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PHYLOGENETIC ANALYSIS OF SOME SPECIES OF *CYPERACEAE* BASED ON DNA INTERNAL TRANSCRIBED SPACERS (ITS) IN IRAQ

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SUMMARY

Cyperaceae is the third-largest monocot family and has ecological and commercial significance worldwide. It is also a perfect model family for evolutionary research due to species diversity and the wide range of variations in lineage diversity. Using a nuclear region internal transcribed spacer (ITS), the current study attempted to identify the three species (*Carex otrubae*, *Bolboschoenus fluviatilis*, and *Eleocharis palustris*) and examine their relationships within the Cyperaceae family. In 2021, gathering of samples transpired from various locations along the Al-Hussainiya River in Iraq and the National Herbarium. According to the findings, the length of the fragments sequenced ranged between 750 and 1000 bp. Results also showed the sequence alignments between the two species (*Carex otrubae* and *Bolboschoenus fluviatilis*) have similarities and differences with those deposited in the Genome Bank. However, the *Eleocharis palustris* showed complete similarity in all regions of the nitrogenous base sequences with species deposited in the Genome Bank.

Keywords: Three species of *Cyperaceae*, ITS, phylogenetic analysis

Key findings: *Carex otrubae* studied species showed the difference, with *C. otrubae* - MN762702.1 and *C. otrubae* - AF284996.1 because of some mutation. The species *B. fluviatilis* showed a difference with *B. fluviatilis* - Q130340.1 diagnosed in South Korea because of the mutation. However, *E. palustris* showed similarities to all other species registered at the National Center for Biotechnology Information (NCBI).

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INTRODUCTION

Cyperaceae is the third-largest grass-like monocot family comprising more than 5600 species found in temperate and tropical regions (Govaerts *et al*., 2020) and has commercial

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and ecological significance (Spalink *et al.*, 2016). Given their large number of species, widespread distribution worldwide, and significant differences in lineage diversity, Cyperaceae has become an excellent model family for studying evolutionary biology (Escudero and Hipp, 2013). The family has enormous species richness and a high genetic diversity level in the tropics. The genus *Carex* L. contains more than 2000 species and primarily comprises a great variety in temperate areas (Govaerts *et al*., 2020; Hamid and Al-Garaawi, 2022). A sizable portion of the observed taxonomic complexity came from using morphological features to determine taxon bounds (Global *Carex* Group, 2015; Jiménez-Mejías *et al*., 2016b). Additionally, considering the complex population and how much data there is to collect, the most species-rich clades tend to experience slower progress in reorganizing angiosperm taxonomy. Large families may require decades and numerous revision cycles (Soreng *et al*., 2017) and become challenging when identifying species, determining homoplasy, and the relationship with satellite genera.

Bolboschoenus fluviatilis, called the river bulrush, is a species of flowering plant in the sedge family (USDA, NRCS, 2015). *Eleocharis palustris,* the Cyperaceae sedge family, includes a perennial mat-forming flowering plant species known as the common spike-rush. It flourishes in marshes throughout North America, Central Asia, Northern and North Africa, and Europe*. E. palustris* is exceedingly varied globally and very difficult to distinguish from other related species (Bureš and Danihelka*,* 2008).

According to Vargas-Ponce *et al*. (2011), the application of molecular systematics has altered the view of the Tree of Life at the taxonomic level. Molecular markers, unaffected by external influences, have emerged as an influential resource for modern taxonomists (Al-Fatlawi *et al*., 2023). The internal transcribed spacer (ITS) of the *rDNA* and the *ndhF* and *trnLF* chloroplast areas are techniques used to analyze the phylogeny using short DNA sequences of standard genome genes. However, a recently discovered taxonomic approach uses DNA from the Cyperaceae family to quickly, efficiently, and precisely identify the species (Feng *et al*., 2015). According to Dong *et al*. (2015) and Wang *et al*. (2015), DNA barcoding has developed into an essential component of both biological systems and the identification of species (Dong *et al*., 2015; Larranaga and Hormaza, 2015) Recent research has suggested that the *rbcL*, *matK*, *trnH*, *psbA-atpF-ycf1-ITS*, and *atpH* genes could serve as prospective standard DNA barcodes for a variety of taxonomic groupings in crop plants. ITS2, a sub-region of the original nuclear ribosomal ITS, has also been proposed as a potential universal DNA barcode to identify the various herbs based on 6600 samples in 4800 species (Chen *et al*., 2010). This identification method resulted from the findings of the researchers who conducted the study. ITS2 appeared more useful for species identification than the complete ITS region (Han *et al*., 2013). It was primarily attributable to its relatively short length and its outstanding PCR amplification efficiency. In addition, Yao *et al*. (2010) found that ITS2 secondary characteristics have the potential to function as molecular, morphological identifiers for species identification. According to Pang *et al*. (2011), the ITS2 sub-region is the most suitable candidate for the role of the preferred molecular marker in plant phylogenetic investigations and species identification.

The application of molecular data, particularly DNA sequencing, has significantly contributed to addressing concerns connected to systemic issues (Shinwari, 2002; Wang *et al*., 2017; Starr *et al*., 2021). The genus Carex has been the subject of several molecular and DNA barcoding research for years, having published these studies. In light of the earlier discussions, the purpose of the presented investigation was to conduct a molecular analysis of several members of the Cyperaceae family by analyzing their ITS nucleotide sequences.

MATERIALS AND METHODS

DNA extraction, amplification, and sequencing of plant material

Sample collection

Samples collection in 2021 took place from different localities of the Al-Hussainiya River and the National Herbarium in Iraq. Obtaining a total of 30 samples of each species (*Carex otrubae, Bolboschoenus fluviatilis,* and *E. palustris*) ensued in this study. Fresh and young leaf samples gathered from the plants of these genera underwent the isolation of genomic DNA (Feng *et al*., 2014).

DNA extraction

DNA extraction from the leaves used the CTAB technique (Doyle and Doyle, 1987). Employing the biodrop equipment validated the DNA's quality. In the process of PCR amplification, amplifying ITS2 sequences used universal primers, specifically ITS1 (F: 5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (R: 5′-TCCTCCGCTTATTGATATGC-3′), previously described by Yao *et al*. (2010) and Feng *et al*. (2015). The primers came from the Integrated DNA Technologies Canada. Conducting the presented research was within the laboratory of the Department of Biology, situated within the College of Science at the esteemed University of Karbala. Carrying out the PCR amplification used a total volume of 25 µl, consisting of 1.5 µl of DNA, 5 µl of Taq PCR PreMix (Intron, Korea), 1 µl of each primer, and 16.5 µl of distilled water. The thermal cycling protocol employed in this study consisted of an initial denaturation step at 94 °C for three minutes, followed by 35 cycles of amplification. Each cycle comprised a denaturation step at 94 °C for 45 sec, an annealing step at 53 °C for one minute, and an extension step at 72 °C for one minute. The final extension step was at 72 °C for seven minutes. The thermal cycler utilized for this procedure was the Gene Amp PCR system 9700, manufactured by Applied Biosystems. Agarose gel electrophoresis employed to separate PCR products utilized a 1.5% concentration (Figure 1). Subsequently, the separated products underwent observation under ultraviolet light (302 nm), facilitated by Intron Korea. The sequence method employed in this study involved conducting direct sequencing after PCR amplification. Macrogen Inc., located in Korea, provided the sequencing service utilized in this process.

Data analysis

Sequences were aggregation with BioEdit Alignment Editor Use and the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://www.ncbi.nlm.nih.gov) to analyze phylogenetics for the data set. The genetic distances and genetic tree analysis proceeded with the MEGA11.0 version (Tamura *et al*., 2013).

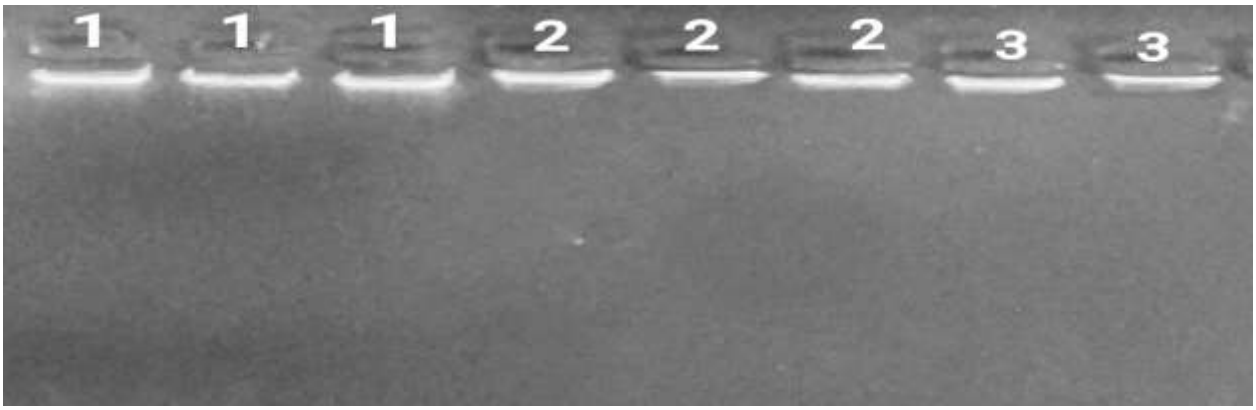


Figure 1. DNA samples isolated and separated by agarose gel electrophoresis at 1.5%.

RESULTS

The total isolated genomic DNA concentration ranges from 300 –to 589 µg, with purity ranging between 1.8 to 1.9 using a biodrop device at a ratio of 260 A280 / A. The molecular size of the samples was 50 to 150 Kb. Carrying out genetic analyses of the three different species, i.e., *Carex otrubae, Bolboschoenus fluviatilis,* and *Eleocharis palustris,* proved successful. The molecular study results detailed the success of the initiator of the target gene utilized in this study. The ITS sequences employed in this analysis ranged from 750 bp in length (Figure 2).

Results of *Carex otrubae* showed the similarity of the studied species with four others (*C. otrubae* MF543762.1, *C. otrubae* MF543761.1, *C. otrubae* DQ 115226.1, and *C. otrubae* MN762512.1) deposited in the Genome Bank. However, it did not match the *C. otrubae* MN762702.1 already in the Genome Bank due to the presence of five mutations that occurred in the nitrogenous bases at different locations and differed from the species *C. otrubae* AF284996.1 with the presence of two mutations (Figure 3).

Creating a genetic tree based on the target DNA sequences in the sample of *Carex otrubae* gained comparison with the DNA sequences deposited in the Genome Bank. The results further showed the phylogenetic tree with seven sequences for various species belonging to the same genus, some identical, while others were different in linking the offspring (Figure 4).

The results of the species *Bolboschoenus fluviatilis* showed a difference in the sequence of some nitrogenous bases of the amplified DNA by PCR using ITS nucleotide sequences. The species *B. fluviatilis* diagnosed in this study revealed closely similar to *B. fluviatilis* diagnosed in South Korea and registered with accession number Q130340.1 at the NCBI. The identities' rate was 99% yet differed because of three mutations present, but similar to *B. fluviatilis* voucher CCDB-18390-B10, registered with accession number MG215956, having identities' rate at 53% (Figure 5).

A genetic tree created based on the target DNA sequences in the sample of *B. fluviatilis* underwent assessment with the DNA sequences deposited in the Genome Bank. The results of the phylogenetic tree showed the presence of three sequences for species belonging to the same genus and species, with some identical to *B. fluviatilis* GO130340.1 (with a rating of 90%), while some different from *B. fluviatilis* MG215956.1 (with a rating of 53%) (Figure 6).

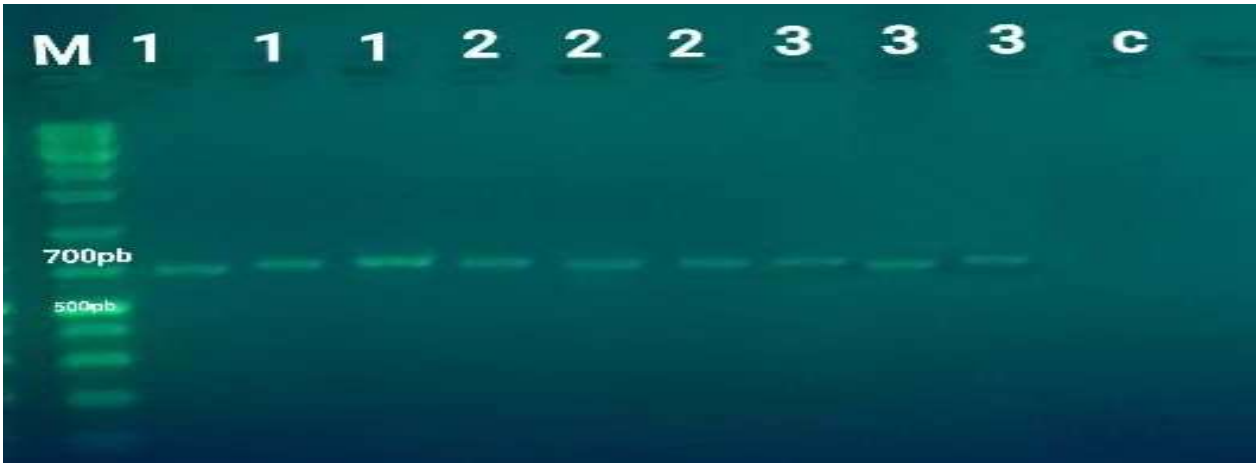


Figure 2. Amplification by PCR and length variations in the ITS regions of plant species *Carex otrubae* 1*, Bolboschoenus fluviatilis* 2*, Eleocharis palustris* 3, control 4, lanes M 10Kbp DNA ladder marker.

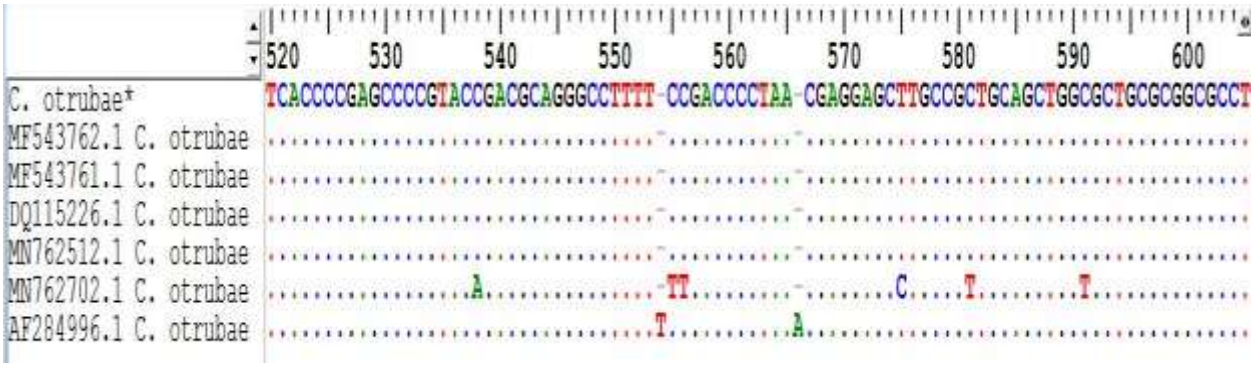


Figure 3. Multiple alignments of ITS nucleotide sequences for the different samples of *Carex otrubae* using the Clustal Omega program.

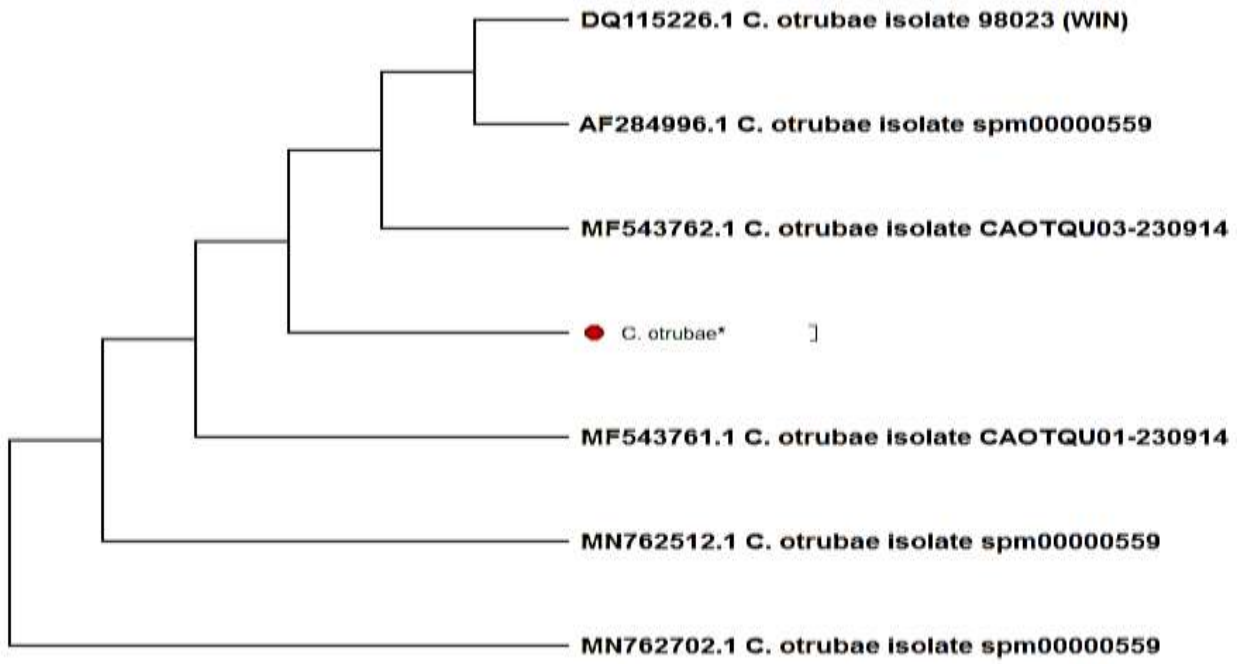
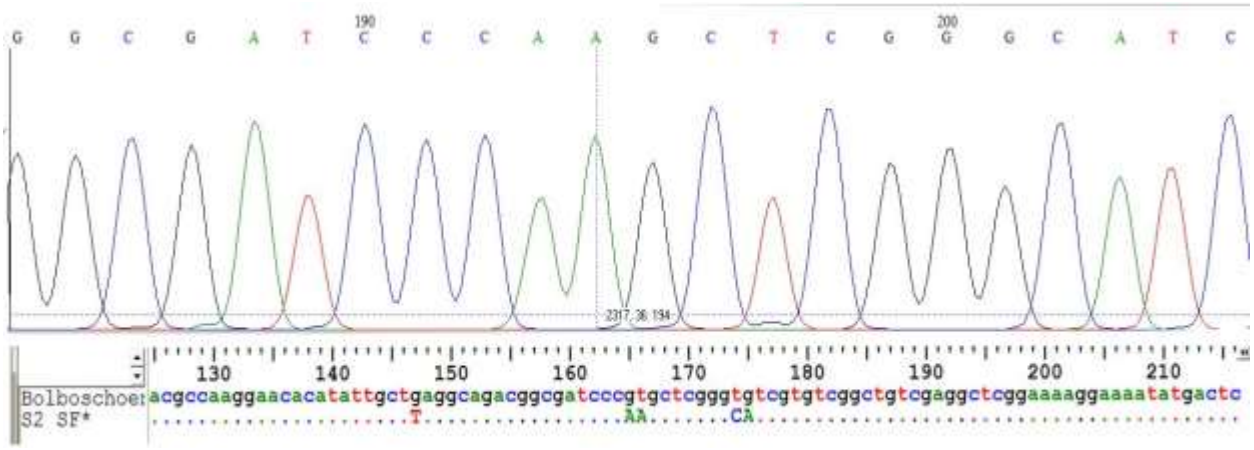
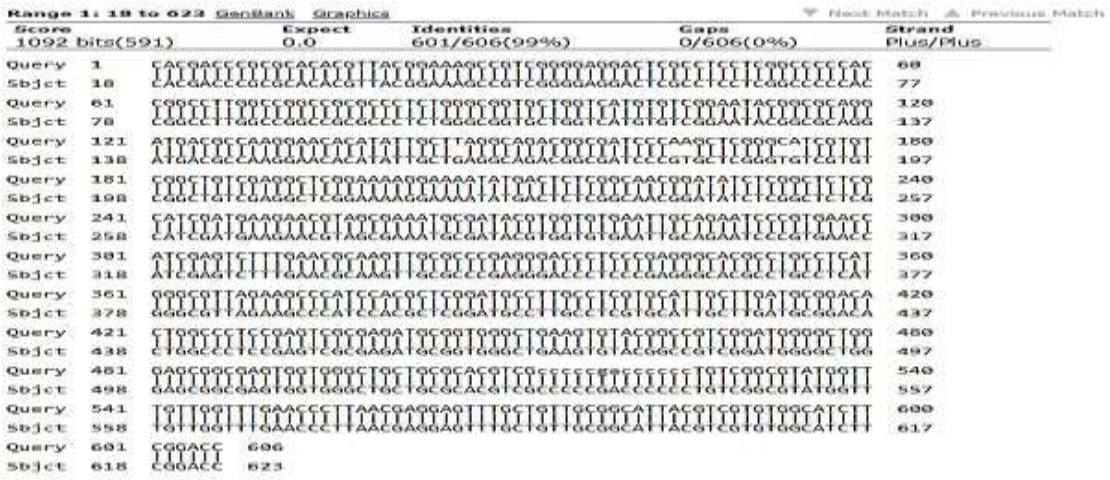


Figure 4. Neighbor-Joining (NJ), a tree showing the genetic relationship of the *C. otrubae* plant identified in this study.

Results of *Eleocharis palustris* showed complete similarity in all regions of the nitrogenous base sequences of the amplified DNA by PCR using ITS nucleotide sequences of the plant species *E. palustris* diagnosed in this study, then registered at the NCBI (Figure 7). A developed genetic tree based on the target DNA sequences in sample *E. palustris* received appraisal with the DNA sequences deposited in the Genome Bank. The phylogenetic tree results showed the presence of eight sequences for various species belonging to the same genus and were completely identical in all the species (Figure 8).



A



B

Figure 5 (A, B). Multiple alignments of ITS nucleotide sequences for the different samples of *B. fluviatilis* using the Clustal Omega program.

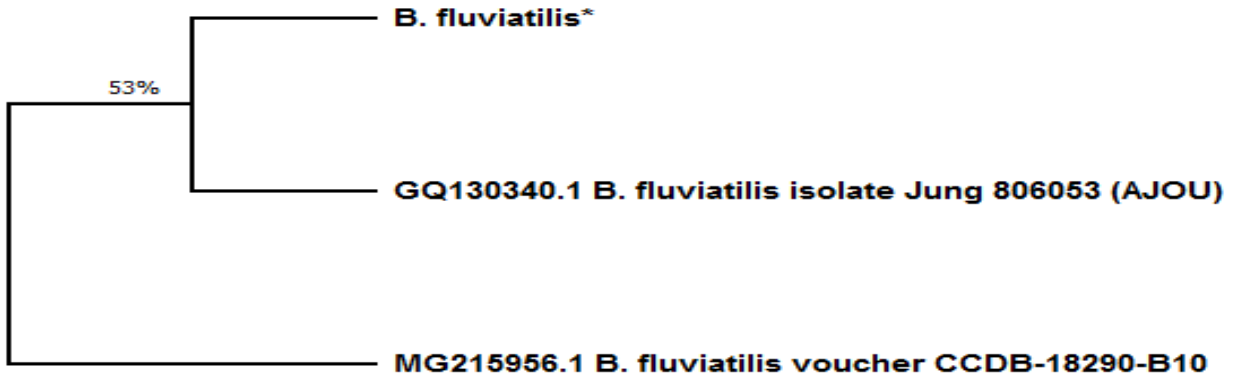


Figure 6. Neighbor-Joining (NJ), a tree showing the genetic relationship of the *B. fluviatilis* plant identified in this study.

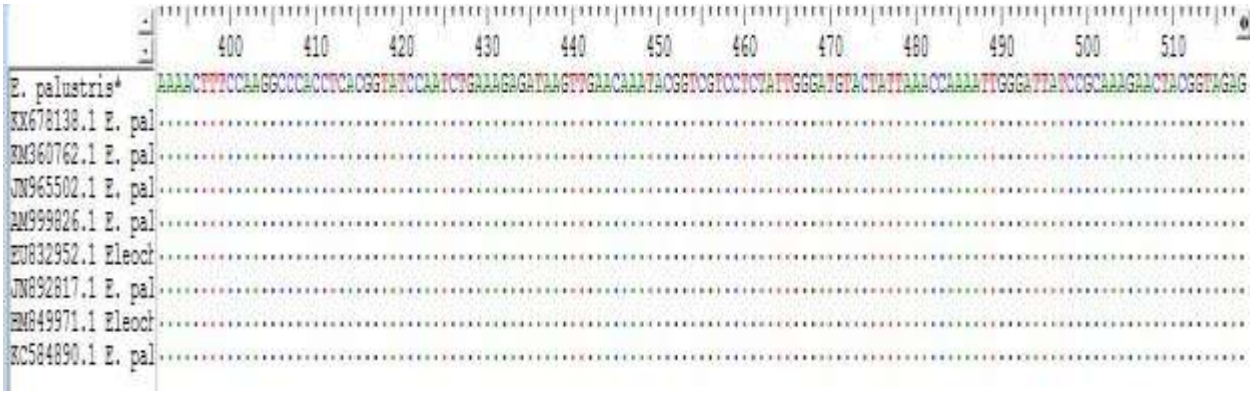


Figure 7. Multiple alignments of ITS nucleotide sequences for the different samples of *E. palustris* using the Clustal Omega program.

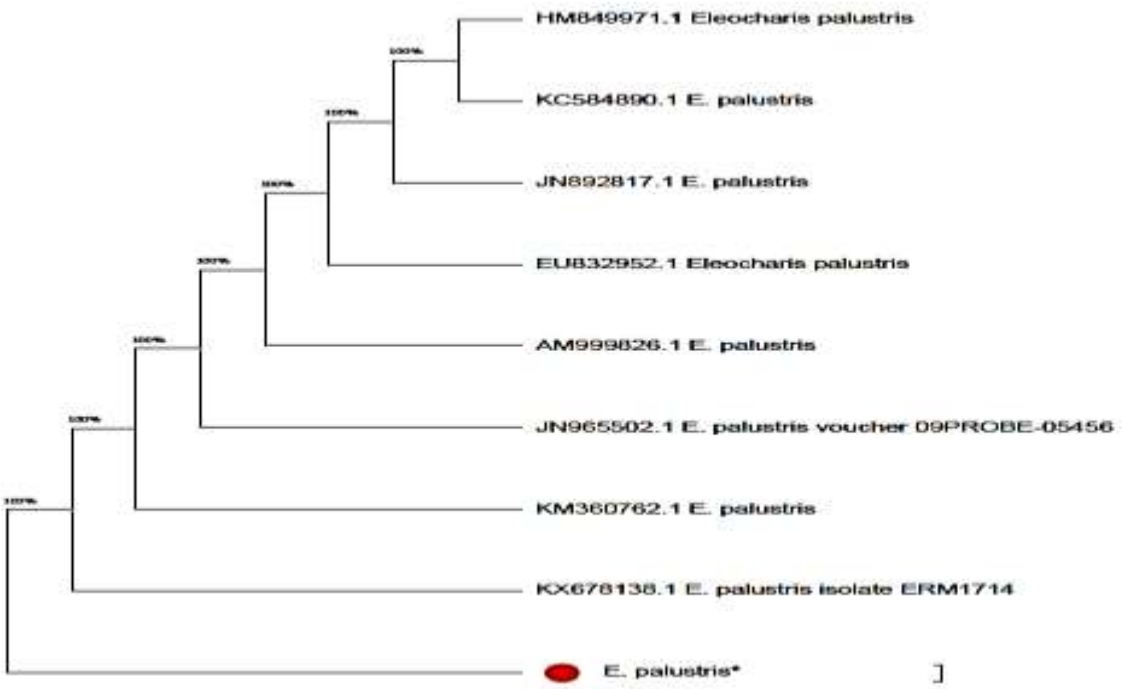


Figure 8. Neighbor-Joining (NJ), a tree showing the genetic relationship of the *E. palustris* plant identified in this study.

DISCUSSION

The abovementioned combinations exhibited a high frequency of developmental impairments, indicating that the interdependent evolution of nuclear and organellar genomes plays a crucial role not only in the process of speciation but also in pre-speciation phenomena. The phenomenon of diverging populations in a clade showed manifestations through alterations in chromosome formulas within species and intraspecific restriction fragment polymorphism of plastomes, according to Abdullah *et al*. (2021). The genetic system within the plant cell, which is also present in eukaryotic cells, is distinguished and

integrated. This system evolves as a whole and imposes further limitations on evolution that are absent in prokaryotes, from findings by Martijn and Ettema (2013). According to Stephan *et al.* (2008), the occurrence of a mutation, whether natural or artificially induced, within any of the genetic compartments of a cell requires a compensatory mechanism that ensures the maintenance of inherent balance, full functionality, and phylogenetic fitness of the entire system. Genetic mutations have the potential to introduce varied traits in an organism. According to Feng *et al*. (2018), if a feature is advantageous, such contributes to an individual's reproductive and survival success.

The genetic sequences of the used samples and those deposited in the Genome Bank gained analysis and were somewhat similar. The comparison of the DNA sequence of the tested samples with the matched DNA sequence determined locations and other details of the amplified PCR product. Revising the information on these sequences will follow once the primer sequences and matching sequences acquire arranging. Heterogeneous variations occurred in the DNA within the amplified sequences by comparing them with the matching series for each analyzed sample. The mutation effect on the Cyperaceae family leads to increasing genetic variations that alter protein function or gene activity and can introduce different traits in the organism.

Although its original purpose was to facilitate species-level identification, DNA barcoding as a tool is valuable. Multiple research investigations have indicated that it could aid in the advancement of studies about taxonomy and biodiversity (Feng *et al*., 2015). The ITS2 region identification has emerged as a potential source of taxonomic signatures in systematic evolution, according to Liu *et al*. (2012) and Feng *et al*. (2015). These signatures can aid in investigating molecular mechanisms underlying plant genome evolution, particularly in compartmental co-evolution, which can ultimately result in population divergence.

CONCLUSIONS

The principal objective is to explore the utility of DNA barcodes, such as the Internal Transcribed Spacer (ITS), for enhancing species identification. Despite potential limitations that may impact result accuracy, the findings indicated that the ITS has the potential to excel in species differentiation within taxonomic classifications.

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