Anales del Jardín Botánico de Madrid 82 (1)
January-June 2025, e155
ISSN-L: 0211-1322, eISSN: 1988-3196
https://doi.org/10.3989/ajbm.624

Is ITS2 more successful in angiosperm DNA barcoding than the entire ITS, ITS1, *matK* and *trnH-psbA* regions? A review

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Abstract. The internal transcribed spacer 2 (ITS2) of the ribosomal cistron, functioning as a mini barcode, has proven successful in numerous angiosperm DNA barcoding studies. However, its efficiency has not been thoroughly questioned in the existing literature. This review statistically evaluates 197 angiosperm DNA barcoding studies that utilized the ITS2 mini barcode. The assessment encompasses not only the ease of amplification, sequencing, and species identification success of the ITS2 region but also a comparison with ITS1, the entire ITS region, *matK*, and *trnH-psbA*. The results align with the previous conclusion that ITS2 can be effectively utilized for angiosperm identification. While the amplification, sequencing, and identification to the genus level of the ITS2 region were high, the success in species identification was not as prominent. No significant differences were observed among the types of material or the primer pairs used in terms of the success of the region. The utilization of only the ITS2 region performed well in comparison to DNA barcode combinations. However, it is noteworthy that none of the reviewed regions can be considered a universal barcode for angiosperms. The results suggest that the ITS1 and ITS2 are equally effective as mini-DNA barcodes, followed by the entire ITS region, while *mat*K and *trnH-psbA* should not be the primary choices.

Keywords. Angiosperms, DNA barcoding, internal transcribed spacers, ITS1, ITS2, ribosomal cistron.

Resumen. El espaciador interno transcrito 2 (ITS2) del cistrón ribosómico, que funciona como un minicódigo de barras, ha demostrado ser eficaz en numerosos estudios de código de barras de ADN de angiospermas. Sin embargo, su eficiencia no se ha cuestionado exhaustivamente en la literatura existente. Esta revisión evalúa estadísticamente 197 estudios de código de barras de ADN de angiospermas que utilizaron el minicódigo de barras ITS2. La evaluación abarca no solo la facilidad de amplificación, secuenciación y éxito en la identificación de especies de la región ITS2, sino también una comparación con ITS1, la región ITS completa, *matK* y *trnH-psbA*. Los resultados concuerdan con la conclusión previa de que ITS2 puede utilizarse eficazmente para la identificación de angiospermas. Si bien los niveles de amplificación, secuenciación e identificación a nivel de género usando ITS2 fueron altos, el éxito en la identificación a nivel de especie no fue tan prominente. No se observaron diferencias significativas entre los tipos de material o los pares de cebadores utilizados en relación al éxito de la región. El uso exclusivo de ITS2 tuvo un buen rendimiento en comparación con las combinaciones de códigos de barras de ADN. Sin embargo, ninguna de las regiones analizadas puede considerarse un código de barras universal para las angiospermas. Los resultados sugieren que las subregiones ITS1 e ITS2 son igualmente eficaces, seguidas de la región ITS completa, mientras que *matK* y *trnH-psbA* no deberían ser las opciones principales.

Palabras clave. Angiospermas, código de barras de ADN, espaciadores internos transcritos, ITS1, ITS2, cistrón ribosómico.

How to cite this article: Aygören Uluer D. 2025. Is ITS2 more successful in angiosperm DNA barcoding than the entire ITS, ITS1, *matK* and *trnH-psbA* regions? A review. *Anales del Jardín Botánico de Madrid* 82: e155. https://doi.org/10.3989/ajbm.624

Title in Spanish: ¿Es ITS2 más eficaz en la codificación del ADN de angiospermas que las regiones ITS, ITS1, *matK* y *trnH-psbA*? Una revisión.

Associate editor: Pablo Muñoz-Rodríguez. Received: 24 May 2024; accepted: 16 January 2025; published online: 08 September 2025.

INTRODUCTION

DNA barcoding is a cost-effective and rapid taxonomic method used to identify organisms through the use of short, standardized gene region(s) (Hebert & al. 2003), particularly where morphological identification is challenging (e.g., Shi & al. 2011; Liu & al. 2017) or not possible due to the condition of material (i.e., growth phase of the plant, sterile materials) (e.g., Liu & al. 2018). This technique is also valuable in identifying cryptic species, controlling the traffic of endemic, and endangered species (i.e., conservation) (e.g., Hebert & al. 2003; Hajibabaei & al. 2007), discriminating herbal medicine, and food ingredients from their cheaper substitutes (i.e., adulterants) (e.g., Xin & al. 2013), tracing dietary components of animals (e.g., Kartzinel & al. 2015), and detecting misidentified species (Han & al. 2010; Kuzmina & al. 2012; Xin & al. 2013; Liu & al. 2017), in conservation, forensic investigations, and biodiversity inventories; for not only professional taxonomists but also non-experts, such as, customs officers and forensic specialists.

The technique has become increasingly popular particularly for the identification of medicinal herbs and their adulterants or substituents. In folk medicine, barks, leaves, and roots are often used, and these plant organs lack morphological characters for accurate species identification (Zhang & al. 2016). Furthermore, traditional medicines are frequently derived from closely related species, posing a challenge for experts identifying these species due to morphological similarities, even if they exist (Sun & Chen 2013; Zhang & al. 2016). Morphological identification is indeed a challenging task that demands professionals a supply that has not kept pace with the expanding market (Pang & al. 2013). Similarly, both macroscopic and microscopic methods, as well as chemical identification methods, are often difficult, requiring expertise, being expensive and time-consuming (Techen & al. 2014). Furthermore, due to the processing of these medicinal herbs, the material contains high amounts of secondary metabolites such as polysaccharides, polyphenols, alkaloids, and flavonoids (Techen & al. 2014).

Newmaster & al. (2013) has demonstrated that fraud in the North American herbal product market is at an excessive level, with most herbal drugs containing unlisted, substituted or even hazardous ingredients. Similar results were reported for other countries (e.g., 60% adulterated products of Indian herbal products, Shanmughanandhan & al. 2016). Since misidentification or fraud of an herbal medicine may lead to reduced efficiency, serious side effects, or even death (Pang & al. 2013; Malik & al. 2019), even a partially effective DNA barcode would be beneficial in many areas, not only for the general public, but also for scientists (Chen & al. 2010).

An ideal DNA barcode should meet the following criteria: i) be a single locus, ii) have a relatively short length (~700 bp), iii) be easily amplifiable with a single pair of universal primers (i.e., possessing relatively conserved flanking regions) and standard PCR conditions, iv) be easily sequenceable and alignable across lineages with minimal manual editing, v) provide high discrimination power with high interspecific and less intraspecific variation (Kress & al. 2005; CBOL 2009; Hollingsworth & al. 2011; Clement & Donoghue 2012; Cowan & Fay 2012; Han & al. 2013). However, unlike animals (i.e., COI/COX1), a universal plant DNA has not been designated to date due to various challenges, including the number of informative characters, extensive hybridization, reticulation, introgression, genome duplication (i.e., polyploidy), manual breeding, and ancient-rapid radiation (Kress & Ericson 2007; Chen & al. 2010). Despite efforts, none of the loci recommended so far fulfills all the above-mentioned criteria across all land plants. Therefore, various combinations of markers, both coding and non-coding, from nuclear and plastid genomes have been proposed (Kress & Ericson 2007). In many cases, the use of a combination of at least two regions or employing nuclear regions with higher substitution rates has been found to be more efficient (Hajibabaei & al. 2007).

While the Consortium for the Barcode of Life (CBOL) (2009) has recommended the use of *matK* + *rbcL* as the standard (i.e., core) DNA barcode over five other coding and non-coding regions (i.e., namely *rpoC1*, *rpoB, psbA-trnH, psbK-psbI*, and *atpF-atpH*), the barcode has only 72% discrimination power in many plant groups, mainly due to the *rbcL* region (CBOL 2009). The *matK* region, however, lacks universal primers and PCR conditions, shows low success rates with degraded materials, and the barcode-pair is not discriminative enough, particularly in woody groups (Clement & Donoghue 2012). Because of the low species discrimination of the initially proposed regions and issues with PCR and primers, the use of supplementary DNA barcodes or replacing standard barcodes with supplementary DNA barcodes has been suggested (Hollingsworth & al. 2011; Clement & Donoghue 2012) from coding and non-coding regions (e.g., ITS, ITS1, ITS2, *trnH-psbA*, *trnL*, *trnL-F*). Later, the use of some of these regions, namely the internal transcribed spacers of nuclear ribosomal DNA (nr ITS/ITS1/ITS2), matK and *psbA-trnH* has proven to be more effective than the standard barcode combination, *matK* + *rbcL*.

The internal transcribed spacer (ITS) region of the nuclear rDNA cistrons has been located between the 18S and 25S coding regions (Selvaraj & al. 2013). Since (in theory) while the mitochondrial genome evolves at the slowest rate, the plastid genome evolves at a moderate pace, and the nuclear genome evolves at the highest rate in plants (Wolfe & al. 1987). This non-functional RNA sequence has proven to be valuable, particularly for low-level plant systematic studies (i.e., genus and species level) (Baldwin 1992; Baldwin & al. 1995). The ITS region consists of three partitions: ITS1, 5.8S and ITS2. While the 5.8S gene is highly conserved, the variability of the ITS region comes from both the ITS1 and ITS2 spacers (Wang & al. 2015).

However, the region also has some undesired qualifications such as the potential of fungal contamination, the need for specific primers, PCR conditions and PCR additives, low PCR efficiency, difficulties in sequence recovery and alignment, gene conversions, pseudogenes, recombination among copies, high mutation rate, numerous indels (insertion-deletions), the presence of paralogous gene copies (i.e., incomplete lineage sorting) and the necessity for cloning in some cases (Wendel & al. 1995; Álvarez & Wendel 2003; Kress & al. 2005; Aguilar & Sánchez 2007; Chen & al. 2010; Vijayan & Tsou 2010; Hollingsworth 2011; Xiang & al. 2011; Cowan & Fay 2012; Han & al. 2013).

Another significant drawback of the region is PCR and amplification problems with degraded material. For instance, Rubinoff & al. (2006) reported that they could amplify only 12% of their herbarium materials, and within this fraction,

some sequences were of very poor quality. Therefore, it has been suggested that the ITS1 and ITS2 subregions possess superior qualifications compared to the entire ITS region (Wang & al. 2016). While the entire ITS or the plastid genome might be more useful for taxonomists, the two subunits could serve as public DNA barcodes suitable for custom officers and forensic examiners (Gao & al. 2010b).

ITS1 and ITS2 are the first and the second internal transcribed spacers of 18S-28S nuclear ribosomal DNA, respectively (Bellemain & al. 2010). In most plants the length of the ITS1 region is generally less than 300 bp (187 to 298 bp), while the length of the ITS2 region is approximately 250 bp (187 to 252 bp). (Selvaraj & al. 2013). These two components of the ITS region have been extensively utilized in low level molecular taxonomic studies due to their ease of amplification (i.e., their short length), sequencing and high nucleotide substitution rates, and these characteristics of ITS1 and ITS2 allow researchers to easily amplify even degraded DNA materials, such as, herbal medicine ingredients, museum specimens and herbarium samples (Selvaraj & al. 2013; Zhu & al. 2015). Additionally, in cases where obtaining the entire ITS region is not possible, ITS1 and ITS2 were suggested as standard barcodes for seed plants (China Plant BOL Group 2011; Yan & al. 2015). It has also been reported that ITS1 and ITS2 provide similar results as DNA barcodes for fungi (Blaalid & al. 2013). Among these two mini-barcodes, while the ITS1 subunit has been reported to exhibit sufficient variability as a DNA barcode (Mishra & al. 2016), it has not gained the same level of popularity as the ITS2 subunit, particularly within the herbal medicine community.

Due to its short length, facilitating amplification even with herbarium specimens, high species resolution, accurate identification, less intraspecific variation, and conserved flanking regions for the development of cost-effective universal primers, the internal transcribed spacer 2 (ITS2) of the ribosomal cistron has been identified as a novel barcode across land plants by Chen & al. (2010) and Yao & al. (2010). Chen & al. (2010) evaluated seven DNA regions, including *psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS for various medicinal plants and their closely related species. Among them, ITS2 demonstrated correct identification at 92.7% and 99.8% at the species and genus levels, respectively, based on more than 6600 samples obtained from 4800 species in 753 genera from 193 families in seven phyla (angiosperms, gymnosperms, ferns, mosses, liverworts, Algae and fungi). Consequently, ITS2 region has been proposed as the core DNA barcode for medicinal plants (Chen & al. 2010) and the high number of ITS2 entries in public DNA databases also makes this region an efficiency candidate (Ankenbrand & al. 2015).

China Plant BOL Group (CPBG) (2011) subsequently verified the authentication of this region as a reliable source (i.e., core plant DNA barcode) in cases where ITS cannot be amplified and/or sequenced. The effectiveness of ITS2 as a plant DNA barcode has been confirmed by many studies (e.g., Han & al. 2013; Chen & al. 2014; Newmaster & al. 2013; Xu & al. 2015; Wang & al. 2016; Braukmann & al. 2017; Ganopoulos & al. 2017). Additionally, the secondary structure of ITS2 has been reported as useful in DNA barcoding studies (e.g., Keller & al. 2009; Shi & al. 2011).

While fungal contamination and the multiple copy problems may still exist (Bell & al. 2016; Cheng & al. 2016), it has been reported that multiple copy nature may not be a problem for the ITS2 region in most cases; this is because the amplified copies could represent the dominant information and ITS2 could be considered a single locus in most cases (Coleman 2009; Gao & al. 2010a; Hou & al. 2013, Xin & al. 2013). Yet, contrasting results have also been suggested (e.g., Aygoren Uluer and Alshamrani, 2019). Moreover, a vast amount of ITS2 data has already been deposited in GenBank (e.g.,72% of known US plant species are already deposited in GenBank, Ankenbrand & al. 2015), making the ITS2 region one of the most popular DNA barcodes.

Over the past years, numerous studies have evaluated the DNA barcoding utility of the ITS2 region (e.g., Chen & al. 2010; Ganopoulos & al. 2017). However, ongoing debates persist regarding its efficacy in the identification of angiosperms. Therefore, the objective of the present paper is to provide a comprehensive perspective on the use of the ITS2 region as an angiosperm DNA barcode. This will involve a comparative analysis with other popular DNA barcodes, including ITS, ITS1, *matK* and *trnH-psbA* through a review of 197 plant DNA barcoding studies that have employed the ITS2 region.

MATERIAL AND METHODS

In this review, the literature on angiosperm plant DNA barcoding studies utilizing the ITS2 region was surveyed. Four databases were searched, namely Google Scholar, JSTOR, BioOne Complete and PubMed using the following keywords: 'ITS2' AND 'internal transcribed spacer' AND 'DNA barcoding'. The review focused on assessing overall

PCR amplification success, sequencing success, reported length and the rate of correct species/genus level identification associated with the utilization of the ITS2 region. The results of these parameters were calculated to provide a comprehensive overview of the effectiveness of the ITS2 region in angiosperm DNA barcoding studies.

Second, the ITS2 region was compared with the top four DNA barcoding regions: ITS1, the entire ITS, *matK* and *trnH-psbA*. The inclusion criteria focused on excluding studies that could not be retrieved despite all efforts and those with integrated approaches, such as, metabarcoding (e.g., Timpano & al. 2020), high-resolution melting (HRM) (e.g., Ganopoulos & al. 2012; Fadzil & al. 2018), single nucleotide polymorphisms (SNP) identification (e.g., Chen & al. 2013; Liu & al. 2016), quantitative PCR (qPCR) (e.g., Howard & al. 2019), cleaved amplified polymorphic sequence (CAPS) (e.g., Peng & al. 2016), TA cloning (e.g., Avarave & Thomas 2021), ITS2 secondary structure (e.g., Umdale & al. 2017; Zhu & al. 2017), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (e.g., Yu & al. 2018), ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS) (e.g., Hu & al. 2020), and studies that coupling ITS2 region with morphology analysis (e.g., pollen morphology, Reunov & al. 2018). Also excluded were studies aimed solely at exploring the authenticity of herbal medicines (i.e., not a comparison of DNA barcoding regions) (e.g., Cui & al. 2020) or exploring the authenticity of herbal medicines or examining specimen age and preservation methods (i.e., not a comparison among DNA barcoding regions) (e.g., Braukman & al. 2017), as well as those without available ITS2 sequences in GenBank (e.g., Ahmed & al. 2022). However, studies were included if they involved the design of new primers (i.e., with the aim of comparing the general effectiveness of the DNA regions, not just the PCR amplification and sequencing success) (e.g., Omelchenko & al. 2022), aimed to test DNA barcodes on various types of materials on i) fresh (e.g., Gao & al. 2010a), dried (e.g., Zhu & al. 2015) or museum material (e.g., Han & al. 2013); ii) unprocessed plant material (e.g., Cheng & al. 2022) or processed herbal products (e.g., Liow & al. 2021); iii) seeds (e.g., Cheng & al. 2022), leaves (e.g., Pang & al. 2011) or fruits (e.g., Chen & al. 2021), or utilized GenBank sequences (e.g., Qin & al. 2017). Machine learning studies (e.g., He & al. 2019; Liu & al. 2023) were also included because they provided clear insights into the efficiency of ITS2 as a DNA barcode.

The compiled data is presented in a supplementary appendix available at https://data.mendeley.com/datasets/xvk-3kdvdfj/1. In this appendix, rows are numbered and listed according to the employed barcoding region (e.g., from 1 to 50, only ITS2 is employed) or according to the most successful DNA barcoding region in the study (e.g., from 50 to 91, ITS2 is the most successful region, from 92 to 97 ITS1 is the most successful region etc.).

Calculations and statistics

First, the overall success rate of the ITS2 region in terms of PCR amplification, PCR sequencing, species and genus level correct identification were calculated by the following formula:

Success Rate = Number of Successes/Total count.

To evaluate the performance of each region, as single barcodes or in a combination, the same formula was used, by calculating the success and failure percentages by considering the number of studies where the region was successfully chosen and the total number of studies.

Second, the correlation between material type (i.e., field trip samples, market samples, field trip + market samples, field trip + herbarium samples) and primer amplification and sequencing success (i.e., universal primers vs. others) were compared using one-way analysis of variance (ANOVA) followed by Hochberg's GT2 test on SPSS v. 28.0. P-values < 0.05 were considered as significant. And third, for the comparisons among different DNA barcodes, namely, ITS2, ITS1, ITS, *matK*, *trnH-psbA* and *trnL-F*, Chi-Square Test for Independence was applied.

RESULTS

PCR amplification, sequencing and identification success of the ITS2 region

Among the 104 studies reporting the PCR amplification success rate for the ITS2 region, six excluded the region due to some problems as indicated below. Nevertheless, the overall PCR success rate stood at 87.40%. Specifically, 69 studies reported a 100% amplification success rate, 12 studies reported a success rate ranging from 90–99%, seven reported a success rate of 80–89%, and the remaining studies reported a PCR success rate between 53–74% (Table 1, Fig. 1).

Table 1. Overall success rates for PCR amplification, PCR sequencing, and species/genus identification of the ITS2 subunit in the reviewed studies. The highest levels of success rates for each parameter are indicated with an asterisk.

Parameter / Success	100%	90–99%	80–89%	0–79%	Overall mean (%)
PCR amplification	69 (66%)*	12 (11.5%)	7 (6.7%)	16 (15.4%)	87.40
Sequencing	61 (62%)*	17 (17%)	7 (7%)	14 (14%)	92.79
BLAST species identification	29 (30%)*	29 (30%)*	12 (12%)	7 (7%)	86.00
BLAST genus identification	49 (75%)*	29 (30%)*	–	5 (8%)	97.00

The mean sequencing success rate was 92.79% across 100 studies. Among these, 61 studies achieved a perfect 100% PCR sequencing success rate, while 17 reported a sequencing success rate ranging from 90–99%. Additionally, seven studies documented an 80–89% success rate, and the remaining studies reported a success rate of less than 70% (Table 1, Fig. 1). The one-way analysis of variance (ANOVA) conducted to test the correlation between material type and primer amplification and sequencing success indicated that there was no significant difference among various sample types and primers (i.e., p-values ranging from 0.48 to 1.00).

In terms of BLAST species identification levels (Table 1, Fig. 1), out of 98 studies, 29 reported a 100% success rate, 29 reported a success rate between 90–99%, 12 reported a rate of 80–89%, and the remaining reported a rate between 9–98% (please note that some studies reported a broad range, such as 9–49%). Nonetheless, the mean species identification rate for the region was 86%. Regarding the Best Match (BM) and Best Close Match (BCM) results, 19 studies reported variable outcomes ranging from 24% to 100%; nevertheless, the average species identification rate stood at 71.22%.

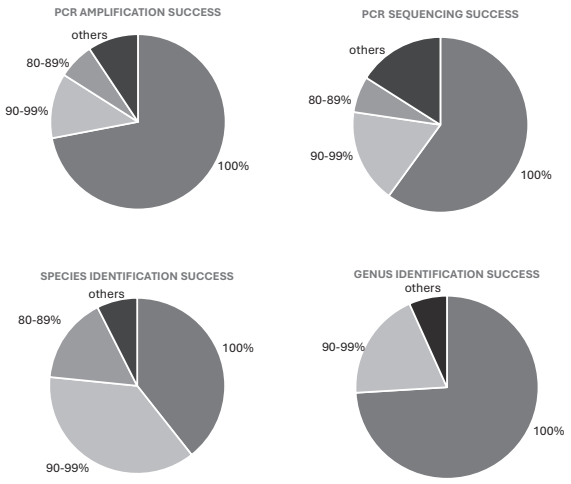

Fig. 1. Overall success rates for PCR amplification, PCR sequencing, and species/genus identification of the ITS2 subunit.

For the genus-level BLAST identification rate, among 65 studies, 49 reported a 100% success rate, while 29 reported a success rate ranging from 90–99%. The remaining studies reported a success rate between 47–100% (please note that some studies reported a wide range, such as up to 100%) (Table 1, Fig. 1). The average success rate was 97%. Similarly, the results of two studies, which indicated the BM or BCM search results for genus-level identification, reported a rate of 95–100%. It is noteworthy that for both species and genus-level identification success, some studies were excluded due to issues such as possible contamination, as reported by Peedales & al. (2016), species identification results covering ferns, as noted by Gong & al. (2018), and insufficient reference data, as mentioned by Al-Juhani & al. (2021).

Comparison with other DNA barcodes

A comparison with the ITS2 DNA barcoding region and others if provided (Tables 2, 3; Figs. 2, 3). The ITS2 region, whether used as a 'single barcode' or in combination with at least one additional DNA barcode, was found to be the most efficient according to the majority of studies reviewed here (66.67%). It was followed by ITS1 (46.88%) and the entire ITS region (41.38%). However, when considering studies indicating the efficiency of the ITS2 region as a 'single barcode', the percentage dropped to 44.44%, with *trnH-psbA* (36.67%) and the *matK* (29.63%) regions following. Notably, the most successful region was the ITS1 subunit, with a 68.42% overall success rate and the entire ITS was the least successful region among others (11.85%) (Tables 2, 3).

Table 2. Success rate of DNA barcodes either on their own or in combination with other DNA barcodes in the reviewed studies.

Barcode	Successfully chosen	Total number of studies	Overall success rate (%)
ITS2	90	135	66.67
ITS1	15	32	46.88
ITS	24	58	41.38
matK	27	93	29.03
trnH-psbA	24	83	28.92
trnL-F	3	20	15.00

Table 3. Success rate of a given DNA barcode when chosen alone as the best DNA barcode in the reviewed studies.

Barcode/s	Successfully chosen	Number of studies	Overall success rate (%)
ITS1	13	19	68.42
ITS2	60	135	44.44
trnH-psbA	11	30	36.67
matK	10	93	29.63
trnL-F	2	13	15.38
ITS	16	27	11.85
trnH-psbA+ITS2	8	83	9.64
rbcL+ITS2	7	94	7.45
ITS+*matK*	3	42	7.14
matK+ITS2	6	93	6.45
ITS2+*matK*+*rbcL*	2	40	5.00
trnH-psbA+ITS	2	42	4.76
rbcL+*matK*	0	80	0

A comparison of the ITS2 region, with ITS, ITS1, *matK* and *trnH-psb*A using the "success rate formula" is presented in Table 4. While this table highlights the ITS2 region as the most successful among others, the results of the Chi-Square test suggest that ITS1 (p-value = 0.62) and the entire ITS (p-value = 0.20) did not show statistically significant differences compared to the ITS2 region in terms of selection as the optimum DNA barcode. On the other hand, *matK* (p-value = 0.0006) and *trnH-psbA* (p-value = 0.0016) were found to be less successful than the ITS2.

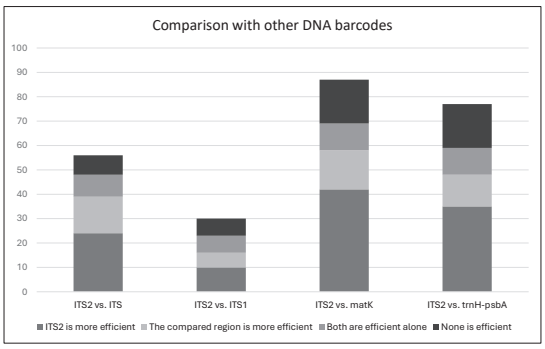

Fig. 2. Results of the comparison of the ITS2 region between ITS (entire), ITS2, *matK* and trnH-*psbA*.

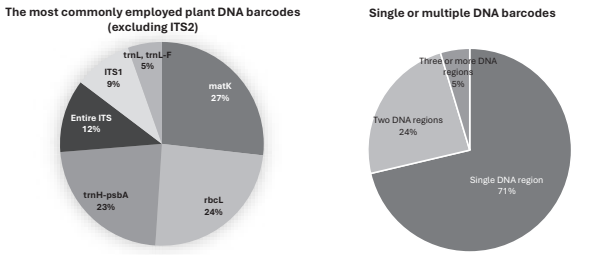

Fig. 3. DNA regions most frequently utilized in the reviewed studies, excluding the ITS2 nuclear region.

Table 4. Comparison of the ITS2 region with other DNA barcoding regions by using the "success rate formula", as a single DNA barcode or in a combination with others. *Chi-Square test.

Result	ITS2 vs. entire ITS	ITS2 vs. ITS1	ITS2 vs. *matK*	ITS2 vs. *trnH-psbA*
ITS2 is more efficient	24 (43%)	10 (33%)	42 (48%)	35 (45%)
The compared region is more efficient	15 (27%)	6 (20%)	16 (18%)	13 (17%)
Both are efficient alone	9 (16%)	7 (23%)	11 (13%)	11 (14%)
None of them is efficient	8 (14%)	7 (23%)	18 (21%)	18 (23%)
Is ITS2 significantly better?*	NO	NO	YES	YES
Total (n) studies reviewed	56	30	87	77

Single or multiple DNA barcode(s)?

In this case, 92 studies (71%) recommended employing only one DNA barcoding region (i.e., ITS, ITS1, ITS2, *matK* and *trnH-psbA*), while the remaining 38 studies (29%) indicated a combination of two (31 studies) or three (six studies) DNA barcodes is sufficient for their angiosperm plant groups. Among these, the most recommended combinations were ITS2 + *trnH-psbA* and ITS2 + *matK*, each suggested by seven studies. This was followed by ITS2 + *rbc*L (six studies), ITS + *matK* and ITS + *trnH-psbA* (three studies for both), and ITS2 + *mat*K + *rbc*L (two studies) (please note that some studies recommended more than one option).

DISCUSSION

The efficiency of the ITS2 region

Internal Transcribed Spacer 2 (ITS2) is a commonly targeted region for DNA amplification in molecular phylogenetic studies, particularly in the context of plant species identification (Chen & al. 2010). However, despite its widespread use, researchers often encounter challenges associated with ITS2 amplification. One prominent issue is the presence of primer mismatches, where the universal or designed primers may not perfectly match the target sequences due to genetic variability (Chase & al. 1993). This can result in reduced amplification efficiency or even complete failure to amplify the ITS2 region from certain samples. Moreover, the presence of pseudogenes in the ITS2 region can confound sequencing efforts (Schoch & al. 2012). Limited DNA yield or poor DNA quality from certain samples can also impede successful ITS2 amplification and sequencing (Tedersoo & al. 2010).

The high success rate of the ITS2 region has been previously reported (e.g., Chen & al. 2010; Han & al. 2013) and subsequently verified by many studies (e.g., Al-Juhani & al. 2021; Tahir & al. 2018). Indeed, the results of the current review demonstrate that the ITS2 region possesses desirable qualifications in terms of PCR amplification and sequencing success rates (≈87% and ≈93% success rate, respectively) (please note that above 70% is accepted as successful). However, in some studies challenges in PCR amplification and sequencing were primarily related to a low amplification rate when using dietary supplements (Baker 2012; Sun & al. 2013), herbal market samples (Molina & al. 2018; Malik & al. 2019), herbarium material (Pe dales & al. 2016); potential incomplete lineage sorting and hybridization/introgression (Zarrei & al. 2015; De Castro & al. 2017), serious endophyte interference (Sun & al. 2012), fair (Gori & al. 2019) and/or multiple bands (Kurian & al. 2020; Sijimol & al. 2020), nonspecific amplification (Lugon & al. 2021), and low success rate of sequencing (Sun & al. 2011). However, for most of these studies, the ITS2 region simply did not yield successful amplification and/or sequencing for the studied group of plants (e.g., Liu & al. 2012; Liu & al. 2017), or the plant taxon under investigation posed a challenging case (e.g., *Quercus* L., Simeone & al. 2013). This could be attributed to certain plant families exhibiting a low sequence recovery rate (e.g., monocots, rosids, asterids) or failing to produce any results (e.g., Pontederiaceae), irrespective of the material's age (i.e., DNA degradation level), preservation method (i.e., herbarium specimens or silica gel-dried samples), or the purported "universal" nature of the ITS2 primers by Chen & al. (2010) not being universally applicable (Kuzmina & al. 2017), and hence not suitable for every plant group. In this case, it is noteworthy that, out of the 13 studies indicating a low amplification/sequencing success rate (i.e., less than 70%), but still considering ITS2 a potential DNA barcoding region (with nine studies either not recommending a DNA barcoding region or suggesting DNA regions beyond the scope of this review), two of them reported that ITS2 remained one of the most successful DNA barcodes for their respective plant groups (e.g., Jiao & al. 2018; Hu & al. 2022). A recent study may contribute to addressing the primer efficiency issues of the ITS2 region, as it demonstrated 95% suitability for plants (Cheng & al. 2016).

In terms of PCR pairs used, the results of the one-way ANOVA test in the current study did not reveal any significant differences among various pairs of primers. However, it is important to note that, even though the studies did not explicitly state, researchers might have tested multiple primer pairs before sequencing the ITS2 region. They could have initially obtained positive results with the first primer pair used (e.g., specific primers for their plant groups) and continued the search for a more efficient one. As we lack information on the specific steps taken in each study to identify suitable primer pairs, it is possible that the results presented here may contain bias.

Fungal/endophyte contamination (i.e., non-specific amplification) problems were reported by only two studies (1%) in this review, specifically from Dioscoreaceae (Sun & al. 2012) and Arecaceae (Lugon & al. 2021) families. Consequently, it seems that non-specific amplification is not a prevalent concern for the ITS2 region (Bell & al. 2016; Cheng & al. 2016). Similarly, problems related to multiple copies of the ITS2 region, such as gene duplication, hybridization, incomplete lineage sorting and polyploidy, have been documented, particularly in taxon-rich plant families (Song & al. 2012; Kuzmina & al. 2017; Xu & al. 2018). However, in the current review, only a few studies (2%) (e.g., Sun & al. 2013; Zarrei & al. 2015; De Castro & al. 2017; Kurian & al. 2020; Sijimol & al. 2020) reported multiple copy issues for their plant groups. It is noteworthy that two of these studies belong to the species-rich plant families previously reported by Kuzmina & al. (2017), namely, Poaceae and Polygonaceae (with the other two belonging to Araceae and Theaceae). In this context, caution is advised concerning possible double peaks and noise (i.e., ambiguous sequences) in sequencing results. While this issue may initially seem attributed to incomplete lineage sorting, hybridization, and/or introgression, it could also indicate contamination or adulterants, particularly in herbal medicines (De Castro & al. 2017). Additionally, P edales & al. (2016) highlighted a low sequencing success rate due to a low amount of DNA and/or PCR inhibitors, emphasizing potential issues related to the material used rather than the amplification and sequencing of the ITS2 region in herbal medicinal products. Kuzmina & al. (2012) also detected paralogous sequences in only approximately 3.5% of their samples, primarily attributable to closely related species.

Secondly, the success of ITS2 amplification can be influenced by the type of material used and its age (Kuzmina & al. 2017). Herbarium specimens, a valuable resource in biodiversity research, often pose challenges for ITS2 amplification due to DNA degradation over time and not only drying but also storage conditions can affect DNA quality, potentially hindering successful amplification (Staats & al. 2011). In the current review, concerning the type of plant material, whether recently collected, herbarium material, or purchased, as supported by the statistical analyses, no significant difference was observed between the PCR amplification/sequencing success and the material type. While in the studies reviewed here, did not provide information about the age of herbarium material, Kuzmina & al. (2017) have reported that herbarium specimens aged over 50 years show less effective sequence recovery rates, not only for the ITS2 nuclear region but also for plastid regions. This

finding was also supported by Molina & al. (2018) and Malik & al. (2019). Similarly, Pedales & al. (2016) reported that PCR amplification of herbal medicines is more challenging than that of field-collected samples; yet, this review did not reveal a significant difference in this case. Further testing is necessary for this issue.

In the present study, it is evident that the ITS2 region demonstrates a high resolution capacity at the genus level, but it is only moderately successful at the species level (please note that above 70% is accepted as successful). Here, it should be noted that for some studies with low identification levels, the main reasons were: possible substitution or contamination (e.g., Pe dales & al. 2016), lack of sufficient sequence data (e.g., Al-Juhani & al. 2021), general identification rates being low for all DNA barcoding regions for that particular group of plants (e.g., Tahir & al. 2018), only one DNA region not being sufficient for the plant group studied (e.g., Han & al. 2021), or just the ITS2 region not being suitable for the plant group, as in most of the studies (e.g., Liu & al. 2012; Sun & al. 2013; Giudicelli & al. 2015). However, it is important to mention that out of 25 studies which indicated low levels of identification success rate (i.e., less than 70%) but a candidate DNA barcoding region (i.e., nine studies did not recommend a DNA barcoding region or some recommended DNA regions out of the scope of this review), eight of them reported that the ITS2 was still one of the most successful DNA barcodes for their plant groups alone (six studies) or in combination (two studies) (e.g., Tahir & al. 2018; Al-Juhani & al. 2021). Furthermore, several studies indicated that the secondary structures of ITS2 would provide extra information to aid in species discrimination (e.g., Gong & al. 2018).

Comparison with other DNA barcodes

Firstly, concerning ITS1 and ITS2, while some studies indicate that the success of the ITS1 and ITS2 subunits is similar in DNA barcoding studies (e.g., Chen & al. 2010; Blaalid & al. 2013; Mishra & al. 2016, 2018), there are also instances where the ITS2 region could be sequenced, but not the ITS1 region (e.g., Enan & al. 2012), or the ITS2 subunit was considerably more successful than the ITS1 region (Nicolè & al. 2011). For instance, Wang & al. (2015) compared the species discrimination power, PCR amplification, and sequencing (in silico) abilities of the ITS1 and ITS2, concluding that ITS1 is a more efficient barcoding candidate. Giudicelli & al. (2015) reported a similar, but slightly higher species identification rate of the ITS1 region over ITS2 for the taxonomically complex *Passiflora* L. Similarly, Wang & al. (2013) indicated that for *Salvia* L. (Lamiaceae), while ITS2 correctly identified only 56% of the taxa, ITS1 successfully identified 81%. Comparable results were also suggested by Chase & al. (2005), Kress & Erickson (2007), Mishra & al. (2016, 2018). In contrast, studies that advocate for employing the ITS2 region over the ITS1 are not rare. For instance, while Selvaraj & al. (2015) reported a higher discrimination ability for the ITS2 region, Chiang & al. (2011) indicated that sequencing the ITS1 region was not successful for *Ricinus communis* L. Indeed, these results somewhat align with the findings of the current study, suggesting that ITS1 and ITS2 subunits are similar to each other in terms of DNA barcoding efficiency. While, overall, the ITS2 mini barcode is more efficient than the ITS1 region, as a single barcode ITS1 is slightly more successful (Tables 2, 3; Fig. 3). However, the difference was not statistically significant; and therefore, further testing is needed.

Secondly, as the ITS2 region constitutes a portion of the entire ITS region and inherently possesses higher phylogenetic informativeness in the reviewed studies (e.g., Muellner & al. 2011; He & al. 2012; Zheng & al. 2014; Nguyen & al. 2020; Devi & al. 2022), this review reveals that, overall, the ITS2 subunit demonstrates slightly higher efficiency than the entire ITS region (Tables 2, 3; Fig. 2), even the difference was not statistically significant (it should be noted that out of seven studies in which the ITS region was reported to be more successful compared to the ITS2 region, only one study employed the universal ITS2 primers, while the others retrieved the region from the entire ITS, which was sequenced with the ITS primers). Interestingly, even in cases where the entire ITS region was advocated as a superior DNA barcoding region due to parsimony-informative sites and discriminatory power, the ITS2 subunit proved nearly as effective as the entire ITS (e.g., Sun & al. 2015; Nguyen & al. 2020). For instance, Wang & al. (2013) reported challenges in sequencing the entire ITS region for the genus *Salvia*, with PCR sequencing failing multiple times due to secondary structures. Han & al. (2013) found the ITS2 region much more successful in amplification compared to the entire ITS region (92% vs. 23%, respectively), even with herbarium material over 90 years old. Sun & al. (2015) reported relatively similar identification rates for the ITS and ITS2 regions for Brassicaceae (i.e., 7% lower for the ITS2). However, the authors emphasized that the ITS region wasn't the ideal barcode, discussing the trade-off between higher identification rates and the challenging sequence recovery of the region compared to the ITS2 subunit. These findings align with previous studies (e.g., Kress & al. 2005; Chen & al. 2010; Han & al. 2013; Liu & al. 2017), indicating sequencing challenges related to the ITS primers.

Thirdly, the ITS2 region emerges as a significantly superior alternative to the *matK* (48% vs. 18%) and the *trnH-psbA* (45% vs. 17%) plastid regions overall (Tables 2, 3; Fig. 2), and this difference was statistically significant. In terms of informativeness, the literature presents conflicting perspectives. Some studies suggest that *matK* and *trnH-psbA* regions

show higher discrimination abilities than the ITS2 region; however, PCR and alignment challenges (i.e., intraspecific variation, alignment and indel problems of the *trnH-psbA* region; PCR amplification and sequencing problems of the *matK* region) render ITS2 a more reliable candidate (e.g., Techen & al. 2014). Conversely, others propose that, compared to highly variable chloroplast regions such as *matK* and *trnH-psbA*, ITS2 is 10-20 times more informative (e.g., Shi & al. 2011). Nevertheless, the general results of the present review overall concur with some previous studies (e.g., Chen & al. 2010; He & al. 2012) asserting that the *trnH-psbA* and *matK* regions lack fundamental DNA barcoding abilities, suggesting they may only serve as supplementary DNA barcodes or for phylogenetic purposes. Furthermore, the shorter ITS2 region demonstrates a sequence recovery advantage in samples aged 10 years or more, in comparison to longer plastid regions such as *matK* and *rbcL*, as previously reported (Kuzmina & al. 2027). The universal primers of the ITS2 region are also advantageous compared to the necessity of multiple or taxon-specific primer requirements of the *matK* region in general (Luo & al. 2010).

Lastly, the examination of 79 studies incorporating the *rbc*L region (or its subunits) reveals that only two studies recommended using this region as a DNA barcode alone, with nine studies proposing its utilization alongside at least one other region. Based on these results, I concur with the findings of certain previous studies (e.g., Aygören Uluer and Alshamrani 2019) asserting that sequencing the *rbcL* region is time and resource-consuming for the majority of angiosperm groups, and thus, its standalone use should be abandoned. Additionally, as previously suggested by researchers (e.g., Hershkovitz & Zimmer 1996; Zhang & al. 2016), the conserved secondary structure information of the ITS2 region could serve as a viable anchor for high taxonomic levels in place of the *rbcL* region (Coleman 2003).

Single or multiple DNA barcode(s)?

Opting for a single DNA barcode offers simplicity in experimental design and data analysis. This approach is particularly advantageous when seeking a straightforward and cost-effective solution, making it suitable for routine species identification purposes (Hebert & al. 2003). On the other hand, the use of multiple DNA barcodes provides several benefits that enhance the accuracy and comprehensiveness of genetic analyses (Hollingsworth & al. 2011). Employing multiple barcodes becomes crucial when dealing with closely related species that share similar genetic sequences (Meier & al. 2006). This approach not only increases accuracy but also improves phylogenetic resolution, offering a more detailed understanding of evolutionary relationships among species (Fazekas & al. 2009; Hollingsworth & al. 2011).

However, majority of the studies reviewed here recommend employing single DNA barcodes (92 studies, 71%) over two-three barcode combinations (38 studies, 29%), which supports the idea that two loci combinations are not better than single locus (Pang & al. 2011) (Tables 2, 3). It is also worth mentioning that out of 27 studies in which more than one locus combinations were used as a DNA barcode, only five of them suggested employing more than two loci (i.e., mainly three loci), the remaining 22 studies adopted two loci combinations. Therefore, the findings of the current review suggest that sequencing more than one locus appears to be an inefficient use of resources and may be necessary only in rare cases.

Interestingly, while 68 studies included the *rbcL* and *matK* regions, none of them reported that this combination could be useful as a DNA barcode for their specific plant group. In contrast, the ITS2 nuclear region was reported as an efficient DNA barcode by 37% of these studies. Therefore, it appears that the initially proposed *matK* + *rbcL* may not be useful for many angiosperm groups, mainly due to low discrimination ability of *rbcL* and PCR/primer problems of *matK* (Clement and Donoghue 2012) and both the ITS1 and ITS2 can easily substitute this core plant DNA barcode (Chen & al. 2010; Wang & al. 2016), except maybe some taxonomically complicated plant groups (e.g., Pang & al. 2011; Simeone & al. 2013; Zarrei & al. 2015; Aygoren Uluer & Alshamrani 2019) because of some problems, such as, the presence of apomixes, polyploidization, hybridization, and manual breeding (Pang & al. 2011). Therefore, the current review advocates for the concept that only for some taxonomically difficult plant groups, such as *Rosa* L., *Alchemilla* L., *Crataegus* L., *Quercus* L. and *Aesculus* L., either combination of several markers or whole genome sequencing may be the only choice (e.g., Wu & al. 2021, but see Chen & al. 2020).

Last but not least, this review has some potential caveats that merit attention. Firstly, the study effectively demonstrates that, in comparison to the entire ITS nuclear region, ITS1, and the plastid *matK* and *trnH-psbA* regions, ITS2 emerges as a more efficient choice as a DNA barcode. However, it's important to note that non-angiosperm DNA barcoding studies were not included in this analysis. Moreover, it should be emphasized that this review specifically delves into DNA barcoding studies utilizing the ITS2 region, not encompassing all DNA barcoding studies. Nevertheless, the primary objective of this study was to conduct a comparative analysis of the ITS2 region with other prominent and informative DNA barcodes, namely *matK*, *trnH-psbA*, ITS, and ITS1. Thirdly, for the sake of comparison, only BLASTn, BM (Best Match), and BCM (Best Close Match) search results were considered, and the concept of the DNA bar-

coding gap was not factored into the analysis. Lastly, a few studies were unable to be included due to reasons such as restricted access or the format of short conference papers, which typically lack detailed results.

In conclusion, these results show that none of the regions reviewed here is a universal barcode for angiosperms, as suggested before by several studies. However, while the ITS1 and ITS2 regions are the most efficient angiosperm DNA barcodes, followed by the entire ITS region. The plastid *matK* and *trnH-psbA* regions are not useful due to amplification, alignment and informativeness problems. Therefore, the ITS1 and the ITS2 should be the first choice as a DNA barcodes; however, if there are phylogenetic problems (e.g., hybridization, concerted evolution) within the plant group the entire ITS region could be a second choice. It is worth noting that two/three locus combinations are suggested by only a few studies reviewed here, which supports the idea that two loci combinations are not better than single locus (Pang & al. 2011). The *matK* + *rbcL* core DNA barcode is not recommended by any of the studies reviewed here. However, because of the above-mentioned caveats, further studies are needed to effectively compare the most popular plant DNA barcodes.

AUTHORSHIP CONTRIBUTION STATEMENT

D. AYGÖREN ULUER: Conceptualization, Data curation, Investigation, Writing–original draft, Writing–review & editing.

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