Egypt. J. Bot. **Vol. 60**, No. 2. pp. 451-460 (2020)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Implications of *rbc*L and *rpo*C1 DNA Barcoding in Phylogenetic Relationships of some Egyptian *Medicago sativa* L. Cultivars

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LUCERNE, alfalfa *(Medicago sativa* L). is a major forage crop of family Fabaceae. The present study was concerned with the genetic discrimination and identification of some *M. sativa* cultivars. Nine *M. sativa* cultivars, representing three different countries (Egypt, Australia and USA), were presented in this study. The genetic relatedness was studied using DNA barcoding of *rbc*L and *rpo*C1 marker genes; Phylogenetic analysis of samples of nine *M. sativa* cultivars was performed and a cluster analysis was derived. Sequence analysis was in complement and efficient in assessing the characterization of genetic relatedness between the studied *M. sativa* cultivars and has revealed that the Egyptian cultivars are more phylogenetically related to the American cultivars (USA 9: Perfect and USA4: Cuf101) and the Australian one (AUS 2: Sirinafa). In conclusion, the results have indicated that DNA barcoding was suitable and crucial for characterization of *M. sativa* at species and cultivar level, which provides an important tool for future analyses of other major important forage crops. Moreover, it might be suggested that breeding lines from *M. sativa* cultivars may develop novel insights and give better understanding of the possibilities of domestication of *M. sativa.* On the same context, some cultivars could be used as important genetic resources for genetic improvement of *M. sativa* in future breeding programs.

Keywords: DNA barcoding, Forage crops, *Medicago sativa* L., Phylogenetic relationship, *rbc*L, *rpo*C1.

Introduction

Lucerne, alfalfa (*Medicago sativa* L.) plays a vibrant role in agricultural sustainability and is regarded as one of the major most important cultivated forage legume crops that originated in the Caucasus region (Michaud, 1988). It belongs to the Fabaceae family, it is famous for its ability to fix nitrogen from air and is widely cultivated worldwide. It is adapted to different climatic conditions (Moreira & Fageira, 2010) and used as a great nutritious feed, especially for dairy cows due to its high protein content (Flajoulot et al., 2005; Geren et al., 2009). The genetic diversity between alfalfa cultivars needs to be further investigated since alfalfa biomass production is highly affected by the similarity/dissimilarity in cultivar genetic backgrounds (Radovic et al., 2010). Alfalfa is an autotetraploid (2n=4x=32) (Stanford, 1951; Armstrong, 1954; Demarly, 1954), allogamous seed-propagated species. Its cross-pollination is due to its self-incompatibility genes which tend to make it hard to obtain a pure inbred line (Ennos, 1983). These factors contribute to alfalfa's genetic variability at individual and population levels.

Deficiency of plant taxa, triggering rich genetic variation, poses a major constraint that significantly limits the improvement programs of plant crops. Heterosis was utilized to improve many important traits and forage yield of alfalfa and to prevent outbreeding depression which might happen in breeding programs before a pure hybrid line is obtained (Jensen et al., 2012).

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Received 24/11/ 2019 ; Accepted 27/1/ 2020
DOI: 10.21608/ejbo.2020.20028.1399
Edited by: Prof. Dr. Soliman A. Haroun, Faculty of Science, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt.

Various molecular markers were employed to study the performance and diversity of the different hybrids.

Distinct plants for breeding purposes were identified by the tool of DNA polymorphism. Selection of superior hybrids from a cross population was achieved by several emerged techniques studying DNA polymorphism (Mardi et al., 2011; Taheri et al., 2018). DNA molecular markers have the advantage of characterizing biodiversity without fear from requiring taxonomic expertise or suffering individual biases. Also, they are not influenced by plant age, physiological conditions, tissue source or surrounding environmental conditions (Bafeel et al., 2012). DNA barcoding offers another resource of molecular markers that are now often used for taxonomic identification, biodiversity records and genetic variation studies (Costion et al., 2011). It can be described as using short unvarying organelle or nuclear DNA sequences (400-800bp) that has adequate sequence variation to identify different taxa and distinguish among species (Ganie et al., 2015). An ideal DNA barcode should be present in all groups of land plants, and show enough sequence variation to differentiate among species, also it should be easily amplifiable and sequenced using only one primer pair (Kress & Erickson, 2007). Diverse regions from the plastid genome, comprising *trnH-psbA* intergenic spacer, *rbc*L, *rpo*C1 and *rpo*B, have been recommended and verified for DNA barcoding of plants with different success rate and different strengths and weakness according to the studied organism (Kress & Erickson, 2007; Singh et al., 2012). *rbc*L gene encodes the large subunit of Rubisco and is widely used in sequence phylogeny and analysis as Rubisco catalyzes the first steps of photosynthetic fixation of carbon and of photorespiration in all photosynthetic organisms (Bathellier et al., 2018). Mutation rate in *rbc*L can be measured (Kool et al., 2012). *rpo*C1 is a chloroplast DNA region, its main advantage is its high amplification success (Chen et al., 2010; Sass et al., 2007) and its slower rate of evolution that other non-coding plastid regions and some other coding plastid genes such as *matK* (Newmaster et al., 2007).

Previous studies have evidenced the need of integration of beneficial and valuable genetically divergent cultivars or cultivar materials of *M. sativa* (Jensen et al., 2012; Robins et al., 2012). However, knowledge on the genetic relationships between *M. sativa* cultivars is so limited, but indispensable for breeding programs and maintenance of forage legume crops aiming for high quality and productivity.

Hereby, the aim of the present study was to sequence regions of *rbc*L and *rpo*C1 chloroplast genes as barcodes to detect the genetic variation between some cultivars of *M. sativa*. Moreover, the effectiveness of combined DNA barcodes in assessing genetic diversity of selected alfalfa cultivars was also investigated.

Materials and Methods

Plant material

A collection of nine *M. sativa* cultivars was kindly obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA), research program of crop genetics, currently at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt. The samples belonged to three countries viz. Egypt, Australia, and United States of America and listed as shown in Supplementary Table 1.

Extraction, purification, and quantification of genomic DNA from M. sativa cultivars

High molecular weight plant Genomic DNA was isolated from 50-100 mg ground and freeze-dried seedlings of *M. sativa* L. cultivars using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and 2% CTAB extraction procedure (Murray & Thompson, 1980). The purity and quantity of extracted DNA were estimated spectrophotometrically using the ND-1000 system (NanoDrop Technologies, Thermo Fisher Scientific Inc.). DNA manipulation and analysis measures related to quantification and purity analysis were achieved according to the Molecular Cloning Laboratory Manual (Sambrook et al., 1989).

DNA barcoding of rbcL and rpoC1 marker gene techniques

The genetic variation and diversity between the studied *M. sativa* cultivars were analyzed by DNA barcoding technique. PCR amplification was taken place in 50µl TRV (a total reaction volume). The thermocycler was programmed to achieve 40 cycles after an initial denaturation cycle for 5min at 94ºC. Each cycle consisted of a

denaturation step at 94ºC for 30sec, an annealing step at 45ºC for 30sec, and an elongation step at 72ºC for 30sec. The primer extension segment was extended to 7min at 72ºC in the final cycle. Amplified PCR products of DNA barcoding loci, were subsequently electrophoresed on 1.5% w/v agarose gel, stained with ethidium bromide (final concentration 100µM/L, Sigma-Aldrich®) in 1X TBE, visualized, and finally documented using a gel documentation and image analysis system according to the Molecular Cloning Laboratory Manual (Sambrook et al., 1989).

Oligonucleotide sequencing of PCR amplicons of rbcL and rpoC1 marker genes

Fractionated PCR amplicons of *rbc*L, and *rpo*C1 marker genes amplified fragments were recovered from agarose gel using QIAquik® PCR PURIFICATION KIT (Qiagen inc., Cat. no. 28106) according to the manufacturer's instructions. Selected and purified amplicons were then cloned into pGEM cloning vector prior to sequencing process. The DNA sequence was determined by automated DNA sequencing method. The automated DNA sequencing reactions were performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE applied Biosystems, USA) in combination with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the Gene Amp 2400 Thermal Cycler, the reaction was conducted in a total volume of 20µl containing 8µl of terminator ready reaction mix, 1µg of plasmid DNA, and 3.2pmol of M13 universal forward primer. The cycle sequencing program was 96°C for 10 seconds, 50°C for 5sec, and 60°C for 4min. The program was repeated for 25 cycles with rapid thermal ramping, the nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 3100 Genetic Analyzer. The data was provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

Data analysis

The banding patterns generated by barcode genes were analyzed to determine the genetic relatedness of the nine examined *M. sativa* samples. Dendrograms were constructed by Dice coefficient's genetic similarity matrix to display accession relationships using the unweighted pair group method with arithmetic mean (UPGMA) of NTSYS version 2.10 (Rohlf, 2000). The principal coordinates analysis (PCoA) was constructed based on Dice coefficient genetic similarity matrix using DCENTER module in NTSYS. Maximum likelihood and delta K (ΔK) values were used to determine the optimum number of groups (Pritchard et al., 2000). Computational analysis of *rpo*C1 and *rbc*L DNA barcodes using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was performed. Sequences were aligned using Align Sequences Nucleotide BLAST and then further analyzed by MEGA5 software.

Results

DNA barcoding loci of rbcL and rpoC1 sequencing results

DNA barcoding loci of two chloroplastic genes (*rbc*L and *rpo*C1) were compared for their amplification, sequencing, and efficacy in assessing the genetic diversity among nine alfalfa cultivars, as listed in Supplementary Table 1. PCR-based amplified products of *rbc*L and *rpo*C1 regions were fractionated by 1.5% agarose gel electrophoresis. The results have showed 100% amplification success across all samples (Fig. 1). Electrophoretic separation of *rbc*L and *rpo*C1 PCR products has revealed sharp DNA amplicons with no byproducts, reflecting and manifesting high specificity of PCR reactions in all studied cultivars. Generated DNA fragments had recorded sizes of 600 and 500 bp for *rbc*L and *rpo*C1, respectively (Table 1). BLAST analysis for each performed sequence of the studied genetic backgrounds (cultivars) was performed to confirm that the *rbc*L and *rpo*C1 sequences were only belonging to *M. sativa* species. BLASTN nucleotide to nucleotide analysis of *rbc*L and *rpo*C1 sequences has revealed strong matching with only *M. sativa* species.

According to data outcomes of sequences alignment, *rbc*L and *rpo*C1 barcoding regions have revealed notable aspects regarding conservation/variation in the analyzed two chloroplastic genes (Supplementary Figs. 1-2). The sequence analysis of *rbc*L PCR products of studied cultivars has shown an accepted level of conservation with some missing nucleotides in between cultivars, especially at the end of each generated sequence (Supplementary Fig. 1). On the same context, a pronounced high level of sequence conservation was recorded through analyzed *rpo*C1 gene products of *M. sativa* cultivars (Supplementary Fig. 2).

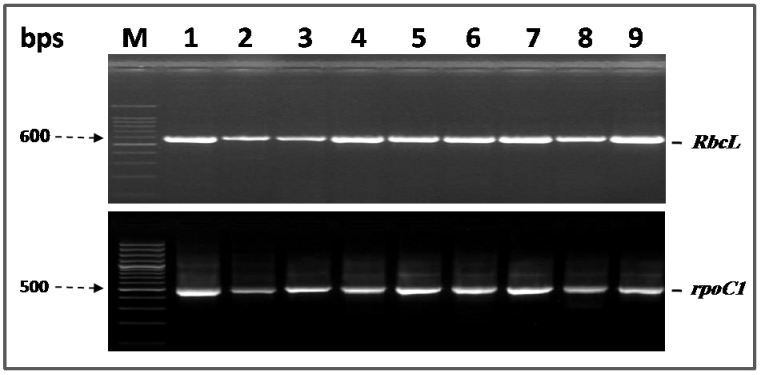

Fig. 1 . Amplification of DNA barcoding loci of *rbcL* and *rpoC1* genes. Agarose gel electrophoresis of amplified PCR products of *rbcL* and *rpoC1* [Black arrowheads indicate the molecular size of amplified DNA barcodes].

TABLE 1. The list of primers' sequences used for PCR amplification barcoding analysis of *Rbc*L and *rpo*C1 marker genes.

Primer Code	Sequence	Product Size	Reference
*Rbc*La-F	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	600bp	Yu et al. (2011)
*Rbc*L724-R	5'-TCGCATGTACCTGCAGTAGC-3'		
*rpo*C1-F	5'- GGCAAAGAGGGAAGATTTCG -3'	500bp	Sass et al. (2007)
*rpo*C1-R	5'- CCATAAGCATATCTTGAGTTGG -3'		

Affirming the quality of presented sequencing data for further implementations, several statistical and bioinformatics measures were executed. Sequence estimates of DNA barcoding loci of *rbc*L (Supplementary Table 2) and *rpo*C1 (Supplementary Table 3) regions have warranted the fineness of generated sequences for employing in further analyses throughout this study. Estimation of genetic variation and/or relatedness were judged using several parameters viz.: number of variable sites (VS), %VS, number of indels, and parsimony informative sites (PIS) were calculated, as shown in Table 2. Sequence estimates have investigated that VS and PIS (327 and 143, respectively) for *rbc*L locus were almost doubled when compared to their corresponding values of *rpo*C1; 98 VS and 38 PIS (Table 2). As a result, the percentage of sequence variation was higher for *rbc*L (62.5%) than for *rpo*C1 (28.4%). In line with the observed genetic variation, pairwise distances resulting from *rbc*L locus were recorded with an average of 15.7%, while the mean was only 4% for *rpo*C1.

To construct a phylogenetic tree representing the similarity and relatedness between *M. sativa* cultivars, pairwise distances from the conserved sequences *rbc*L and *rpo*C1 were used. Constructed phylogenetic trees were generated based on *rbc*L, *rpo*C1, their combined data similarity identity matrices created by CLUSTAL 2.1 by using UPGMA algorithm. The cluster of *rbc*L clearly discriminated between alfalfa sequences (Fig. 2A, Supplementary Table 4). However, it showed a degree of cultivar individualization. In case of *rpo*C1, the cultivars were clustered in a manner that may have some differences than that of *rbc*L (Fig. 2B, Supplementary Table 4). Both clusters were in complement to separate the Egyptian cultivars in two different sub-clusters in each case. Hereby, for more concluding interpretation of presented investigations and to refine the potential of genetic relationships between studied alfalfa cultivars, the *rbc*L and *rpo*C1 barcodes were combined (Fig. 2C). The later approach has emerged a net cluster demonstrating the relatedness of the Australian cultivar to the Egyptian ones. Moreover, the Egyptian cultivars were clustered together and seemed to be more phylogenetically related to the American cultivars, specifically with USA9 and USA4 (Fig. 2C, Supplementary Table 4). The same findings were obtained using principal coordinates analysis (PCoA) of 30 and 10 % of variation by coordinates 1 and 2, respectively which resulted in grouping the Egyptian cultivars together.

TABLE 2. Genetic variation estimates of DNA barcoding regions of *rbc*L and *rpo*C1 genes

Barcoding gene locus	No. of taxa	Range of aligned length (bases)	Total matrix taxa	Undetermined characters	Missing %	Variable sites (VS)	VS %	Parsimony informative sites (PIS)	Average pairwise distance
*Rbc*L	9	516-587	4644	624	13.4	327	62.5	143	0.157
*Rpo*C1	9	354-480	4320	366	8.4	98	28.4	38	0.0047

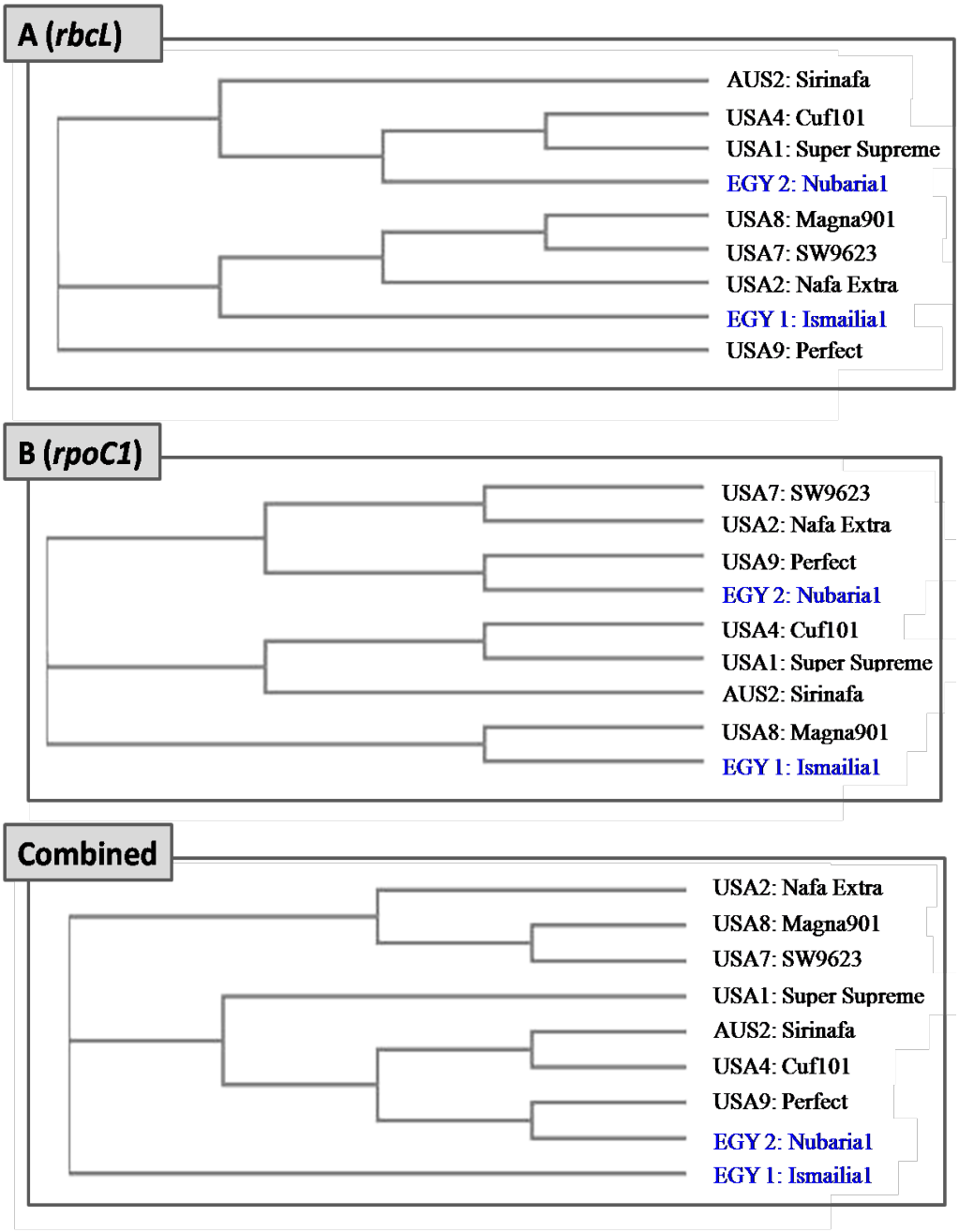

Fig. 2. Cluster analysis of DNA barcoding regions of *M. sativa* cultivars resulted from similarity matrices of A) *rbc*L, B) *rpo*C1, and C) their combination [Dendrogram for the nine examined *M. sativa* cultivars, constructed using Unweighed Pair-group, showing the level of genetic material. Arithmetic mean (UPGMA) and computed similarity matrices were performed according to Dice coefficient].

Discussion

Assessment of genetic diversity of M. sativa by DNA barcoding of rbcL and rpoC1

Measurement of genetic variation in germplasm of plants is pivotal since a large and varied genetic background permit for selection of genotypes harboring desirable traits. Because alfalfa is a self-incompatible and allogamous species (Tucak et al., 2010), the efficacy of genetic diversity greatly helps in breeding programs. The benefits of utilizing DNA barcoding have widely enriched studies of several disciplines viz., ecological biodiversity, domestication of highly important strategic and economic crops, food quality, and the characterization of both invasive and new species (Suriya et al., 2018). Although universal plant-specific or species-specific primers are difficult to find but DNA barcoding protocols for species identification have immensely and speedily developed (Gong et al., 2018). Therefore, the present study has positioned DNA barcoding approach to assess the genetic similarity/diversity in nine *M. sativa* cultivars from Egypt, Australia, and USA. Based on the accurate perspectives on the level of nucleotide, barcoding technique was used to reveal the phylogenetic relationships between the nine alfalfa cultivars.

DNA barcoding of M. sativa rbcL and rpoC1 and its phylogenetic implications

DNA barcoding can provide a preliminary indication of the scope and nature of genetic diversity inter- and/or intra-population(s). Besides that, it assists in comparative studies of population diversity of several plants (Hajibabaei et al., 2007). A high rate of a positive selection among different lineages in *Ilex* (Aquifoliaceae) was demonstrated (Yao et al., 2019). Amandita et al. (2019) has shown that *rbc*L barcoding has a much higher level of sequence recoverability than *matK* (95% and 66%, respectively) which in turn have had a great ability to differentiate between flowering plants. Moreover, *rpo*C1 was proven to differentiate between different populations of the species *Galphimia* that produce the chemical compounds galphimines from others non-producing species. DNA barcoding investigation was confirmed by thin layer chromatography to identify the different chemical profiles (Gesto-Borroto et al., 2019). Both *rbc*L and *rpo*C1 regions were used individually and in combination in several plant studies (Hollingsworth et al., 2009; Singh et al., 2012; Inglis et al., 2018; Gao et al., 2019). In this study, the cluster of *rbc*L clearly discriminated between alfalfa sequences. However, it showed a degree of cultivar individualization. In case of *rpo*C1, the cultivars were differently clustered. For more confirmation and to obtain a conclusive relatioship, a combined dendrogram based on both *rbc*L and *rpo*C1 was constructed and resulted in a net cluster demonstrating the relatedness of the Australian cultivar to the Egyptian ones. Moreover, the Egyptian cultivars were clustered together and seemed to be more phylogenetically related to the American cultivars, specifically with USA9 and USA4. Thus, in this study, using *rbc*L gene as barcode was more conclusive than *rpo*C1 in detecting genetic variability among the tested *Medicago* cultivars. This result indicates its fitness as one of the candidate DNA barcoding loci for examining plant biodiversity. Some earlier studies have supported this opinion. It was reported that *rbc*L is the topmost characterized gene among chloroplast regions (Fazekas et al., 2008). In addition, Hollingsworth et al. (2011) declared that *rpo*C1 had a good sequence quality, but with low discriminatory power. However, other studies did not recognize *rbc*L as a suitable locus for DNA barcoding because of its lower sequence polymorphism and genetic distance than *matK* locus (Hosein et al., 2017). Taken together, the genetic relationship revealed from DNA barcoding of some loci of chloroplast genome (*rbc*L and *rpo*C1 genes) were conclusive and have shown an efficient, integral and vital considerable genetic discrimination between studied *M. sativa* cultivars. This study has emphasized and accessed the invalidity of a notion that the genetic diversity of studied cultivars is obeyed by the rule of geographical distribution tendency. Moreover, the Egyptian cultivars seemed to be more phylogenetically related to the American cultivars (USA 9: Perfect and USA4: Cuf101) and the Australian (AUS 2: Sirinafa) one, as shown in Fig. 3.

Conclusion and Future Prospective

This study has investigated some alfalfa cultivars of economic importance as useful sources of genetic information in breeding program(s) in the developing countries to engender new possibilities of producing lines harboring high forage quality and productivity. To draw a more comprehensive insights concerning assessing genetic diversity/ relatedness, utilizing additional DNA barcoding sequences (viz., cox1, trnL-F, matK, ndhF,

atpB, and ITS genes) is suggested. Moreover, including IRAP molecular markers may specify the high insertional activity for the evaluated retrotransposons (RTNs) in the genome of *M. sativa* cultivars.

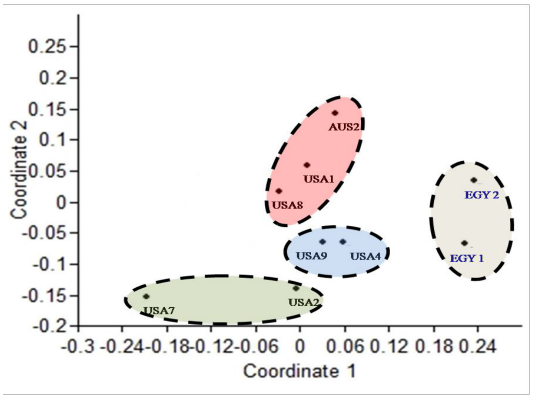

Fig. 3. Molecular characterization and genetic relationships between studied *M. sativa* cultivars as revealed by *rbcL* and *rpoC1* genes [Scatter diagram showing principal coordinate analysis (PCoA), blotting the first two principal coordinates in the selected species to visualize the similarities/dissimilarities of data].

Acknowledgments: The authors are grateful to postgraduate studies and research affairs, Faculty of Science, Ain Shams University, Cairo, Egypt for their keen support and providing possible research grant to Faculty affiliates to obtain valuable materials and reagents without which this work would have not appeared to light. The authors are also appreciating the help of Prof. Dr. Maher Mohamed Shehata, Professor of Molecular Genetics, Cytology and genetics Unit, Faculty of Science, Ain Shams University, Cairo, Egypt for his careful revision and valuable comments and keen advises during preparation of the first draft of manuscript and also for Dr. Sara Aly, Cytology and genetics Unit, Faculty of Science, Ain Shams University, Cairo, Egypt for her kind support and keen help to access the quality of sequencing using Mega 5 software.

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آثار ترميز الحمض النووى (DNA Barcoding) لجينات *rpoC1* و *rbcL* فى العلاقات الوراثية بين بعض اصناف البرسيم *Medicago sativa* L. المصرية

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يعتبر نبات البرسيم *Medicago sativa* L. من نباتات الأعلاف الهامة التى تنتمى إلى العائلة القرنية Fabaceae وتتعلق هذه الدراسة بالتمييز الوراثي وتحديد بعض أصناف البرسيم وقد عرضت في هذه الدراسة تسعة أصناف من البرسيم تمثل ثلاثة بلدان مختلفة (مصر وأستراليا والولايات الأمريكية). تم اجراء ترميز الحمض النووي لدراسة الأصناف المختارة و تمت دراسة الاختلافات الوراثية بين الأصناف المختلفة باستخدام ترميز الحمض النووي من الجينات *rbcL* و*rpoC1* وتم تنفيذ التحليل الوراثي لتسع عينات مختاره من أصناف *Medicago sativa* لاشتقاق التحليل العنقودى Cluster analysis وكان تحليل التسلسل مكملا وفعالا في تقييم توصيف الترابط الجيني بين أصناف البرسيم المدروسة. وفي الختام، أشارت النتائج إلى ان ترميز الحمض النووي كان على الأرجح مناسبا ومحوريا لتوصيف الأنواع والأصناف من البرسيم مما يوفر أداة هامة للتحليلات المستقبلية للمحاصيل العلفية الهامة الأخرى وعلاوة على ذلك، فانه من المقترح أن خطوط التربية من أصناف البرسيم قد تظهر رؤى جديدة تعطى فهم أفضل لتوطين التنوع الوراثى لنبات البرسيم، وفي نفس السياق، يمكن استخدام بعض الأصناف كموارد وراثية مهمة للتحسين الجيني ل *Medicago sativa* في برامج التكاثر المستقبلية.