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Isozymes, RAPD and ISSR Variation in *Melilotus indica* (L.) All. and *M. siculus* (Turra) B.G. Jacks. (Leguminosae)

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Abstract: Isozymes and DNA fingerprinting techniques including RAPD and ISSR were used to locate the genetic variations between the morphologically similar two *Melilotus* Egyptian species i.e. *M. indica* and *M. siculus*. Three isozymes and five primers for each of the two PCR-based techniques; RAPD and ISSR were used. The studied two species showed comparable isozymes patterns. They are displayed only five polymorphic loci and this could be attributed to environmental conditions. RAPD and ISSR analyses showed a polymorphism percentage of 71.4% & 60%, respectively. The recorded variation between the two studied species gives extra support for their current treatment as two different species.

Key words: Molecular Markers, *Melilotus,* Lauraceae, RAPD, ISSR, Fingerprinting

INTRODUCTION

Melilotus is a forage genus of the Leguminosae and comprises about 19 annual or biennial species. It has the advantages of high seed yields, tolerating temperature extremes, superior nitrogen fixation rates in comparison to other legumes and is beneficial in crop rotations [1-4]. The genus is native to Eurasia and North Africa where the four Egyptian species are naturally grow and some times are cultivated [5-9]. Of these *Melilotus albus* Medik and *M. officinalis* Lam. are very rare, whereas *M. indica* (L.) All. and *M. siculus* (Turra) B.G. Jacks. are very common.

Except the morphological based work of Isely [10] and Stevenson [6], no other taxonomic studies have been made on the interspecific relationships of *Melilotus*. However, the majority of species have intermediate forms that are unlikely to be caused by environmental effects or hybridization [4]. The two Egyptian species i.e. *Melilotus indica* (L.) All. (= *M. parviflorus* Desf.) and *M. siculus* (Turra) B. G. Jacks. (= *M. messanensis* (L.) All.) display this finding as they could only distinguish from each other by the larger size and wrinkled pods of *M. siculus*, whereas all other characters are similar. Both species are glabrous annual grass; Stem 20-60 cm, angled, procumbent or ascending, much branched from the base, with branches, very often, the further development of main stems; 3-foliolate leaves; obovate or orbicular dentate leaflets; nearly entire stipules, the lower leaves of 1-2.5×0.5-1.8 cm with petiole 3-6 cm; Inflorescence with 4-10 flowers, lax or compact, with stalk of 0.3-1 cm, shorter than the sheet contiguous; Flowers erect-patent or pendulum; pedicels 1-2 mm, reflections; Calyx 2-3 mm, teeth broadly triangular, acuminate, unequal, equal or longer than the tube; Corolla 4-5 mm, yellow gold, standard and wings shorter than the keel; Fruit 7-8 (9) × 3-3.5 mm, with 1-2 seeds, oblong-oval, flattened [7, 9]. Furthermore, both species have the same somatic chromosome number of 2n = 16 [11, 12].

Various DNA fingerprinting techniques have been extensively utilized to discriminate the taxa at specific and varietals levels. These include isozymes, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR). Isozymes are widely used for genotype identification to evaluate genetic integrity [13, 14]. Either RAPD or ISSR provide good taxonomic markers to determine the rates and patterns of change occurring in DNA or proteins; and to reconstruct the evolutionary history of genes and organisms [15-17]. In the present work Isozymes, RAPD and ISSR markers are used in order to locate the genetic variations and relationships between the two Egyptian species *Melilotus indicus* and *M. siculus*.

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MATERIAL AND METHODS

Melilotus indica (L.) All. (= *M. parviflorus* Desf.) was collected from Al-Maadeia village of Rosetta District, Egypt, whereas *M. siculus* (Turra) B. G. Jacks. (= *M. messanensis* (L.) All.) was collected from Borg-Al-Arab, Alexandria, Egypt. Their identification and nomenclature follows Täckholm [7] and Boulos [9]. Voucher specimens have been deposited at the Herbarium of Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Egypt. For the isozymes analysis, α-and β-esteras (Est.) and aldehyde oxidase (Ao.) were used. Isozymes were separated in 10 % Native-polyacrylamide gel electrophoresis as described by Stegemann *et al.* [18]. For isozyme extraction, 0.5 g of fresh leaves was homogenized in 500 µl extraction buffer using a mortar and pestle; centrifuged at 10 000 rpm for five minutes; the supernatant was kept at –20°C until use. For electrophoresis, 50 µl of extract was mixed with 25µl of treatment buffer and 50 µl of this mixture was applied to the well. In gels staining, protocols of Scandalios [19] were used for α and β-Est.; Wendel & Weeden [20] for Ao. Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed.

In the molecular analysis, five primers for each of the two PCR-based techniques; RAPD and ISSR were used. DNA extraction was performed using protocols of Dellaporta *et al.* [21]. For RAPD analysis, five 10-*mer* random DNA oligonucleotide primers (Operon Technologies, Inc, USA) of arbitrary sequences were independently used in PCR reactions as described by Williams *et al.* [22]. Codes and sequences of these primers were listed in Table 2. Amplifications were performed in 50 µl reaction volume containing: 0.2 mM dNTBs, 1.5 mM $MgCl_2$, 5.0 µl 10X buffer, 0.2 µl Primer, 3.0 µl template DNA (50 ng/ µl) and 0.3 µl Taq DNA polymerase (5U/ µl). Each of the reaction mixtures was overlaid with a drop of light mineral oil per sample. Amplifications were carried out in Perkin Elmer thermocycler. The optimal conditions for PCR amplification were as follows: an initial 4 minutes denaturation step at 94 °C followed by 37 cycles of 1 minute at 94 °C, 1 minute at 37 °C and 2 miutes at 72 °C, with a final extension step at 72 °C for 8 minutes. A volume of 15 µl of the RAPD products were electrophoresed in 1.2 % agarose gel and run was performed at 100 V for about 60 minutes in pharmacia submarine (20cm x 20cm). The bands were visualized on UV trans-illuminator and photographed by Polaroid camera.

Table 1: The used taxa of the present study and their localities

No.	Taxa	Localities
1	*Melilotus indica* (L.) All. (= *M. parviflorus* Desf.)	Tabia, Rashied
2	*M. siculus* (Turra) B. G. Jacks. (= *M. messanensis* (L.) All.)	Boussaily, Rashied

Table 2: The presence (+) and absence (-) of bands in each of α- esterase, β-esterase and aldehyde oxidase isozyme profiles of the tudied taxa.

Isozyme	band	1	2
α-esterase	α-est 1	+	+
	α-est 2	+	+
	α-est 3	+	+
	α-est 4	+	+
	α-est 5	-	+
β-esterase	β-est 1	+	+
	β-est 2	+	+
	β-est 3	+	-
	β-est 4	-	+
	β-est 5	+	+
	β-est 6	+	+
	β-est 7	-	+
	β-est 8	-	+
Aldehyde oxidase	Ao 1	+	+
	Ao 2	+	+
	Ao 3	+	+

ISSR analysis was carried out in a total reaction of 50µl containing 0.2 mM (dNTPs), 1.5mM $Mgcl_2$, 5.0 µl 10X buffer, 0.2 µl primer (50 pmoles), 3.0 µl template DNA (50ng/ µl), 0.3 µl Taq DNA polymerase (5U/ µl), in sterile water up to 50 µl. PCR amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min. at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min., an annealing step at 47°C for 1 min. and an elongation step at 72°C for 2 min. The primer extension segment was done for 7 min. at 72°C in the final cycle.

For scoring the isozymes, RAPD and ISSR data, clear and distinct bands were scored as (+) or (-) for presence and absence, respectively. Differences in bands intensity among profiles of the different samples were not considered.

RESULTS AND DISCUSSION

Photos of the produced banding patterns by application of the isozyme, RAPD and ISSR techniques on the two studied taxa of *Melilotus* are shown in Figures 1, 2 & 3, respectively. Their scored bands are given in

Table 3: A list of RAPD and ISSR primers and their nucleotide sequences

RAPD		ISSR	
Primer	Sequence	Primer	Sequence
OP-B02	5-TGATCCCTGG -3	HB10	(GA)6 CC
OP-B03	5-CATCCCCCTG -3	HB12	(CAC)3 GC
OP-B05	5-TGCGCCCTTC -3	HB13	(GAG)3 GC
OP-B06	5- TGCTCTGCCC-3	HB14	(CTC)3 GC
OP-B10	5-CTGCTGGGAC -3	HB15	(GTG)3 GC

Table 4: The presence (+) and absence (-) of amplified DNA bands in the generated RAPD profiles of the studied taxa

RAPD primer	M. s	1	2
B02	1532	+	+
	976	+	+
	585	+	+
	456	+	+
B03	992	+	-
	843	+	-
	655	-	+
B05	1515	+	-
	1072	-	+
	785	+	+
	345	+	-
B06	1211	-	+
	1075	-	+
	831	-	+
	700	+	-
	600	+	-
	500	+	-
B10	1065	+	-
	817	-	+
	600	+	-
	358	+	+

Table 5: The presence (+) and absence (-) of amplified DNA bands in the generated ISSR profiles of the tudied taxa

ISSR primer	M. s	1	2
HB10	1566	+	-
	1492	-	+
	1260	-	+
	1046	+	-
	853	-	+
	800	+	-
	891	-	+
	493	+	-
	407	-	+
HB12	1754	+	-
	1603	-	+
	1488	-	+
	1086	+	+
	847	+	+
	800	-	+
	645	+	+
	467	-	+
HB13	1332	+	-
	821	+	+
	700	+	+
	650	-	+
	300	+	-
	200	+	-
	100	+	+
HB14	1140	+	+
	958	-	+
	753	+	+
	647	+	+
	600	+	-
	500	+	-
HB15	1600	+	-
	1097	+	+
	876	+	+
	733	+	+
	622	+	+

Table 6: The number and types of bands and percentage of the total polymorphism in the Isozymes, ISSR and RAPD.

Type	Monomorphic bands	Polymorphic bands	Total	Polymorphism %
Isozyme	13	5	18	27.77
RAPD	6	15	21	71.40
ISSR	14	21	35	60.00

Tables 3, 4 & 5, respectively, whereas number and types of the amplified DNA bands and percentage of the total polymorphism are given in Table 6. Among the studied three isozymes (Fig. 1; Tables 2 & 6), only the banding patterns of α- and β-esterase revealed slight polymorphism with a percentage of 27.77 % between the two studied species. Hannan & Orick [23] in their study on the genetic variation between two *Iris* species based on isozyme analysis declared that, the presence of similar isozyme patterns is evidence for the presence of geologically single origin of both species from a single gene pool. On the other hand, Dolan [24] when examining *Streptanthus morrisonii* species complex based on isozyme analysis pointed out that, the divergence for a few morphological and life-history characters can occur in response to environmental conditions, but without divergence at allozyme loci. In the present study, *Melilotus indica* and *M. siculus* showed comparable patterns of isozymes. They are displayed only five polymorphic loci according to the interpretation of the produced banding patterns. The detectable isozyme variation at few loci and the observation that the isozymes found in *M. indica* exhibited similar electrophoretic mobilities to those of *M. siculus* lead to accept the hypothesis of a geologically recent origin of *M. indica* from a single, genetically represented by few individuals of *M. siculus* gene pool and this could be attributed to environmental conditions.

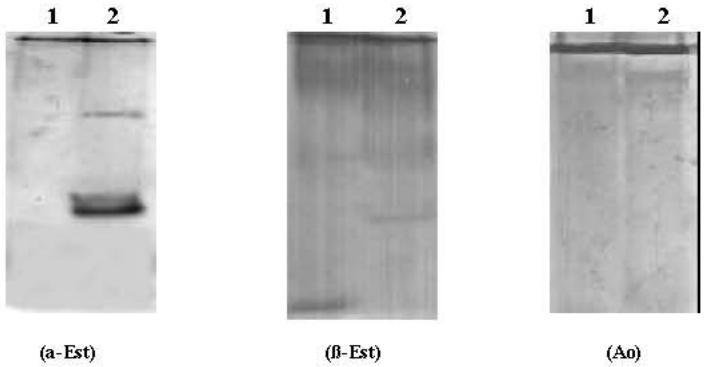

Fig. 1: Electrophoritic banding patterns of α–esterase (α-Est), β–esterase (β-Est) and aldehyde oxidase (Ao) isozymes for *Melilotus indica* (1) and *M. siculus* (2)

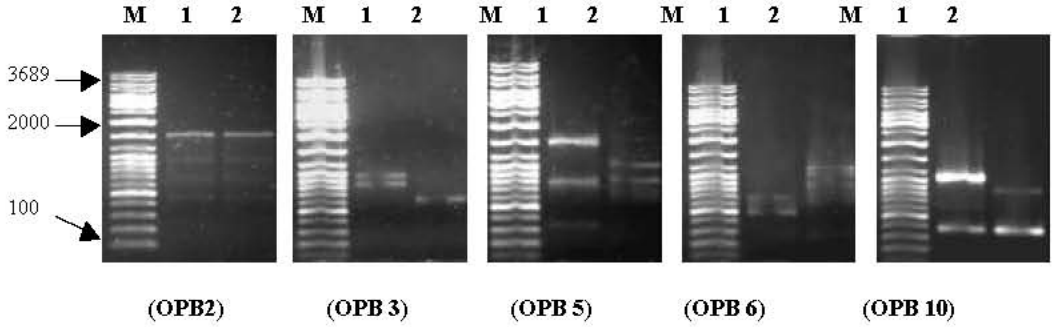

Fig. 2: RAPD profiles of the orange *Melilotus indica* (1) and *M. siculus* (2) generated by the OP-B02, 3, 5, 6 and 10 primers; M = marker.

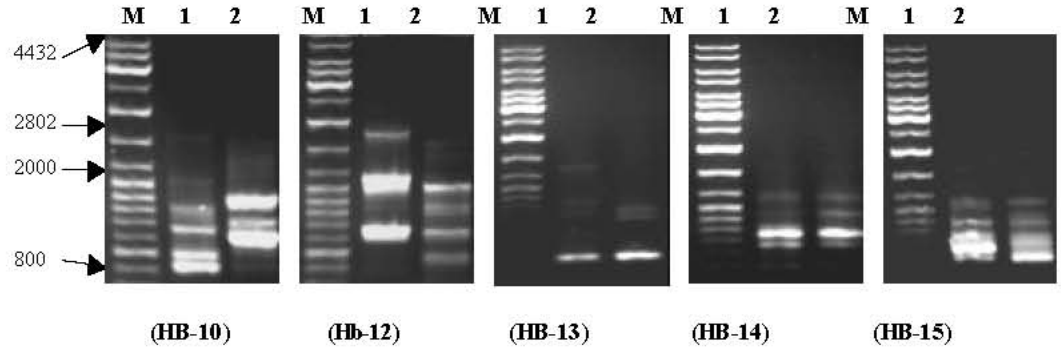

Fig. 3: ISSR profiles of *Melilotus indica* (1) and *M. siculus* (2) generated by the HB10, 12, 13, 14 and 15 primers; M = marker.

The amplification of randomly selected gene loci (RAPD-PCR) is usually a more sensitive method to detect genetic variation in plant species compared to gene product level methods e.g. isozymes. Previous studies on other species have demonstrated that RAPD markers can be of great value for measurement of variation and establishing of genetic similarities at the intra- and interspecific levels [25]. RAPD analysis (Fig. 2; Tables 3 & 6) revealed that, the four primers i.e. B3, B5, B6 and B10 are succeeded to generate polymorphic and reproducible amplification products. Out of 21 scored bands in the RAPD-PCR profiles, only six monomorphic bands are recognized, whereas the remaining fifteen bands are polymorphic. Nine unique bands distinguished *Melilotus indica* (1) from *M. siculus* (2). A high level of polymorphism percentage of 71.4% is observed between

the two species based on such analysis, which reflects a considerable variation between the two species. Thus, the recorded variation between the two studied species may give extra support for their current treatment as two different species. On the other hand, such results show that RAPD technique is useful for discrimination of the two studied *Melilotus* species.

Nagaoka & Ogihara [26] and Aboel-Atta & Ahmed [27] reported that either RAPD or ISSR markers are not affected by the environmental conditions and that ISSR markers are more specific than those of RAPD in determining the genetic variation between taxa. Furthermore, Julie and Pablo [28] reported that the products generated from 5' anchored primers should exhibit more co-dominant polymorphism than RAPD. In the present study, the polymorphism level revealed by ISSR primers was higher than that of RAPD primers as shown in Table 7, which supports these views. All the used five ISSR primers (Fig. 3; Table 3 & 6) are succeeded to generate polymorphic and reproducible amplification products. 35 fragments are generated by the five primers, from which 21 are monomorphic and could be used to discriminate between the two species, whereas the remaining 14 bands are polymorphic. A considerable level of polymorphism i.e. 60% is observed between the two species based on this analysis, which is further confirming the considerable high level of dissimilarity between the two studied species.

In a recent review, Bussell et al. [29] reported that the appropriate level for the application of arbitrarily amplified dominant markers, which include ISSRs are useful below taxonomic levels at which the variable sequences can provide sufficient information. These authors further stated that the markers might be useful for phylogenetic analyses involving species that are closely related and represent recent radiations. Likewise, Ge *et al.* [30] concluded that, the genetic differentiation between two related species as revealed by ISSR markers might be due to a possible vicariant evolutionary event from a single common ancestor through the fragmentation of its natural distribution range. In the present study, the recorded distinct genetic differentiation based on ISSR products between *M. indica* and *M. siculus* confirms such conclusion. Because the level of genetic variation in selectively neutral marker loci is mainly determined by mutation and genetic drift [31], the level of variation detected for marker loci will not necessarily be a direct reflection for such level that determines adaptability or individual fitness [32]. Therefore, samples from different habitats should be considered in the future studies on the genus *Melilotus*.

In conclusion, previous descriptions of *Melilotus* species were mainly based on morphological features leading to nomenclature issues. The produced isozyme data clarified that the morphological similarity of the two studied species could be as a result of resemblance in their environmental conditions. However, the recorded high values of polymorphism percentage as revealed by the used PCR-based techniques indicated a considerably high degree of variability between both species which confirms their taxonomic treatments as two independent species. The usefulness of DNA based fingerprinting as a powerful alternative to determine the genetic diversity and relationship between the species is further confirmed.

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