# Evaluation of Breeding Programs Susceptibility for Two Important Forage Crops Using DNA Barcoding

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## ABSTRACT

**This investigation was carried out to identify and evaluate *Medicago sativa* and *Trifolium alexandrinum* probability for breeding program based on two bar-coding genes (rbcl and Cox1 genes). Identification of *Medicago sativa* Baladi 1 was performed through rbcl and Cox1 genes. *Medicago sativa* Baladi 1 was identified as *Medicago sativa* voucher G00199095 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast (Sequence ID: KJ204375.1) and *Medicago sativa* voucher Ahrendsen_23 for rbcl and Cox1 genes respectively.**

**Identity values were recorded with 90% of identity for alfalfa, Baladi 1 Genotype ribulose – 1/5 – bisphosphate carboxylase / oxygenase large subunit (rbcl) gene (sequences ID: KJ206375.1) also, identity values were recorded with 91.24% of identity for alfalfa Baladi 1 Genotype, cytochrome c oxiddase bubunit I gene (cox 1) (sequence ID: KJ 204375.1).**

***Trifolium alexandrinum* Helaly genotype was identified as *Trifolium alexandrinum* (Sequence ID: HM850407.1) and *Trifolium alexandrinum* voucher K-016Hv (Sequence ID: KU234213.1) as rbcl and Cox1 genes respectively. Affiliation of genetic origin was detected for *Trifolium alexandrinum* with 100 % of similarity with origin source which indicate highly possibility for applying breeding programs comparing with *Medicago sativa* which reflect the lowest genetic similarity with origin source.**


**Key Words: DNA Barcoding; rbcL; Cox 1; *Trifolium alexandrinum*; *Medicago sativa*; NCBI BLAST.**

## INTRODUCTION

DNA sequences to identify organisms have been proposed as a more ancient approach than traditional taxonomic practices (Blaxter, 2004; Tautz *et al*., 2003). Kress *et al*. (2005) have demonstrated the effectiveness of such DNA bar-coding in angiosperms using nrDNA and non-coding cpDNA sequences. In Trifolium, extensive germplasm collections of most wild-collected species exist (Morris and Greene, 2001).

Trifolium is a member of the large clad of legumes lacking one copy of the chloroplast inverted repeat, the IRLC (Lavin *et al*., 1990; Liston, 1995). Molecular phyllogenetic studies have identied a strongly supported "vicioid clad" within the IRLC composed of the tribes Trifolieae and Fabeae

Molecular polymorphism with random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) were employed to determine taxonomic relationships among 25 samples representing nine species of Orobanche L. (Orobanchaceae) dendrogram produced by the analysis of the molecular data (RAPD and ISSR) resembled that constructed by NJ dendrogram for the morphological variation Sahrawy and Karakishi (2015) evaluated the use of two chloroplast regions, trnL and rpoC1, and a nuclear internal transcriber region, ITS2, for their efficiency to barcode the main Mediterranean leguminous crops. Twenty-five legume species were studied. Species identification based on the sequence similarity approach was performed using the GenBank database. The DNA regions trnL and ITS2 successfully (100%) discriminated the Mediterranean crop legume species used, while rpoC1 identified only 72% of them. Furthermore, the use of the trnL region enabled the discrimination of even very closely related species, like Phaseolus lunatus and P. coccineus or Vicia faba subsp major with V. faba subsp minor, which are so closely related that even in NCBI they were both referred as Phaseolus vulgaris and V. faba, respectively. trnL and ITS2 are efficient DNA bar-coding target regions in order to discriminate Mediterranean leguminous crops and provide a reliable and efficient tool for the scientific, agricultural and industrial community. (Madesis *et al*., 2012).

Badr (2001) examined Trifolium alexandrinum using AFLP data. The data support a close relationship of T. alexandrinum accessions from Syria and Egypt to *T. apertum*, T. *berytheum*, and *T. salmoneum* ability of these species to cross freely indicates that *T. salmoneum* and *T. berytheum* may be regarded as the primary ancestors from, which man domesticated Egyptian clover through artificial selection in Syria. Following domestication, the earlier forms of the crop species could have been taken into rain-fed cultivation in Palestine and irrigated cultivation in Egypt. In this regard, the domestication of Egyptian clover may be analogous to other crops, such as barley and wheat, which were also domesticated in the Fertile Crescent and taken into cultivation in the Nile Valley. It appears that genetic improvement of the crop occurred in Egypt

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after cultivation, and that the varieties that were developed in Egypt were later distributed worldwide.

Parsimony and Bayesian phyllogenetic analyses were conducted based on nuclear ribosomal DNA internal transcribed spacer and chloroplast trnL intron sequences obtained from 218 of the ca. 255 species of Trifolium, representatives from 11 genera. Incongruence between the nrDNA and cpDNA results suggests six cases of apparent hybrid speciation, and identifies the putative progenitors of the allopolyploids *T. dubium*, a widespread weed, and *T. repens*, the most commonly cultivated clover species (Ellison *et al*., 2006).

Origin and ancestry of Egyptian clover ( Trifolium alexandrinum L.) As revealed by AFLP markers. The origin and ancestry for Egyptian clover, Trifolium alexandrinum, was examined using AFLP data. The data support a close relationship of T. alexandrinum accessions from Syria and Egypt to T. apertum, T. berytheum, and T. salmoneum. However, cross ability and geographic distributions suggest that T. apertum is an unlikely progenitor. In contrast, T. salmoneum appears to be the most probable progenitor for Syrian material of Egyptian clover, although a close relationship to T. berytheum was also revealed. The ability of these species to cross freely indicates that T. salmoneum and T. berytheum may be regarded as the primary ancestors from, which man domesticated Egyptian clover through artificial selection in Syria. Following domestication, the earlier forms of the crop species could have been taken into rain-fed cultivation in Palestine and irrigated cultivation in Egypt. In this regard, the domestication of Egyptian clover may be analogous to other crops, such as barley and wheat, which were also domesticated in the Fertile Crescent and taken into cultivation in the Nile Valley. It appears that genetic improvement of the crop occurred in Egypt after cultivation, and that the varieties that were developed in Egypt were later distributed worldwide. Kergoat et al., (2004) reconstructed partial sequences of three mitochondrial genes (12S rRNA, cytochrome b, and cytochrome c oxidase subunit I) phylogeny of European seed beetles (Bruchidae) belonging to the genera Bruchus Linnaeus and Bruchidius Schilsky. Adult beetles examined in this study were obtained from larvae bred from seeds directly collected in the field. Parsimony, maximum likelihood, and Bayesian inference were used to infer phylogenetic relationships among species. Both genera, Bruchidius and Bruchus, formed monophyletic groups in all analyses.

Sequence of the chloroplast-genome encoded rbcL gene from Medicago sativa cv. Regen S was compared to pea. Alfalfa shares 94.1% nucleotide sequence homology with pea for 1721 bases spanning the gene beginning 213 bases upstream of the coding sequences through 83 bases into the 3′ flanking region ending at position 1508. Pea sequences are highly divergent from alfalfa after this point. The deduced amino acid sequence is 94.3% homologous to that of pea, with 56% (15/27) of the substitutions non-conservative (Aldrich *et al*., 1987). Also, DNA barcodes from most herbal products (91%) were recovered and all leaf samples (100%), with 95% species resolution using a tiered approach (*rbcL* + *ITS2*). Most (59%) of the products tested contained DNA barcodes from plant species not listed on the labels. Although we were able to authenticate almost half (48%) of the products, one-third of these also contained contaminants and or fillers not listed on the label. Product substitution occurred in 30/44 of the products tested and only 2/12 companies had products without any substitution, contamination or fillers. Some of the contaminants we found pose serious health risks to consumers (Newmaster *et al*., 2013).

**The aime of the present study was to**:

- Use DNA Barcoding to Identify Medicago Sativa Baladi 1 and Trifolum Alexandrinum Helaly Genotypes.
- Evaluation of Breeding programs susceptibility for Medicago Sativa Baladi 1 and Trifolium alexandriunum, Helaly Genotypes using DNA Barcoding (rbcL and Cox 1) genes.

## MATERIALS AND METHODS

The Seeds were obtained from the Forage Crops Research Department (ARC) (Medicago Sativa – alfalfa, Baladi 1 and Trifolum alexandrium, Egyptian clover Helaly).

**METHODS**:

**Sequence Database for DNA Bar-coding:**

Identification and comparing sequences under study was carried out at National Center for Biotechnology Information (NCBI) database.

**Taxon sampling and origin of sequences.**

Two Leguminosae samples (*Trifolium alexandrinum* and *Medicago sativa*) were studied including in reference database.

**DNA extraction, amplification and sequencing.**

Freshly collected specimens were stored on silica prior to extraction. DNA was extracted using the Gene JET Genomic DNA purification kit (Thermo-Scientific) following the manufacturer's protocol. As shown in table (1), two plastid regions were amplified, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene and cytochrome c oxidase subunit 1 gene (Cox1) with specific primer according to Kergoat, *et al*., 2004 and Cai *et al*., 2008, Gurdon *et al*., 2014,

**Table 1. Specific Primer sequence under study**

| | | Primer sequence | Length | Tm | GC% |
|---|---|---|---|---|---|
| *Trifolium alexandrinum* | Rbcl | CAAGGCTTTGCGTGCTCTAC | 741 | 59.83 | 55.00 |
| | | TATCGCGGCAATAGTGAGCC | | 60.32 | 55.00 |
| | Cox1 | ATATTGCCCATAGAGGCCCTTC | 289 | 59.69 | 50.00 |
| | | GCATAGTGATTGCTCCTGCT | | 58.04 | 50.00 |
| *Medicago sativa* | Rbcl | CGGCTACCGATGGACTTACC | 339 | 59.97 | 60.00 |
| | | GTTCCACCCTCTTCCAGACG | | 60.04 | 60.00 |
| | Cox1 | TATGGTTTGCCGGCGATGAT | 759 | 60.18 | 50.00 |
| | | TTGTAATTGCCCCTGCCAGT | | 59.89 | 50.00 |

Young *et al*., (2011) for *Medicago sativa* and *Trifolium alexandrinum.* Amplified products were separated by gel electrophoresis (1.0% Agarose). Obtained RT-PCR products were purified from Agarose gel and quantities spectrophotometrically preparing for sequencing experiment through ABI Prism 7000 instrument based on manufacturer procedure

**Nucleotide sequence accession numbers.**

Nucleotide sequences of bar-coding genes (rbcl and Cox1 genes) were submitted to identified through NCBI BLAST program (http://www.ncbi.nlm.nin.gov/BLAST/) as a single sense-strand contiguous sequence for each of Baladi 1 and Helaly genotypes. PCR products were directly sequenced in 2 directions of each fragment with a Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an automated ABI 3730 sequencer (PE Applied Biosystems). The sequences were aligned using the CLUSTAL W program.

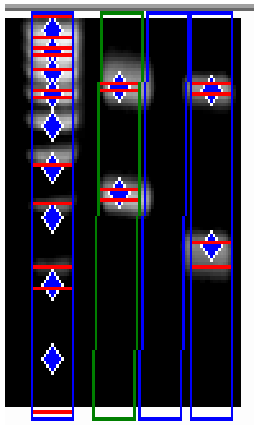
## RESULTS AND DISCUSSION

**Specific gene detection technique:**

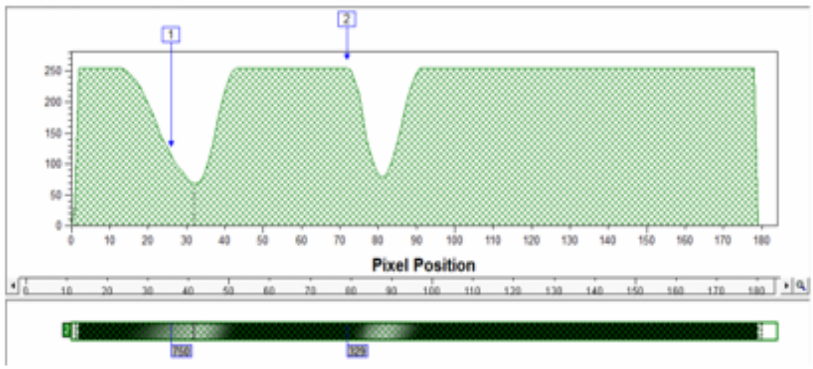
Main purpose of this investigation is identifying and evaluating *Medicago sativa* and *Trifolium alexandrinum* probability for breeding program. Thus, two bar-coding genes (rbcl and Cox1 genes) were employed for identification. Based on alignment data with reference genes, genetic similarity were evaluated and possibility for breeding program were evaluated for *Medicago sativa* and *Trifolium alexandrinum.*

Photograph (1 and 2) show molecular weight parameters. Thus, specific fragments lengths were detected for each of *Medicago sativa and Trifolium alexandrinum*.


**Photograph 1. Specific PCR products for 1. *Medicago sativa* Baladi 1 genotype and *2. Trifolium alexandrinum* Helaly genotype with 339, 759 bp and 741, 289 bp for *rbcl* marker gene and cytochrome c oxidase subunit 1 gene respectively**

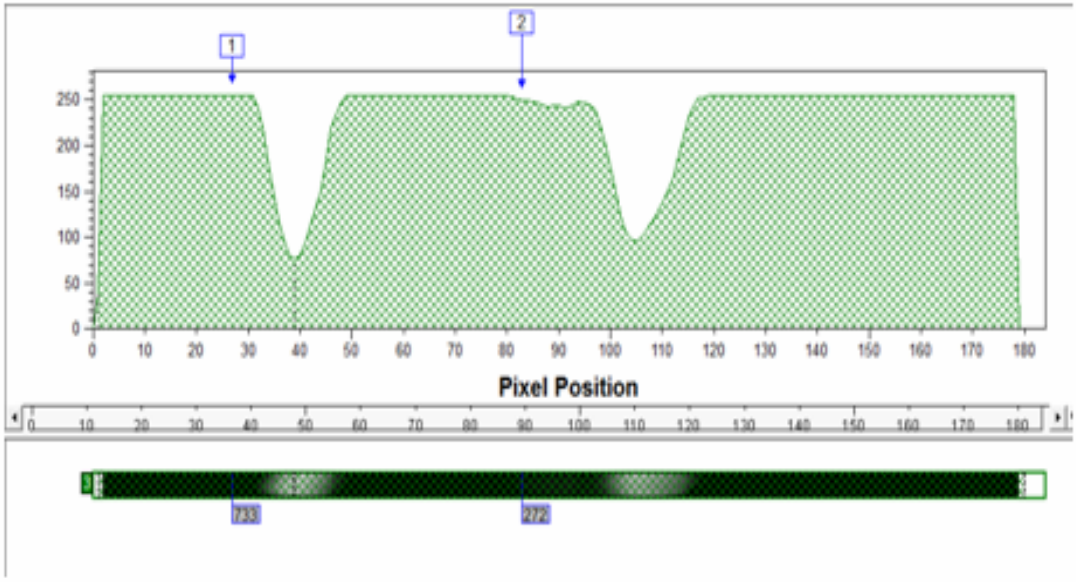


**Photograph 2. Detection of specific PCR products for *Medicago sativa* Baladi 1 genotype *and Trifolium alexandrinum* Helaly with 339, 759 bp and 741, 289 bp for rbcl marker gene and cytochrome c oxidase subunit 1 gene respectively**




| Band No | Position | Volume | Peak Height | Area | Band % | Lane % | MW | Rf |
|---|---|---|---|---|---|---|---|---|
| 1 | 26 | 192404.00 | 114.56 | 992.00 | 14.83 | 14.83 | 750.125 | 0.142 |
| 2 | 72 | 1105307.00 | 254.72 | 4736.00 | 85.17 | 85.17 | 329.030 | 0.393 |

**Photograph 3. Specific PCR products for Medicago sativa Baladi 1 genotype with 339, 759 bp**




| Band No | Position | Volume | Peak Height | Area | Band % | Lane % | MW | Rf |
|---|---|---|---|---|---|---|---|---|
| 1 | 26 | 5582.00 | 80.22 | 64.00 | 0.53 | 0.45 | 741.119 | 0.152 |
| 2 | 68 | 1043127.00 | 253.88 | 4512.00 | 99.47 | 84.09 | 285.195 | 0.398 |

**Photograph 4. Specific PCR products for *Trifolium alexandrines* Helaly with 741, 289 bp**

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CLUSTAL O(1.2.4) multiple sequence alignment


EMBOSS_M-Rbcl         cgcaacctggagttccggctgaagaagcaggtgcagcggtagctgccgaacgagctttct
EMBOSS_M-ori-rbcl     CGCAACCTGGAGTTCCGGCTGAAGAAGCAGGTGCAGCGGTAGCTGCCGAATCTTCCACTG
                      ************************************************  : * :

EMBOSS_M-Rbcl         ggacatggacggcatcggctaccgatggacttaccagtcttgatcgttataaaggacgct
EMBOSS_M-ori-rbcl     GGACATGGACAACTGTGTGGACCGATGGACTTACCAGTCTTGATCGTTATAAAGGACGCT
                      **********..*:  *   ************************************

EMBOSS_M-Rbcl         gctaccacatcgaacctgttgctggagaagagactcaatttattgcttatgtagcttatc
EMBOSS_M-ori-rbcl     GCTACCACATCGAACCTGTTGCTGGAGAAGAGACTCAATTTATTGCTTATGTAGCTTATC
                      ************************************************************

EMBOSS_M-Rbcl         ccttagacctttttgaagaaggttctgttactaacatgtttacctccattgtaggtaatg
EMBOSS_M-ori-rbcl     CCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACCTCCATTGTAGGTAATG
                      ************************************************************

EMBOSS_M-Rbcl         aacgctttctcaaggccttgcgtgctctacgtctggaagag-ggtggaaccccgttgctt
EMBOSS_M-ori-rbcl     TATTTGGGTTCAAGGCCTTGCGTGCTCTACGTCTGGAAGATTTGCGAATCCCCGTTGCTT
                      :*      ******************************   * *.*:***********

EMBOSS_M-Rbcl         atgttaaaactttccaaggtgaggtctcttgaatccaagt
EMBOSS_M-ori-rbcl     ATGTTAAAACTTTCCAAGGT-------------------
                      ******************
```

**Figure 1. Comparison alignments between rbcl marker gene for *Medicago sativa* Baladi 1 genotype and rbcl reference sequence**

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>EMBOSS_M-Rbcl
    cgcaacctgg agttccggct gaagaagcag gtgcagcggt agctgccgaa cgagctttct      60
    ggacatggac ggcatcggct accgatggac ttaccagtct tgatcgttat aaaggacgct     120
    gctaccacat cgaacctgtt gctggagaag agactcaatt tattgcttat gtagcttatc     180
    ccttagacct ttttgaagaa ggttctgtta ctaacatgtt tacctccatt gtaggtaatg     240
    aacgctttct caaggccttg cgtgctctac gtctggaaga gggtggaac  cccgttgctt     300
    atgttaaaac tttccaaggt gaggtctctt gaatccaagt                           340
//
```

**Figure 2. Rbcl marker gene sequence for *Medicago sativa* Baladi 1 genotype**
**(DNA Barcoding of alafalfa Baladi I Genotype (rbcL) gene)**

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>EMBOSS_M-Cox
     TCAAATTCTT GGTGGGAATC ATCAACTTTA TAATGTTTTA ATAACGGCTC ACGCTTTTTT          60
     AATTCTCTTC TTTATGGTT TGCCGGCGAT GATAGGTGGA TCTGGTAATT  GGTCTGTTCC         120
     GATTCTTATA GGTTTTGAA  ACATGGCATT TCCACGATTA AATAATATTT CATTCTGGTT         180
     GTTGCCACCA AGTCTCTTGC TCCTATTAAG CTCAGCCTTA GTAGAGGTGG GTAGCGGCAC         240
     TGGGTGGACG GTCTATCCGC CCTTAAGTGG TATTACCAGC ACCTATTTTC GAGCAGTTGA         300
     TTCAGCAATT TCTAGTCTTC ATCGTTTCAT CCATTTTAGG TTCTATCAAT TTTATAACAA         360
     CTATCTCCAA CATGCGTGGA TTTTACACAT CTATGCATAG ATCACCCCTA TTTGTGTGGT         420
     CCGTTCCAGT AACAGCATTC CCACTTTTAT TATCACTTCC GGTACTGGCA GGGGCAATTA         480
     CAATGTTATT AACCGATCGA AACTTTAATA CAACCTTTTC TGATCCCGCA CCCATTACCT         540
     GGACTATCTG ATACCAGCAT CTCTTTCGGT TCTTCGGTCA TCCAGAGGTG TATATTCCAA         600
     TTCTGCCTGG ATCCGGTATC ACGGCATTTC TCGTTTCGAC TTTTTCGGGA AAACCGGTCT         660
     TCGGGTATCT GGGAGGGGA  TATGCCATGA TCAGTATAGG TGTTCTTGGA TTAGGGGCTT         720
     GGGCTCATCA TATGTTTACT GTGGGCTTAG ACGTTGATAC CC
```

**Figure 3. Cytochrome c oxidase subunit 1 gene (Cox1) marker gene sequence for *Medicago sativa* Baladi 1 genotype**

**(DNA Barcoding of alfalfa Baladi 1 Genotype (Cox 1) gene)**

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CLUSTAL O(1.2.4) multiple sequence alignment

EMBOSS_M-Cox      TCAAATTCTTGGTGGGAATCATCAACTTTATAATGTTTTAATAACGGCTCACGCTTTTTT
sequence1         ----------------------------------------ataacggctcacgctttttt
                                                          ********************

EMBOSS_M-Cox      AATTCTCTTCTTT-ATGGTTTGCCGGCGATGATAGGTGGATCTGGTAATTGGTCTGTTCC
sequence1         aatgatctttttatggttatgccggcgatgataggtggatctggtaattggtctgttcc
                  ***  **** ***   * * ***************************************

EMBOSS_M-Cox      GATTCTTATAGGTTTT-GAAACATGGCATTTCCACGATTAAATAATATTTCATTCTGGTT
sequence1         gattcttataggtgcacctgacatggcatttccacgattaaataatatttcattctggtt
                  ************       ***************************************

EMBOSS_M-Cox      GTTGCCACCAAGTCTCTTGCTCCTATTAAGCTCAGCCTTAGTAGAGGTGGGTAGCGGCAC
sequence1         gttgccaccaagtctcttgctcctattaagctcagccttagtagaggtgggtagcggcac
                  ************************************************************

EMBOSS_M-Cox      TGGGTGGACGGTCTATCCGCCCTTAAGTGGTATTACCAGCACCTATTTTCGAGCAGTTGA
sequence1         tgggtggacggtctatccgcccttaagtggtattaccagccattctggaggagcagttga
                  ************************************    * *     **********

EMBOSS_M-Cox      TTCAGCAATTTCTAGTCTTCATC---------GTTTCATCCATTTTAGGTTCTATCAATTT
sequence1         ttcagcaatttctagtcttcatctatctggtgtttcatccattttaggttctatcaattt
                  **********************       *****************************

EMBOSS_M-Cox      TATAACAACTATCTCCAACATGCGTGGATTTTACACATCTATGCATAGATCACCCCTATT
sequence1         tataacaactatctccaacatgcgtggacctggaatgactatgcatagatcaccctatt
                  ***************************  *   *   **********************

EMBOSS_M-Cox      TGTGTGGTCCGTTCCAGTAACAGCATTCCCACTTTTATTATCACTTCCGGTACTGGCAGG
sequence1         tgtgtggtccgttccagtaacagcattcccacttttattatcacttccggtactggcagg
                  ************************************************************

EMBOSS_M-Cox      GGCAATTACAATGTTATTAACCGATCGAAACTTTAATACAACCTTTTCTGATCCCGCACC
sequence1         ggcaattacaatgttattaaccgatcgaaactttaatacaacctttttctgatcccgcagg
                  ************************************************************

EMBOSS_M-Cox      CATTACCTGGACTATCTGATACCAGCATCTCTTTCGGTTCTTCGGTCATCCAGAGGTGTA
sequence1         agggggagaccccatattataccagcatctctttcggttcttcggtcatccagaggtgta
                        * ** * ******************************************

EMBOSS_M-Cox      TATTCCAATTCTGCCTGGATCCGGTATCACGGCATTTCTCGTTTCGACTTTTTCGGGAAA
sequence1         tattccaattctgcctggatccggtatcataagtcatatcgtttcgacttttcgggaaa
                  ****************************       * ********************

EMBOSS_M-Cox      ACCGGTCTTCGGGTATCTGGGA-GGGGATATGCCATGATCAGTATAGGTGTTCTTGGATT
sequence1         accggtcttcgggtatctaggcatggtttatgccatgatcagtataggtgttcttggatt
                  ***************** **    **  **********************************

EMBOSS_M-Cox      AGGGGCTTGGGCTCATCATATGTTTACTGTGGGCTTAGACGTTGATACCC----------
sequence1         tcttgtttgggctcatcatatgtttactgtgggcttagacgttgatacccgtgcctactt
                      * ******************************************

EMBOSS_M-Cox      ----------------------------------------
sequence1         caccgctgctaccatgatcatagctgtccccacaggaatt
```

**Figure 4. Comparison alignments between cytochrome c oxidase subunit 1 gene for *Medicago sativa* Baladi 1 genotype and rbcl reference sequence**

Identification of *Medicago* sativa Baladi 1 genotype was performed through rbcl and Cox1 genes. Figure (1) shows comparing rbcl marker gene for *Medicago* indicated identification as *Medicago sativa* voucher G00199095 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast (Sequence ID: KJ204375.1).

To evaluate genetic stability for *Medicago sativa* baladi1, rbcl marker gene for *Medicago sativa a*nd rbcl original sequence were compared. Interestingly, comparison data showed that, 90 % of genetic similarity was detected between rbcl marker gene for *Medicago sativa* and rbcl reference sequence. (Fig. 2).

For further confirmation cytochrome c oxidase subunit 1 gene (Cox1) marker gene was applied for identification *Medicago* sativa Baladi 1 genotype (Fig.3) *and* indicated as *Medicago sativa* voucher Ahrendsen_23 cytochrome c oxidase subunit 1 gene, complete cds; mitochondrial.

Highly genetic similarity was founded between cytochrome c oxidase subunit 1 gene (Cox1) for *Medicago sativa* and c oxidase subunit 1 gene (Cox1) for reference sequence and estimated with 91.24% (Fig.4).

Suspected *Trifolium alexandrinum* Helaly genotype was identified based on ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, *Trifolium* sample was identified as *Trifolium alexandrinum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast (Sequence ID: HM850407.1) with 100% of genetic identity (fig.5).

To esstimate genetic relationship between *Trifolium alexandrinum* and genetic origin of *Trifolium alexandrinum*, alignment results were analyzed between ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene for *Trifolium alexandrinum* Helaly genotype and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) reference gene. Thus, 95.92 % of genetic similarity was recorded (fig.6).

In the light of rbcl marker identification gene, comparing cytochrome c oxidase subunit 1 gene (Cox1) marker gene for *Trifolium alexandrinum* indicate identification as *Trifolium alexandrinum* voucher K-016Hv cytochrome c oxidase (COI) gene, partial cds; mitochondrial (Sequence ID: KU234213.1) with 100 % of genetic similarity (figure 7).

Preserve the originality was detected (fig.8) through comparing cytochrome c oxidase (COI) gene, partial cds; mitochondrial sequence with cytochrome c oxidase (COI) gene, partial cds; mitochondrial reference sequence and showed completely identical similarity with 100 % of genetic similarity.

It is important to note that DNA-based identification in *Trifolium* would be much more challenging without the availability of a comprehensive global monograph and biological information for most of the genus (Gillett and Taylor, 2001). Such a robust taxonomic foundation is lacking for the great majority of the world's species

```
>EMBOSS_Tri-Rbcl
     ACCACATCGA GCCGGTTGCT GGAGAAGAAA CTCAATTTAT TGCTTATGTA GCTTATCCCT        60
     TAGACCTTTT TGAAGAAGGT TCTGTTACTA ACATGTTTAC CTCCATTGTA GGTAATGTAT       120
     TTGGGTTCAA GGCTTTGCGT GCTCTACGCC TGGAAGATTT GCGAATCCCC GTTGCTTATG       180
     TTAAAACTTT CCAAGGTCCT CCTCACGGAA TCCAAGTTGA GAGAGATAAA TTGAACAAGT       240
     ATGGACGTCC CCTATTGGGA TGTACTATTA AACCTAAATT GGGTTTATCC GCTAAGAATT       300
     ACGGTAGAGC AGTTTATGAA TGTCTACGCG GTGGACTTGA TTTTACAAAA GATGATGAAA       360
     ATGTGAACTC CCAACCATTT ATGCGTTGGA GAGACCGTTT CTTATTTTGT GCCGAAGCTA       420
     TTTATAAATC ACAGGCCGAA ACGGGTGNNN TCACGGAATT NNNNNNNNNN NNNNNNNNNN       480
     NNNTTCCGGT GCGGTTGTTT GGCTGTATTT GCAAGAGAAT TGGGCGTTCC TATAGGCCAC       540
     TAATGCAGGA CTACCTAACA GGCGGATTCA CTGCAAATAC TACCCTGGCT CACTATTGCC       600
     GCGATAATGG TCTACTTCTT CATATCCACC GTGCAATGCA TGCAGTTATC GATAGACAGA       660
     AAAATCATGG TATGCACTTT CGTGTATTAG CTAAAGCGTT ACGTTTGTCT GGTGGAGATC       720
     ATATTCACGC CGGTACTGTA G                                                741
//
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**Figure 5. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) marker gene sequence for *Trifolium alexandrinum* Helaly Genotype**

**(DNA Barcoding of Egyptian clover Helaly Genotype (rbcL) gene)**


**Figure 6. Comparison alignments between rbcl marker gene for *Trifolium alexandrinum* Helaly genotype and rbcl reference sequence**

```
>EMBOSS_Tri-Cox
     TCTTTCAGCT AATATTGCCC ATAGAGGCCC TTCTGTTGAT TTAGCTATTT TTAGATTACA      60
     TTTAGCTGGT GTATCATCAA TTTTAGGAGC AATTAATTTT ATTACTACCA TGATTAATAT     120
     ACGACCTATT GGTATACAAT TAGATAAACT TCCTTTATTT GCTTGGTCAG TTTTAATTAC     180
     TGCTATTTTA CTTCTGCTTT CCCTCCCTGT ATTAGCAGGA GCAATCACTA TGCTTTTAAC     240
     AGATCGAAAT ATTAATACTT CATTTTTTGA CCCTGCAGGA GGTGGGGAT                 289
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**Figure 7. Cytochrome c oxidase subunit 1 gene (Cox1) marker gene sequence for *Trifolium alexandrinum* Helaly genotype**

**(DNA Barcoding of Egyptian clover Helaly Genotype (Cox 1) gene)**

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CLUSTAL O(1.2.4) multiple sequence alignment


EMBOSS_Tri-Cox      TCTTTCAGCTAATATTGCCCATAGAGGCCCTTCTGTTGATTTAGCTATTTTTAGATTACA
sequence1           tctttcagctaatattgcccatagaggcccttctgttgatttagctatttttagattaca
                    ************************************************************

EMBOSS_Tri-Cox      TTTAGCTGGTGTATCATCAATTTTAGGAGCAATTAATTTTATTACTACCATGATTAATAT
sequence1           tttagctggtgtatcatcaattttaggagcaattaattttattactaccatgattaatat
                    ************************************************************

EMBOSS_Tri-Cox      ACGACCTATTGGTATACAATTAGATAAACTTCCTTTATTTGCTTGGTCAGTTTTAATTAC
sequence1           acgacctattggtatacaattagataaacttcctttatttgcttggtcagttttaattac
                    ************************************************************

EMBOSS_Tri-Cox      TGCTATTTTACTTCTGCTTTCCCTCCCTGTATTAGCAGGAGCAATCACTATGCTTTTAAC
sequence1           tgctattttacttctgctttccctccctgtattagcaggagcaatcactatgcttttaac
                    ************************************************************

EMBOSS_Tri-Cox      AGATCGAAATATTAATACTTCATTTTTTGACCCTGCAGGAGGTGGGGAT
sequence1           agatcgaaatattaatacttcatttttgaccctgcaggaggtggggat
                    *************************************************
```

**Figure 8. Comparison alignments between cytochrome c oxidase subunit 1 gene for *Trifolium alexandrinum* Helaly genotype and rbcl reference sequence**

The results obtained for identification and evaluation of Similarity with the original genetic base are in agreements with the results of Ganopoulos *et al*., (2012). They applied Barcode-DNA High-Resolution Melting (Bar-HRM) analysis method using the universal nuclear plant DNA barcoding region ITS2 for the identification, adulteration and quantification of the main pasture species. Bar-HRM detected *Medicago lupulina* adulterants in *Trifolium pratense* seeds as low as 1:100. In conclusion, Bar-HRM analysis could be a faster with higher resolution and cost-effective alternative method to authenticate forage and pasture species and quantitatively detect the purity of their seeds or their feed products. More light was added to our findings Gillett and Taylor, (2001). They applied DNA-based identification in *Trifolium* would be much more challenging without the availability of a comprehensive global monograph (Zohary and Heller, 1984) and biological information for most of the genus. Such a robust taxonomic foundation is lacking for the great majority of the world's specie

Effectiveness of several genes (cox1, rbcL, 18S and ITS rDNA) were assessed to distinguish cryptic species within the model morphospecies Cox1 divergence was usually much greater than rbcL divergence and always much more variable than 18S rDNA. ITS rDNA sequences were more variable than cox1, but well-known problems concerning intragenomic variability caution against its use in identification. More information and less sequencing effort mean that cox1

can be a very useful aid in diatom identification. The usefulness of cox1 for determining phylogenetic relationships among tree topologies were very similar, although support values were generally lower for cox1 (Evans et al., 2007). With agreements to our findings, Hawkins et al., (2015) DNA metabarcoding and melissopalynology were able to detect the most abundant floral components of honey and plant Taxt. There was 92% correspondence for the plant taxa that had an abundance of over 20%. However, the level of similarity when all taxa were compared was lower, ranging from 22-45, and there was little correspondence between the relative abundance of taxa found using the two techniques. DNA metabarcoding provided much greater repreatability, with a 64% taxa match compared to 28% with melisspalynology.

Altschul *et al*., (1990) introduced BLAST tool for finding sequence similarity (Basic Local Alignment Tool). BLAST approximates alignments that optimize a measure of local similarity, the Maximal segment pair score. Such an alignment may be thought of as minimizing the evolutionary distance or maximizing the similarity between two sequences compared. BLAST employs a measure based on well – defined mutation scores to compare two sequences, whether DNA or amino acid sequences to discover sequence homology. Pairwise alignment is deciding if a pair of sequences is evolutionary related or not. Pairwise similarity scores for the sequences that be fed into a cluster analysis or tree calculating program. The tree is calculated to place more similar paris of sequences closer together on the tree than sequences that are less similar.

## CONCLUSION

This work aims at evaluating and identifying *Medicago sativa* and *Trifolium alexandrinum* (two important Forage crops) for breeding programs susceptibility via two bar-coding genes (rbcl and Cox1 genes).

Identification of *Medicago* sativa Baladi 1 genotype was performed through rbcl and Cox1 genes identified as *Medicago sativa* voucher G00199095 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast (Sequence ID: KJ204375.1) and *Medicago sativa* voucher Ahrendsen_23 for rbcl and Cox1 genes respectively. Moreover, *Trifolium alexandrinum* Helaly genotype identified as *Trifolium alexandrinum* (Sequence ID: HM850407.1) and *Trifolium alexandrinum* voucher K-016Hv (Sequence ID: KU234213.1) as rbcl and Cox1 genes respectively. *Trifolium alexandrinum* showed more success for breeding program comparing with *Medicago sativa* as a result of genetic similarity superiority with origin sequences.

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## الملخص العربي

## تقييم قابلية نوعين من محاصيل العلف لبرامج التربية باستخدام تقنية DNA Barcoding

عبد العزيز طلعت بندق

في هذه الدراسة تم عمل شفرة كودية مميزة لكلاً من البرسيم المسقاوي صنف الهلالي والبرسيم الحجازي صنف بلدي ١ وكذلك تم تقييم صلاحية كل من البرسيم المسقاوي صنف الهلالي والبرسيم الحجازي صنف بلدي ١ لبرامج التربية باستخدام تقنية الباركود DNA وهذا عن طريق جينين يستخدمان في إعطاء شفرة كودية مميزة لكل منهما وهما .(rbcl and Cox1 genes). ولقد أظهرت النتائج أن استخدام جين (rbcL) في تعريف البرسيم الحجازي صنف بلدي ١ بأنه *Medicago sativa* voucher G00199095 ribulose -1,5- bisphosphate carboxylase /oxygenase large subunit (rbcL)gene وأعطى نسبة تشابه بلغت ٩٠% بالمقارنة بالأصل الوراثي (Sequence ID: KJ204375.1) وأيضاً أمكن تعريف البرسيم الحجازي صنف بلدي ١ باستخدام جين (Cox 1) وأوضحت النتائج أنه Medicago Sativa Voucher Ahrendsen-23 وأعطى نسبة تشابه بلغت ٩١,٢٤% بالمقارنة بالأصل الوراثي (Sequence ID : KJ 204375.1) أما بالنسبة للبرسيم المسقاوي صنف الهلالي فقد أظهرت النتائج تعريفه باستخدام جين (rbcL) بأنه *Trifolum Alexandrinum* (Sequence ID : HM850407.1) وأعطى نسبة تشابه بلغت ٩٥,٩٢% بالمقارنة بالأصل الوراثي (Sequence ID : HM850407.1) وأيضاً أظهرت النتائج تعريفه باستخدام (Cox 1 Gene) بأنه *Trifolum alexandrinum* voucher K-016 HV (Sequence ID : KU234213.1) وأعطى نسبة تشابه ١٠٠% بالمقارنة بالأصل الوراثي (Sequence ID : HM850407.1) وفي ضوء النتائج المتحصل عليها تم استخدام التشابه الوراثي مع الأصل الوراثي كمقياس للصلاحية لبرامج التربية وأوضحت النتائج ربما صلاحية البرسيم المسقاوي صنف الهلالي لبرامج التربية كنتيجة لتطابقه بنسبة ٩٧,٩% مع الأصل الوراثي بالمقارنة بالبرسيم الحجازي بلدي ١ الذي بلغت نسبة تطابقه مع الأصل الوراثي ٩٠,٦%.