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DNA Fingerprint of *Alkanna tinctoria* Subspecies in Misurata, Libya

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Abstract: The genus *Alkanna* is a member of the family Boraginaceae and is found throughout temperate and subtropical areas of the world, with a major centre of distribution in the Mediterranean region. Two subspecies of *Alkanna tinctoria* L. (*Alkanna tinctoria tripolitana* and *Alkanna tinctoria tinctoria*) are endemic to Libya. Flower size and flower colour were used only as taxonomic characters in determining the subspecies, therefore, the purpose of this paper to use another tool for determining the two subspecies of *Alkanna tinctoria* L. and to investigate DNA markers for the two subspecies. Ten random 10-mer primers were used to detect genetic variation between the two subspecies. The results of primer OP-O05, primer OP-O06, primer OP-O12, primer OP-O17 and primer OP-O19 indicated the presence of molecular markers to the *Alkanna tinctoria tinctoria* and monomorphic bands while Primer OP-O10, primer OP-O13 and primer OP-O17 resulted in the production of molecular marker for *Alkanna tinctoria tripolitana* and also the appearance of monomorphic bands. So the results showed the occurrence of some molecular genetic markers that could be useful in the recognition and the taxonomical description of the plants.

Key words: RAPD-PCR · Genetic markers · Taxonomy · *Alkanna tinctoria*

INTRODUCTION

The genus *Alkanna* is a member of the family Boraginaceae and is found throughout temperate and subtropical areas of the world, with a major centre of distribution in the Mediterranean region [1]. The economic importance of this species is unclear. Nevertheless, it is presumed to be used in the medical and dying industries, like the other members of the family Boraginaceae [2]. Some *Alkanna* species are used as potherbs and for dye. *A. tinctoria* is used to stain wood and marble and to colour medicines, wines and cosmetics [1] (Heywood, 1978). *Alkanna tinctoria* L. is a procumbent to ascending, setose to hispid, perennial herb, (5-) 10-20 (-25) cm tall. Basal leaves oblong-linear, 30-10 (150) x 5-10 (15) mm; lower cauline leaves, somewhat shorter than basal, cordate at base, upper cauline elliptic-ovate, entire, acute to subacute. Bracts oblong to lanceolate, 6-10 (15) x1-5mm. Calyx oblong-lanceolate, 5-7 x1.5-2 mm in flower, increasing to 7-12 mm in fruit, ± densely covered with setiform hispid hairs. Corolla blue 6-8 (-9) mm in diameter (*ssp. tinctoria*) or white 8-12 mm in diameter (*ssp. tripolitana)*, glabrous, tube somewhat longer than the calyx, limb 6-10(-12) mm in diameter, lobes rounded, somewhat spathulate. Anthers c. 1 mm long, filaments somewhat shorter than the anthers. Nutlets c. 2 mm in diameter, ± obconic, irregularly reticulate to tuberculate [3].

The two subspecies are endemic to Libya. Distinguished from type subspecies by its white and usually somewhat larger flowers. However, both the taxa intergraded with each other in flower size but the flower colour is fairly constant. Further, the type subspecies has a wide distribution, while the subspecies *tripolitana* seems to be confined to North West parts of Libya [3].

Using the Polymerase Chain Reaction (PCR), single-copy genomic sequences were readily amplified by a factor of more than 10 million with high specificity and DNA segments up to 2,000 base pairs [4, 5]. Williams *et al.* [6] described DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. These polymorphisms, simply detected as DNA segments amplified from one parent but not the other, are inherited

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in a Mendelian fashion and can be used to construct genetic maps in a variety of species. They suggested that these polymorphisms to be called RAPD markers (Randomly Amplified Polymorphic DNA).

The segregation of the RAPD markers of alfalfa using PCR with single 10-mers of arbitrary sequence was analyzed by Echt *et al.* [7] by using 19 primers, they found 28 polymorphic fragments out of 37 amplified ones in the ratio of 76%. These fragments segregated as dominant Mendelian traits. From these results, they reported that RAPD markers were useful for the rapid development of genetic information in species like alfalfa where little information currently exist or is difficult to obtain [8]. Sivolap *et al.* [9] identified 26 wheat cultivars of different ecogeographical origins depending on RAPD and SSRP analysis of the DNA and they developed procedures for composing genetic formula for wheat cultivars and their certification.

This study aimed to distinguish and detect molecular genetic markers between two subspecies of *Alkanna tinctoria* L. (*Alkanna tinctoria tinctoria* and *Alkanna tinctoria tripolitana*) collected from Misurata, Libya.

MATERIALS AND METHODS

The two subspecies of *Alkanna tinctoria* L. (*Alkanna tinctoria tinctoria* and *Alkanna tinctoria tripolitana*) collected from Zoraik commune, Misurata, Libya.

DNA Extraction and RAPD PCR Reaction Condition: DNA extraction from plant tissues was carried out using DNeasy plant Mini Kit (Qiagen Inc.). Ten 10-mer random primers presented in Table 1 were procured from Company (Qiagen Inc, UK) and used for RAPD analysis. The reaction mixtures were overlain with a drop of light mineral oil per sample. Amplification was carried out in a thermocycler programmed for RAPD amplification reaction was performed in a final volume of 50 µl containing 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 9.0), 2 mM dNTPs, 10 mM primer, 50 ng of template DNA and 0.5 U of *Taq* polymerase (Qiagen Inc, UK). Reactions were performed in a Perkin Elmer 2400 thermocycler programmed for 42 cycles. RAPD-PCR was performed according to Williams *et al*. [6] as one cycle of 94°C for 4 min (denaturizing), 94°C for 1 min, 37°C for 2 min and 72°C for 2 min (annealing) and with a final extension of 10 min at 72°C. PCR products were analyzed using 1.2% agarose gel electrophoresis and visualized with 10 µg/µl ethidium bromide staining [10]. The sizes of the fragments were estimated based on two DNA ladders of 100 to 12000 bp (GIBCOBRL, USA) and 12000 to 250 bp (Stratagene, USA). Fragments were detected on UV-transilluminator and photographed using Gel Doc 2000 Bio-Rad.

Table 1: Random primer names and their sequences for RAPD-PCR analysis

Primers name	Sequence
OP-O01	5'-GGCACGTAAG-3'
OP-O04	5'-AAGTCCGCTC-3'
OP-O05	5'-CCCAGTCACT-3'
OP-O06	5'-CCACGGGAAG-3'
OP-O10	5'-TCAGAGCGCC-3'
OP-O12	5'-CAGTGCTGTG-3'
OP-O13	5'-GTCAGAGTCC-3'
OP-O16	5'-TCGGCGGTTC-3'
OP-O17	5'-GGCTTATGCC-3'
OP-O19	5'-GGTGCACGTT-3'

RESULTS

Ten random 10-mer primers were used to detect genetic variation between *Alkanna tinctoria tripolitana* and *Alkanna tinctoria tinctoria*. The number of fragments for each primer varied from 1 to 6 and the sizes of amplified fragments ranged from 377 to 2536 bp (Fig. 1 and Table 2). Primer OP-O01 revealed no species-specific markers in the two subspecies and two monomorphic bands were detected in both subspecies with 1375 and 431 bp respectively (Fig. 1 and Tables 2, 3, 4). Primer OP-O04 indicated no molecular markers. There displayed four monomorphic fragments ranged from 1909 to 377 bp and no species-specific markers could be detected in the two subspecies.

The results of primer OP-O05 indicated the presence of two molecular markers to the *Alkanna tinctoria tinctoria* with molecular size of 1697 and 524 bp respectively and two monomorphic bands (Fig. 1 and Tables 2, 3, 4). The results of primer OP-O06 showed the presence of one marker for *Alkanna tinctoria tinctoria* with molecular size of 1078 bp and the appearance of three monomorphic bands with molecular size of 1349, 865 and 536 bp, respectively (Fig. 1 and Tables 2, 3, 4). Primer OP-O10 resulted in the production of one marker for *Alkanna tinctoria tripolitana* (887 bp). Also the results showed the appearance of two monomorphic bands (789-589 bp) (Fig. 1 and Tables 2, 3, 4). The results of primer OP-O12 revealed the presence of four markers

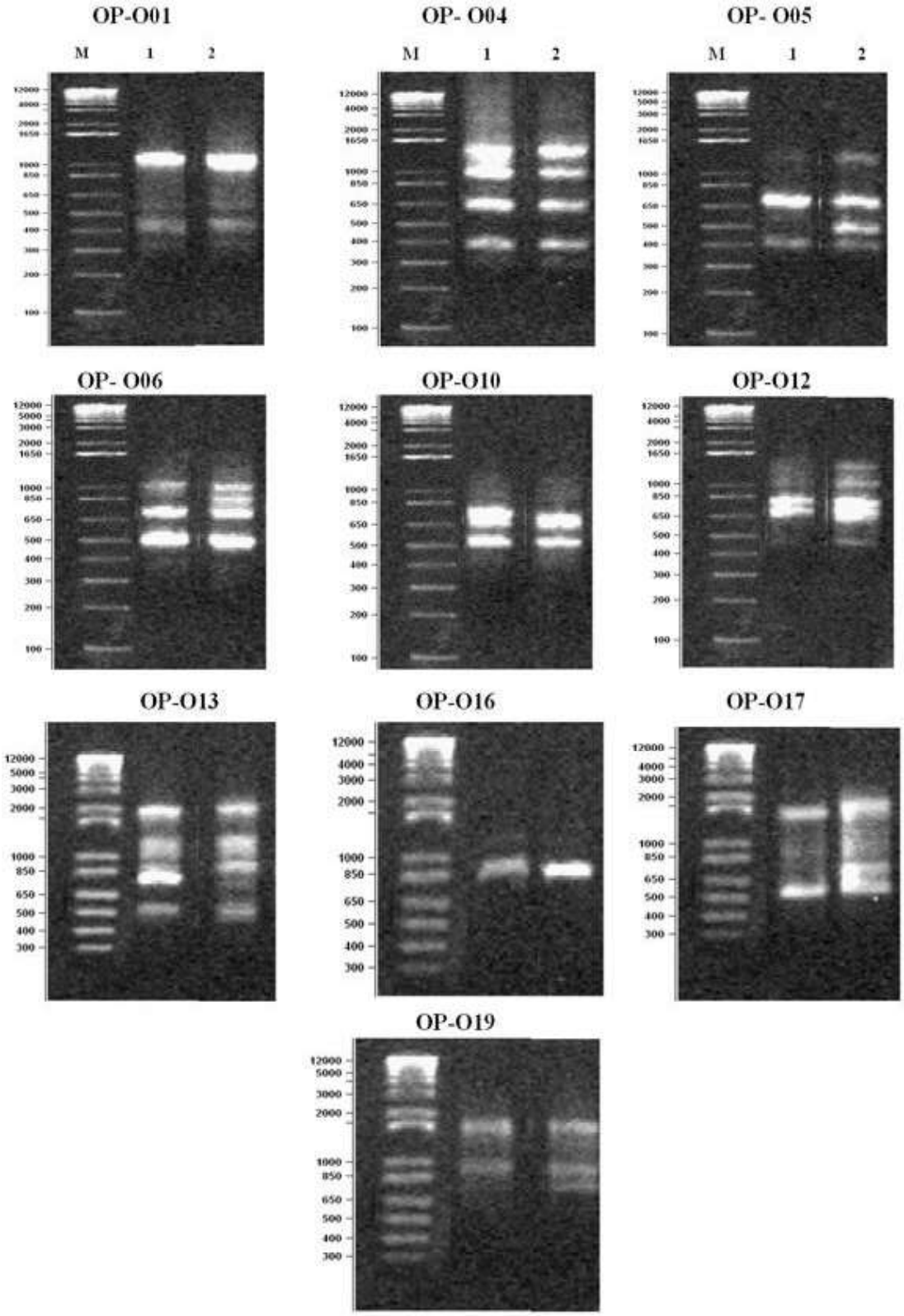

Fig. 1: DNA polymorphism of two subspecies of *Alkanna tinctoria* L.; 1- *Alkanna tinctoria tripolitana* and 2- *Alkanna tinctoria tinctoria* generated by the ten random 10-mer primers. M refers to DNA standards

Table 2: DNA polymorphism using RAPD analysis using ten random 10-mer primers for two subspecies of *Alkanna tinctoria* L.; *Alkanna tinctoria tripolitana* and *Alkanna tinctoria tinctoria*

		Plant subspecies	
Primers name	Molecular size (bp)	*A. tinctoria tripolitana*	*A. tinctoria tinctoria*
OP-O1	1375	+	+
	431	+	+
OP-O4	1909	+	+
	1303	+	+
	719	+	+
	377	+	+
OP-O5	1697	-	+
	838	+	+
	524	-	+
	405	+	+
OP-O6	1349	+	+
	1078	-	+
	865	+	+
	536	+	+
OP-O10	887	+	-
	789	+	+
	589	+	+
OP-O12	1818	-	+
	1354	-	+
	977	+	+
	832	+	+
	708	-	+
	477	-	+
OP-O13	2536	+	+
	1501	+	+
	1276	+	+
	999	+	+
	810	+	-
	492	+	+
OP-O16	1007	+	+
OP-O17	2336	-	+
	1970	+	-
	779	-	+
	564	-	+
	520	+	-
OP-O19	2102	+	+
	1063	+	+
	807	-	+

Table 3: DNA monomorphic and polymorphic bands using randomly amplified polymorphic DNA (RAPD markers) by the ten random 10-mer primers among the two subspecies of *Alkanna tinctoria* L.

		Polymorphic bands	
Primer's name	Monomorphic bands	DNA markers	Total No. of bands
OP-O01	2	0	2
OP-O04	4	0	4
OP-O05	2	2	4
OP-O06	3	1	4
OP-O10	2	1	3
OP-O12	2	4	6
OP-O13	5	1	6
OP-O16	1	0	1
OP-O17	0	5	5
OP-O19	2	1	3

Table 4: Molecular markers of the DNA by using randomly amplified polymorphic DNA (RAPD markers) by the ten random 10-mer primers for the two subspecies of *Alkanna tinctoria* L.

		Samples	
Primer's name	Molecular weight (bp)	1	2
OP-O5	1697	-	+
	524	-	+
OP-O6	1078	-	+
OP-O10	887	+	-
OP-O12	1818	-	+
	1354	-	+
	708	-	+
	477	-	+
OP-O13	810	+	-
OP-O17	2336	-	+
	1970	+	-
	779	-	+
	564	-	+
	520	+	-
OP-O19	807	-	+

(1818-1354-708-477 bp) for *Alkanna tinctoria tinctoria* and also the occurrence of two monomorphic bands with molecular size of 977 and 832 bp respectively (Fig. 1 and Tables 2, 3, 4). A marker of 810 bp was shown against primer OP-O13, where it was present in *Alkanna tinctoria tripolitana* and also the appearance of five monomorphic bands a (Fig. 1 and Tables 2, 3 and 4).Primer OP-O16 results showed only one monomorphic band with molecular size 1007 bp. (Fig. 1 and Tables 2, 3, 4). The results of primer OP-O17 revealed the presence of two markers (1970-520 bp) for *Alkanna tinctoria tripolitana* and also the occurrence of three markers for *Alkanna tinctoria tinctoria* with molecular size of 2336, 779 and 564 bp respectively (Fig. 1 and Tables 2, 3, 4). Primer OP-O19 resulted in the production of one marker for *Alkanna tinctoria tinctoria* (807 bp). Also the results showed the appearance of two monomorphic bands (2102-1063 bp) (Fig. 1 and Tables 2, 3, 4).

DISCUSSION

The morphology of *Alkanna tinctoria* subspecies were studied by Jafri and El-Gadi [3], however molecular markers as well DNA fingerprint are limited and no reports could be found. Consequently, the present study is considered the first report for characterizing the two *Alkanna tinctoria* L. under study. *Alkanna tinctoria* L is an endemic taxon of Libya. No information on *Alkanna tinctoria* L was found in the literature except for some morphological properties [3] such as shape of stem, shape of leaf, corolla, bracts and nutlet, structure of anthers. Flower size and flower colour were used as taxonomic

characters in determining the subspecies, therefore, the purpose of this paper to use another tool for determining the subspecies and to investigate DNA markers for the *Alkanna tinctoria* subspecies. DNA molecular markers have been employed as a tool in many studies such as genetic variability and gene flow analysis, species identification and to detect molecular markers for economically important traits, a logical step for efficient gene mapping and genotyping of individuals, useful tools for the rapid development of genetic information in plants, important to distinguish between different species by comparing polymorphism in genomic fingerprints [11-24]. Thus, RAPD-PCR is a reliable tool for identifying and characterizing *Alkanna tinctoria tripolitana* and *Alkanna tinctoria tinctoria* subspecies.

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