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Review Article

DNA Barcoding Technique – A Molecular Biology Tool for Plant Identification - A Review

Paula-Maria Galan[1,2], Livia-Ioana Leti[1,2] and Silvia Strajeru[1]

[1]*Plant Genetic Resources Bank "Mihai Cristea", Suceava, Romania*

[2]*Alexandru Ioan Cuza University, Iasi, Romania*

Abstract

Plant organisms represent the lungs of the Earth. Plants are considered a real and important source of energy for the existence of species and the geochemical and climatic evolution of our planet. Plant species are essential for human survival, are the main source of food, provide raw materials for many industries and have various medical applications. Knowledge, understanding and protection of plant biodiversity are essential for human life on Earth, especially in the current context of global warming. Thus, the discovery of new plant species and their assessment increases our knowledge about the world we live in and helps us realize their im-portance and role. The DNA barcode technique is a widely used tool for identifying new species, evaluating them and for taxonomic classification. Interest in this technique has grown increasingly; therefore there is now an online database, where about 10 million DNA barcodes are available. Identifying a universal DNA barcode in plants is a real challenge, even if we are talking about using a single locus barcode or multilocus barcode. At the present, regions of the plastid (*matK, rbcL, trnH-psbA*) and nuclear (*ITS*) genome are used for this purpose. Thus, this activity of identifying a universal DNA barcode for the plant kingdom is ongoing.

Keywords: *DNA barcode, plant, biodiversity, matK, rbcL, ITS, plastid genome, nuclear genome.*

Introduction

Knowing, understanding, discovering nature and trying to live in harmony with it, will allow both, us and our descendants enjoy everything around us. The Earth is home to millions of species, each adapted to different environmental conditions, with unique species in certain areas and with a density of biodiversity which varies according to the global area. In 2011, the Census of Marine Life estimated that the total number of species on Earth is around 8.7 million, but mankind has known only 1.3 million. Of these, 6.5 million are species on land and 2.2 million are in the ocean (https://www.sciencedaily.com/releases/2011/08/110823180459.htm).

According to a report by the Royal Botanic Gardens, Kew, in the United Kingdom, there are 391,000 species of vascular plants, known to science worldwide, of which 369,000 species of flowering plants. About 2,000 species of plants are discovered every year, unfortunately some of them are already threatened with extinction (https://ourworldindata.org/biodiversity-and-wildlife).

The world we live in, is developing at a dizzying speed, like in a roller coaster. Everything is affected by technology and industrialization: man, animals, plants. Every day, worldwide, a lot of species of plants and animals disappear, of which man does not know and will never discover. In a report published on May 6, 2019, IPBES (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services),

***Corresponding Author:**
Paula-Maria Galan;
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states that, nature is in global decline and that the rate of extinction of species in the plant and animal kingdoms is at its highest level of all time (https://www.un.org/sustainabledevelopment/blog/2019/05/nature-decline-unprecedented-report/).

An important branch of biological research is that of genetic diversity of different species. This is important and provides information of great value to human society. The knowledge gained about genetic diversity is an important of information source for agriculture, plant breeding programs and the creation of food security strategies. Hence this strong need to conserve plant genetic resources, worldwide, but also at the level of each country. So, for the purpose of preserve the plant germoplasm in *ex situ*, genebanks were emerged.

The use of the molecular biology technique for the analysis of diversity or for the identification of new species, is the best approach in this regard. The knowledge, achieved from the analysis of genetic diversity has a significant impact in genebanks management and breeding program of germoplasm, in the identification and elimination of duplicates, but perhaps, the most important aspect is that it's the basis for establishing the core collection (Ma, K.H. *et al*., 2009). The use of DNA based molecular marker has paved the way for practical changes in the species identification techniques (Botstein, *et al*., 1980). The rapid advances in molecular biology in recent decades, have provided researchers with several new techniques to identify different plant species easily and reliably. These techniques have been used successfully to answer questions about genetic diversity (Karp, *et al*., 1996), (Karp, *et al*., 1997). Among these we list a few: random amplification of polymorphic DNA (RAPD) (Virk, *et al*., 1995); restriction fragment length polymorphism (RFLP) (Miller and Tanksley, 1990), (Zaharleva, *et al*., 2001), (Byrne, *et al*., 1998); amplified fragment length polymorphism (AFLP) (El-Esawi, *et al*., 2016), (Xuexia, L. *et al*., 2008); sequence-tagged simple sequence repeats (SSRs) (Tuler, *et al*., 2015), (Ali, A. *et al*., 2019) or DNA barcoding (Nicolè, *et al*., 2011), (Mishra, *et al*., 2017), (Wu, F. F. *et al*., 2020), (Gogoi and Bhau, 2018), (Carneiro, *et al*., 2019). The DNA barcode technique is a relatively recent technique, that has significantly contributed to the understanding of the composition of biological communities around the world, as well as to the monitoring and understanding of the boundaries of species (DeSalle and Goldstein, 2019). The principle of the DNA barcode is to use short DNA sequences to discriminate and identify samples (Hebert, *et al*., 2003). The mitochondrial gene cytochrome oxidase I (COI, cox1) has been accepted as the global standard for metazoans (Hajibabaei, *et al*., 2007) and the internal transcribed spacer of nuclear ribosomal DNA (nrITS) has been widely accepted as a universal barcode marker for fungi (Schoch, *et al*., 2012). When we talk about plants, things get more complicated. The mitochondrial genome (mtDNA) evolves slowly, therefore the level of variation is not sufficient to recognize species (Hollingsworth, P.M. and all., 2009). Following several researches, there were 7 plastid regions, considered as candidate molecular markers in plant. Of these, four regions are coding (*matK, rbcL, rpoB* and *rpoC1)* and another three are intergenic spaces (atpF-atpH, trnH-psbA and psbK-psbI), but no good results were obtained, using single locus or multilocus sites for wheel plants (Hollingsworth, P.M. and all., 2009). However, CBOL recommends the 2-locus combination of *rbcL+matK* as the plant barcode. The purpose of this review is to highlight the importance of using DNA barcodes for plant biodiversity studies, but also to present the advantages and disadvantages of using different DNA markers in this direction.

DNA Barcoding Technique, Applications, Perspectives

In 2003, Canadian biologist, Paul D. N. Hebert (Centre for Biodiversity Genomics, University of Guelph, Canada), developed the DNA barcode method, which was invented in 1980s, for arthropods, by Carl Woese and

colleagues (Woese, *et al*., 1990). The DNA barcode technique is characterized by the use of short DNA sequences, from representative genes, either from the organellar genome (chloroplastic, mitochondrial) or from nuclear genome (Ali, M.A. *et al*., 2015), (Kress, *et al*., 2005), (Purty and Chatterjee, 2016). The method is widely used to identify species using short orthologous DNA sequences, called DNA barcodes. At the same time, this technique, has been proposed to identify juveniles, associate sexes, enhance forensic or facilitate biodiversity (Kress, *et al*., 2005). DNA barcodes have many practical applications in plants, but not only (Table 1).

Table 1: Applications of the DNA barcoding technique

Application	References
Species monitoring and authentication in herbal products Determination of plant species used in herbal medicines with adverse drug reactions	(De Boer, *et al*., 2015), (Ya-Na, LV, *et al*., 2020)
Food authentication Characterization of the food microbiome and food safety Customization of healthy diets	(Galimberti, *et al*., 2019)
Taxonomic sorting tool and discriminate taxa in families with complex taxonomy	(Govender and Willows-Munro, 2019), (Kress, 2017),(Sass, *et al*., 2007), (Du, *et al*., 2011), (Theodoridis, *et al*., 2012)
Environmental monitoring and assessment (detecting invasive species, species surveillance) Wildlife protection (wildlife crime, exotic products) Quality assurance and control (fraud, adulteration)	International Barcode of Life, (Kress, 2017)
Identification of cryptic plants	(Lahaye, *et al*., 2008), (Miwa, *et al*., 2009), (Jie, L. *et al*., 2011)
Studying the genetic diversity of plant germplasm, as well as discriminating new individuals.	(Ali, M. A., 2016), (Kress, 2017)
Identification of the plant component in the diet of herbivores	(Valentini, *et al*., 2009), (Navvaro, *et al*., 2010), (Stech, *et al*., 2011), (Jurado-Rivera, *et al*., 2009)
Identification and characterization of medicinal plants	(Alkaraki, *et al*., 2021), (Zhou, *et al*., 2018), (Agrawal, *et al*., 2016), (Kreuzer, *et al*., 2019), (Little, 2014)

The information obtained from the experiments, provides especially to taxonomists, extremely valuable information about diversity, finally giving the possibility to realize a global inventory of the species from the vegetal kingdom (Ali, M. A., 2016). According to, the International Barcode of Life, plant barcoding studies use one or few plastid regions (e.g., *matK, rbcL* or *trnH-psbA* spacer and other) and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Although the use of this method of identifying and discriminating plant germplasm has many advantages, its introduction was not immediately accepted by botanists, until a few years later (Nicolè, *et al*., 2011). In the last decade, the study of plants, through the technique of DNA barcodes has accelerated and the interest of researchers in this direction has increased. This can be seen in the number of published works. A simple search (on September 5,

2022) on the *PubMed* platform, using the keywords "DNA barcoding'' and "plant", for the period 2005-2022, returned 2,132 publications from a variety of journal. Thus, their number has steadily increased since the introduction of this technique (Figure 1).

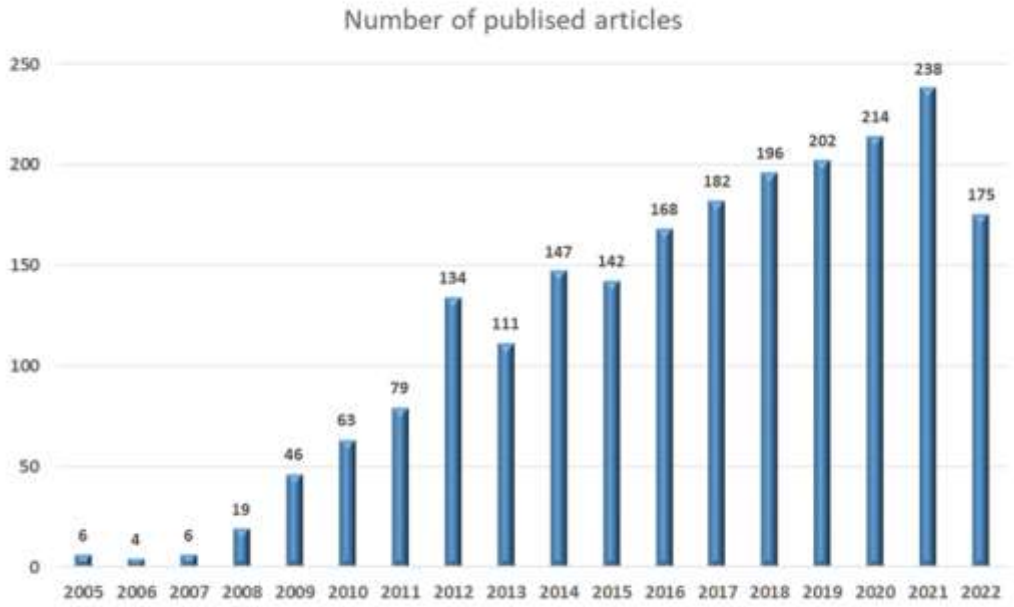

Figure 1: Publications per year registered in *PubMed*, containing "DNA barcoding" and "plant", as keywords (obtained September 5, 2022)

This technique involves going though steps (Figure 2), such as isolating DNA from samples of interest, amplifying the target DNA barcode region, sequencing the PCR products, by using the Sanger technique and the finally, comparing them to the references in the database, to find the matching species (Ekrem, *et al*., 2019). Therefore, the barcodes are kept in an online library, which allows comparisons between the analyzed and unidentified organisms until that moment and the previously identified taxon sequences (Grant, *et al*., 2021). This contributes significantly to understanding the boundaries of species (DeSalle and Goldstein, 2019).

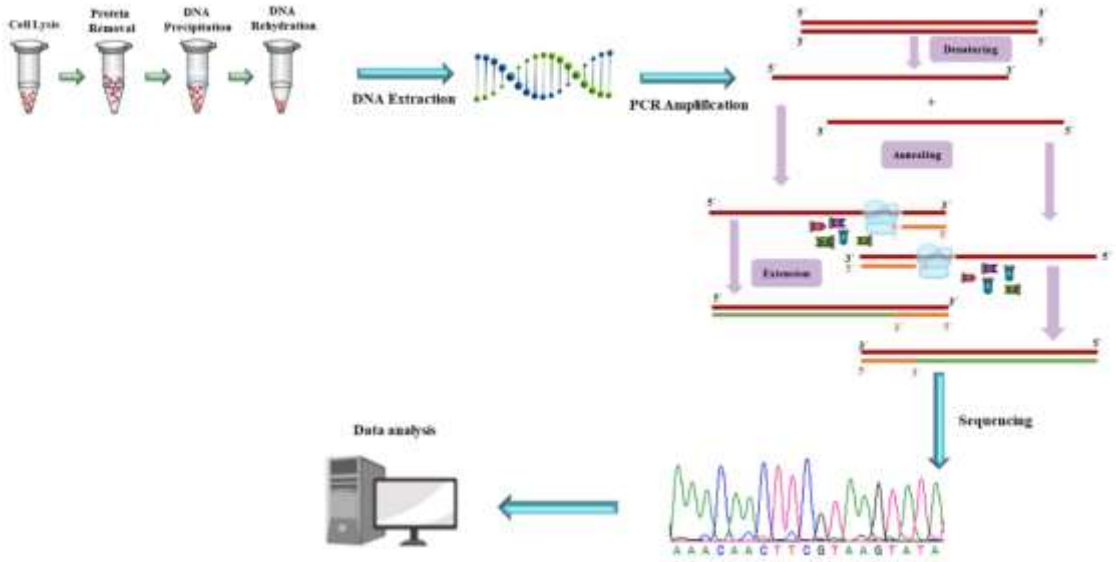

Figure 2: Steps involved in the DNA barcoding technique-from sample to barcode data analysis

In 2005, a web platform for DNA barcodes was created, known as, Life Barcode Data Systems (BOLD or BOLD Systems) and its first major project was to provide DNA barcodes for 500,000 species by 2015. The BOLD online platform, can provide more information, such as sequence quality, but also a basic analysis that has the effect of generating graphs that provide information on inter and intraspecific genetic distances, tree reconstructions, haplotype distribution or basic identification of unknown samples, by matching with the closely related individual (Ratnasingham and Hebert, 2007). The reference library is constantly expanding (Table 2), so at present (October 2021), it has a number of 10,037,483 specimens with barcodes (for 327,787 different species, of which about 70,000 are plant species). Of course, most organisms do not have a DNA barcode, but the regions with the most species studied from this perspective are North America and Europe (Grant, *et al*., 2021).

Table 2: Number of specimens with sequences, specimens with barcodes, species and species with barcodes from different phylum, from BOLD System platform (Source: Taxonomy BOLD Systems)

Phylum	Specimen records	Specimens with sequences	Specimens with barcodes	Species	Species with barcodes
Bryophyta	22,311	20,992	6,526	3,777	1,815
Chlorophyta	17,991	13,231	5,563	3,656	1,796
Lycopodiophyta	1,335	1,142	698	337	279
Magnoliophyta	441,905	399,440	241,595	122,719	67,781
Pinophyta	7,284	6,944	5,523	844	785
Pteridophyta	11,633	9,579	9,221	3,932	3,780
Rhodophyta	56,168	40,537	28,065	5,383	3,346

There are three conditions that a DNA barcode should meet in order to be considered ideal: 1) for the development of high-specific universal primers, it is necessary that the regions flanking the sequence of interest be sufficient; 2) the region of interest must be relatively short in length to allow the sequencing process to run smoothly so that the number of manual edits of the nucleotide sequence is as small as possible; 3) to allow a hight discrimination between the analyzed samples (Hollingsworth, P.M. and all., 2009). DNA barcodes have become an extraordinary tool, used by researchers to identify and assess life on our planet. At the same time, the advances in molecular biology are staggering and the emergence of new technology, which could replace the method of assessing organism by DNA barcodes, is inevitable. Over time, this issue has been intensely debated and analyzed. Thus, a group of researches performed a SWOT analysis of the method of evaluating individuals based on DNA barcodes (Grant, *et al*., 2021). They are marked the most important strengths, weaknesses, threats and apportunities. The authors state that this technique allows rapid identification of analyzed species; allows solving uncertain issues related to taxonomy, at the same time compared to traditional species identification technique, this method has led to an increase in cost and time efficiency. On the other hand, the principal weaknesses of this molecular biology techique are the multiple failed amplifications, the standardization of the method is difficult between organism groups and in some cases, barcode's amplifications are done with multiple markers. The main threat mentionated by Grant, *et al*. is the possibility of the emergence of a new technology, but an important point view in this way is the economic resources, this technique is expensive and it's needed a stable infrastructure. The SWOT analysis of DNA barcoding techiques disclose several opportunities: biomonitoring activity can be automated, allows increasing international access to DNA resources, on the other hand

this technique can estimate species diversity from complex and enviromental samples.

Single-Locus DNA Barcodes

matK

matk or *Ribosomal RNA Maturase Kinase* is a plastidial gene from plants. The gene *matK* has an unknown function, but it is considered that it would have the role for splicing the *trnK* precursor, and that is because of its position (Figure 4), inside the *trnK* gene(Zoschke, *et al*., 2010). The chloroplast *matK* barcode is considered to been an essential marker for the discrimination of species or taxa (De Mattia, *et al*., 2011). Unlike othe DNA barcodes, matK has an extraordinary ability to phylogenetically differentiate samples from each other (Müller, *et al*., 2006). The *matK* gene is characterized as having a high evolutionary rate, obvious interspecific divergence as well as a low transition/transversion rate and suitable length (Selvaraj, *et al*., 2008), (Min and Hickey, 2007). At the same time, the *matK* gene is considered the closest plant analogue to the cytochrome c oxidase subunit 1 (CO1) gene animal barcode. Located between bp 205-1046 (including primer sites), the *matK* barcode regions consist of approximately 842 bp region at the center of the gene, the plastid genome, completely sequenced from *Arabidopsis thaliana* (Hollingsworth, *et al*., 2011). Phylogenetic analysis has shown that the matK gene, compared to other candidate genes for DNA barcodes, has shown a strong parsimony informative character (Okoth, *et al*., 2016). The amplification and obtaining of *matK* amplicons, with PCR reaction, is difficult to achieve, even with universal primer sets (Xiwen Li, *et al*., 2015), (Rajphriyadharshini and Weerasena, 2020). For angyosperm, the *CBOL Plant Working Group (The Consortium for the Barcode of Life)* (Hollingsworth, P.M. and all., 2009) showed a success rate of nearly 90% in amplifying DNA, when using a single primer pair. However, we cannot say the same thing about gymnosperms, whose amplification rate is significantly lower (83%). For cryptogams the situation is much worse, the rate of amplification being 10%. For different groups of taxa, different primer pairs are required, but this is not a rule, as sometimes universal primer pairs also work even better (Hollingsworth, *et al*., 2011), (Chase, *et al*., 2007). For example, for amplification of the *matK* gene, two pairs of universal primers are frequently used (Table 3).

Table 3: Universal primer sequences for the candidate genes for barcoding in plants

Barcode markers	Universal primers set 5′-3′		Reference
	Forward primer	Reverse primer	
matK	***3F_KIM*** CGTACAGTACTTTTGTGTTTACGA G	***1R_KIM*** ACCCAGTCCATCTGGAAATCTTGGTTC	(Carneiro, *et al*., 2019),(Hollingsworh, P.M. and all., 2009), (Li, H.Y. *et al*., 2012),(Yan, L.J. *et al*., 2015), (Yan, H.F. *et al*., 2015), (Wu, F. *et al*., 2019), (Gostel, *et al*., 2020), (Amandita, *et al*., 2019)
	390_f CGATCTATTCATTCAATATTTC	***990_r*** GGACAATGATCCAATCAAGGC	(Carneiro, *et al*., 2019),(Gamage, *et al*., 2006),

			(Hollingsworth, P.M. and all., 2009),(Bhagya, C. *et al*., 2021) (Mishra, *et al*., 2017), (Gostel, *et al*., 2020), (Amandita, *et al*., 2019)
rbcL	***rbcLa_f*** ATGTCACCACAAACAGAGACTAAAGC	***rbcL_r*** GAAACGGTCTCTCCAACGCAT	(Carneiro, *et al*., 2019),(Yan, H.F. *et al*., 2015), (Amandita, *et al*., 2019)
trnH-psbA	***psbA3'f*** GTTATGCATGAACGTAATGCTC	***trnHf*** CGCATGGTGGATTCACAATCC	(Nicolè, *et al*., 2011),(Tate an Simpson, 2003), (Li, H,Q. *et al*., 2012),(Yan, L.J. *et al*., 2015), (Gostel, *et al*., 2020).
	fwdPA GTTATGCATGAACGTAATGCTC	***revTH*** CGCGCATGGTGGATTCACAATCC	(Mishra, *et al*., 2017)
ITS	***ITS3*** GCATCGATGAAGAACGTAGC	***ITS4*** TCCTCCGCTTATTGATATGC	(Li, H.Q. *et al*., 2012), (Nicolè, *et al*., 2011)

According to Hollingsworth (Hollingsworth, *et al*., 2011), one of them (3F/1R; K. J. Kim unpublished) has PCR amplification and sequencing rate up to 70% among angyosperms. To increase the amplification rate, but also the sequencing rate, by up to 10% a pair of secondary primers (390F/ 1326R) can be also used (Cuénoud, *et al*., 2002). Using the same pair of primers (390F/1326R), Bhagya, *et al*., (Bhagya, C. *et al*., 2021) showed that the PCR amplification success rate for *Cinnamomum* species was 100% and at the same time, for the *matK* region, good quality forward and reverse sequencing data was obtained, for all the samples analysed. For some of the bryophyte and fern groups, the *matK* gene is not recoverable with the available primers pairs, most being designed for angyosperms. Amplification of the *matk* gene in ferns is a real challenge, because genome rearrangements suggest that the gene is not flanked by the *trnK* exons conserved in clades, which makes it difficult to achive the complete *matk* sequence, from which barcode primers sequences can be designed (De Groot, *et al*., 2011), (Kuo, *et al*., 2011). In a study, conducted by Lahaye, *et al*. (Lahaye, *et al*., 2008), they amplify the *matK* gene of 1667 angiosperm plant samples, using universal primers sets (390F and 1326R) and achived a success rate of 100%. The same result was obtained in 2002 (Cuénoud, *et al*., 2002), when the *matK* gene was amplified with the same universal primers, 390F and 1326R, to study the inter- and infrafamilial phylogenetic relationships in the *Caryophyllales* order, studying 127 taxa. For the study of genetic diversity and genetic traceability in bean germplasm, the potential of the DNA barcode was tested, using the *matk* marker. The results were not as expected: multiple failed amplifications and low sequence quality (Nicolè, *et al*., 2011). Others have reported

similar difficulties. Using the *matK* barcode, to identify 92 species from 32 diverse genera of land plants (251 samples), the resultats were not as expected, the success rate being of only 65% (Fazekas, *et al*., 2008). In the nutmeg family (Myristicaceae), *matK* can discriminate

Figure 4: The *matK* region, from the chloroplastic genome, as found in most angyosperms

the species from the *Poaceae* family (67,92%) (Yao, P.C. *et al*., 2017). *MatK* is suitable for most families when it comes to the family resolution of the species. For four families *(Onagraceae, Polemoniaceae, Boraginaceae, Caprifoliaceae), matK* delivered perfect resolution (100%). However, we cannot say the same about the *Asteraceae* and *Poaceae* families, where the percentage is lower, within the species, 67,17% and 75,25% respectively (Braukmann, *et al*., 2017). Mishra, *et al*. (Mishra, *et al*., 2017) evaluated 222 individuals, representing 41 *Terminalia species* DNA barcode reference library and for barcode *matK* the highest inter-specific sequence divergence was reported (0.689). The effectiveness of the DNA barcoding technique was tested to identify *Dipterocarps* in Sumatra, Indonesia, the chloroplastic gene *matK* being used for this purpose. The results showed that the sequencing rate of the *Dipterocarpaceae* family for *matK* was 81%. It proved effective to identify samples belonging to the *Anthoshorea, Hopea, Richetia* and *Parashorea* groups, but inefficient to resolve the relationships within the *Rubroshorea* clade (Carneiro, *et al*., 2019). *Matk* barcode showed a high level of discrimination among *Vicia* species (Van de Wouw, *et al*., 2003),(Wu, F.F. *et al*., 2020),(Raveendar, *et al*., 2017). For Indonesian medicinal plant identification is recommanded *matK* region and *ITS2* and *rbcL* can be used as complementary regions or alternative (Cahyaningsih, *et al*., 2022). For the plant family *Fabaceae*, *matK* gene is considered a good and promising cadidate for DNA barcoding studies (Abdelsalam, *et al*., 2022). From the facts presented above, we can say

less than 49% of species (Newmaster, *et al*., 2006). A study of species from two families growing in the same region, showed that *matK* can better discriminated species from the Chenopodiaceae family (93,6%), but less in the case of

that the *matK* marker is not a suitable universal barcode for all plants land.

rbcL

rbcL is a large subunit of ribulose-1,5-bisphosphate cataboxylase-oxygenase (RuBisCo) (Purty and Chatterjee, 2016). RuBisCo is an enzyme involved in the process of photosynthesis in plants, participating in the first major step of carbon fixation, the process being represented by the conversion of carbon dioxide into glucose (Feller, *et al*., 2008) and is known to be the most abundant protein on earth (Dhingra, *et al*., 2004), (Okoth, *et al*., 2016). Currently, in phylogeny studies within the angiosperm's family and subclass, the *rbcL* gene is widely used (Kang, *et al*., 2017). The most characterized plastid coding region in GenBank is *rbcL*, with wide representation from all major groups, and therefore provides a good baseline for comparison with other plastid genes (Newmaster, *et al*., 2006). Unlike *matK,* the *rbcL* gene is easy to amplify and sequence for most land plants, which is great (Li, X. *et al*., 2015). Moura, *et al*., (Carneiro, *et al*., 2019) reported that the success rate of sequencing, for the rbcL region, with universal primers, in the *Dipterocarpaceae* family was 83,7%. However, a major disadvantage, would be that it has the lowest divergence, compared to other plastid genes in flowering plants, due to the slow evolution (Kress, *et al*., 2005). This obviously limits, the use of this marker, due to its modest power of discrimination, especially in studies at the species level (Hollingsworth, P.M. and all., 2009), (Lahaye, *et al*., 2008), (Gao, *et al*., 2010). In the complete *A. thaliana* plastid genome sequencing, the *rbcL* barcode consists of a 599 bp region at the

5' end of the gene, located at the 1-599 bp (including primer sites) (Hollingsworth, *et al*., 2011). Kress and al.,(Kress, *et al*., 2005) proposed the use of the *rbcL* plastid marker to applying barcoding to flowering plants. But this was not a good choice, as *rbcL* showed the lowest divergence of any of the plastid regions used in the study (0.83%). Other reported the same. The *rbcL* was used for DNA barcoding evaluation of *Vicia* and the results were not as expected. it had the lowest values for intraspecific distance (0,0005) and interspecific distance (0,0146) (Wu, F.F. *et al*., 2020). For the discrimination of *Cinnamomum* species, the researchers used a single-locus DNA barcode (*rbcL*). The PCR reaction and the sequencing of the samples had a success rate of 100%. Less variability was reported in the *rbcL* region compared to other plastid markers. The variability in the *rbcL* was limited to only one site, at the 56th position, found in only one sample (*C. ovalifolium* accession 001) (Bhagya, C. *et al*., 2021). *rbcL* was found with the lowest number of variable sites (22/636) and highly conserved (95,9% identical sites) for 222 individuals of 41 *Terminalia* species. The poor performance of *rbcL* as a locus of choice trough their study in closely related groups of *Lysimachia L.* (Myrsinaceae), was also reported by Zhang, *et al*. (Zhang, *et al*., 2012). The same statemet, was raportated by Aneva, *et al*., for *Thymus* species, *rbcL* showed the lowest discrimination power in this case (Aneva, I. *et al*., 2022).

trnH-psbA

Over time, a variety of loci have been suggested as DNA barcodes for plants. *trnH-psbA* is a non-coding intergenic region with high rates of insertion/deletion (Kress and Erickson, 2007) and at the same time it is the most widely used plastid barcode (Li, X. *et al*., 2015). The two genes, that delimit the non-coding region *trnH-psbA,* are involved in different physiological processes in plants. The *psbA* gene *(photosystem II protein D1)* is localized in the *LSC* (large single copy) region of the chloroplastic genome and is involved in the process of photosynthesis (Chacon, 2017), while *trnH* is the gene of histidine transfer RNA (trnH) (Degtjareva, *et al*., 2012). The length of the *trnH-psbA* sequence varies depending on the group of plants. So the values are different in eudicotyledons, gymnosperms, monocotyledons, mosses and ferns ranged from 152 bp to 851 bp, 283 bp to 1006 bp, 151 bp to 905 bp, 103 bp to 265 bp and 167 bp to 547 bp, respectively (Pang, *et al*., 2012). According to Degtjareva, *et al*., (Degtjareva, *et al*., 2012) the *trnH-psbA* is characterized by a high content of AT bp (69,77%), which is not surprising, because the non-coding regions of the plastid genome of plants have this characteristic. Because it evolves rapidly, *trnH-psbA* is considered to be the most promising candidate for use as a third barcode (*matK* and *rbcL* occupying the first two places) in plants (Kress and Erickson, 2007), (Hollingsworth, P.M. and all., 2009). The longer *trnH-psbA* regions can be difficult to retrieve, because of the premature termination of sequencing reads caused by mononucleotide repeats (polyG repeteas or polyT repeats in *Poaceae* (Bieniek, *et al*., 2015)), so they are necessary taxon-specific internal sequencing primers designed to obtain high quality bidirectional sequences (Ebihara, *et al*., 2010),(Zhang, *et al*., 2012), (Mishra, *et al*., 2017). *trnH-psbA* has also been used to identify species in different taxonomic groups. The *trnH–psbA* region correctly identified 35.5%, 51.1%, 45.7%, 72.2%, and 75.0% of 633 gymnosperm,13.727 eudicotyledon, 3054 monocotyledon, 277 moss, and 292 fern sequences at the species level, respectively, using BLAST softaware (Pang, *et al*., 2012). The length of the *trnH-psbA* intergenic spacer can significantly vary even in related species, as it is characterized by a large number of inversions and indels, which complicates alignment in some genera, such as the genus Gentiana. The *trnH-psbA* is therefore not recommended as a DNA barcode for *Gentiana* (Liu, J. *et al*., 2016). Nontheless, the *trnH-psbA* is considered to be a promising barcode DNA, with regard to establishing phylogeny relationships or discriminating samples, from certain families, especially *Fabaceae* families (Gao, *et al*., 2013), (Loera-Sánchez, *et al*., 2020). The *trnH-psbA* barcode can discriminated samples which

belongs *Ocimum*, but not *Thimus* specie. For achieve a high resolution of results, it requires that the *trnH-psbA* intergenic spacer to be used in two-locus or three-locus barcode systems (Kress, *et al*., 2005), (Chase, *et al*., 2007), (Wang, J. *et al*., 2021).

ITS

For phylogenetic studies or sample discrimination, a variety of loci have been proposed as DNA barcodes for plant, including regions of the plastid genome (non-coding spaces and coding genes) and the nuclear genome (intergenic spaces and coding genes) (Figure 5). One of the most popular seqeunce and powerful phylogenetic markers, at the species level, which proves a high level of interspecific divergence, is the ITS spacer. The internal trnscribed spacer (ITS) is located at the level of the 18S–5.8S–26S nuclear ribosomal cistron (Álvarez and Wendel, 2003). The nuclear ribosomal RNA gene appear in thousand of copies and are organized in tandem arrays, as a rule clustered in two separate genomic loci (Elder, J. F. and Turner, 1995). The 5S rDNA locus encodes 5S RNA genes, and the 45S rDNA locus contains genes for 18S, 5.8S, and 26S rRNA. These genes are separated by the internal transcribed spacers ITS1 and ITS2. The untranscribed intergenic spacers (IGS), extending from 3 'end of 26S rRNA to the 5' end of the 18S rDNA coding regions, separate the 45S transcription units (Rogers and Bendich, 1987). In the mature rRNA, the ITS are not incorporated, their function being to encode signals for proper processing of the rRNA transcript (Hillis and all. 1991). Also, the sequence ITS1-5,8S-ITS2 is used in studies to identify phylogenetic relationships (Hřibová, *et al*., 2011), (Álvarez and Wendel, 2003). CBOL suggests that the intergenic transcribed spacer should only be used as an additional place for the discrimination of samples (Hollingsworth, P.M. and all., 2009).

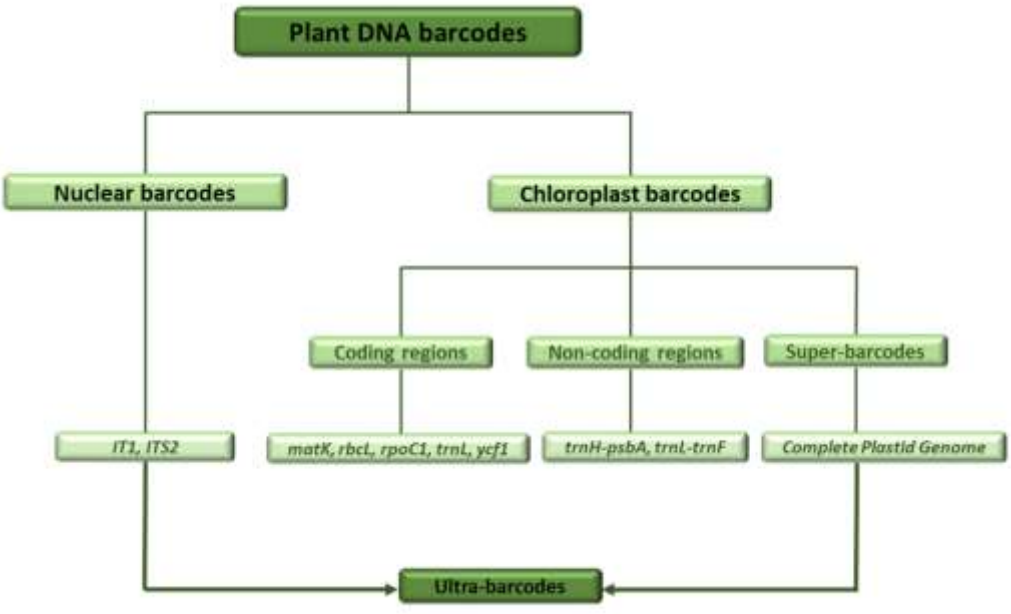

Figure 5: Schematic illustration of DNA barcode markers used for phylogenetic studies

It is known that in plants the 5,8S rDNA and the secondary structure of functional, ITS2 spacer are highly conserved, in contrast to the ITS1 spacer (Hershkovitz and Zimmer, 1996), (Jobes and Thien, 1997). Over time, a number of limitations have been exposed regarding the use of this region in studies based on the DNA barcoding technique. A number of limitations were presented by Hollingsworth, *et al*.: difficulties of amplification and sequencing, incomplete concerted evolution and fungal contamination. Fungi are eukaryotic organisms that contain the ITS sequence in their genome, which can be

preferentially amplified and easily confused with the plant sequence (Hollingsworth, 2011). The ITS region that can be incorporated into the core barcodes, when direct sequencing of the region is possible, because this has a higher discriminatory power than plastid barcodes (Li, D.Z. *et al*., 2011). According to Silva, *et al*., the nuclear ITS data set of *Phaseolus vulgaris* proved, poorly informative, in contrast to the cpDNA regions (Nicolè, *et al*., 2011). For phylogenetic reconstructions, especially between closely related taxa, high intragenomic variation, may limit the usefulness of ITS rDNA (Vollmer and Palumbi, 2004). The discriminatory power of the ITS2 is lower compared with the entire ITS1-5,8S-ITS2 region (Liu, J. *et al*., 2011). One of the most commonly reported issues is the difficulty of amplifying the ITS region, even if universal primer sets are used. In a study conducted in 2009, a success rate of PCR amplification and sequencing of 41% was reported, after analyzing a number of 285 tropical trees (Gonzalez, *et al*., 2009). To identify and distinguish the Shi-Liang tea species, from closely related species, ITS2 sequences were examined and the results showed that these regions can be used as DNA barcodes successfully (Ma, S. *et al*., 2017). For improving the PCR success rate of *ITS2* region, it is possible to use some additives, like DMSO, 7-deaza-dGTP, betaine or formamide. Using 5% DMSO, it was raport an imporving of PCR succes rate with 91,6%, follwed by the second hight percent, 75%, with 1M betaine. The lowest PCR success rate (16,6%) was observed with 3% formamide (Varadharajan and Parani, 2020). The ITS region is the best DNA barcode, that provides 100% resolution for species of *Dendrobium* (Singh, *et al*., 2012). For the *Euphorbia* genus, ITS1 showed the best results as regard of power of discrimination, followed by two other DNA barcodes, ITS2 and the entire ITS region (El-Banhawy, *et al*., 2020). ITS2 is considered superior to plastid markers, but 7% and 10% lower than the ITS region (Li, D.Z. *et al*., 2011), (Sun, *et al*., 2015). On the other hand, ITS2 is considered a very good alternative to ITS, due to its length, it allows a much better sequencing and the use in different studies has been a success (White, *et al*., 1990), (Coleman, 2003), (Gao, *et al*., 2010), (Chen, *et al*., 2010), (Yao, H. *et al*., 2010). According to Oshingboye, ITS is an attractive DNA barcode, but which can be used in combination with other molecular markers, to provide a better resolution for the analyzed samples (Oshingboye and Ogundipe, 2018).

Other Widely Used Plastid Barcodes

At the moment, the DNA barcoding technology, for studying the molecular phylogeny of plant is based on the use of loci from the chloroplastic genome, because it has a low evolutionary rate, in contrast with the nuclear loci (Dong, *et al*., 2012). Another reason, why chloroplastic markers are chosen is the ease of PCR amplification and sequencing, compared to nuclear markers (Hurst and Jiggins, 2005). Beyond the candidate barcodes described above, for phylogenetic reconstructions at various taxonomic levels, many fragments of coding regions, intergenic spacers and introns have been used, like *atpB, atpB-rbcL, ndhF, rpl16, trnL-F, trnS-G, psbK-psbI, rpoC1, rpoB, trnL, ycf5, ycf1, atpF-atpH* (Li, J. 2008), (Hollingsworth, *et al*., 2011), (Vijayan & Tsou, 2010). However, it turned out these chloroplast regions are not suitable for plant DNA barcoding at lower taxonomic levels because of insufficient variation (Li, X. *et al*., 2015). For example, the *atpF-atpH* and *psbK-psbI* intergenic spacers is not been widely used in plant systematic and phylogenetics studies, because the CBOL Plant Working Group state that, *psbK-psbI*, despite high levels of discriminatory power, has a lower sequence quality and universality, while *atpF-atpH* has showed relatively modest discriminatory power, intermediate sequence quality and universality (Hollingsworth, P.M. and all., 2009). *ycf1* can be the most promising plastid DNA barcode of land plants, because it is the most variable plastid genome region (Dong, *et al*., 2015). The combination of *rbcL+matK+trnH-psbA* has a lower resolution than *ycf1* (the second largest gene in the chloroplast genome with a length of apx. 6000 bp), in the analysis of a sampling covering 420 species from 67 families that included mosses, gymnosperm and angiosperms (Dong, *et al*., 2015). For the species of

the *Paris* genus, this gene also showed good results in the analysis (Song, *et al*., 2017). However, at the same time, for the *Kalidium* genus of the *Chenopodiaeae* family, the *ycf1* gene, did not show a high discriminating ability (Liang and Wu, 2017). According to Kess and al., the *atpB-rbcL*, *ycf6-psbM* and *psbM-trnD* intergenetic spacers have a high interspecific variability (Kress, *et al*., 2005). The phylogenetic relationships and low resolution among the main lineages of the *Dipterocarpaceae* family was observed for the *trnL-F* intergenic spacer (Carneiro, *et al*., 2019). For phylogenetic analyses of the *Orchidaceae* family amplification and sequencing of the *ndhF* and *ycf1* regions is recommended, this genes can be used in future studies as barcodes for orchid (Li, H. *et al*., 2021). It is difficult to find a universal bar code for the plant kingdom similar to that of the animal kingdom.

Candidate Multi-Locus DNA Barcodes

There are many attempts to identify a universal plant barcode, similar to that of the animal kingdom, COI, but this goal has proven to be much more difficult to achieve, using single loci (Kress, *et al*., 2005), (Kress and Erickson, 2007), (Fazekas, *et al*., 2008), (Lahaye, *et al*., 2008). Many researchers affirm that multi-locus DNA barcodes are needed to identify some samples or to study their phylogeny (Table 4) and at the same time the results are much better compared to studies using single locus DNA barcodes (Carneiro, *et al*., 2019), (Gogoi and Bhau, 2018), (Wu, F.F, *et al*., 2020), (Mishra, *et al*., 2017), (Hollingsworth, P.M. and all., 2009), (Lahaye, *et al*., 2008). Many combinations of plastid loci have been proposed, including *ITS+rbcL* or *ITS+matK* (Gogoi and Bhau, 2018), *matK+rbcL+trnH-psbA* (Bhagya, C. *et al*., 2021), *rbcL+matK+ITS* or *rbcL+matK* (Mishra, *et al*., 2017), *ITS+trnH-psbA+trnL-trnF* or *ITS+matK+trnH-psbA+trnL-trnF* (Wu, F.F. *et al*., 2020). Due to the straightforward recovery of the *rbcL* region and the discriminatory power of the *matK* sequence, the CBOL Plant Working Group recommended *matK+rbcL* as the universal barcode combination (Hollingsworth, P.M. and all., 2009). Although CBOL Plant Working Group claims that the *matK+rbcL* combination has the best power of discrimination, many studies disagree with this theory (Li, H.Q. *et al*., 2012), (Clement and Donoghue, 2012), (Yan, H.F. *et al*., 2011). For the *Primula* genus, the *matK+ITS* combination was recommended, because it is the best barcode for discrimination (Yan, H.F. *et al*., 2015), while *ITS+trnH-psb+matK* has the best power of discriminating *Rhododendron* species (Yan, L.J. *et al*., 2015). The multilocus DNA barcodes as *matK* + *ycf1* and *ndhF* + *ycf1* can be used for identification at the genera and species levels for *Orchidaceae* famiy plants (Li, H. *et al*., 2021). However, so far, no combination of loci has been identified, which could provide a good power of discrimination, for all samples in the plant kingdom.

Chloroplast genome as a Super-Barcode: A New Way for Plant Discrimination

Research has shown over time, that the identification of plant species with great accuracy has an extremely important role in conserving biodiversity (Struck, *et al*., 2018). For a long time, the characterization and differentiation between plant species was done only from a morphological point of view, but the appearance of molecular markers revolutionized the way of identifying plants, having a substantial contribution in the field of systematics. However, a well-functioning molecular marker is not available for all plant species in the plant kingdom, especially for the closely related individuals (Wu, L. *et al*., 2021). In the field of phylogeny, the chloroplastic genome is a powerful and versatile tool (Zhai, *et al*., 2019). Due to the low capacity for discrimination given by molecular markers, several researchers have proposed the use of the entire genome in studies of discrimination against individuals (Li, X. *et al*., 2015). In 1986, he first cp-genome-*Nicotiana tabacum* was sequenced (Shinozaki, *et al*., 1986). At the moment, more than 5000 complete chloroplast genomes are available on the NCBI organelle genome database which means only 1.27% of total plant species worldwide. In angiosperms, the plastid genomes is circular and has a quadripatite structure composed of a large single copy

region (LSC), small single copy region (SSC) and a pair of inverted repeats (IRs) (Figure 6) and ranges in size from 72 to 217 kb (Yan, H.F. *et al*, 2011). For identification of plant species, especially for taxonomi-cally difficult taxa such as the genera Citrus (Struck, T.H. *et al*., 2017), Oncidium (Wu, L. *et al*., 2021) , Gossypium (Zhai, W. *et al*., 2021), Pterocarpus (Shinozaki, K. *et al*., 1986) , Fritillaria (Guo, X. *et al*., 2007), Cinnamomum (Bhagya, C. *et al*., 2021) or Quercus (Hollingsworth, *et al*., 2016), the complete plastid genome as a super-barcode was suggested.

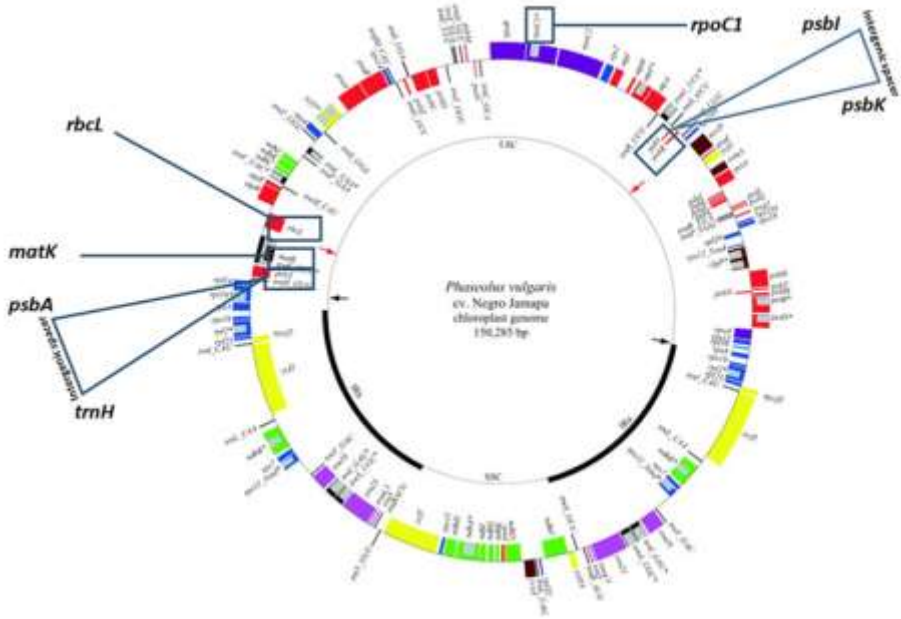

Figure 6: Schematic map of the *Phaseolus vulgaris* plastome and locations of potential candidate plant DNA barcodes in chloroplast genome (Source: (Guo, *et al*., 2007))

Also, cp-genome has a higher interspecific and lower intraspecific divergence and small size, in contrast with the nuclear genome (Li, X. *et al*., 2015). Although it is a very powerful tool of discrimination, the use of chloroplastic DNA as a super barcode also has a major disadvantage: the cost of achievement. Despite being expensive for *de novo* sequencing, The Roche/454 sequencing platform currently offers the longest sequence readings and is a very good choice. However, a cheaper alternative method is the *Illumina* platform (Li, X. *et al*., 2015). The study of the entire chloroplastic genome has become the best technique in the study of the relationships between closely related plant species. In the last period, the number of published articles that use the complete sequencing of the plastid genome in various phylogeny studies has increased a lot (Hollingsworth, *et al*., 2016). But more recently, the ultra-barcoding technique is used, which involves complete sequencing of the plastid genome and nuclear ribosomal DNA sequences (Ji, *et al*., 2020), (Kane, *et al*., 2012).

Genera	Candidate Single Locus DNA Barcodes	Candidate Multi Locus DNA Barcodes	Remarks	References
Gentiana	*rbcL* *matK* *trnH-psbA* *ITS* *ITS2*	*matK+ ITS* *rbcL + matK+ ITS*	*trnH-psbA* gave the highest inter- and intra-specific divergence of all the single regions. the combination of *rbcL* and *matK* is not recommended in	(Liu, J. *et al*., 2016)

			this case because had the lowest genetic divergence and could only poorly discriminate species in this genus. *ITS* showed the best performance of all five barcodes for discriminating species of *Gentiana.*	
Terminalia	*ITS* *rbcL* *matK*	*ITS+rbcL* *matK+ITS* *rbcL+matK* *rbcL+matK+ITS*	*trnH-psbA* was a problematic barcode, with multiple failed amplifications and low sequence quality, so it was removed from analyse. *matK* and *ITS* showed the highest variability. the greatest intraspecific variation within the species, was proven by the combination of *rbcL+matK+ITS.* The combination of *ITS* and *matK* was concluded to be the best match with a 94.44% identification frequency.	(Mishra, *et al*., 2017)
Vicia	*ITS1* *ITS2* *matK* *rbcL* *trnH-psbA* *trnL-trnF*	*matK+rbcL* *ITS+matK+trnL-trnF* *ITS+trnH-psbA+trnL-trnF* *ITS+matK+trnH-psbA+trnL-trnF*	either *matK* or *trnH-psbA* are the most suitable DNA barcode regions for Vicia. *ITS* and *ITS1* showed a more limited ability to identify *Vicia* species, compared with *matK* and *trnH-psbA.* *rbcL* and *trnL-trnF* regions were less variable and less effective in the molecular identification of Vicia species. the combination of *matK* and trnH-psbA is the best barcode for *Vicia.*	(Wu, F.F. *et al*., 2020)
Phaseolus	*matK* *rbcL* *ITS1* *ITS2* *trnH-psbA* *trnL*	-	*matK* had multiple failed amplifications and low sequence quality and was removed from the study. *trnH-psbA* intergenic spacer and *trnL* intron contributed little or not at all toward resolving the genetic identities of landraces and varieties. for genetic identification of varieties within *P. vulgaris*, DNA barcoding is not recommended.	(Nicolè, *et al*., 2011)
Triticum	*matK*	-	In discriminating closely related	(Osman and

			Triticum species, the *matK* sequence has an important role, being used in detecting the evolutionary history of *Triticum* species. *matK* is suitable to resolve the phylogenetic problems in *Triticum* specie.	Ramadan, 2019)
Cinnamomum	*matK* *rbcL* *trnH-psbA*	*matK+rbcL+trnH-psbA*	using either single sequence barcode of either *matK* or *trnH-psbA* or combined barcodes of all three regions, it is not enough to resolve the phylogeny problem of *Cinnamomum*.	(Bhagya, C. *et al*., 2021)
Nepenthes	*ITS* *rbcL* *matK*	*ITS+rbcL* *ITS+matK* *rbcL+matK*	of the single-locus DNA barcodes, ITS had the highest success rate for correct species identification and the lowest rbcL, but all single- locus barcodes had low levels of species discrimination, in contrast with multilocus barcodes. the combination *of ITS+matK* showed the maximum success rate to discriminate species.	(Gogoi and Bhau, 2018)
Codonopsis	*ITS* *rbcL* *matK* *trnH-psbA*	*ITS+matK* *ITS + trnH-psbA* *matK + rbcL* *rbcL + trnH-psbA* *ITS + matK + rbcL*	the best performance for discriminating samples is presented by the combination of *ITS* and *matk.* *rbcL* presented the lowest percentages of informative and variable sites and the lowest discrimination ability. *ITS* presented the highest percentage of variable sites, had greater intra- and inter-specific divergence, the highest discrimination rates.	(Wang, D.Y. *et al*., 2017)
Clerodendrum	*ITS2* *matK,* *rbcL* *ycf1*	*ITS2 + matK* *TS2 + matK + ycf1* *rbcL + ycf1*	the highest success rate for correct identification of samples had the combination *ITS2+matK.* the combination *rbcL+ycf1* had the lowest discriminatory rate. among the single locus, *rbcL* had lowest level of discrimination and *matK*, followed by *ITS2* showed relatively high levels of discriminations success rate.	(Gogoi, *et al*., 2020)
Ficus	*rbcL* *ITS*	*matK+rbcL*	*ITS* showed relatively high levels of species discrimination.	(Li, H.Q. *et al*., 2012)

	matK		*rbcL* had the lowest discrimination levels.	
Primula	*rbcL* *matK* *trnH-psbA* *ITS* *ITS2*	*rbcL* + *matK* *rbcL* + *matK* + *trnH-psbA* *rbcL* + *matK* + *ITS* *rbcL* + *matK* + *ITS2* *rbcL* + *matK* + *trnH-psbA* + *ITS* *rbcL* + *matK* + *trnH-psbA* + *ITS2*	the highest DNA barcoding gap was delivered by the combination *rbcL+matK+trnH-psbA+ITS* *ITS* showed the highest discriminatory power. *rbcL* provided the lowest discrimination rate. *rbcL+matK* showed the poorest discriminatory power.	(Yan, H.F. *et al*., 2015)
Hypericum	*rbcL* *matK* *trnH-psbA*	*rbcL* + *matK* *rbcL* + *matK* + *trnH-psbA*	*matK* and *trnH-psbA* barcodes in combinations have successfully	(Pyrka, *et al*., 2021)
Paris	*psbA-trnH* *rpoB* *rpoC1* *rbcL* *matK* *ITS2*	*rbcL+matK*	the combination of *rbcL+matK* is not recommended for authentication in this. ITS2 can be used to identify medicinal plants of genus *Paris*.	(Zhu, *et al*., 2010)
Viburnum	*matK* *rbcL* *trnH-psbA* *nrITS*	*rbcL+ matK* *rbcL+ matK + trnH-psbA* *rbcL+ matK + nrITS*	in this case, the combination *rbcL+matK*, does not have a great discriminating power. *trnH-psbA* and *nrITS* have a much higher discriminatory power.	(Clement and Donoghu, 2012)

Conclusions

In the process of species identification, both when talking about the species in the animal and plant kingdoms, DNA barcoding plays a very important role, being considered the beginning of a revolution in this direction, having a lot of applications, such as identifying species, drawing the boundaries of species delimitation, identifying cryptic species, determining populations diversity, phylogenetic reconstruction or applications in the field of biodiversity conservation (Imtiaz, *et al*., 2017). The biggest disadvantage of the DNA barcoding plant is the lack of a universal marker. Although many studies have been conducted over time to identify a universal DNA barcode in plants, this has shown that there is currently no universal marker, such as the COI (cytochrome c oxidase I) marker for the animal kingdom. Moreover, we can talk about several DNA barcodes that can be used for genetic diversity studies or the identification of individuals, depending on the family, genus or species of the plant.

CBOL proposed to identify a single DNA sequence that can be used for all plants. But no single-locus DNA barcode can achieve the goals set in a study. Although *matK* has been proposed in this regard, several studies have shown that this region is difficult to amplify (even if universal markers are used) and to sequence (Li, X. *et al*., 2015), (Rajphriyadharshini and Weerasena, 2020), (Nicolè, *et al*., 2011). At the same time, it is possible that some species in the plant kingdom do not contain this sequence, as is the case with algae (Li, X. *et al*., 2015). Other single-locus DNA barcodes, such as rbcL, ITS or trnH-psbA, used individually, do not provide enough information in phylogeny studies or to identify individuals, in all plants.

The difficulty of obtaining a universal DNA barcode for plants, like COI from animals, is due to the deficiency of adequate variation within single loci (Fazekas, *et al*., 2008), (Lahaye, *et al*., 2008), (Kress and Erickson, 2007),(Chase, *et al*., 2007). The studies showed

that multi-locus markers are much more successful in species identification and in plant phylogeny studies, than single-locus markers. In this case, CBOL recommended the combination of *matK+rbcL* as a universal barcode for plants. But much research has contradicted the effectiveness of this combination (Yan, H.F. *et al*., 2015), (Zhu, *et al*., 2010), so there is no suitable multi-locus marker for universal plant identification. Therefore, different combinations of markers are useful for different plant genera (*matK+trnH-psbA* was recommended for *Vicia* (Wu, F.F. *et al*., 2020) and *Hypericum* genera (Pyrka, *et al*., 2021), or *ITS +matK* for *Terminalia* genus (Mishra, *et al*., 2017)).

If a few years ago, sequencing the entire chloroplastic genome was a rather difficult goal to achieve, especially due to the high costs of today, due to continuous advances in sequencing technology, these genomes are used as super barcode DNA, for the identification of individuals or in phylogeny studies. The information provided from the decoding of the plastid genome has a much better accuracy, thus allowing the objective conclusion of the hypotheses (Jiao, *et al*., 2019). Recently, the term ultra-barcode DNA has been introduced, which involves the sequencing and use of encoded information from the chloroplastic genome and ribosomal DNA (Ji, *et al*., 2020).

In conclusion, we can say that DNA barcodes are an extremely important genetic tool, in terms of solving problems related to diversity, identification of unknown samples or related to the conservation of plant biodiversity. Although, no single-locus or multi-locus marker, universally valid for all plants, has been identified at present, the steps in this direction continue and the identification of a single plant DNA sequence, similar to that in animals (COI) would revolutionize the use of DNA barcodes in plant species. However, the future seems to be more towards the use of super-barcodes or ultra-barcodes, which is an extraordinary thing, because the volume of information achieved and the discriminatory power, in this case, are much higher. Unfortunately, this can only be done by those who benefit from a considerable budget, the sequencing costs being, still, quite high.

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