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Application of gene sequences in plant phylogenetic inferences

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Abstract

Molecular phylogenetic is the branch of phylogeny that analyzes hereditary molecular diversity, mainly in DNA sequences, to increase data on an organism‘s evolutionary relationships. Due to the taxonomic levels of the study, various molecular markers are applied in molecular phylogeny. The selection of molecular instrument is of paramount matter to ensure that a proper level of variation is meliorated to respond the phylogenetic question. In this review, we have been trying to discuss about gene markers used in the plant phylogeny at various taxonomic levels. The current gene markers used in phylogeny include: the ribosomal nuclear genes, low copy nuclear genes and the extra-nuclear genome (mitochondrial and chloroplastic genomes). Conserved regions could be used at higher taxonomic levels in phylogenetics studies and regions with more changes could be applied between closely related taxa. One of the most common sequences for studying the phylogenetic relationships at the generic and infrageneric taxonomic levels in plants is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron. Chloroplastic gene sequences have been used extensively at the family level and above but chloroplast non-coding sequences such as introns and intergenic spacers are used more frequently at lower taxonomic levels. Low-copy nuclear genes are most useful at the interspecific and intraspecific levels where cpDNA and/or nrDNA cannot provide adequate resolution. Evidence offers that for more strongly reconstruction of phylogeny, several discrete genes are needed. Now, uses of next generation sequencing (NGS) techniques are reported. Techniques for NGS are an alternative to prevalent methods that let access to hundreds of DNA regions.

Key words: rDNA, cpDNA, Low-copy gene, Plant phylogeny, Taxonomic level.

INTRODUCTION

Molecular phylogeny is the study of evolutionary relationships between organisms using molecular data. Due to the taxonomic levels of the study, various molecular markers are applied in molecular phylogeny (Small *et al*., 2004). Gene markers used in phylogeny include: the nuclear genome (ribosomal and non-ribosomal genes), the extra-nuclear genome (mitochondrial and chloroplastic genomes) and low copy nuclear genes (Yli-Mattila *et al*., 2000). The mitochondrial genome because of rapid changes in its structure is rarely used in plant phylogeny studies (Palmer *et al*., 2000). It is clear that low-copy nuclear genes are most useful at the low taxonomic levels where cpDNA and nrDNA cannot provide adequate information (Sanjur *et al*., 2002). The selection of molecular marker is very important to ensure that a proper level of variation is indicated. The gene regions with high mutation rates are proposed for inferring phylogenetic questions at lower taxonomic levels, while protected areas are used at high taxonomic levels. In this review, we have discussed about gene markers used in the plant phylogeny at various taxonomic levels.

What is the importance of mole-cular marker in phylogenetic studies?

In combination with clear methods for phylogenetic reconstruction, molecular data have changed meaning

of relationships at all levels of the taxonomic hierarchy. Different molecular markers are required for different taxonomic levels because of different rates of sequence evolution among genomes, genes and gene regions. The selection of molecular tool is of higher value to ensure that a suitable level of variation is cashed to answer the phylogenetic questions such as inferring taxon delimitation and determination of evolutionary relationships among organisms. The appropriate choice of molecule for analysis of phylogenetic relationships is significant for the reliability of the results (Small *et al*., 2004).

The requirements affecting the best selection of markers

The requirements affecting this choice include the following: (a) the molecule should not be too variable in evolution nor too stable to effectively distinguish the taxa of interest; (b) the molecule should occur within all the taxa; (c) the molecule should have a single origin predating the divergence of the taxa; (d) the molecule should change in evolution at a constant stochastic rate; (e) the phylogeny of the molecule should reflect the phylogeny of the whole organism (Yli-Mattila *et al*., 2000); (F) a single-copy gene may be more useful than multiple-copy gene; at the interspecific and intraspecific levels (g) primers should be available to selectively amplify the marker gene (Kjer, 1995) and (H) homology is also a fundamental meaning at the evolutionary genomics. Recognization homology relationships between sequences is the first fundamental phase in many biological research, and more particularly in inferring orthologs and paralogs genes (Fitch, 2000). When the similarity between two genes is due to divergency, they are orthologous genes. But if the similarity is due to the gene duplication in their last common ancestor, they are paralogous genes (Nei and Kumar, 2000). In the reconstruction of the phylogenetics trees, a full gene complex should be used, and if it was limited to use a gene set, that set should be orthologous gene. Using paralogous genes may cause the genetic tree and species tree to be incompatible (Vandamme, 2009). Horizontal transmission such as hybridization of closely related taxa, horizontal gene transfer and organelle acquisition through endosymbiosis is also important (Philippe *et al*., 2011). Homologs resulting from horizontal gene transfer between two organisms are termed xenologs. Xenologs can have different functions, if the new environment is vastly different for the horizontally moving gene (Fitch, 1970). Horizontal gene transfer (HGT) or lateral gene transfer (LGT) is the transition of genes from a species to another one through processes different from ancestral inheritance (or vertical transfer). HGT has been identified, as one of the major evolutionary forces motive prokaryote evolution (Worning *et al*., 2000). Phylogeny is generally represented as a tree because vertical evolution is the primary mechanism of inheritance for genetic material. However, the existence of horizontal transmission makes phylogenetic trees only practical approximations, which will probably be replaced by phylogenetic networks in the long term (Philippe *et al*., 2011).

Gene markers that are used in the plant phylogeny

Gene markers used in plant phylogeny include: the nuclear genome (ribosomal and non-ribosomal genes), the extra-nuclear genome (mitochondrial and chloroplastic genomes) and low copy nuclear genes. Because of its commonly slow rate of sequence evolution and rapid rate of structural evolution mtDNA usually has been ignored by plant systematists as a possible source of datum (Palmer *et al*., 2000). Mitochondrial DNA, because of its scarcity relative to cpDNA in most species, its greater complexity in size and its multiple molecular forms, has been less commonly used in phylogenetic studies than cpDNA. Mitochondria1 genomes are somewhat larger and more variable and may also show physical variations on a basic unit structure within a plant (Sanjur *et al*., 2002).

Application of chloroplast genomes in phylogenetics studies

Chloroplast genome organization is highly conserved within land plants (Kim and Jansen, 1995). Land plant cpDNAs usually contain 110–130 different genes. The majority of these genes (about 80) code for proteins, mostly involved in photosynthesis or gene expression; the remainder are transfer RNA (about 30) or ribosomal RNA (about 4) genes (Martin *et al*., 2013).

The genome of the chloroplast is composed of four regions: large single copy, small single copy and two inverted repeat regions that are located between large and small single copy areas (Jansen and Palmer, 1987). Although the gene content and organization of the chloroplast genome is evolutionarily conservative (Palmer, 1991) changes occur which are important in terms of phylogeny studies (Wicke *et al*., 2011). For example, the phylogenetic relationships of the Orchidaceae were determined using whole chloroplast genomes (Dong *et al*., 2018). Complete chloroplast genome at *Quercus* genus was used to revive phylogenetic relationships, identify the unique characteristics of the *Q. acutissima* cp genome and confirm non-monophyletic relationships in genus *Quercus* (Li *et al*., 2018). Also the relationships within

the Genus *Citrus* were elucidated with the analysis of 34 chloroplast genomes (Carbonell-Caballero *et al*., 2015). Even the complete chloroplast genome of *Tetragonia tetragonioides* was sequenced to study phylogeny and evolutionary relationships within Caryophyllales order (Choi *et al*., 2018).

Different evolutionary rates in diverse parts of cpDNA

Generally, in plants the mitochondrial genome evolves at the slowest rate, the chloroplast genome at a slightly faster rate and the nuclear genome at the fastest rate (Gao *et al*., 2008). The evolution rate of cpDNA is 2-3 times slower than the rate in nuclear genes and 20 times slower than animal mtDNA sequences, but 3-4 times faster than plant mitochondrial genes (Raubeson and Jansen, 2005). For this reason cpDNA gene sequences (e.g. *rbcL*, *atpB*, *matK* and *ndhF*) have been used extensively at the family level and above (Li, 2008). For example, phylogenetic relationships among families of Liliales were better defined than in a previous molecular analysis using four plastid loci: *matK*, *rbcL*, *atpB* and *atpF-H* (Kim *et al*., 2013). Also Molecular phylogenetics of Phyllanthaceae inferred from plastid *atpB, matK, 3'ndhF and rbcL* (Kathriarachchi *et al*., 2005).

Non-coding sequences such as introns (e.g. *rpL16*, *rpoCl*, *rpSl6*, *trnL*, *trnK*) and intergenic spacers (e.g. *trnT-trnL*, *trnL-trnF*, *atpB-rbcL*, *psbA-trnH*) are used more frequently at lower taxonomic levels (Wilson, 2009). For example, phylogenetic relationships of *Aristida* and relatives (Poaceae, Aristidoideae) has been investigated based on noncoding chloroplast *trnL-F* and *rpl16* (Cerros-Tlatilpa *et al*., 2011). Phylogenetics of *Impatiens* and *Hydrocera* (Balsaminaceae) has been investigated using chloroplast *atpB-rbcL* spacer sequences (Janssens *et al*., 2006). The *trnH–psbA* intergenic spacer region and its combinations have utilized as plant DNA barcodes (Pang *et al*., 2012). Phylogenetic relationships in subtribe Poinae (Poaceae, Poeae) have been investigated based on nuclear *ITS* and plastid *trnT-trnL-trnF* sequences (Gillespie etal., 2008). Molecular phylogeny of 35 generally accepted genera in Maleae (Rosaceae) was established using 15 chloroplast regions including *atpB-rbcL*, *matK, ndhF, petA-psbJ, psbA-trnH, psbM-trnD, rbcL, rpl16, rpl20-rps12, rps16, trnC-ycf6, trnH-rpl2, trnL-trnF, trnS-trnG*, and *ycf1* (Sun *et al*., 2018). Seven cpDNA non-coding regions (*atpB-rbcL, psbA-trnH, psbD-trnT, rpl32-trnL, rps16* intron*, rps16-trnK,* and *trnL-trnF*) were amplified and sequenced for a revised molecular phylogeny of *Typha* (Typhacecae) in order to infer the biogeographic history of the genus (Zhou *et al*., 2018). Given the relatively slow rate of cpDNA evolution, even non-coding cpDNA regions often fail to provide significant phylogenetic information at low taxonomic levels (Peterson *et al*., 2010).

Ribosomal DNA (rDNA) is the most exploited source for molecular systematic data

Ribosomal characters are the most exploited source of molecular data for systematic studies of organismal relationships (Hilu *et al*., 2008). In eukaryotes, the rDNA cistron encodes the 18S,5.8 S and 26S rRNAs, separated by the two internal transcribed spacers (ITS1 and ITS2), and flanked by the 5‘ and 3‘external transcribed spacers (5‘-ETS and 3‘-ETS). The cistron occurs in hundreds to thousands of copies, each separated by the intergenic spacer (IGS sensu stricto), at one to a few chromosomal loci. The 18S sequence forms the ribosomal small subunit, while the 5.8S and 26S form most of the large subunit (Rogers and Bendich, 1987).

Phylogenetic analysis of 18S rDNA sequences provide a critically required independent data set for the assessment of higher-level relationships in many instances. However, because of a conservative rate of evolution, analysis of these sequences alone will not provide the adequate resolution (Hershkovitz *et al*., 1999). The rate of evolution of 18S rDNA is about one-third to one-half that of rbcL, providing enough variable nucleotide positions to identify major clades. An incorrect concept about 18S rDNAs is that its sequences are highly disposed to insertion and deletion, so these indels make 18S rDNA sequences difficult to align while indels largely occur in a few helical regions and so cause few alignment problems, even across the angiosperms. The angiosperm I8S rDNA is a mosaic of highly conserved and highly variable regions. These conserved and variable domains were observed not only across the angiosperms, but also within specific clades, suggesting that some regions evolve quite rapidly and may be under limited selective presure. A transition/transversion ratio of roughly 2: 1 was observed in the angiosperms and within several specific clades (Soltis *et al*., 1998). A series of studies investigated the utility of 18S rDNA for phylogenetic inference in plants (Hamby and Zimmer, 1988, 1992; Nickrent and Franchina, 1990; Nickrent and Soltis, 1995; Kron 1996; Soltis *et al*., 1997). These studies provided important early insights into phylogenetic relationships. The phylogeny of land plants has been inferred from 18S rDNA sequences (Soltis *et al*., 1998)**.**

Within the ITS, there is a largely ignored coding region, the 5.8S rRNA gene. Similar to the other coding

regions, the 5.8S region evolves relatively slowly but, because of its location within the ITS, it is generally used only as an alignment tool. Although 5.8S appears to have evolved somewhat faster than 18S, the small size of this molecule limits its utility in angiosperm phylogenetics (Hershkovitz and Lewis, 1996).

26S rDNA sequences are composed of conserved core regions, which are alignable across kingdoms, and divergence domains or expansion segments which evolve more rapidly and are the loci of the majority of length mutations for this gene, presumably due to reduced functional constraints. Protected core areas could be applied at higher taxonomic levels and expansion parts could be used among more closely related taxa. Plant 26S rDNA sequences contain significant phylogenetic signals in both conserved core regions and expansion segments. These sequences are easily aligned, evolve 1.6 to 2.2 times as fast as 18S rDNA, and yield 3.3 times the number of phylogenetically informative characters. Conserved core regions evolve 0.59 to 0.82 times as fast as entire 18S rDNA sequences and provide approximately the same number of phylogenetically informative characters and therefore, should be appropriate for phylogeny reconstruction at taxonomic levels similar to those detected with 18S rDNA. Expansion segments evolve at a rate of 6.4 to 10.2 times as fast as the conserved core regions of 26S rDNA; they may need to be appropriately weighted or perhaps excluded, from phylogenetic analyses at much higher taxonomic levels but they appear to be informative at or below the interfamilial level in angiosperms (Kuzoff *et al*., 1998). Furtheremore, because of the much longer length of the 26S rDNA region (~3400 bp, compared with ~1800 bp for the 18S rDNA), complete sequencing of the 26S rDNA is behind the 18S region sequencing (Maia, 2014). The utility of partial 26S rDNA sequences was illustrated in early studies (Hamby and Zimmer1992; Bult and Zimmer 1993; Bult *et al*., 1995; Stefanovic *et al*., 1998).

A phylogeny inferred from 26S rDNA sequences showed that *Cuscuta* is a derived member of Convolvulaceae. *Cuscuta* is a parasitic angiosperm that has been considered alternatively either as a genus within Convolvulaceae or as a monogeneric family in its own right (Neyland, 2001). Nuclear 26S rDNA sequences were used to corroborate and test previously published matK-rbcL-based hypotheses of phylogenetic relationships in Cornales (Fan and Xiang, 2003). An example of phylogenetic utility of 26S rDNA sequences below the genus level is phylogenetic relationships within *Cornus* (Cornaceae). The 26S rDNA sequence data of *Cornus* consist of 12 expansion segments spanning 1034 bp. These expansion segments evolve about four times as fast as the conserved core regions (Fan, 2001).

A large combined data set of complete 18S-26S rDNA sequences yields a very rational, but poorly supported, overall picture of angiosperm phylogeny. In summary, targeted sequencing of 18S/26S rDNA is not supported by NGS. However, it has been suggested that the 18S/26S rDNA data be used rather than simply discarded; because these regions provide useful phylogenetic information and are abundant in next-generation sequencing runs (Maia *et al*., 2014).

Ribosomal ITS sequence is one of the most popular sequences for plant phylogenetics

One of the most common sequences for studying the phylogenetic relationships at the generic and infrageneric taxonomic levels in plants is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron (Baldwin *et al*., 1995). The 18S, 5.8 S and 26s rRNAs are separated by the two internal transcribed spacers (ITS1 and ITS2). The discrimination power of ITS1 is higher than ITS2 (Hollingsworth *et al*., 2011). ITS sequences have been widely used in molecular evolution studies in plants including several taxonomic levels (Bellarosa *et al*., 2005; Kan *et al*., 2007; Queiroz *et al*., 2011; Ghada *et al*., 2013; Fan *et al*., 2014; Kim *et al*., 2015; Doh *et al*., 2016; Almerekova *et al*., 2017; Terra *et al*., 2017; Azani *et al*., 2017; Murphy and Tranel, 2018). Even, using of ITS sequence has been able to resolve the relationships at the population level. We studied the systematics of *Onobrychis* genus (Fabaceae) and in line with it, molecular phylogeny of different species was investigated. For example, we used ITS sequences for separating subsections in *Onobrychis* sect. *Onobrychis* (Toluei *et al*., 2012). Variation of Iranian *Onobrychis carduchorum* populations is an example of using ITS sequences data in population level (Toluei *et al*., 2013a). In the molecular characterization of *Onobrychis altissima* populations from Iran, *O. chaldoranensis* was descripted based on morphology and ITS sequences (Toluei *et al*., 2013b). Also molecular phylogeny and ecogeography of *Onobrychis viciifolia* Scop. evaluated with ITS sequences and different populations of *O. viciifolia* were separated (Toluei *et al*., 2013c). We used ITS sequences along with trnL-F of the plastid genome at *Moltkia* and *Anchusa* genera to revive phylogenetic relationships, introduce new species and determine non-monophyletic or monophyletic groups (Toluei, 2018). Furthermore, other studies in population level have been done such as phylogenetic status of *Oreophysa*

microphylla (Fabaceae-Galegeae) based on ITS region (Kazempour Osaloo *et al*., 2006). Also infrageneric relationships in *Astragalus* was investigated based on ITS sequences (Kazempour Osaloo *et al*., 2003, 2005). Nuclear ribosomal ITS sequences were used to access intraspecific genetic diversity in 23 species of the genus *Passiflora.* The results indicate that ITS may be a useful tool for the evaluation of intraspecific genetic variation in *Passiflora* (Mäder *et al*., 2010).

Since 18S–26S rDNA arrays reside in the nuclear genome, ITS sequences are biparentally inherited and are thus distinguished from the cpDNA loci in widespread use (Baldwin, 1992). Some of the earlier studies demonstrated how valuable this property is for revealing past cases of reticulation, hybrid speciation, and parentage of polyploids (Kim and Jansen, 1994, Rieseberg and Wendel, 1993).

Because there are hundreds to thousands of nuclear rDNA repeats in plant genomes, they are more easily isolated than most low-copy nuclear loci, requiring little experimental expertise to successfully amplify. Excluding gymnosperms, both the high copy number and the small size of the target DNA fragments facilitate ITS amplification by PCR, even permitting the use of ancient material, herbarium specimens, and samples other than from living material (Maggini *et al*., 2000). The conservation of specific nucleotides in secondary structures of ITS is a useful tool to improve the alignments (Keller *et al*., 2010). Analyses of ITS secondary structures obtained from the *Passiflora* species was a valuable source of information for improvement of alignment, considering the difficulties of aligning species from the various subgenera (Mäder *et al*., 2010), and the same strategy could be used in other plant genera that present a complex taxonomy (Giudicelli *et al*., 2017).

Utility of low-copy nuclear gene sequences in plant phylo-genetics

Low-copy nuclear genes have a great potential to compensate cpDNA and nrDNA for the improvement resolution and robustness of plant phylogenetic reconstruction. Low-copy nuclear genes, are used in plant phylogenetic studies in solving the evolutionary dynamics of nuclear gene families. Most protein coding nuclear genes of plants have exons and introns. Exons, which are usually under strong purifying selection that eliminates deleterious mutations, have relatively slow rates of nucleotide substitution. Unlike chloroplast genes, introns of low-copy nuclear genes have rather vast rates of nucleotide replacement (Sang, 2002). A nuclear gene with regions diverging at variable rates can potentially provide phylogenetic markers at various taxonomic levels (Zimmer and Wen, 2015). Highly conserved low-copy nuclear genes are easy to clone and align, and are more phylogenetically informative than widely used organellar genes. These nuclear genes have multiple intrinsic qualities enabling them to be acceptable markers for reconstructing angiosperm phylogeny, even eukaryotic relationships, further providing new sights into the evolutionary relationships of angiosperms (Zhang *et al*., 2012). Molecular phylogenetic analysis of important genes will enhance new girth to systematic and evolutionary studies of plant variety (Sang, 2002).

Interfamilial and higher levels

Nuclear ribosomal DNA, chloroplast genes and mitochondrial genes have proven useful at high taxonomic levels because they evolve at relatively low rates. Some low-copy nuclear genes have been applied to survey relationships at such high taxonomic levels. For instance, The phytochrome genes, answered as a useful marker for realization the primary diversification of angiosperms (Donoghue and Mathews, 1998). Five low-copy nuclear genes (SMC1, SMC2, MSH1, MLH1 and MCM5) of 91 angiosperm species from 46 orders and three gymnosperm species were obtained for resolving angiosperm phylogeny (Zhang *et al*., 2012). A total of 96 single-copy nuclear gene loci were identified from the KOG (eukaryotic ortholo-gous groups) database for identification of nuclear low-copy genes and their phylogenetic utility in rosids, most of which were first used for phylogenetic analysis of angiosperms (Wang *et al*., 2015). Single copy genes were used to infer phylogenetic relationships between major seed plant taxa. In total, 3072 single-copy genes in 31 gymnosperms and 2156 single-copy genes in 34 angiosperms were identified. All studied seed plants shared 1469 single-copy genes, which are generally involved in functions like DNA metabolism, cell cycle and photosynthesis (Li *et al*., 2017).

Intergeneric level

In contrast with the interfamilial and interspecific levels, nuclear ribosomal DNA has been less employed for phylogenetic studies at the intergeneric level. This is perchance because sequences of the small and big subunits of nrDNA diverge too slowly to yield enough phylogenetic data among closely related genera, while the internal transcribed spacers diverge too quickly to be aligned unambiguously among distantly related genera (Sang, 2002). Low copy nuclear genes have provided useful phylogenetic markers at the intergeneric level. Galloway *et al*. (1998) tested the phylogenetic utility of

the arginine decarboxylase gene (Adc) in Brassicaceae. Relationships among these genera are well resolved and supported (Galloway *et al*., 1998). The phylogenetic relationships of tribe Areceae were determined using two low-copy nuclear genes including malate synthase (MS) and phosphoribulokinase (PRK) genes (Lewis and Doyle, 2002).

Interspecific level

At the interspecific level, sequences of chloroplast DNA, including noncoding regions, usually diverge too slowly to resolve close relationships (Sang, 2002). ITS sequence variation is not always sufficient to resolve closely related species. Thus, nuclear genes with rapidly evolving introns, are needed for a full resolution of interspecific phylogenies. For example, Alcohol dehydrogenase genes (*Adh*) were used to study relationships within the genera *Paeonia* and *Gossypium* (Sang *et al*., 1997). The phylogenetic resolution of 19 previously published nuclear regions in Leguminosae was reassessed using 18 species from two clades of the Caesalpinieae representing both distantly related genera and closely related species. Phylogenies reconstructed from the intron-spanning regions AIGP, SHMT, AT103 and EIF3E showed the best phylogenetic potential for studies of closely related species of caesalpinioid legumes and were congruent with ITS and plastid data (Babineau *et al*., 2013). Phylogenetic reconstruction using four low-copy nuclear loci strongly supports a polyphyletic origin of the genus *Sorghum* (Hawkins *et al*., 2015). Single-copy nuclear intron markers were developed for species-level phylogenetics with a case study in Paullinieae (Sapindaceae)(Cherry *et al*., 2017).

Intraspecific level

Plant organelle genes diverge too slowly to resolve population-level relationships. Low-copy nuclear genes with rapidly evolving introns provide an appealing source of DNA sequence data for phylogenetic reconstruction at the intraspecific level (Schaal and Olsen, 2000). Genetic variation at the intraspecific level was assesed in two congeneric species of *Cereus* (Cactaceae: Cereeae). The low copy nuclear gene phytochrome C (*PhyC*) was used as nuclear genes complement of the plastid DNA in phylogenetic studies (Silva *et al*., 2016).

Which markers are most effective in plant phylogenetics?

Plastid DNA and nuclear rDNA are used more than other genes in phylogenetic studies and are amplified simply with universal primers. Low-copy nuclear genes have not been widely pursued because the difficulty in their isolation and characterization (Small *et al*., 2004). Single-copy nuclear genes are biparentally inherited, thus they can expose evolutionary kinship for diploid (Cronn *et al*., 2003) or polyploid (Small *et al*., 1998; Liu *et al*., 2001; Cronn *et al*., 2002b) species. Low-copy genes are rarely topic to concerted evolution (Cronn *et al*., 1999; Senchina *et al*., 2003). The last decade has been centralized on low copy nuclear genes for investigating evolutionary relationships between species because chloroplast genome is not adequate to resolve relationships among closely taxa in some groups (Rieseberg *et al*., 2000; Ishikawa *et al*., 2009; Soltis *et al*., 2009). Recently, single-copy genes were identified as molecular markers for phylogenetic study in seed plants (Li *et al*., 2017). Chloroplast and nuclear ribosomal genes are dominant sequences for plant phylogenetic analysis. Until lately, a few low copy nuclear genes were applied in such studies. Single and low copy nuclear genes have quickly evolutionary rates. Now, use of next generation sequencing (NGS) techniques that are easier and cheaper has been reported (Zimmer and Wen, 2015). Evidence offers that for stronger surveies of phylogenetic trees and branch times, several discrete genes are needed. Most phylogenetic trees for non-model organisms are based on single sequences or only a few regions use prevalent sequencing methods. Techniques for enormous parallel sequencing or next generation sequencing (NGS) are alternatives to prevalent methods that let access to hundreds of DNA regions. These methods were used to resolve the phylogenetic inconformity found in *Polystachya* Hook (Abreu *et al*., 2018). Mitochondrial genes are suitable sequences for investigating some distant evolutionary relationships because of their slow rate of evolution (Hiesel *et al*., 1994). Chloroplast genes are not always the best choice for phylogenetic studies. Non-coding sequences may be better. It should be tried to identify regions with the enough variation rate (Cascales *et al*., 2017). DNA barcoding is the procedure of recognition of species based on nucleotide diversity of short DNA regions (Little and Stevenson, 2007). It has been developed rapidly in recent years, and become a useful tool for biodiversity investigation, molecular phylogeny and evolution (Hollingsworth *et al*., 2011; Kang *et al*., 2017). DNA barcoding methods rely on the usage of chloroplast gene sequences. Because of the low evolutionary rates of chloroplast genes, there are few choices appropriate for molecular studies on angiosperms at low taxonomic levels and DNA barcoding of species. In plants, due to the hardness in the discovery of a universally passable barcode, it is yet to be well established (Jurado-Rivera *et al*., 2009). In 2009, the Consortium for the Barcode

of Life (CBOL) plant working group proposed the chloroplast gene rbcL and matK as the core barcodes of plant species, as well as intergenic sequence trnH-psbA and the nuclear gene ITS as the supplement barcodes (Hollingsworth *et al*., 2011; Dechbumroong *et al*., 2018).

CONCLUSION

A gene with regions diverging at variable rates can potentially provide phylogenetic markers at various taxonomic levels. Conserved regions could be used at higher taxonomic levels and areas with more changes could be employed among more closely related taxa. At any taxonomic level, if cpDNA and nrDNA phylogenies are poorly resolved, weakly supported, and/or incongruent with each other, low-copy nuclear genes should be considered. Low-copy nuclear genes are most useful at the interspecific and intraspecific levels where cpDNA and/or nrDNA cannot provide adequate resolution. Although there are large numbers of phylogenetic markers available, the researcher should not be limited only to these genes. In fact, there is a need for developing additional markers for phylogenetic analysis.

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