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Plant DNA Barcodes

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Abstract—The search for universal plant DNA barcodes has proved to be a big challenge. A single locus (or combination of several loci) that can be used for species identification has yet to be revealed, though the (*rbcL* + *matK* + ITS) combination recommended in 2009 as the standard makes it possible to assign a species to the corresponding genus. The variability of some markers differs in different taxonomic groups and usually makes it possible to select the DNA barcode (sometimes even a mini-barcode) for a specific group, especially for applied tasks. Next-generation sequencing (NGS) methods make it possible to obtain a large number of extended DNA barcodes (sequences of complete chloroplast genomes and ribosomal genes), which allows the researcher to overcome the limitations of standard DNA barcodes. It is important that NGS technologies significantly enhance the possibility of the use of herbarium specimens. The search for plant DNA barcodes is ongoing.

Keywords: plant DNA barcoding, species discrimination, chloroplast DNA, next-generation sequencing
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INTRODUCTION

In science and practice, there is a constant need for the accurate identification of a species or genus of plant samples, as well as the determination of the origin of plant products. However, the lack of experienced experts often does not allow this need to be satisfied. Thus, the field of the potential application of DNA barcoding in botany and expert services is very wide. In science, it is used to distinguish species that are hard to be identified (such as tree species with a short bloom period or rarely blooming), to make a more precise identification of the species composition of local floras and plant communities, and to reveal new species, including cryptic ones. DNA barcoding has also a wide and expanding range of practical applications, including the protection of biodiversity and rare species and the prevention of their collection and illegal sale; the control of plant raw materials, herbal teas, honey, and other commercial products; the control of weeds, invasive species, and allergy-causing plants, etc.

At the same time, from the very beginning of the implementation of the international CBOL (Consortium for the Barcodes of Life) program, plant DNA barcoding became a big challenge. Since the standard DNA barcode for most animals, a fragment of the mitochondrial *CO1* gene, was inapplicable for plants because of the low and uneven mutation rate of plant mitochondrial DNA, it was decided to choose a barcoding region among the chloroplast regions of the genome. A special group (Plant Working Group CBOL) was organized to search for plant DNA barcodes. Several promising chloroplast DNA sequences were considered; these candidates included both encoding sequences (*matK*, maturase; *rpoB*, β subunit of RNA polymerase; *accD*, β' subunit of acetyl-CoA carboxylase; *ndhJ*, a nicotinamide dehydrogenase subunit); *ccsA*, encoding protein involved into the cytochrome c biosynthesis; *rbcL*, the large subunit of ribulose-bisphosphate carboxylase, EC 4.1.1.39) and noncoding sequences (*trnH–psbA*, *atpF–atpH*, and *psbK–psbI* spacers); the ITS regions of nuclear ribosomal genes were also later added to the candidate sequences. These regions were tested on different plant groups to evaluate the quality of their amplification with universal primers and the level of plant species discrimination. As a result, scientists soon understood that no single region was able to serve as a barcode for all plants; for this purpose, they have to find a combination of DNA regions. The initial stages of the search and the features of the regions that were finally selected as DNA barcodes were described earlier in detail (Shneyer, 2009). Note that the search took significantly more time than planned, and it appears to remain unfinished.

In 2009 the CBOL Executive Committee proposed a plant DNA barcode consisting of a combination of two chloroplast DNA regions representing fragments

of the *rbcL* and *matK* genes with lengths of about 600 and 800 nucleotides, respectively (Hollingsworth et al., 2009). Some studies reported that this combination allowed good species discrimination (Burgess et al., 2011), though the resolution level for some genera, such as *Crocus*, *Berberis*, and *Primula*, was obviously insufficient (Seberg and Petersen, 2009; Roy et al., 2010; Van et al., 2011). A special comparative study was performed by Chinese scientists, who used samples of 6286 plants of 1757 species and 141 genera to sequence five different DNA regions considered to be potential DNA barcodes. A combination of *matK* and *rbcL* markers was species-specific in 49.7% cases (Li et al., 2011). Some studies reported an increased intrageneric resolution in the case of the addition of either an ITS region (family Palmaceae, Jeanson et al., 2011) or *trnH–psbA* spacer (family Labiatae, De Mattia et al., 2011). Some authors have already proposed the addition of the well-studied ITS region to DNA barcodes (Feliner and Rosselló, 2007). Some Chinese authors analyzed ITS data for thousands of species from different families and found ITS2 to be a very efficient DNA barcode (Ren et al., 2010; Gao et al., 2010). As a result, it was proposed in 2011 to add nuclear ITS1 and ITS2 regions and the chloroplast *trnH–psbA* spacer, which was called an alternative DNA barcode, to the list of standard DNA barcodes and to use them if needed (Hollingsworth, 2011; Li et al., 2011). Obviously, these markers had some limitations; it was noted that good results could be probably obtained with highly variable low-copy-number nuclear genes, but, due to their unequal variability in different taxa and some other problems (Shneyer, 2009), none of them could be chosen to be a DNA barcode.

In subsequent years, numerous studies were carried out with the use of a complete or almost complete set of recommended barcodes, sometimes with addition of other loci (mainly those from the chloroplast genome). The objects were chosen based on both systematic (species of the same or close genera or family) and floristic (species from the same territory or location) principles. Though the *rbcL* gene was shown to be amplified better than other genes, it resulted in a rather low resolution; its combination with other markers does not always improve species discrimination, though it usually made it possible to assign a species to the corresponding genus. Amplification of the *matK* gene requires a larger number of primers (especially in the case of mosses and ferns), but it usually provides a higher resolution. The *trnH–psbA* spacer is characterized by a large number of indels and inversions; its length can significantly vary even in closely related species, which complicated the alignment in some genera, such as the genus *Gentiana* (Liu et al., 2016). At the same time, in the case of no or few such changes, this marker, alone or in combination with ITS, is able to provide sometimes a better resolution than *rbcL* and/or *matK* (Costion et al., 2011; Parmentier et al., 2013; Christina and Annamalai, 2014; Huan et al., 2018). However, in some other studies, the *trnH–psbA* spacer just slightly improved the resolution obtained for the *rbcL* + *matK* loci (Burgess et al., 2011).

As the number of completely sequenced chloroplast genomes deposited in the GenBank increased, the search for more variable regions providing better resolution than the standard DNA barcodes was intensified. Dong et al. (2015) reported that the *ycf1* gene (the second largest gene in the chloroplast genome with a length of ~6000 bp) demonstrated better resolution than the *rbcL* + *matK* + *trnH–psbA* combination in the analysis of a sampling covering 420 species from 67 families that included mosses, gymnosperms, and angiosperms. This gene also showed good results in the analysis of species of the genus *Paris* (Song et al., 2017). However, the studies of oaks in China (Yang et al., 2017) and plants from the genus *Kalidium* of the family Chenopodiaeae (Liang et al., 2017) did not show a high discriminating ability for this gene.

The authors of many studies stated that all chloroplast markers used in their work provided poor species discrimination (Roy et al., 2010; Arca et al., 2012; Federici et al., 2013; Alves et al., 2014; Wu et al., 2017). Significant intraspecific and low interspecific variability and a lack of barcode gaps between species were observed. The testing of seven DNA barcoding chloroplast markers for discriminating 71 species from the genus *Salix* completely failed, since a unique barcode was obtained for only one species (Percy et al., 2014). According to the authors of the study, the most probable explanation of this fact was a selective sweep, when the selection of a useful mutation results in the selection of concomitant alleles with the deletion of other alleles and, therefore, a corresponding reduction of the total polymorphism. Another explanation notes the low gene flow between populations (Petit and Excoffier, 2009; Federici et al., 2013), since the gene flow provided by seeds alone (note that organelles are transferred mainly by seeds) is less than that provided by seeds and pollen. Therefore, a more intensive search for nuclear markers was recommended for more successful species discrimination by DNA barcoding (Naciri et al., 2012).

Indeed, many studies demonstrated that the nuclear ITS region provided better resolution than chloroplast markers (Vivas et al., 2014; Liu et al., 2016). The study of a Brazilian species from the poorly studied family Sapotaceae, which is represented mainly by tropic evergreen trees and shrubs and is characterized by short-lived (i.e., rarely accessible) flowers required for species identification, showed that ITS provided the highest resolution, which was not improved by the addition of chloroplast markers (Vivas et al., 2014). At the same time, an intragenomic ITS polymorphism due to incomplete concerted evo-

lution was sometimes observed in allopolyploid species (Zarrei et al., 2015).

Many studies of medicinal plants, especially applied studies, showed that the ITS2 region provides better species discrimination than ITS1 and even ITS1 + 2 (Chen et al., 2010; Han et al., 2012; Michel et al., 2016; Wang et al., 2016). Species of the family Apiaceae, which include some medicinal plants, are poorly discriminated by nonexperts. Species-specific primers for the ITS2 region were developed for those Apiaceae species that can be confused with medicinal species, and a system was created for the examination of the corresponding plant material (Kim et al., 2016). Nevertheless, this situation is not common. The resolution of the ITS1 + 2 region, both alone and in combination with other DNA barcodes, was evaluated for *Primula* species (Yan et al., 2015) and taxa of the family Lauraceae (Liu et al., 2017). According to the results, it surpassed the resolution of the ITS2 region, both alone and in combination with other barcodes. The ITS1 region provided better species discrimination than ITS2 and ITS1 + 2 in the study of mosses of the *Racomitrium canescens* complex (Stech et al., 2013) and the group of legume plants from the subtribe Cassiinae (Mishra et al., 2016). Thus, the usability of marker regions, even with an equal rate and character of their evolution, may vary depending on the task of the study.

In some cases, within the same study the best species discrimination was achieved with the use of different loci for different plant groups. For example, a study of species from two families growing in the same region showed that ITS better discriminated species from the family Poaceae, while *matK* worked better with species of the family Chenopodiaceae (Yao et al., 2017). Different results were obtained from a study of two genera, *Ocimum* and *Thymus*, belonging to the same Nepetoideae subfamily of the family Lamiaceae. Species of both genera were not discriminated by the *rbcL* and *matK* loci, while the *trnH–psbA* locus discriminated *Ocimum* but not *Thimus* species. The most large-scale plant DNA barcoding project ("DNA Barcoding of Vascular Plants of Canada") included 96% of 5108 plant species and used three loci, *rbcL*, *matK*, and ITS2. All of these barcodes assigned the species to the correct genus (91–98% of the samples studied), but they were characterized by different levels of species discrimination: 81% for *matK*, 72% for ITS2, and only 44% for *rbcL* (Braukmann et al., 2017). The discrimination of species from Canadian Arctica was inferior to species from the southern forest regions, though the latter were characterized by a higher species richness.

In some studies none of the standard DNA barcodes or their combinations resulted in successful species discrimination. Such a situation was observed for some mosses (Hassel et al., 2013), the genus *Calligonum* (Li et al., 2014), and hawthorn *Crataegus* (Zarrei et al., 2015). The limitations of standard barcodes became the subject of numerous discussions (Hollingsworth et al., 2016; Coissac et al., 2016). These standard barcodes allows quite sufficient species discrimination for many applied tasks, but they are not able to identify all plant species or even to discriminate all species within large taxonomic groups or regional floras.

The resolution level provided by standard markers was especially low for evolutionarily young groups, such as Macaronesian species of the genus *Lotus* (Ojeda et al., 2014). An attempt to use two low-copy nuclear genes to discriminate species within this group (Hawaiian species from two genera, *Clermontia* (family Campanulaceae) and *Cyrtandra* (family Gesneriaceae)), was unsuccessful (Pillon et al., 2013). Due to high intraspecific variability, conserved ancestral alleles, and some other factors, these markers discriminated species even worse than chloroplast regions. Despite the numerous attempts to use standard DNA barcodes and some additional chloroplast regions, no sufficient species discrimination was achieved for the genus *Araucaria* from the New Caledonia islands, a region known as a biodiversity hotspot (Escapa and Catalano, 2013; Gaudeul et al, 2014; Kranitz et al., 2014). As a result, it was proposed to use complete chloroplast sequences for species identification. Even several years ago, nobody could dream of it. However, new technologies, such as next-generation sequencing (NGS), provided scientists with such an opportunity. The complete chloroplast genomes of 11 of 13 *Araucaria* species grown on the island represented by two to three samples were sequenced with NGS technology (Ruhsam et al., 2015). Researchers also sequenced 11 single-copy and low-copy nuclear genes and compared the species discrimination obtained with chloroplast genomes (~147 000 nucleotides) and 11 nuclear genes (>6000 nucleotides in total). In the first case, the discrimination was higher, though it still did not completely satisfy the authors, since more than half of the species were not monophyletic (Ruhsam et al., 2015). The authors concluded that the discrimination of young and actively radiating species requires a larger number of variable nuclear genes. Nevertheless, the use of complete chloroplast genomes was considered to be very attractive.

Soon after the development of NGS technology, the obtained sequences were proposed for use in DNA barcoding (Nock et al., 2011). However, NGS analysis is very expensive, so new and cheaper approaches were developed to obtain these sequences (Stull et al., 2013). NGS methods are characterized by the depth of sequence coverage (sequencing depth), an index showing how many times a nucleotide was read; the higher the index was, the higher was the chance to avoid errors during the assembly of the whole genome or its region. An approach called genome skimming was proposed. In this approach, a small depth of sequencing and rather low quality of total DNA reads

are virtually chosen and set; however, they still remain sufficient for a satisfactory assembly of repeated regions (ribosomal genes and organelle genomes). As a result, ~1 Gb of sequences can be obtained (Straub et al., 2012). This approach makes analysis cheaper and makes it possible to examine thousands of samples. In addition, it allows researchers to exclude rare variants and errors of different origins. Thus, genome skimming is likened to the Sanger sequencing, which is also unable to detect rare variants (Bakker et al., 2016). As shown above, the most variable and, therefore, the most discriminating markers are often different for different taxonomic groups of plants. The chloroplast genome sequence includes regions of standard DNA barcodes, as well as other genes and spacers. The most variable of these can be chosen for the corresponding plant group.

Another approach was proposed for the sequencing of low-copy nuclear genes: target enrichment, i.e., the obtainment of required gene sequences from genome DNA samples with oligonucleotide probes followed by their NGS sequencing (Nicholls et al., 2015; Hollingsworth et al., 2016).

Thus, with progress in sequencing technologies, the ideas for the use of a new genome data format for DNA barcoding appeared. These ideas finally resulted in the formulation of the so-called a twin track approach, which included (1) the accumulation of standard DNA barcodes and their libraries and (2) the active development and application of enlarged barcodes via genomic skimming (Coissac et al., 2016). An enlarged or extended barcode represents the sequences of the chloroplast genome, plus ribosomal genes obtained by NGS.

DNA barcoding often requires the use of herbarium samples, the total number of which in the world reaches dozens of millions. At the same time, these samples often provide only short (250–400 nucleotides) DNA fragments. The extraction of a sufficient amount of good-quality DNA requires a thorough selection of reagents and extraction methods (Särkinen et al., 2012). It was shown that successful DNA extraction depends not only on the sample age (it is significantly lower for samples older than 50 years) but also on the methods of sample drying, the pesticides used for the treatment of storage facilities, and the taxonomic group of the plant. For example, DNA extraction was observed to be very problematic for herbarium samples of plants from the family Boraginaceae (Kuzmina et al., 2017). As a result, the so-called DNA mini-barcodes (short DNA sequences less than 200 nucleotides in length) were developed for the Sanger analysis of materials, which presumably contain strongly damaged DNA (old herbarium samples, treated plant raw materials, canned plant product).

The search for such regions in the *rbcL* gene was performed *in silico*; the resulting sequences made it possible to identify samples up to the family level (Little, 2014). When the sequences of chloroplast genomes are known, particular solutions of applied tasks in relation to a given species are found from the search for the most variable DNA regions for these species. For example, *Panax ginseng* can be reliably discriminated from other related species with the following regions: 60 nucleotides of the *ycf1a* gene, 100 nucleotides of the *ycf1b* gene, or 280 nucleotides of the *rps16* gene (Dong et al., 2014).

An important advantage of the NGS methods is the possibility to read sequences of nuclear and chloroplast markers of several species at one time; this is especially essential for taxonomic authentication of herbal supplements and the control of their quality (Ivanova et al., 2016).

Since NGS methods use fragmented DNA, they were tested for their possible use in the analysis of herbarium samples (Bakker et al., 2016). The results of this test were positive, i.e., they allow researchers to obtain expanded barcodes from old samples (especially standards). Analysis of the DNA of museum (including herbarium) samples is now designated by a special term, "museomics" (Chomicki and Renner, 2015).

Degraded DNA presents not only in herbarium samples but also in medicinal herbal teas and other plant raw materials. NGS methods were seen to have a high efficiency as compared to Sanger sequencing for the examination of herbal medicinal products (Ivanova et al., 2016).

In the early beginnings of the DNA barcoding era, scientists understood and discussed the probable impossibility to discriminate closely related species by DNA barcodes (Shneyer, 2009). When it becomes possible to use DNA barcodes representing complete chloroplast sequences and ribosomal gene regions sequenced by NGS, some scientists propose to extend the application of DNA barcoding to the discrimination of even at lower taxonomic levels (subspecies and varieties) and to call it ultra-barcoding (Kane et al., 2012).

To date, the search for optimal DNA barcodes of plants continues.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement of the welfare of animals. This article does not contain any studies involving animals or human participants performed by any of the authors.

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