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THE MOLECULAR AND PHYLOGENETIC CHARACTERIZATION OF THE *TAGETES ERECTA* L. AND *COSMOS SULPHUREUS* CAV. USING DNA BARCODING AND BIOINFORMATICS TECHNIQUES

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Abstract

The correct identification of key botanical specimens such as *Tagetes erecta* and *Cosmos sulphureus* Cav. is critical to ensure their safety, efficacy, and appropriate use in herbal medicines. DNA barcoding utilizes sequence variation within a specific and uniform region of the genome (referred as "barcode") in order to achieve precise species identification. The DNA barcodes "matK" and "rbcL" were employed in order to ascertain the phylogenetic relationships of *Tagetes erecta* and *Cosmos sulphureus* Cav. Once DNA extraction was completed, its amplification was done by Polymerase Chain Reaction (PCR). The determination of DNA quality was conducted using agarose gel electrophoresis. The PCR amplification was subsequently followed by BTseq, a Next Generation Sequencing Technique after which the length of DNA fragment of *Tagetes erecta* was found to range between 700 and 800 base pairs. The sequencing data obtained from the rbcL and matK primers was subjected to the BLAST search in the NCBI database. The closely related sequences corresponding to each plant sample were downloaded and utilized for further analysis. The calculation of pairwise nucleotide identity was performed using the MEGALIGN software, developed by DNA Star to conduct phylogenetic analysis. The MEGA X software was used in the construction of a maximum likelihood tree. The phylogram was cross-referenced with the existing data obtained from the fossil record of plant species. The results revealed that the rbcL primer was highly suitable for DNA barcoding of *Tagetes erecta* plants, whereas the matK primer was the most appropriate choice for identifying *Cosmos sulphureus* plants.

Keywords: barcode; BTseq; DNA sequencing; MEGA X; rbcL primer

Introduction

The use of plants in medicine has been of great importance throughout history, primarily owing to their capacity to produce secondary metabolites that possess notable biological properties. According to Achika *et al.* (2014), the Asteraceae family exhibits a diverse array of properties mainly due to their anti-inflammatory, antimicrobial, antioxidant and hepatoprotective activities. *Tagetes erecta* and *Cosmos sulphureus* are classified within the family "Astraceae", which is a prominent assemblage of angiosperms, encompassing around 1000 genera and 23,000 species on a global scale (Czarnecka and Denisow, 2014).

The Mexican Marigold, scientifically known as *Tagetes erecta*, is herbaceous plant that exhibits an erect growth habit and completes its life cycle within a year. It can attain a maximum height of approximately 180 cm. It is predominantly cultivated for traditional utilities like the production of garlands, notably in the subcontinental region of India and Pakistan (Gupta *et al.*, 2012). In wild types, the coloration is characterized by a vibrant shade of yellow, while cultivated types exhibit a range of hues between lemon-yellow to deep brown-red and having the ability to withstand arid soil conditions as well as

clay-based substrates (Setshogo, 2005). The roots of Mexican Marigold secrete substances that consist primarily of flavonoids, amines, amides, phenols, and ketones. These compounds possess insecticidal and nematicidal properties (Olabiya *et al.*, 2007). The utilization of Mexican Marigold plants in Nigeria resulted in a noteworthy reduction of gall formation on cowpea roots as well as a decrease in the population of soil nematodes (Olabiya *et al.*, 2007). As a result, it was proposed that Mexican Marigold plants could be employed as an effective strategy for managing nematode pests affecting cowpea crops.

During the past two decades, DNA identifiers, which are brief, standardized DNA fragments, have been devised for species identification (Hebert *et al.*, 2003). In the early 21st century, DNA barcodes were initially devised as universally recoverable DNA segments for species identification and were implemented for animals (Hebert *et al.*, 2004). The purpose of DNA barcodes is to identify a singular DNA segment that can be used to identify all living taxa. The identification of specimens using barcodes is dependent on the target species possessing sufficient genetic differentiation to permit species separation despite the presence of morphological similarities.

Cosmos sulphureus is a plant that moderately reseeds with abundant seed germination when grown on sandy and loamy soils (Ghayal *et al.*, 2018). This plant is commonly utilized for various medicinal purposes like enhancing blood circulation, functioning as an anti-aging agent, alleviating body heat, fortifying bone marrow and treating infections caused by pathogenic microorganisms (Bindurani *et al.*, 2013).

The MatK and trnH-psbA demonstrate consistent genetic variability in relation to plastidial markers. However, in accordance with the CBOL Plant Working Group, matK was chosen as the best marker because trnH-psbA sequence sizes and alignments were problematic (Chase *et al.*, 2005). This locus has the least divergence of all plastid genes in flowering plants (Kress *et al.*, 2005). The need to amplify additional markers may increase the cost and time required for taxonomic identification; consequently, some researchers have chosen a combination of two regions (matK and rbcL) as a compromise that best accommodates the DNA barcoding criteria.

Bioinformatics tools allow users to manipulate the topologies of phylogenetic trees readily and interactively. matK and rbcL DNA sequences were used to ascertain the phylogeny of *Tagetes erecta* and *Cosmos sulphureus* in this study. The purpose of this study was to evaluate the molecular and phylogenetic diversity of Maxican Marigold using DNA barcoding and the most recent bioinformatics tools.

Materials and Methods

Identification of *Tagetes erecta L.*

The collection of *Tagetes erecta* L. and *Cosmos sulphureus* Cav. was made at the Government Graduate College of Science, Wahdat Road, Lahore, Pakistan. For the authentication of the identification of the plants, eminent professors of plant taxonomy were contacted and accession numbers were obtained.

Retrieval of Primer Sequence, Synthesis

The 'rbcL' gene primer sequence was retrieved by the procedure devised by Asmussen and Chase (2001). On the other hand, the'matK' gene primer sequence was chosen by the method of Ford *et al.* (2009). These sequences were sent to a commercial source "CELEMICS BTSeq, Seoul, Korea" for primer synthesis.

Dilution of Primers

Dilutions were made as per table 1 and to ensure that all of the lyophilized DNA was settled to the bottom of the primer tubes, the material was centrifuged at 8000 rpm for 30 seconds followed by the addition of sterile water. Samples were then left for 10 minutes in a water bath at 60°C after which, the tubes were re-centrifuged for 30 seconds at 8000 rpm.

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Table 1: Dilution of the primers of 'rbcL' and 'matK'.

Sr. No.	Primer Name	M.W	nmol	Addition of water for 100mM stock	Working Primer Dilution (10mM) Stock Dilution+Sterile water		Total working primer 10mM volume
					100mM stock volume	Water volume	100ul
1	rbcL-F	6072	30	300ul	10ul	90ul	100ul
2	rbcL-R	6076.5	30	300ul	10ul	90ul	100ul
3	matK-F	6354.5	30	300ul	10ul	90ul	100ul
4	matK -R	5737.8	30	300ul	10ul	90ul	100ul

Collection of Leaf Samples

A thermos flask containing liquid nitrogen was used. Young leaves of the above-mentioned genera were chosen from the field. A sterilized scalpel blade was used to incise the plant leaves. After being removed, the leaves were wrapped in aluminium foil, tagged, and preserved in a liquid nitrogen containing flask before transportation to the Plant Bio-Tech Lab, Department of Botany, Govt. College of Science, Wahdat Road, Lahore, Pakistan.

DNA Extraction of *Tagetes erecta* L. and *Cosmos sulphurous* Cav.

The technique reported by Doyle and Doyle in 1990 was used to recover genomic DNA from *Tagetes erecta* L. and and *Cosmos sulphurous* Cav. leaves.

PCR amplification of *Tagetes erecta* L. and *Cosmos sulphurous* Cav. for matK gene

DNA extracted from *Tagetes erecta* L. and *Cosmos sulphurous* Cav. was utilized as a starting material for PCR. The PCR process employed specific primers: the forward primer 5′TAATTTACGATCAATTCATTC-3′ (matK Forward Primer) and the reverse primer 5′CTTCCTGTAAAGAATTC-3′ (matK Reverse Primer). After PCR, the amplified fragments were separated on 1% agarose gel and were examined for gene amplification using UV light.

PCR amplification of *Tagetes erecta* L. and *Cosmos sulphurous* Cav. for rbcL gene

The extracted DNA was utilized as a template for PCR, using specific primers: the forward primer 5′ATGTCACCACAAACAGAAAC-3′ (rbcL Forward Primer) and the reverse primer 5′TCG CAT GTA CCY GCA GTT GC3′ (rbcL Reverse Primer). The resulting PCR fragments were separated on a 1% agarose gel and examined under UV light to verify gene amplification. The PCR product, along with a 100-bp DNA ladder, was loaded into the well of the gel, which was prepared for this purpose.

Processing of the PCR Product for Sequencing

Prior to proceeding with sequencing, the samples underwent gene cleaning using the Thermo Scientific GeneJET PCR Purification Kit (cat. # K0702). This purification kit was used to remove any impurities and unwanted components from the PCR products, ensuring that the gene samples were of high quality and ready for sequencing.

Labelling of Samples and Primers for DNA sequencing

Labelled samples and primers (Table 2) were sent to a commercial source, specifically "CELEMICS BTSeq" based in Seoul, Korea, for next-generation sequencing.

Table 2: Labelling of samples of *Tagetes erecta* and *Cosmos sulphureus*

Sr. No.	Plant Name	Label	Primer Label
1	*Tagetes erecta L.*	TE-1-M	matK_F matK_R
2	*Tagetes erecta L.*	TE-2-M	matK_F matK_R
3	*Tagetes erecta L.*	TE-3-M	matK_F matK_R
4	*Cosmos sulphurous Cav.*	CS-1-M	matK_F matK_R
5	*Cosmos sulphurous Cav.*	CS-2-M	matK_F matK_R
6	*Cosmos sulphurous Cav.*	CS-3-M	matK_F matK_R
7	*Tagetes erecta L.*	TA-RB-1	rbcL F rbcL. R
8	*Tagetes erecta L.*	TA-RB-2	rbcL_F rbcL R
9	*Tagetes erecta L.*	TA-RB-3	rbcL_F rbcL R
10	*Cosmos sulphurous Cav.*	CO-RB-1	rbcL_F rbcL R
11	*Cosmos sulphurous Cav.*	CO-RB-2	rbcL_F rbcL R
12	*Cosmos sulphurous Cav.*	CO-RB-3	rbcL_F rbcL R

Bioinformatics Analysis

After receiving the results of sequencing, the following Bioinformatics tools were applied to the data for analysis:

NCBI BLAST

A sequence data file was generated and was subjected to NCBI BLAST to assess its similarity index. Subsequently, some identical sequences were downloaded, along with sequences containing the 'rbcl gene' and 'matK gene' from various taxa.

Multiple Alignments

The downloaded sequences were organized into a Notepad sequence file, after which MegaX software was employed to align these sequences (Table 3 and 4).

Construction of Phylogenetic Neighbourhood Tree

The aligned sequences were trimmed at both ends to ensure an equal number of nucleotides for all selected sequences. Subsequently, a Mega format file was generated, which was then utilized to construct a phylogenetic neighbourhood tree.

Table 3: Paraphrase: Multiple alignment of 'Tagetes erecta' with other related species of the rbcL gene

Sr. #	Plant Label	Accession No.	Nucleotide Length	Gene
1	*Tagetes erecta L. (GCS)*	MS 19	727	rbcL
2	*Tagetes erecta*	MN203535	724	rbcL
3	*Tagetes erecta*	MN309813	724	rbcL
4	*Tagetes minuta*	NC_065038	724	rbcL
5	*Flaveriasonorensis*	HQ534145	724	rbcL
6	*Mikania salviifolia*	MT793843	724	rbcL
7	*Mikania cordata*	NC_053251	724	rbcL
8	*Mikania purpurascens*	MT793853	724	rbcL
9	*Ginkgo biloba*	MS 21	633	rbcL
10	*Rudbeckia laciniata var*	MN518844	724	rbcL
11	*Sphagneticolatrilobata*	MS 20	735	rbcL
12	*Parthiniumhysterophorus*	MS 18	707	rbcL
13	*Helianthus anuus*	MS 17	719	rbcL
14	*Solanum melonglena*	MS 16	740	rbcL
15	*Duturametel*	MS 15	735	rbcL
16	*Solanum nigrum*	MS 14	725	rbcL
17	*Solanum lycopersicum*	MS 13	726	rbcL
18	*Nicotiana plumbaginifolia*	MS 12	744	rbcL
19	*Capsicum annum*	MS 11	725	rbcL
20	*Luffa aegyptiaca*	MS 10	725	rbcL
21	*Lagenaria siceraria*	MS 9	726	rbcL
22	*Ocimumtenuiflorum*	MS 8	690	rbcL
23	*Mentha spicata*	MS 7	742	rbcL
24	*Hibiscus rosa sinensis*	MS 6	726	rbcL
25	*Hibiscus rosa sinensis*	MS 5	735	rbcL
26	*Stevia rebaudiana*	MS 25	719	rbcL
27	*Launaea nudicaulis*	MS 26	717	rbcL
27	*Tagetes lemmonii*	NC_061912	724	rbcL
28	*Ageratinaareolaris*	MW048343	724	rbcL
29	*Eriophyllumlanatum*	MH183145	724	rbcL

Results and Discussion

DNA Extraction and quantification of *Tagetes erecta* and *Cosmos sulphureus*

Following extraction, the presence of DNA of *Tagetes erecta* and *Cosmos sulphurous* was confirmed on agarose gel. Fig. 1 a and b show the comparative outcomes of genomic DNA bands.

PCR Amplification of rbcL and matK gene in *Tagetes erecta* and *Cosmos sulphureus*

The genomic DNA extracted from *Tagetes erecta* and *Cosmos sulphureus* plants was used for PCR amplification of the rbcL gene. Using full-length gene

primers, a PCR product of 1300 bp was obtained. The resulting PCR products were then separated on a 1.0% agarose gel. It was observed that an amplification with the rbcL gene occurred in case of *Tagetes erecta*, while there was no evidence of amplification for *Cosmos sulphureus* (Figure 2a and b).

Table 4: Multiple alignment of *Cosmos sulphurous* with other related species of matK gene

Sr. #	Plant Label	Accession No.	Nucleotide Length	Gene
1	*Cosmos sulphurous Cav.*	MS 4	1032	matK
2	*Cosmos sulphureus*	EU049362	1029	matK
3	*Coreopsis basalis*	AY551492	1029	matK
4	*Coreopsis notha*	EU049357	1029	matK
5	*Heterospermapinnatum*	EU049363	1021	matK
6	*Thelespermafilifolium*	KP126886	1029	matK
7	*Cosmos bipinnatus*	NC_046828	1029	matK
8	*Coreopsis senaria*	EU049360	1029	matK
9	*Bidens pilosa*	MN433104	1029	matK
10	*Bidens asymmetrica*	NC_047268	1029	matK
11	*Chenopodium album*	MS 1	1017	matK
12	*Euphorbia prostrata*	MS 2	1023	matK
13	*Euphorbia heterophyla*	MS 3	1025	matK
14	*Ecliptaprostrata*	MS 22	1024	matK
15	*Lactuca sativa*	MS 23	1014	matK
16	*Spinach_matk*	MS 24	1017	matK
17	*Chuquiraga spinosa*	EU385338	1902	matK
18	*Chuquiragajussieui*	MG553773	1103	matK
19	*Maclediumzeyheri*	EU385375	1911	matK
20	*Pasaccardoagrantii*	EU385384	1447	matK
21	*Tarchonanthuscamphoratus*	EU385403	1900	matK
22	*Pseudostifftiakingii*	JN837405	2515	matK
23	*Ianthopappuscorymbosus*	KF989851	2469	matK

Table 5: DNA Quantification of *Tagetes erecta* and *Cosmos sulphureus*

Sr. No	Plant Name	Label	Concentration (ng/µl)
1	*Tagetes erecta L.*	TE-1	300
2	*Tagetes erecta L.*	TE-2	310
3	*Tagetes erecta L.*	TE-3	289
4	*Cosmos sulphurous Cav.*	CS-1	250
5	*Cosmos sulphurous Cav.*	CS-2	260
6	*Cosmos sulphurous Cav.*	CS-3	270
7	*Tagetes erecta L.*	TA-4	310
8	*Tagetes erecta L.*	TA-5	300
9	*Tagetes erecta L.*	TA-6	296
10	*Cosmos sulphurous Cav.*	CO-4	280
11	*Cosmos sulphurous Cav.*	CO-5	260
12	*Cosmos sulphurous Cav.*	CO-6	250

On the other hand, the genomic DNA of both the plants was used for the PCR amplification of matK gene (Fig. 1a and b). The amplification of matK gene was clearly observed in *Cosmos sulphurous* but was absent in case of *Tagetes erecta* (Fig. 2b).

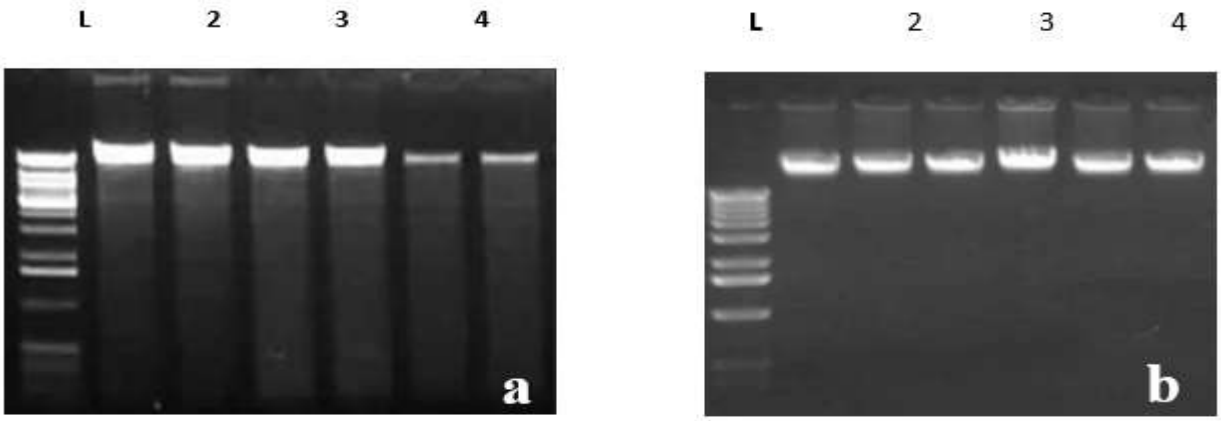

Fig. 1 a) Lane L : 1kb DNA ladder; Lane:2-7: Genomic DNA of *Tagetes erecta.* **b)** Lane L : 1kb DNA ladder; Lane 2-7: Genomic DNA of *Cosmos sulphurous.*

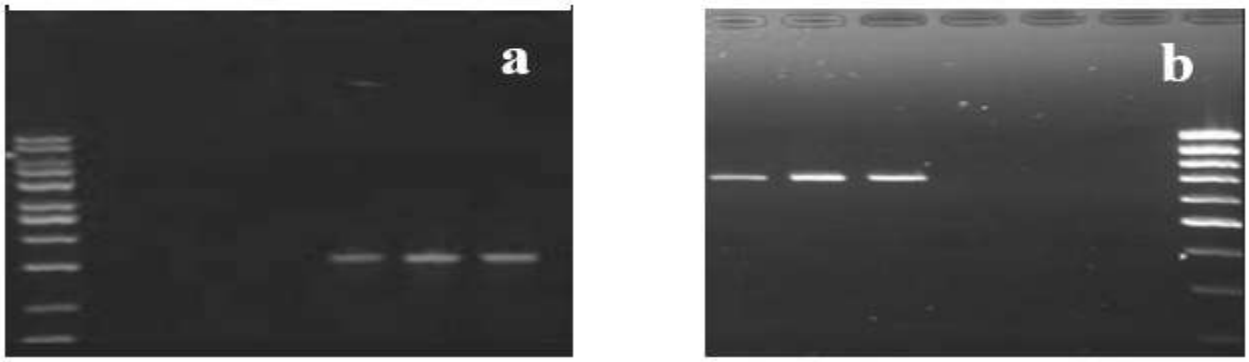

Fig. 2 **a)** 1kb DNA ladder;Lane 2-4: PCR Amplification of rbcL in *Cosmos sulphurous*; Lane 5-7: PCR amplification of rbcL in *Tagetes erecta*. **b)** 1kb DNA ladder;Lane 1-3: PCR Amplification of matK in *Cosmos sulphurous*; Lane 4-6; PCR amplification of rbcL in *Tagetes erecta*.

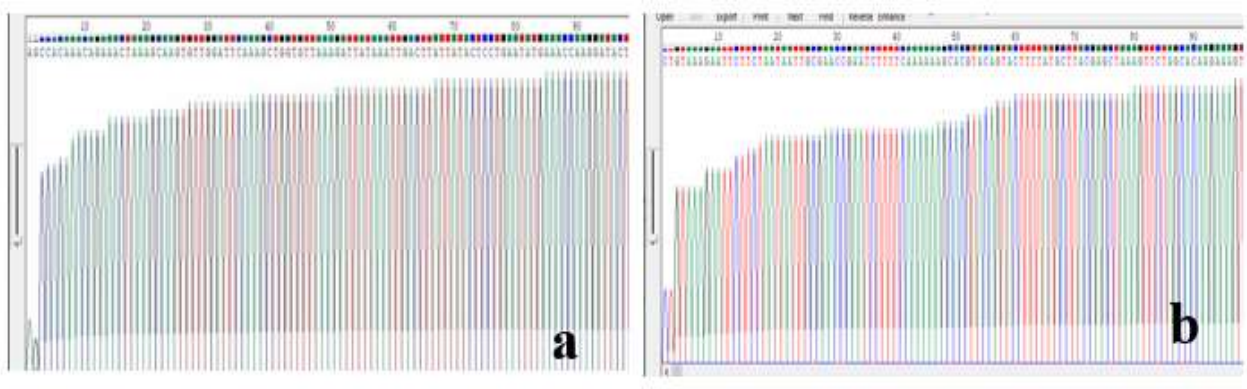

Fig. 3 (a) MS 19 *Tagetes erecta sequencing peaks* with rbcL gene. **(b)** MS 4 sequencing peaks of *Cosmos sulphurous* with matK gene

DNA Sequencing

BTSeq is NGS based. DNA taken through PCR products was then proceeded to randomly fragment the DNA. The fragmented DNA was tagged with proprietary barcodes, and was further sequenced via NGS. After NGS, we collected thousands of reads, and with this data the reference sequence was mapped. The assembly generated reads (100X sequencing depth) were used to create one consensus sequence (Fig. 3a and b).

Multiple Sequence Alignment

After nucleotide BLAST the sequences of closely matched species were aligned by Mega X. Software (Fig. 4a and b).

Pairwise Nucleotide Identity

To determine the relationship of *Tagetes erecta* (GCS) rbcL to other previously reported rbcL gene sequences of the same plant and other angiosperms, the sequence used herein, was subjected to pair wise nucleotide analysis using the Sequence ClustalW, available in the DNASTAR package. *Tagetes erecta* GCS used in this study showed maximum homology at 99.3% with rbcL gene of *Tagetes lemmonii* (Accession number NC061912), 99.7% *Tagetes erecta* (Accession number MN203535), 98.8% *Ageratina* (Accession number MW048343), 98.8 *Flaveriasonorensis* (Accession number HQ534145) (Fig. 5a). *Cosmos sulphureus* GCS used in this study showed (Fig. 5b) maximum homology at 98% with matK gene of *Coreopsis basalis* (Accession numberAY551492), 97.7% with *Coreopsis notha* (Accession numbere EU049357), 97.8% with *Heterosperma pinnatum* (Accession number EU049363) and 98.2% with *Thelesperma filifolium* (Accession number KP126886).

Maximum Likelihood Phylogenetic Tree

Phylogenetic analysis of DNA sequence of *Tagetes erecta* (GCS) rbcL gene facilitated the placement of rbcL gene in distinct clade, well distant from already reported gene sequences (Fig. 6a). Similarly, result obtained for phylogenetic analysis of DNA sequence of *Cosmos sulphurous* (GCS) matK gene aided the placement of matK gene in distinct clade that is well distant from already reported gene sequences (Fig. 6b).

Discussion

The task of identifying organisms based on their morphological characteristics poses a significant challenge (Tan *et al.*, 2020). This study involved the morphological characterization of *Tagetes erecta* and *Cosmos sulphureus*, as well as the molecular identification of these plants through the utilisation of a DNA barcoding approach.

Primers for *Tagetes erecta and Cosmos sulphureus* used in this study were forward primer of rbcL 5′-ATGTCACCACAAACAGAAAC-3′ and reverse primer of rbcL 5′-TCG CAT GTA CCY GCA GTT GC-3′, these primers were previously used by Asmussen and Chase (2001). On the other hand,

sequence of matK forward 5′-TAATTTACGATCAATTCATTC-3′ and matK reverse primer 5′-CTTCCTCTGTAAAGAATTC-3′ was used for DNA barcoding in plants by Ford and co-worker, (2009). The product size of DNA amplification for rbcL gene was 800 bp and for matK gene was 1500 bp. Ristaino *et al.* (1998) studied PCR amplification of ribosomal DNA for species identification in the plant reported similar results.

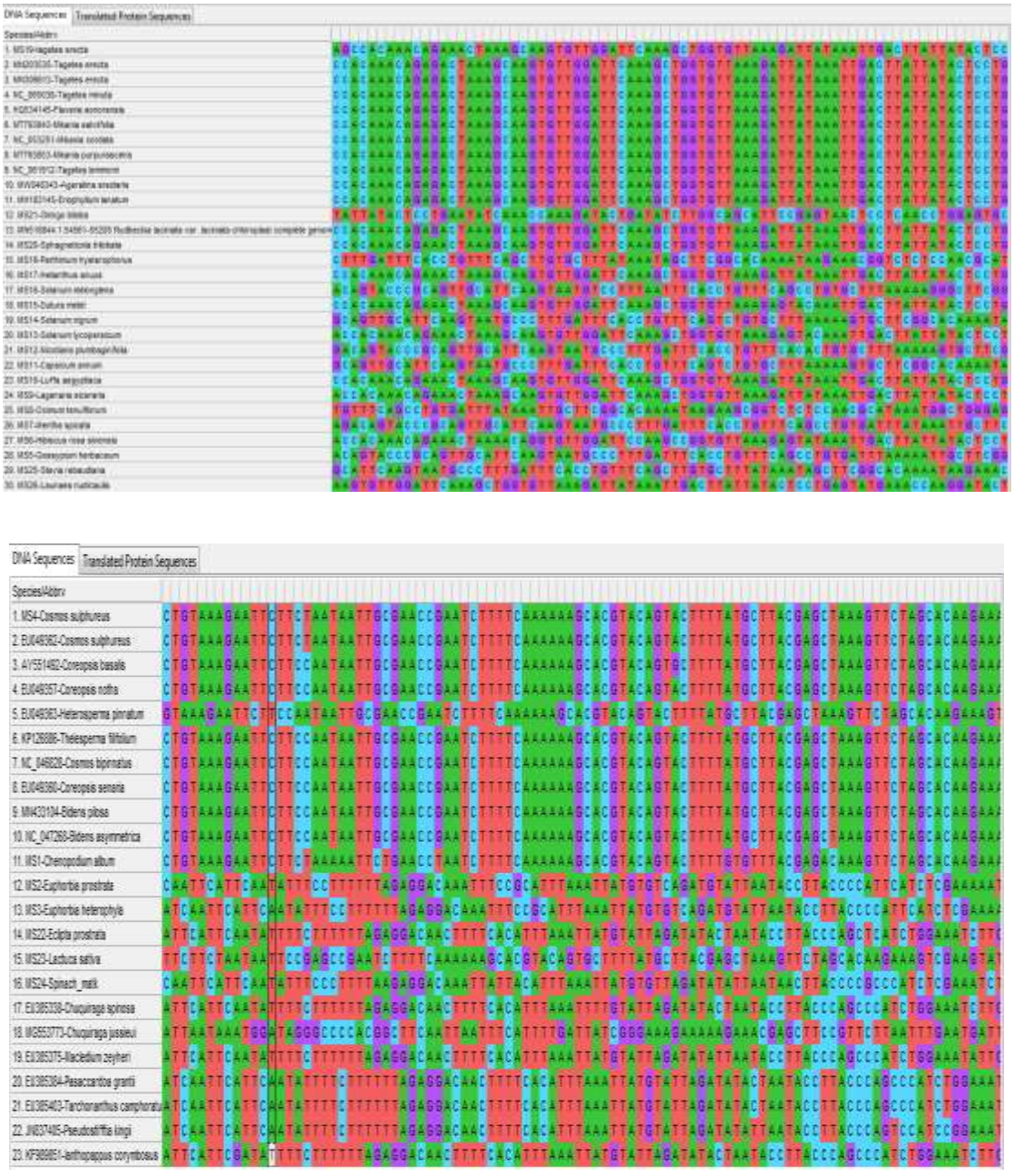

Fig. 4: Aligned sequence of closely related specie of **a)** *Tagetes erecta* plant **b)** *Cosmos sulphureus* plant

At present it was found that the amplification of rbcL gene occurred in case of *Tagetes erecta*. Previously, Jiang *et al*. (2020) also reported the presence of rbcL genes in the chloroplast of Marigold plants and suggested that the rbcL region may serve as a location with a high frequency of genetic mutations, making it valuable for conducting phylogenetic analysis and facilitating in developing molecular markers. Although, there was no amplification occurred regarding rbcL genes in *Cosmos sulphureus*. However, it is noteworthy that the amplification of the matK gene was seen, in contrast to recent findings in the literature indicating weak matK amplification in vascular plants compared to rbcL genes (Ho *et al*., 2021).

Tagetes erecta GCS used in this study showed maximum homology with rbcL gene of *Tagetes lemmonii*, *Tagetes erecta*, and *Ageratina*. *Tagetes erecta* is also closely related to *Flaveria* as described in the previous study (Swenson and Bremer, 1999). On the other side, *Cosmos sulphureus* GCS used in this study showed maximum homology with rbcL gene of *Coreopsis basalis*, *Coreopsis notha*, *Heterosperma pinnatum* and, *Thelesperma filifolium*.

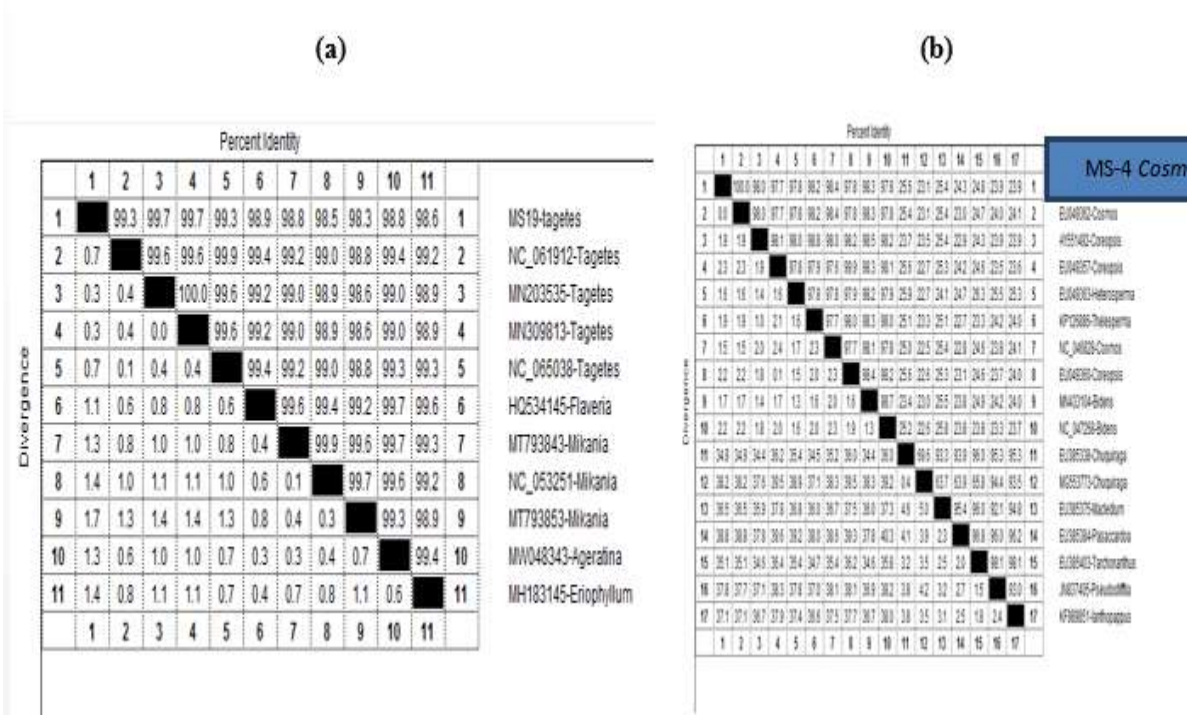

Fig. 5 The plant **a)** *Tagetes erecta* shows maximum homologies with its related species. Pairwise nucleotide identity of rbcL gene used in available in Genebank. Sequences were aligned using MegAlign (DNASTAR) software **b)** *Cosmos sulphurous* shows maximum homologies with its related species. Pairwise nucleotide identity of matK gene used in available in Genebank. Sequences were aligned using MegAlign (DNASTAR) software**.**

Conclusion

On the basis of the present study, it can be concluded that there is the striking closeness of *Tagetes erecta and Cosmos sulphurous* thus elucidating the phylogenetic relationship between the two species. With the help of DNA barcoding, we can reduce the labour for the identification and also accuracy to choose the correct medicinal plant. Also, rbcL has been proved to be the best barcode region for the identification of *Tagetes erecta* plant and matK is proved to be the best barcode region for the identification of *Cosmos sulphurous* plant.

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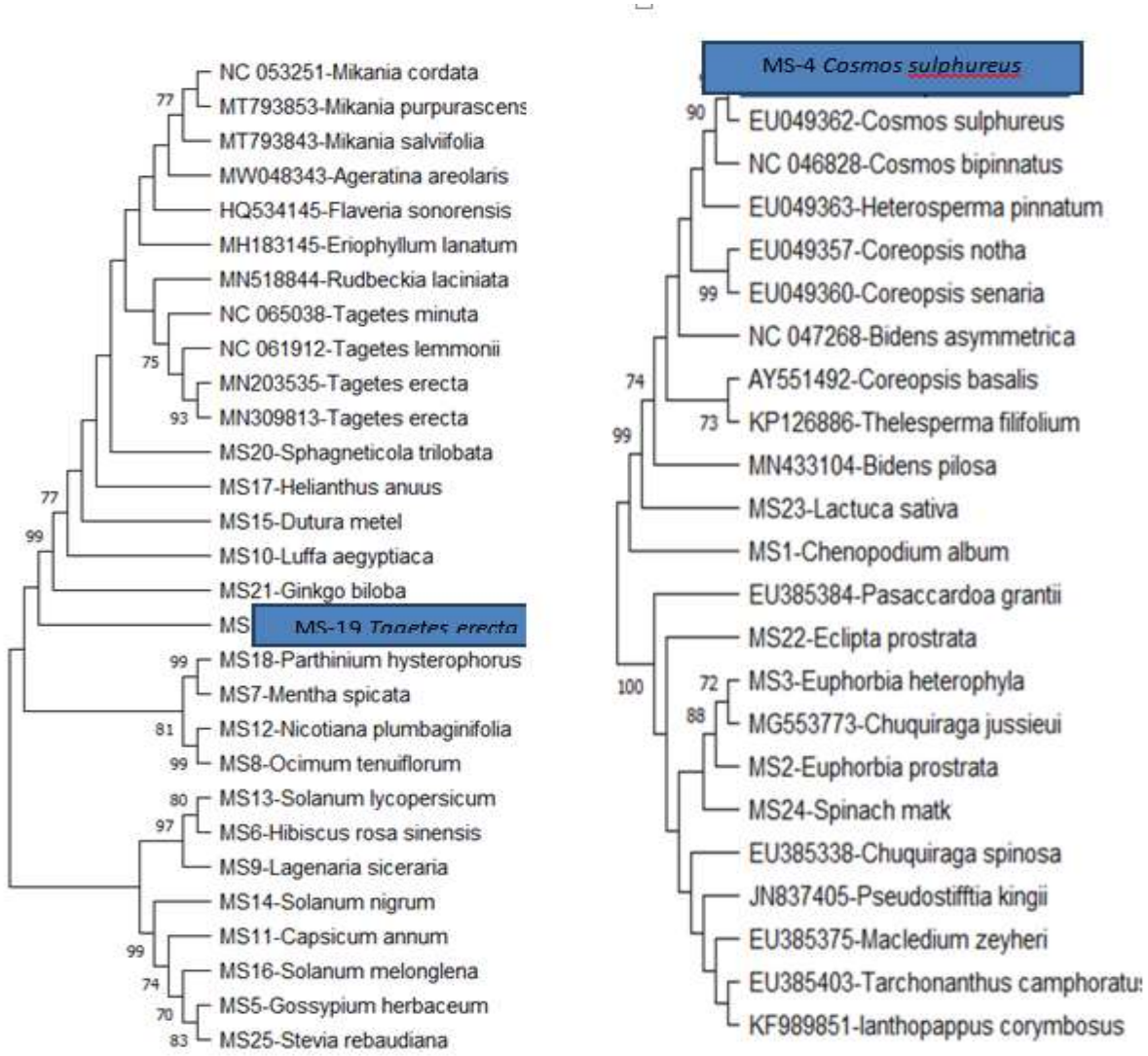

Fig. 6 a) Maximum likelihood (ML) phylogenetic tree reconstructed for the *Tagetes erecta* plant with rbcl gene available in GenBank, which representative used in the present study. The bootstrap values are shown for nodes for 1,000 replicates. **b)** Maximum likelihood (ML) phylogenetic tree reconstructed for the *Cosmos sulphurous* plant with matK gene available in GenBank, which representative used in the present study. The bootstrap values are shown for nodes for 1,000 replicates.