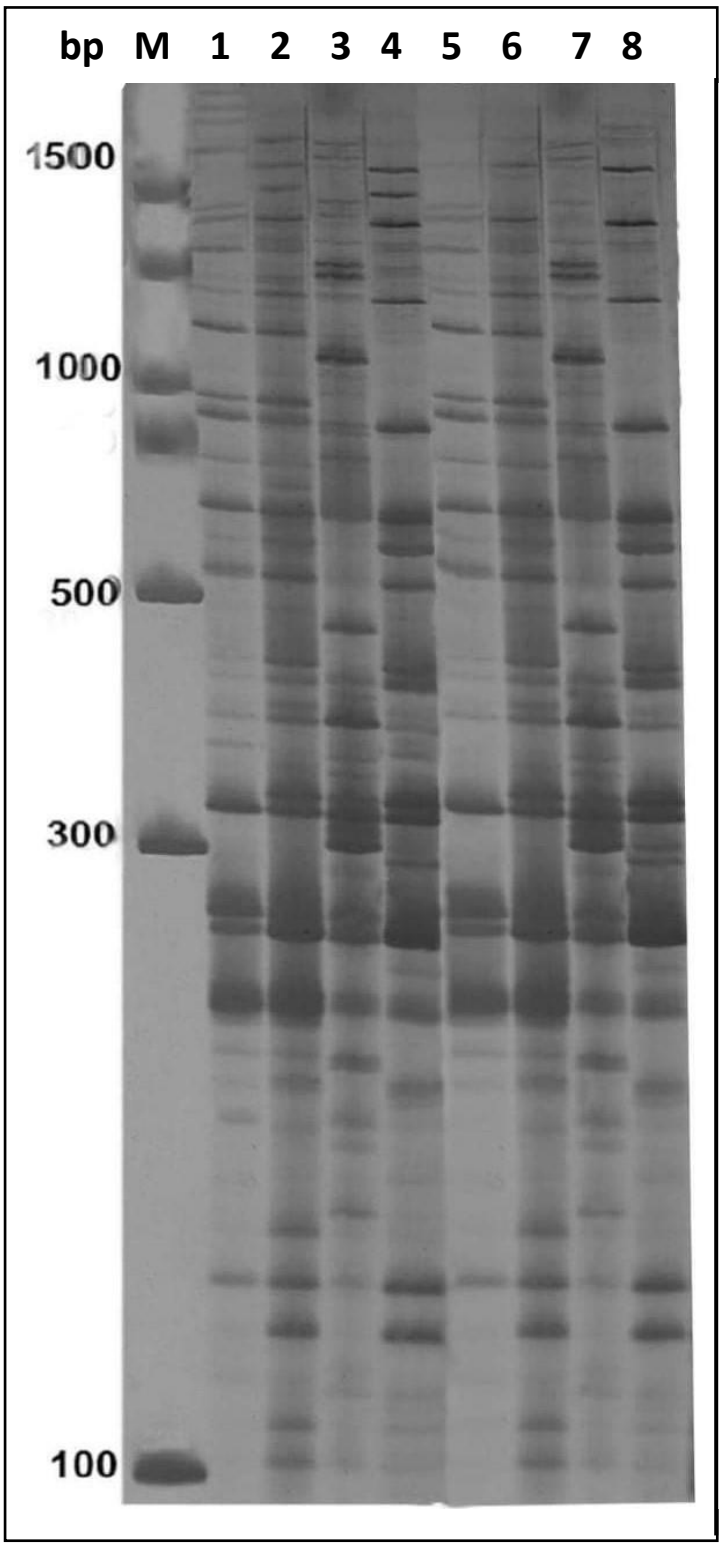

Fig. 2 AFLP banding profile of *A. spinosus* using four primer combinations
(E-ACT/M-CAA (lane 1&5), E-ACG/M-CAC (lane 2&6), E-ACT/M-CTT (lane 3&7) and E-ACG/M-CAA (lane 4&8)).
where Lane 1, 2, 3,and 4 for sample (A), Lane 5, 6, 7 ,(and 8 for sample (B
M: DNA ladder (100-1500 bp).

Table 2. AFLP primer combinations, selective nucleotides, total number of bands, amplified fragments size range, polymorphic bands, and polymorphism percentage.

Primer Name	Total number of bands	Range of band size (bp)	Number of monomorphic bands	Number of polymorphic bands	Polymorphic percentage (%)
E-ACT/M-CAA	34	112-1838	29	5	14.7
E-ACG/M-CAC	39	100-1743	35	4	10.3
E-ACT/M-CTT	40	112-1743	39	1	2.5
E-ACG/M-CAA	35	106-1743	31	4	11.4

Table 3. Similarity matrix between *A. spinosus* samples based on AFLP analysis.

	A	B
A	**1.0**	
B	**0.95**	**1.0**

Table 4. Data obtained from AFLP on Egyptian *A. spinosus* species using four primer combinations.

Parameters and their abbreviations		AFLP
Number of markers	***U***	**4**
Number of non-polymorphic bands	n_{np}	**134**
Number of polymorphic bands	n_p	**14**
Average number of polymorphic bands/assay unit	n_p/U	**3.5**
Number of loci	***L***	**148**
Number of loci/assay unit	n_u	**37**
Total number of effective alleles	N_e	**159.51**
Min of PIC	***PIC***	**0.97**
Max of PIC	***PIC***	**0.98**
PIC value	***PIC***	**0.99743939**
Fraction of polymorphic loci	***β***	**0.09**
Assay efficiency index	A_i	**39.88**
Effective multiples ratio	***E***	**3.50**
Marker Index	**MI**	**3.49**
Total Banding pattern	***Bp***	**11**
Effective number of patterns/ assay unit	***P***	**2.75**

Furthermore, the obtained results showed that, the computed similarity between the two collected samples of *A. spinosus* is found to be very high (0.95) among overall primer combinations regardless of different collection periods. This finding agrees with Savelkoul *et al.* [19] , who stated that establishing similarity, typing, and identification may be generalized.

For example, patterns with 90-100% homology are thought to have been produced by the same strains, while patterns with 60-90% homology indicated that different strains of the same species created those patterns, and isolates produced patterns with 40-60% homology from the same genus but different species. Less than 40% homology allows for identifying isolates from different genera.

Additionally, collection of plant samples of *A. spinosus* species in two successive growth periods confirmed the validity of the molecular fingerprint of the species that was the subject of the study as well as the reliability and consistency of AFLP technique. Due to the fact that molecular markers remain constant and visible in all tissues regardless of the growth, differentiation, development, or level of a cell's defence. Molecular markers offer a number of benefits over conventional phenotype-based substitutes.

Additionally, they are not impacted by environmental factors, pleiotropic effects, or epistatic effects [20]. Furthermore, the minute variations (polymorphisms) between the two collected *A. spinosus* plant samples observed in the present study could be explained by the fact that when the DNA fingerprints of related samples are compared, both common and polymorphic bands would be observed.

These differences (polymorphisms) are observed in an otherwise identical fingerprint. Therefore, the detected polymorphisms in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence such as mutations or creating a restriction site, and insertions, deletions, or inversions between two restriction sites [15, 16].

6. Conclusion

In conclusion, the current research confirms the capability and effectiveness of the AFLP technique to produce a full image of the genome of *Astragalus spinosus* (Forssk.) Muschl species which is considered an excellent tool for genetic analysis. Therefore, creating a molecular reference system for the accurate identification of *A. spinosus* plant species appears to be achievable.

7. Abbreviations

AFLP: Amplified fragment length polymorphism; *Astragalus spinosus: A. spinosus;* RAPD: Random amplified polymorphic DNA; RFLP: Restriction fragment length polymorphism**;** SSR: Microsatellites or simple sequence repeats; SNP: Single nucleotide polymorphisms; PCR: Polymerase chain reaction; PIC: Polymorphism information content.

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