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THE POTENTIAL VALUE OF DNA BARCODING OF SOME LOCAL Zea L. AND *Trifolium Tourn*. ex L. CULTIVARS

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Biodiversity data play a vital role in preserving and using agrobiologically resources for food and agriculture (FAO 1994, 2006). The data include plant descriptions that can be used to accomplish and apply collections. Biodiversity strengthens the evidence of the frequency of species genetic diversity and affects various variables, including agroecological and ecogeographical effects, for di-

verse market destinations. The genetic relationships among taxa exhibit significant changes that may involve multiple disciplines, including plant improvement and breeding programs, genetic diversity, species diversification, and systematic approaches (Mahdy *et al*., 2021). Species richness is a crucial indicator of biodiversity recognized in terms of biodiversity. The diversification of economic crops can provide opportunities to reduce exposure risks due to biotic and abiotic stresses, including climate change and loss of biodiversity. This approach depends mainly on improving novel genotypes, sustainably utilizing gene pools, screening various risks and challenges, and measuring biodiversity (Mahdy and Ahmad, 2023). Owing to the lack of large-scale sampling of many representative genera and species, the diversity and phylogeny of the family have been compromised by restricted access to research covering plant gene bud diversity, in addition to the considerable similarities among the same gene pool (Mahdy and Rizk, 2023).

The most cultivated economical cultivars, specifically local and threatened genotypes in Egypt, belong to *Fabaceae* and *Poaceae* families. Most of their species are annual and perennial herbaceous species. These plants have a broad sense of diversification and various uses and are naturalized in some regions of Egypt. Both have excellent systems for highlighting biodiversity, subsequently providing excellent genetic resources for food, agriculture, genetic improvement, and conservation (Cheng *et al*., 2020).

Currently, food security and agriculture have emerged as global priorities due to the adverse effects of climate change (Mousavi-Derazmahalleh *et al*., 2019). Several variables, including the degree of intraspecific genetic variation, influence how well crops adapt to environmental stresses. Genomic approaches represent an excellent opportunity to evaluate biodiversity and implement future crop improvement programs (Raza *et al*., 2019). DNA barcoding is a powerful tool for studying systematic and taxonomy, population structure, and biodiversity (Antil *et al*., 2023 and El-Banhawy *et al*., 2021). DNA barcoding is a genetic method that highlights direct and indirect biodiversity indicators (Kress, 2017). This technique allows taxon identification without using morphological cues throughout relatively small standard genes (Selvaraj *et al*., 2013). In addition, Little, (2010) reported the efficiency of the barcode quality index (B), which is a statistical indicator of sequencing success and provides contig coverage and sequence quality.

Desalle *et al*., (2005) explored topical enthusiasm over the development of an initiative to sequence the DNA of all nominated species, which has generated two significant areas of contention as to

how this 'DNA barcoding' initiative should proceed. Many genes, such as *rbc*L, *mat*K, *rpo*C1, *trn*H-*psb*A, and ITS, are used in plant identification (CBOL Plant Working Group**,** 2009). Ribulose-1,5-diphosphate (*rbc*L) is a cpDNA marker that has a high level of similarity between entries and contains 534 conserved sites and 7 variable sites. Moreover, a cross-sectional mutation was identified for G/T at position 335, followed by transitions at 362 (A/G), 368 (A/G), 371 (C/T), and 391 (C/T), revealing the evolution of plant lineages (Ude *et al*., 2019). Genetic variations among the six Egyptian clover (*Trifolium alexandrinum* L.) genotypes were evaluated using molecular markers to detect more polymorphic loci and for fingerprinting (Zayed *et al***.,** 2011; El-banna and Ghazy, 2017 and Bondok 2019) identified *T. alexandrinum* cv. Helaly (HM850407.1) and *T. alexandrinum* voucher K-016Hv (KU234213.1) as the *rbc*L and *Cox*1 genes, respectively. *Trifolium alexandrinum* had greater genetic similarity.

This work involved a representative sample of some field crops in Egypt and was utilized to estimate the discriminatory power and sequence quality of selected barcode markers. The aims of this study are to evaluate whether the currently accepted core barcode markers such as *mat*K, *rbc*L, and *rpo*C1 genes are suitable for estimating biodiversity; estimate the potential value and use of those barcode markers to clarify species and genotype limits within hot spots of Egypt; and exam the technical feasibility of DNA barcode markers for identifying and managing economically important cultivars.

MATERIAL AND METHODS

Genotypes material and marker sampling

Targeted fieldwork in Egypt, convoyed by the Forage Crops Research Department, Field Crop Research Institute (FCRI), Agricultural Research Center (ARC), Giza, Egypt, was used to collect the samples, which represented more than 50% of the cultivated area in Egypt. The genotypes are listed in Table (1), and the supporting information is included in the current study.

Following the recommendations of the CBOL Plant Working Group (2009), three core barcode markers were sequenced: namely, *rbc*L, *mat*K, and *rpo*C1 (Table 2).

DNA extraction, purification, and amplification

Fresh leaf tissues of the six studied genotypes were collected and dried in silica gel following total DNA extraction (Mahdy 2018; Mahdy *et al***.,** 2017 and Chase and Hills, 1991). DNA extraction was carried out using the DNeasy TM Plant Kit (Qiagen, Inc.) according to the manufacturer's protocol. The reaction mixture was composed of 1x buffer (Promega), a 15 mM of $MgCl_2$, a 0.2 mM of dNTPs, 20 combinations of each pri-

mer, a 1 U of Taq DNA polymerase (GoTaq, Promega), a 40 ng of DNA, and ultrapure water to a final volume of 50 µL. Samples were amplified in the Perkin-Elmer/GeneAmp PCR System 9700 (PE Applied Biosystems). PCR was programmed for 40 cycles followed by an initial denaturation cycle for 5 min/94ºC; each cycle adjusted to a denaturation step at 94ºC/30sec., an annealing step at 50ºC/30sec., and an elongation step at 72ºC/1 min. The primer was extended at 72ºC/7 min in the final cycle. The products were electrophoresed in a 1.2% agarose gel that stained with ethidium bromide (0.5 µg/ml) in 1X TBE buffer on 95 volts. The sizes of the PCR-amplified products were standardized using a 100 bp of DNA ladder. The PCR products were visualized under an UV light and photographed by using a gel documentation system (Bio-Rad 2000) for the next steps. The PCR products were cleaned through preparation via the PCR protocol.

Sequencing

The products were sequenced in an automatic ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols submitted by the manufacturer. The forward primer was used for every sample. The products were purified from the separate terminators via the ethanol precipitation protocol. The samples were resuspended in a ddH_2O and electrophoresed in an ABI 3730xl sequencer (Microgen Company). Six novel DNA sequences corresponding to the six studied genotypes were deposited in the World Gene Bank, and their accession numbers are shown in Table (3).

Data assembly and analysis

The barcode quality index (B30) was estimated according to the methods of Little (2010) and Jeanson *et al.* (2011). The sequences were subjected to BLAST and Align Sequences Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/ BLAST).

RESULTS AND DISCUSSION

Barcode quality

The quality of the three core barcode sequences is presented in Fig.(1) based on the B30 barcode quality index. Three barcode sequences had a B30 value above ca. 0.50. The best sequence quality was obtained for the core barcode of *mat*K marker (B30= 0.66), followed by the core barcode of *rpo*C1 marker (B30= 0.57). The barcode quality considered the quality of the sequence and the interference between complementary DNA strands of the same sequence. According to the B30 index values, a sequence quality of 1 indicates the maximal and highest complementary sequence quality. Except for the absence of both quality values and monostrand sequences, B30 values for each sequence were calculated, and the dissimilarity in the sequence quality of each marker was tabulated.

Sequence divergence and matching success

All the three genes succeeded in amplifying through the six different genotypes. The *rbc*L and *rpo*C1 regions were amplified through *Zea Mexicana* (Schrad.) Kuntze, while *mat*K, and *rpo*C1 regions were amplified from the genotypes of *T. alexandrinum* (Table 3). Sequence alignment analysis revealed that a 100% of the genus *Zea* L. hits were from 630 bp for Baladey, 615 bp for Gemmiza, 624 bp for Early Teosinte, and 627 bp for Sakha Teosinte genotypes in the *rpo*C1 gene. The *rbc*L gene was aligned to fragments of 567, 564, 552, and 570 bp for Baladey, Gemmiza, Early Teosinte, and Sakha Teosinte genotypes, respectively (Table 3). The maximum scores were ranged from 1003 to 965 and from 1164 to 1112 for the *rbc*L and *rpo*C1 sequences, respectively. The AT percentages were ranged from 55.6 to 56.5% for the *rbc*L sequence and from 55.9 to 58.7% for the *rpo*C1 sequence. Moreover, the GC percentages were ranged from 43.5 to 44.4% for the *rbc*L sequence and from 41.3 to 44.1% for the *rpo*C1 sequences. The matK gene was amplified at 744 and 570 bp for Giza 6 and Serw 1 genotypes, respectively. Both genotypes were amplified at 465 bp for *rpo*C1 sequence. The *mat*K gene had an AT% greater than the GC%, with a score of 1430 as presented in Table (3).

BLAST indices, similarity matrices and hierarchical clustering

Zea mexicana was tested using two cpDNA markers, namely; RNA polymerase subunit (*rpo*C1) and 1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L). Phylogenetic analysis identified *Z. Mexicana* at the cultivar level. The data obtained by BLAST and the phylogenetic tree are presented in Tables (4&5) and Fig. (2). The highest similarity values were represented. The *rbc*L and *rpo*C1 genes alignments against NCBI GenBank generated a query coverages of 98.3 and 99.11%, respectively, of the four genotypes of *Z. Mexicana* (Tables 4&5). The highest similarity value recorded against other *Zea* species was reached a 100%: such as *Coix aquatic* Roxb., *C. lacryma-jobi* L., and *Z. diploperennis* Iltis, Doebley and R. Guzmán, by *rpo*C1 gene, as well as *Z. mays* L., and *Z. diploperennis* by *rbc*L gene (Tables 4&5).

In addition, the *rbc*L and *rpo*C1 sequences showed a 100% of similarity with those of *Z. mexicana*. The genotypes of *Z. mexicana* were successfully identified by *rbc*L gene, whereas *rpo*C1 gene was effective at the genus level. The phylogenetic trees were generated by similarity matrices, as shown in Fig. (2), to illustrate the clustering of closely related samples under study together with their related species. The combined phylogenetic analysis is also presented in Fig. (2), revealing that the three tested markers supported the identification and biodiversity of the four *Z. Mexicana* genotypes, revealing that the most closely related taxa were *Z. diploperennis* and *Z. mays*.

BLAST and phylogenetic tree results for the two *T. alexandrinum* geno-

types, Giza 6 and Serw 1 (Tables 6&7 and Fig. 3), revealed high similarity values with other genotypes of the genus *Trifolium* Tourn. ex L. *Trifolium tomentosum* L. showed the highest similarity value (99.16%), as well as *T. glomeratum* L. (97.11%) with the *matK* gene. *T. repens* L. (97.85%) showed the highest similarity value with the *rpoC1* gene compared with the other taxa. The newly generated sequences of the three tags, *mat*K, and *rpo*C1, were used as barcodes. Alignment of the *matK* and *rpoC1* sequences against GenBank accessions resulted in query coverage values that were ranged from 95.11 to 97.85% for the *rpoC1* gene and from 94.25 to 99.16% for the *matK* gene (Tables 6&7). For Giza 6 and Serw 1 genotypes, sequencing of the *matK* and *rpoC1* regions of *T. alexandrinum* yielded 744 and 465 bp (the length of the query) for Giza 6 genotype, respectively, as well as 570 and 465 bp for Serw 1 genotype, respectively. Sequence alignment analysis revealed that all of *T. alexandrinum* had scores of 1430 (for Giza 6 and Serw 1) or 841 and 854 bp (for Giza 6 and Serw 1) according to the *matK* and *rpoC1* markers, respectively. Finally, the *matK* and *rpoC1* markers were detected in *Trifolium* at both the species and genus levels.

The phylogenetic trees are represented in Fig. (3) which showed the agglomeration of closely related taxa and dispersal of relatively distantly related species. The combined phylogenetic analysis revealed that *matK* and *rpoC1* markers included the genus *Trifolium*, and the most closely related varieties were *T. repens*, *T. glomeratum*, and *T. tomentosum*.

The results indicated that barcode markers provide a prospecting tool and a powerful way to identify genotypes. These findings suggested that DNA barcoding may be useful for identifying genotypes belonging to various taxa. High-quality DNA sequences are required for successful application of barcodes (CBOL Plant Working Group, 2009 and Little, 2010). In the present study, the mean sequence quality values (mean B30 value) were ranged from 0.50 to 0.66. The B30 values that are consistent with those obtained in previous studies were ranged from 0.40 to 0.90 (Little, 2010 and Jeanson *et al*., 2011). These values also agreed with the criteria of the CBOL (CBOL Plant Working Group, 2009 and Little, 2010).

Plant identification is the first step toward conservation, classification, and breeding approaches. Recently, DNA barcoding has been successfully introduced for plant authentication as an essential base for various disciplines and for determining conservation priorities (Ampatzidis and Vougioukas, 2009 and Bondoc, 2013). This technique provides a proper way to aid in plant genetic resource conservation (Geary and Bubela, 2019 and Zeinalabedini *et al*., 2021). Therefore, a short genetic sequence of a standard part of the genome could be sufficient. Phylogenic analysis and BLAST analysis revealed high similarity values with closely related taxa, reaching a 100%. It was reported earlier that identification by DNA

barcoding at the species level is successful when the similarity rate includes a single species and scores are higher than a 98%. On the other hand, at the genus level, it is successful when all BLAST scores were similar to more than a 95% and were included in a single genus (Geary and Bubela, 2019). The highest similarity values in this study were recorded between the query samples and the GenBank database data, which ranged between 99.03 and 100%.

The obtained *rbc*L sequences were 99.5% similar to those of the closely related species *Zea*. Therefore, the identification of *Z. Mexicana* was successful at both the species and genus levels by the *rbc*L sequence and at the genus level only by the *rpo*C1 sequence. The phylogenetic trees supported the inclusion of the *Z. Mexicana* genotypes below the genus *Zea* and revealed similarity with other closely related species, such as *Z. mays*. Combined phylogenetic analyses generated from cpDNA revealed the evolutionary relationships between and within species (Zeinalabedini *et al*., 2021). The results presented here showed successful identification of plant cultivars at the genus level, species, or both. Therefore, plant identification methods are based on nucleotide data from the taxa of obtained interest during DNA barcoding (Zeinalabedini *et al*., 2021). However, additional DNA markers that can be used to determine plants more accurately may be needed. This study evaluated the possible use of DNA barcoding to document genotypes of interest, especially endemic and endangered ones, by assigning them to the proper classification position and breeding program.

Moreover, Angers *et al*., (2016) cleared the use and development of a bioinformatics concept to process the genome sequences available to automatically examine large numbers of input candidates, identify the targets of novel nuclear barcodes, and design related primer pairs. Furthermore, Liang *et al*., (2020) reported that the use of a barcode system is crucial for efficiently identifying and determining maize lines. On the other hand, Annor *et al*., (2020) explored heterotic groups as an essential prerequisite for developing magnificent maize hybrids. Improving these cultivars has been a great challenge, especially for improving early germplasm quality.

The traditional identification methods used for studying conservation, biodiversity, and classification disciplines are insufficient and need to be more accurate. Recently, DNA barcoding has been alternatively introduced to certify germplasms as a significant approach for determining evolutionary, biodiversity, and ecological conservation priorities (Krawczyk *et al*., 2018 and Enan and Ahamed, 2014). DNA barcoding is an adequate method for identifying various plant taxa and has potential value for identifying and conserving plant genetic resources (Fazekas *et al*., 2008; Yang *et al*., 2017 and Zhi-Fang *et al*., 2021).

DNA barcoding involves identifying and characterizing *T. alexandrinum*,

which are rarely preserved by the barcode method; this is a recent approach for preserving germplasm and measuring the differences between them. It is an excellent model for identifying genotypes and is known at the global level. DNA strand barcoding was carried out through the using of both the *mat*K and *rop*C1 markers from the plastid genes of the Giza 6 and Serw 1 genotypes. It is a multicut and good method in the middle and Upper Egypt plants. Moreover, Serw 1 genotype is a salinity-tolerant variety that can resist relatively high soil and water salinities.

BLAST matching of *T. alexandrinum* revealed a similarity more than 95%, and well-matched the other species of the genus *Trifolium* (Madesis *et al*., 2012). In this study, the highest similarity values were oscillated from 99.03 to 99.38% in NCBI data bases. The *mat*K and *rpo*C1 sequences showed a similarity of 98.21% with respect to closely related species from data base, which matched the level of species and genus similarity according to the *rbc*L sequences and genus similarity according to only the *mat*K and *rpo*C1 sequences in NCBI. The phylogenetic trees revealed that *T. alexandrinum*, which was found in the genus *Trifolium*, was the closest species of *Trifolium*. These sequences are more effective to understand the evolutionary relationships between and within germplasms (Steiner 2006; Fazekas *et al*., 2008 and Xiong *et al*., 2020). The results demonstrated that the identification of *T. alexandrinum* was successful at the species, genus, or both levels when nucleotide data was available for the equivalent species in the database (Cai *et al*., 2008 and Sveinsson and Cronk, 2014). However, additional markers need to be combined for advanced authentication.

SUMMARY

Genetic divergence and biodiversity are vital for food and agricultural use. Crop diversification has been challenged by the limited available research that investigating the genotypes and varieties of biodiversity plants. *Fabaceae* and *Poaceae* are the largest plant families in Egypt, and their members are widely cultivated due to their significant economic value. Six genotypes belong to *Fabaceae* [(two genotypes of *Trifolium alexandrinum* L.), and *Poaceae* (four genotypes of *Zea mexicana* (Schrad.) Kuntze)] were used to study the status of the biodiversity of cultivated crops using three DNA barcode markers, namely; *mat*K, *rbc*L, and *rpo*C1 genes. The resulting data revealed that *rpo*C1 marker was successfully amplified across all the cultivars. Three genes had a barcode quality index (B30) above 0.50, and the best sequence quality was assigned to *mat*K marker (B30= 0.66), followed by *rpo*C1 marker (B30= 0.57). The *rbc*L marker was successfully amplified only through *Z. mexicana*, while *mat*K marker was amplified only through *T. alexandrinum.* The results provided a helpful evidence for biodiversity and could be used for subsequent crop improvement programs. The results showed that *mat*K and *rpo*C1 markers had the best sequence

quality and were convenient for enhancing many different areas of biodiversity.

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Table (1). List of the studied genotype names with common abundance.

ID	Taxa	Genotype
1	*Trifolium alexandrinum* L.	Giza 6
2		Serw 1
3	*Zea mexicana* (Schrad.) Kuntze	Baladey
4		Gemmiza
5		Early Teosinte
6		Sakha Teosinte

Table (2). Sequences and product sizes of the three barcoding primers. (F) Forward primer; (R) Reverse primer..

Name	Sequence		Size (bp)
***mat*K**	**F**	CGATCTATTCATTCAATATTTC	900
	R	TCTAGCACACGAAAGTCGAAGT	
***rbc*L**	**F**	ATGTCACCACAAACAGAGACTAAAGC	600
	R	TCGCATGTACCTGCAGTAGC	
***rpo*C1**	**F**	GGCAAAGAGGGAAGATTTCG	550
	R	CCATAAGCATATCTTGAGTTGG	

Table (3). Estimation of genetic variation and biodiversity using DNA barcoding.

Barcod marker	Taxa	Genotype	Length (bp)	Accession number	Identity (%)	Score	GC%	AT%
*rpo*C1	*T. alexandrinum* L.	Giza 6	465	OK474799	97.7	841	41.9	58.1
		Serw 1	465	OK474800	99.7	854	41.3	58.7
*rpo*C1	*Z. mexicana* (Schrad.) Kuntze	Baladey	630	MZ962636	100	1164	44.1	55.9
		Gemmiza	615	MZ962637	100	1136	43.9	56.1
		Early Teosinte	624	MZ962638	100	1153	43.9	56.1
		Sakha Teosinte	627	MZ962639	98.7	1112	43.8	56.2
*mat*K	*T. alexandrinum* L.	Giza 6	744	OK413869	100	1430	328.	67.2
		Serw 1	570	OK413870	100	1430	29.8	70.2
*rbc*L	*Z. mexicana* (Schrad.) Kuntze	Baladey	567	OK032610	99.1	1003	44.2	55.8
		Gemmiza	564	OK032611	98.9	1011	43.7	56.3
		Early Teosinte	552	OK032612	98.9	987	43.5	56.5
		Sakha Teosinte	570	OK032613	97.1	965	44.4	55.6

Table (4). *Zea mexicana* genotypes DNA barcode of related plant species with similarity percentages were downloaded from the GenBank database using *rbc*L gene.

Baladey genotype					**Gemmiza genotype**				
Plant species	**Accession no.**	**E-value**	**Query coverage (%)**	**Similarity (%)**	**Plant species**	**Accession no**	**E-value**	**Query coverage (%)**	**Similarity (%)**
Zea mays L.	MW537013.1	0.0	100	100	Zea mays L.	MW537015.1	0.0	100	100
	MW537012.1	0.0	100	100		MW537014.1	0.0	100	100
	MW537008.1	0.0	100	100		MW537013.1	0.0	100	100
	MW537007.1	0.0	100	100		MW537012.1	0.0	100	100
	MW537006.1	0.0	100	100		MW537010.1	0.0	100	100
	MW537005.1	0.0	100	100		MW537009.1	0.0	100	100
	MW537011.1	0.0	100	99.84		MW537008.1	0.0	100	99.84
	XM_035965460.1	0.0	100	99.84		MW537007.1	0.0	100	99.84
	XM_035964225.1	0.0	100	99.84		MW537006.1	0.0	100	99.84
Zea diploperennis ltis Doebley & R.Guzmár	MT610091.1	0.0	100	99.84		MW537005.1	0.0	100	99.84
Early Teosinte genotype					**Sakha Teosinte genotype**				
Plant species	**Accession no.**	**E-value**	**Query coverage (%)**	**Similarity (%)**	**Plant species**	**Accession no**	**E-value**	**Query coverage (%)**	**Similarity (%)**
Zea mays L.	MW537015.1	0.0	100	100	Zea mays L.	MW537015.1	0.0	98	98.72
	MW537014.1	0.0	100	100		MW537014.1	0.0	98	98.72
	MW537013.1	0.0	100	100		MW537013.1	0.0	98	98.72

	MW537012.1	0.0	100	100		MW537012.1	0.0	98	98.72
	MW537010.1	0.0	100	100		MW537010.1	0.0	98	98.72
	MW537009.1	0.0	100	100		MW537009.1	0.0	98	98.72
	MW537008.1	0.0	100	100		MW537008.1	0.0	98	98.72
	MW537007.1	0.0	100	100		MW537007.1	0.0	98	98.72
	MW537006.1	0.0	100	100		MW537006.1	0.0	98	98.72
	MW537005.1	0.0	100	100		MW537005.1	0.0	98	98.72

Table (5). *Zea mexicana* genotypes DNA barcode of related plant species with similarity percentages were downloaded from the GenBank database using *rbo*C1gene.

Baladey genotype					**Gemmiza genotype**				
Plant species	**Accession no.**	**E-valu**	**Query coverage (%)**	**Similarity (%)**	**Plant species**	**Accession no.**	**E-valu**	**Query coverage (%)**	**Similarity (%)**
Coix aquatica Roxb.	MT942628.1	0.0	98	99.1	*Brassica rapa* L.	EU334403.1	0.0	100	98.94
Coix lacryma-jobi L.	MT471102.1	0.0	98		*Glyphochloa forficulata* (C.E.C.Fisch.) Clayton	MK593552.1	0.0	100	
Elionurus muticus (Spreng.) Kuntze	MT610077.1	0.0	98		*Hemarthria uncinata* R.Br.	MT610063.1	0.0	100	
Elionurus tripsacoides Willd.	MT610053.1	0.0	98		*Hemarthria compressa* (L.f.) R.Br.	MT610055.1	0.0	100	
Hemarthria altissima (Poir.) Stapf &C.E.Hubb.	MT610054.1	0.0	98		*Hemarthria altissima* (Poir.) Stapf &.E.Hubb.	MT610054.1	0.0	100	
Hemarthria compressa (L.f.) R.Br.	MT610055.1	0.0	98		*Mnesithea lepidura* (Stapf) de Koning &C Sosef	MT610086.1	0.0	100	
Hemarthria uncinata R.Br.	MT610063.1	0.0	98		*Mnesithea selloana* (Hack.) de Koning & Sosef	MT610052.1	0.0	100	
Mnesithea lepidura (Stapf) de Koning & Sosef	MT610086.1	0.0	98		*Coix aquatic* Roxb.	MT942628.1	0.0	99	98.93
Mnesithea selloana (Hack.) de Koning & Sosef	MT610052.1	0.0	98		*Elionurus muticus* (Spreng.) Kuntze	MT610077.1	0.0	99	
Zea diploperennis Iltis, Doebley & R.Guzmán	MT610091.1	0.0	98		*Zea diploperennis* Iltis, Doebley & R.Guzmán	MT610091.1	0.0	99	
Early Teosinte genotype					**Sakha Teosinte genotype**				
Plant species	**Accession no.**	**E-valu**	**Query coverage (%)**	**Similarity (%)**	**Plant species**	**Accession no.**	**E-valu**	**Query coverage (%)**	**Similarity (%)**
Coix aquatic Roxb.	MT942628.1	0.0	100	98.9	*Brassica rapa* L.	EU334403.1	0.0	100	96.56
Elionurus muticus (Spreng.) Kuntze	MT610077.1	0.0	100		*Coix lacryma-jobi* L.	MT471100.1	0.0	100	
Hemarthria uncinata R.Br.	MT610063.1	0.0	100			MT471101.1	0.0	100	

Loxodera bovonei (Chiov.) Launert	MT610092.1	0.0	100			MT471102.1	0.0	100	
	MT610100.1	0.0	100		*Glyphochloa forficulata* (C.E.C.Fisch.) Clayton	MK593552.1	0.0	100	
Loxodera caespitosa (C.E.Hubb.) B.K.Simon	MT610094.1	0.0	100		*Hemarthria altissima* (Poir.) Stapf & C.E.Hubb.	MT610054.1	0.0	100	
Mnesithea lepidura (Stapf) de Koning & Sosef	MT610086.1	0.0	100		*Hemarthria compressa* (L.f.) R.Br.	MT610055.1	0.0	100	
Sorghum bicolor (L.) Moench	MW999225.	0.0	100		*Hemarthria uncinata* R.Br.	MT610063.1	0.0	100	
Sorghum sorghoides (Benth) Q.Liu & P.M.Peterson (basionym *Cleistachne sorghoides* Benth.)	MT610082.1	0.0	100		*Mnesithea lepidura* (Stapf) de Koning & Sosef	MT610086.1	0.0	100	
Zea diploperennis Iltis, Doebley & R.Guzmán	MT610091.1	0.0	100		*Mnesithea selloana* (Hack.) de Koning & Sosef	MW537005.	0.0	98	98.72

Table (6). *Trifolium alexandrinum* (Giza 6 and Serw 1) DNA barcode of related plant species with similarity percentages were downloaded from the GenBank database using *rpo*C1 gene.

Plant species for Giza 6 genotype	**Accession no.**	**E-value**	**Query coverage (%**	**Similarity(%)**
T. repens L.	MT120812.1	0.0	100	95.74
	KP126863.1	0.0	100	
	KC894706.1	0.0	100	
	JN617154.1	0.0	100	
	MT506238.1	0.0	100	
T. patens Schreb. (syn. *T. aureum* Thuill.)	KC894708.1	0.0	100	95.11
T. boissieri Guss.	KJ788284.1	0.0	100	
T. grandiflorum Schreb.	KC894707.1	0.0	100	
T. repens L.	EU750362.1	0.0	100	
T. resupinatum L.	MN857161.1	0.0	100	
Plant species for Serw 1 genotype	**Accession no.**	**E-value**	**Query coverage(%**	**Similarity (%)**
T. repens L.	MT120812.1	0.0	100	97.85
	KP126863.1	0.0	100	
	KC894706.1	0.0	100	
	JN617154.1	0.0	100	
	MT506238.1	0.0	100	
T. patens Schreb. (syn. *T. aureum* Thuill.)	KC894708.1	0.0	100	97.20
T. boissieri Guss.	KJ788284.1	0.0	100	
T. grandiflorum Schreb.	KC894707.1	0.0	100	
T. repens L.	EU750362.1	0.0	100	96.99
T. resupinatum L.	MN857161.1	0.0	100	

Table (7). *Trifolium alexandrinum* (Giza 6 and Serw 1) DNA barcodes of related plant species with similarity percentages were downloaded from the GenBank database using *mat*k gene.

Plant species for Giza 6 genotype	**Accession no.**	**E-value**	**Query coverage (%)**	**Similarity (%)**
T. alexandrinum L.	MN857160.1	0.0	100	97.11
T. glomeratum L.	JX505831.1	0.0	100	
T. ochroleucon Huds.	MW073059.1	0.0	100	
T. squamosum L.	JN895608.1	0.0	100	
T. breweri S.Watson	MF963502.1	0.0	100	94.67
T. macrocephalum (Pursh)Poir.	MF963504.1	0.0	100	
	MF963693.1	0.0	100	
T. ochroleucon Huds.	MW073072.1	0.0	100	
T. canescens Willd.	MW073062.1	0.0	99	94.25
T. caucasicum Tausch	AF522119.1	0.0	100	
Plant species for Serw 1 genotype	**Accession no.**	**E-value**	**Query coverage (%)**	**Similarity (%)**
T. tomentosum L.	JX518122.1	0.0	100	99.16
T. hirtum All.	MW073078.1	0.0	100	95.74
	AF522124.1	0.0	100	
T. lupinaster L.	AF522127.1	0.0	100	
T. beckwithii W.H.Brewer ex S.Watson	AY386946.1	0.0	100	95.61
T. gracilentum Torr. &A.Gray	AF522123.1	0.0	100	
T. kingii S.Watson	MF963503.1	0.0	100	
T. longipes Nutt.	MF963516.1	0.0	100	
T. nanum Torr.	AF522129.1	0.0	100	
T. macrocephalum (Pursh) Poir.	MF963693.1	0.0	100	95.48

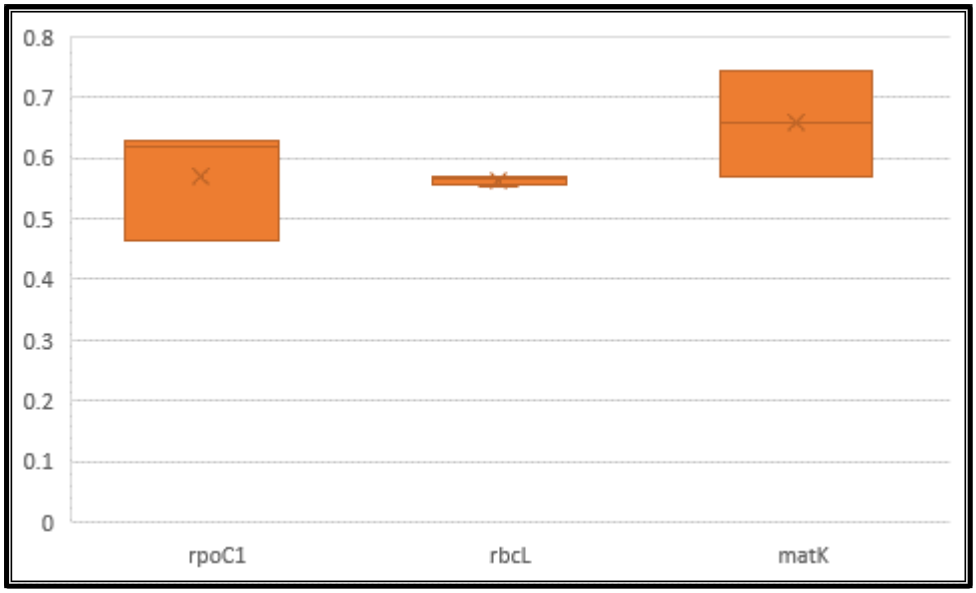

Fig. (1). Box plots of sequence quality for each barcode used marker.

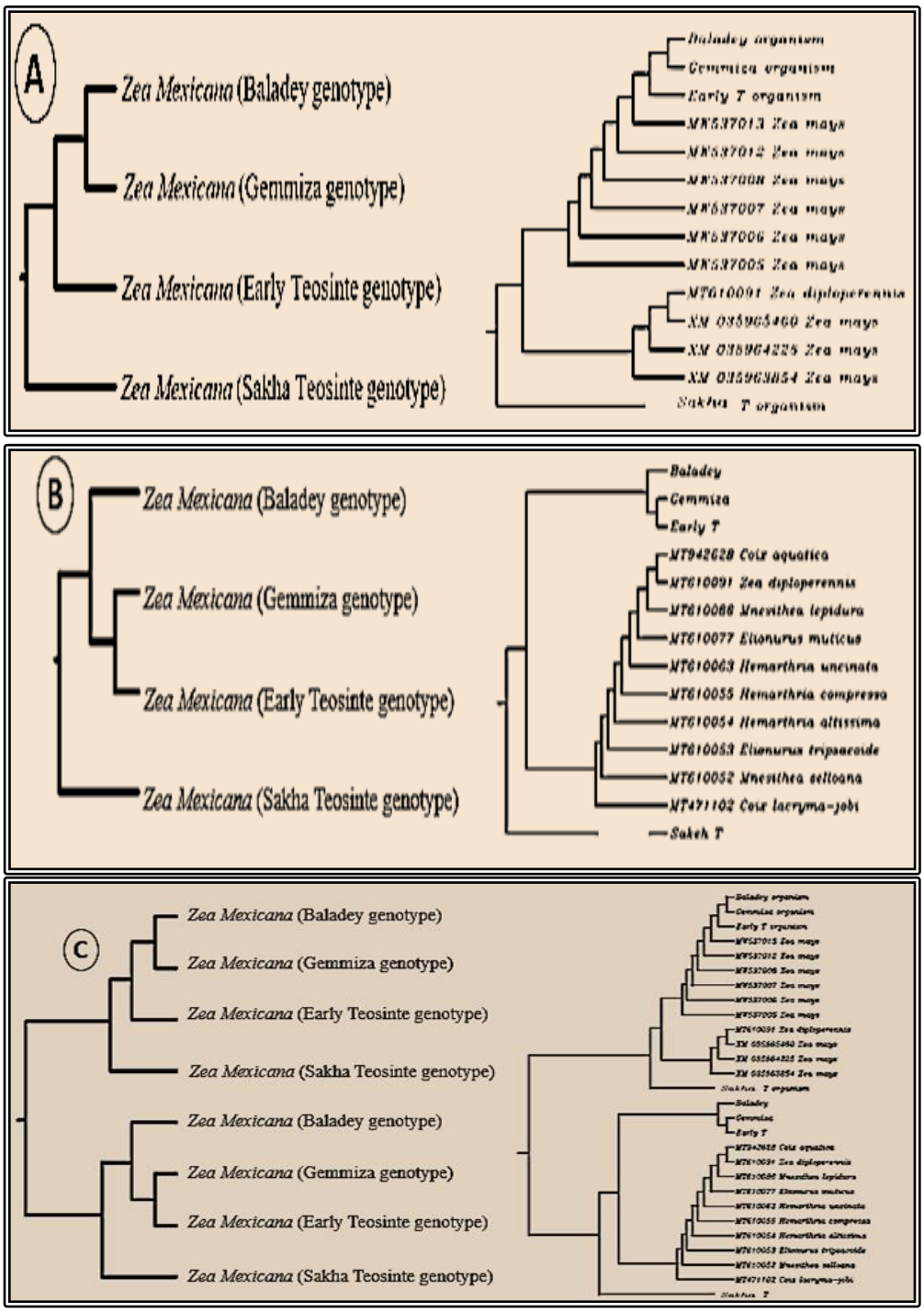

Fig. (2). Phylogenetic trees of the *Zea mexicana* genotypes against the GenBank database. (A) *rbc*L; (B) *rpo*C1; (C) *rbc*L *and rpo*C1 markers.

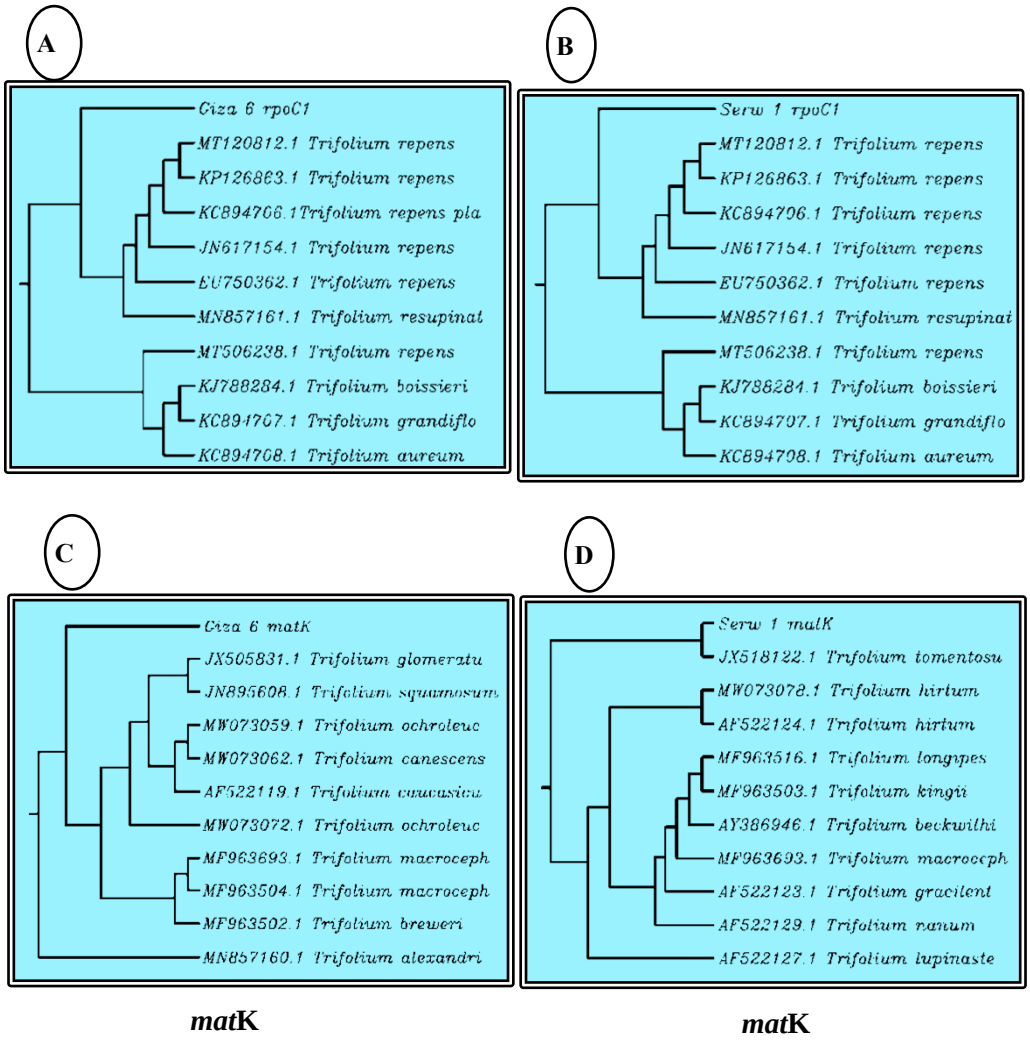

Fig. (3). Phylogenetic trees of the *Trifolium alexandrinum* genotypes against the GenBank database. (A) *T. alexandrinum* L. (Giza 6, *rpo*C1 gene); (B) *T. alexandrinum* L. (Serw 1, *rpo*C1 gene); (C) *T. alexandrinum* L. (Giza 6, *mat*K gene); (D) *T. alexandrinum* L. (Serw 1, *mat*K gene) with 10 genotypes from the GenBank database.