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Searching for DNA Barcodes in Plants

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Abstract: The utility of large scale standardized DNA sequencing and bioinformatics tools are now increasing in the area of plant systematics and evolution while a short DNA segment can be effectively used for the identification of species. However the consensus is still lacked about the precise DNA segments that are to be used for identification. DNA barcoding was proposed as a technique for recognizing and identifying a eukaryotic species through comparison of sequences of a standard short DNA fragment referred as a DNA barcode from an unknown specimen to a library of reference sequences from known species. An optimal DNA barcode region is a small fragment which is present in all representatives of the major taxonomic group which is accompanied by invariable nucleotide sequence in all members of the same species, but with enough variation to discriminate among the species. DNA barcoding is accomplished in animals in which Cytochrome c oxidase gene [CO1] is utilized as a barcode but finding a barcode in plants is proved to be a challenge. Several fragments of DNA [*rbcL, matK, rpoB, rpoC, trnh-psbA* etc] or combination of these segments have been proposed for identification of plants and systematic studies. Internal transcribed spacer region of nuclear ribosomal DNA are also evolving as a potential barcode for the major plant groups. With no doubts DNA barcoding will be reshaped in the future. The present review highlights the potential of DNA barcoding for the characterization of land plants and future prospects.

Key words: Barcode · DNA Sequencing · *Rbcl* · *Matk*

INTRODUCTION

Species classification and identification is generally been considered as a job of expert taxonomists who tend to provide a nomenclatural backbone that serves as a basic ingredient in all kinds of biological studies. Today life scientists have to solve many complex issues like maintaining the biodiversity, ensuring the biosecurity, to stay aside from pandemics and protect the species. Accomplishment of these goals greatly depends on the global partnerships and relies on our ability to rightfully identify the specie [1]. Identification, authentication and drawing lines among the species correctly is critical for the diversity of life due to its capability to determine either these organisms belong to the same parent or not [2]. In spite, the knowledge with expert taxonomist, they may not cover all the taxon identifications upon a non-specialist request.

In biology identification is the keystone [3] therefore, different techniques and tools are used to make identifications and place different organisms into groups which is based on certain characteristics. Species identification hinge on the knowledge of taxonomists for whom it's very difficult to identify all the taxon of organisms requested by the non-taxonomists. To circumvent these hurdles the project 'DNA Barcode of Life' was developed aiming to standardizing, rapid and inexpensive methods that can be used by non-professionals.

DNA barcoding is an innovative technique which allows identification of an organism at any stage and aims to provide accurate, rapid and automatable identification of species using a short, standardized DNA fragment referred as DNA barcode of a gene region and comparison of such internal specie tag of an unknown sample to a library of known sequences.

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It helps to identify organism at any point of growth from a very lesser sample of tissue, fresh or conserved many years ago.

Theoretically any constant, nonzero sequence dissimilarity that discriminates between the two species can work as DNA barcode. Moreover, for a DNA barcode there is no need of demonstration of homology mutations like that require for the phylogenetic marker. In simple words for the species identification low level of difference is enough to discriminate among the species if no phylogenetic relationship is available.

What Does DNA Barcoding Mean?: As mentioned by Hebert *et al*. [4] the diversity of life calculated by number of species is overwhelming. Using current techniques of description and publication, sometimes it takes decades by the plant experts to define in the 10million-15million species. In order to overcome these hurdles DNA barcoding is considered as short cut that would speed up the identification of species. These are short segments of DNA of (800-1400 bp] belong to a gene sequence quickly evolving so that to differentiate species and also have flanking regions that are sufficiently conserved to facilitate the barcode region to be examined by the universal primers. DNA barcoding can be not only used for previously identified species but it can also describe species that are controversial and morphologically confusing which are not yet assigned its classification.

"DNA barcoding" aims to develop an inexpensive, fast and standardized method for specie identification that is accessible to the other non-taxonomists [1]. DNA barcoding possess the notion that a short standardized DNA sequences referred as "DNA barcode" that can differentiate species, as some times between species genetic variation go above than within species [4]. A massive DNA barcode library will be serving as a standard to which the barcode of the test specimen, which are actually the unidentified or falsely identified samples from the herbalists, gardens and forests, will be compared [5].

The identification of plants through molecular techniques can be used in many scientific and applied sciences. It would help in finding new species of plants as well as animal groups. An ideal DNA barcode regions is that which is a small stretch of nucleotides existing in all species of the main taxonomic group. The region that is used as a DNA barcode must be flanked by little variable regions so that the universal primers created for this region can amplify it easily.

The Barcode of Life Project: The intention behind this project was to create a system for eukaryotic organisms that can be adopted worldwide inventory based on a standard molecular approach. It was initiated in 2003 by researchers at the University of Guelph in Ontario, Canada and promoted in 2004 by the international initiative, with the secretariat at the National Museum of Natural History in Washington. In 2003 at the University of Guelph in Ontario, Canada it was started [http:// www.barcoding.si.edu] and further supported by the international 'Consortium for the Barcode of Life' (CBOL] through an international initiative in 2004. The CBOL comprise of five working groups, namely Database Working, Technology Development Working Group, Data Analysis Working Group, DNA Working Group and plant working group [PWG CBOL). They formed a project comprising researchers from seven countries and 11 organizations. Kew Royal Botanical Garden (London] was the chief organizer and key coordinator. The number of its member organizations increased to 150 from 45 countries including natural history museums, herbaria, botanical gardens, university departments, private companies and government organizations.

The motivation behind the DNA barcode project is not to construct the tree of life or to do molecular taxonomy [6, 7] but relatively to form a simple analytical tool centered on strong taxonomic knowledge that is gathered in the reference library of DNA barcode [8]. Singham and Hebert, [9] reported that the DNA barcode of life Data System [BOLD, http://www.boldsystems.org] has constantly been created since 2004 and was officially established in 2007. This Data system helps in the acquisition, analysis, storage and publication of DNA barcode archives.

Five most capable regions were selected for plants for more work by the PWG CBOL site [www.kew.org/barcoding] by the end of 2005. These regions comprise of matK (maturase), rpoB (RNA polymerase â subunit), accD (â subunit of acetyl-CoA carboxylase, an enzyme of fatty acid biosynthesis), rpoC1 (RNA polymerase â' subunit) and ccsA (previously known as ycf5; the gene encoding a protein involved in Cytochrome c biosynthesis).

At the third International Barcoding Conference Plant Working Group (PWG] of the consortium for the Barcode of life (CBOL) recognized the use of mat K and rbcl as the fundamental barcodes while Itpb and ITS2 as additional regions for the identification of plants.

Barcode for Land Plants: The procedure of selecting DNA barcode in plants is more difficult in plants compared to the animals, the CO1 gene is a good barcode in plants only for algae (DNA barcoding is required due to its higher degree of morphology and difficult to characterize), like some of its genera of red [10], brown [11] and diatoms [12]. The genome of plants is usually considered as having by huge structural rearrangements of the mitochondrial genome and having imported sequences from nuclei and chloroplasts [13]. Unlike the animals mitochondrial genome the rates of substitution and structural rearrangement in the mitochondrial genome greatly vary among different plants [14, 15]. Due to these issues the plants mitochondrial genome is rarely used in plants evolution at species level [16]. Due to these kind of issues the mitochondrial gene CO1 cannot be used as an effective barcode for plants.

DNA barcoding is a competent procedure in many animals groups for species identification by sequencing the mitochondrial gene CO1 [4]. Unlike the animals the mitochondrial DNA genes CO1 cannot be used for the plants due to some reasons. The main reason is that in pants the synonymous substitution is very low, about 50 to 100 fold lesser than the genes of mammalians, lower 2 to 3 fold in chloroplast genes and 10 to 20 fold lesser than in genes of nucleus [17, 18].

Kress *et al*. [19] initially search the plastid regions that resulted in the 7 leading candidate regions [20, 21]. Among these 7 barcode regions four belongs to the coding regions [rbcl, rpo, matK, rpo and rpoC1], while the remaining 3 are goes to the noncoding spacers [trnH–psbA, psbK–psbI and atpF–atpH]. In the search for an efficient barcode region different researchers suggested many combinations of these loci but fail to make any consensus [22]. Absence of agreement on these kind of issues hindered advancement in plant barcoding. We know that in animals there is a well establish DNA barcode region of Cytochrome c oxidase region CO1 of mitochondria. While on the other hand, finding a DNA barcode region in plants is more disputing job. Usually there is world widely no DNA region for the plants that carry all required benchmarks. So for this purpose a two or multi step methodology should be applied.

Different sections of the chloroplast genome like (matk, rbcl, trnH-psbA, rpoB and rpoC) in such an order that two or three fragments of these were proposed with higher capability but more demonstrating samples should be selected for getting the best contestant.

Nuclear rRNA genes have internal transcribed regions called spacers [ITS] can also be taken as DNA barcode because it is highly variable and is mostly used in phylogenetic studies but it also has some short comings.

Contribution to the Taxonomy: The DNA barcoding will turn the complicated Linnaean taxonomic system reachable and will aid to different masses like conservationists, ecologists and those organizations which are responsible for the controlling of invasive species, pests and for the food safety.

Beside the assigning samples to identified species, the DNA barcoding technique will speed up the process of finding new species via helping the taxonomists to constantly classify the specimens and underlining the contrary groups that may be new species.

By taking advantage of all these skills the DNA barcoding promises a lot of opportunities to the taxonomists to increase their work and finally get a fully completed worldwide biological record.

As whenever any new technology emerges it also creates so many controversies. Although this technology has great possible remunerations both to the experts and consumers of taxonomy, in some scientific areas it has been disputed [23, 24]. Some opponents considered DNA barcoding as against the taxonomy," and debating the enactment of this technology will point to the death of the system that takes 250 years in building. In our view this concept arise due to some miss understandings about the work of DNA barcoding. In reply to arguments like this we want to highlight many positive effects of this technology for taxonomy and biodiversity.

Standards for the Selection of Efficient Barcode Region: Selection of DNA barcode seems quite an easy task but that is not the case. Selection of a suitable locus as barcode from plants is complex and difficult. Up till now, there is not as such consensus among the scientific community regarding a universal plant barcode but they are already envisioning the applications of such plant molecular markers that can be employed in a wide paradigm of research. It is proposed that beside the help in plant taxonomy, plant barcode can aid to the international trade of species on the brim of extinction. It can be used in the quantitative comparisons of genetic diversity. A conserved region such as *rbcL*, can be exploited for forest dynamics and functional evolution of traits [5].

As compared to animals, finding a standard plant barcode is being an issue yet to be resolved. Many scientists have already acknowledged that a single genetic marker is not enough to differentiate exactly one plant species from another but one has to find sets of multiple markers which can discriminate plant species from one another. [25]

Kress and Erickson [5] have indicated some very important factors that should be kept in mind before conceiving and choosing a plant DNA barcode.

To become an efficient DNA barcode, the gene area must have fulfilled following conditions:

- The barcode must be capable enough to differentiate the specie which alternatively means that a barcode should possess enough specie level resolution
- Should have conserved flanking regions so that regions that can be used to develop universal primer sets for plethora of taxon so that it can be used in universal PCR amplifications.
- A barcode should possess short sequence for easiness in DNA extractions and reliable amplification by PCR.
- Lastly a plant barcode should be handled effectively with bioinformatics tools and analysis.

It is difficult to find a 4 in 1 barcode, because of the tradeoff relationship that is prevalent because the universal primers can generally be found for the highly conserved region flanked by the hyper variable intergenic spacers. It should be supportive to bidirectional sequencing [26].

Different region of the genome are recommended to be best barcodes for plants but none of them is very much acknowledged among the taxonomists. The reason behind this disagreement is due to some shortcomings confronted by the plastid marker comparative to plant CO1 and since of a measurable circumstance that what region of the gene should be used as a plant barcode is not yet existed.

For a DNA barcode to perform well, the variation between the species must be as much clear so that they may be differentiate easily from each other, nevertheless it must be little adequate within the species that a rich threshold amongst the inter and intra specific distinction can be observable. Two things are very important characteristics of a DNA barcoding loci, one is the presence of conserved flanking segments to support the routine amplification through highly different groups and enough internal variability to allow species differentiation but with a relatively low level of intra specific variation.

Moreover, other positive features of a good DNA barcode is its short length that helps in sequencing with which a sub optimal material, in the absence of heterozygosity the polymerase chain reaction occur trailed by sequencing but not cloning, absence of problematic DNA sequences, like that regions with many microsatellites DNA, which cause reduction in sequence quality, universal capacity of the sequence to get amplified/sequenced with standardized primers and easy alignablity and ability to mend efficiently from the herbarium samples and other forms of degraded material [25].

Other Proposed Barcodes in Plants: Following the debate various plant barcode combinations were proposed by scientists. However there are many types of DNA barcodes that can be used as universal but still it is not yet decided that which one is the best one for any new species [23, 27]. The process of exploration for a potential DNA barcode is done on testing a few plants that covers only a few plants from distantly related terrestrial plants [19, 29, 30].

Generally the genes taken from angiosperms as barcode are rpoB, matK, rpoC1, accD, ndhJ and YCF5and while in non-angiosperms ndhJ, matK, rpoB, accD and rpoC1 are used (http://www.rbgkew.org.uk/ barcoding/index.html).The plastid gene matk was suggested as universal Barcode for those plants that bears flowers because of his high capabilities of identifying the variation, easy amplification and alignment [29].

New master [27] studied matk and the intergenic spacer trnH-psbA in Myristicaceae and Seberg and Petersen [22] in Crocus and established them as suitable land plant barcodes. In angiosperms the internal transcribed spacer areas of nuclear ribosomal DNA [ITS] is normally suggested as barcodes due to the facts that these are frequently vastly variable at the species and generic level and within single individual different copies are frequently present. Even though ITS can act as a best barcode in many plant taxa and can be a suitable additional locus, there are many circumstances where they show incomplete concerted evolution and intra-individual variation reduces his ability to become as a universally acceptable plant barcode.

Kress *et al*. [19] also recommended the trnH-psbA plastid intergenic spacer region could become an appropriate candidate as universal barcode for land plants. Simultaneously, Consortium for the Barcoding of Life Plants Working Group (CBOL-PWG) studied a number of other genomic regions as candidate for barcode ignoring trnH-psbA due to its complex form of molecular evolution. Taberlet et al. [30] suggested the chloroplast intron trnL region as a possible barcoding candidate gene in angiosperms. Further, three regions viz. psbK-psbI, atpF-atpH and matK were proposed [20]. De ley *et al*. [31] contended that non-coding regions trnH-psbA and atpF-atpH should be considered as suboptimal barcodes owing to presence of microsatellites.

It is evident from the studies that the plastid genome is very slowly evolving compare to other genomes and shows intra molecular recombination [14], so there for it was assumed that more than one barcode is needed to provide sufficient variation so that this technique can work properly [33, 29]. Kress and Erickson [22] suggested to conglomerate the original trnH-psbA barcode from Kress *et al*. [19] along with rbcL (which is not variable enough at species level for many plant groups). This grouping is also have the capability to potential be used as a universal barcode.

Hollingsworth *et al*. [33] calculated the key seven candidate plastid regions, rpoB, rpoC1, matK, rbcL, atpF-atpH and psbK psbI and trnH-psbA among the three different groups of land plants. It was noted that rpoC1is the best universal locus and was very good amplified among all three groups. The trnH - psbA exhibited highest universality of the noncoding sections of the DNA. In specific taxonomic groups, for the identification of species, the coding locus shows good performance. Through these groups, not a single locus exhibited high degree of resolvability and universality. The distribution of Inter-specific sequences from single loci was mutual. But when these several loci were shared, very less number of barcodes were shared amongst species. Very less amount of enhancements on these were gained using many new three-locus groupings involving matk, trnH-psbA, rbcl, rpoC1 and trnH-psbA, however no single combination clearly overtook all others.

This report further points to the necessity to carry out enormous work towards standardizing the candidate genes in each plant species. Chase *et al.* [28] proposed to make the universal barcodes with the combination matk + rpoc1+ rpoB and matk + rpoc1 + trnH & psbA. The combination of two-locus of rbcL+ matK has been suggested for the terrestrial plants as the main barcode [34]. The five loci, viz. rps4, rpoC1, trnH-psbA, trnL- trnF and rbcl in bryophytic plants, were amplify easily and sequence and indicated substantial inter specific genetic variability, showing that these could become a potentially useful DNA barcodes. TherpoC1 and rbcL was considered as superlative performing single loci in the coding regions [35].

Recently, CBOL (Consortium for the Barcode Life) has approved the use of *rbcL* and *matK*, plastid loci as official barcode for the land plants while waiting for further studies on *trnH-psbA* to be used as barcode [34, 36].

Conserved Chloroplast DNA: Basically plants possess three genomes i.e. nucleus, chloroplast and mitochondria. Chloroplast DNA [cpDNA] possesses the most preferred DNA sequences for phylogenetic investigation. The reasons are: (1) it is relatively easy to purify, characterize, clone and sequence [37] (2) it evolves at a conservative rate [38] providing an excellent window of genetic resolution for phylogenetic studies among major taxonomic groups; and (3) it has a large body of information on the molecular structure and organization to study evolution of chloroplast itself [37].

***rbcL*:** The *rbcL* gene that codes for "RUBISCO"; ribulose-1, 5-bisphosphate-carboxylase/oxygenase a free enzyme present in stroma is present in the single copy region of chloroplast genome and the coding region is separated by intergenic spacer [600-800] nucleotides [39]. Among the different loci of plastids, *rbcL* is the most well characterized gene and is sufficiently reported for the recovery of bidirectional sequences of high quality. It is now considered as an integral component for specie discrimination [26]. The *rbcL* gene sequence is applied on a wide variety of taxa to resolve the taxonomic issues.

The *rbcL* based DNA barcoding has been successfully used to resolve the taxonomic confusions on the familial and higher level. Similarly it has been effective to resolve the issues on lower (inter/intra generic) levels like in Cupressaceae, Cornaceae, Ericaceae, Geraniaceae etc. Sometimes being too conserved, *rbcL* gene may not resolve the closely related genera [40].

MatK: Another widely used barcode for plants is another cpDNA gene region *matK* which codes for the only maturase of higher plants while the *matK* exon being located within the *trnk* intron. The *matK* gene is about

1600 bp [41]. It is among the preferred choices in the systematic study of higher plants because of greater number of non-synonymous mutations, indels (insertions and deletions) and nucleotide substitution [42-44]. This gene also possesses a high ti/tv [transition/transversion] ratio. Some scientist refrained from the use of *matK* in broader taxon size for instance in overall angiosperms relationships because of these reasons. Similarly in case of *matK* taxon specific primers might be required which discourage its use [45].

DNA Barcoding Applications in Plants: The applications of DNA barcoding can be fragmented into following categories:

The major application, is to contribute in the process of recognizing unknown species to known species. In plants the DNA barcoding is likely to deliver understandings in taxonomy at species level in those taxa which have simple morphologies, which are very broadly distributed, those which are miniscule in size and those which are not adequately characterized due to low attention by the taxonomists to understand the diversity they have.

Due to the DNA barcoding research the valuable understanding in diversity of cryptic species becomes possible [46, 47]. Generally many of the important features that are necessary for the plants identification are missing in the bryophytes.

Using DNA barcoding knowledge of defining the species boundaries in plants bearing seeds is enhanced, backing to the finding of enigmatic species or helping as an independent arbiter between competing taxonomies [35, 48].

Many experts of their professions are currently involved in the identification of plants e.g. foresters, conservationists, taxonomists, ecologists, agriculturalists, scientists, custom and quarantine officers [25].

Apparent positions behind this include (1) differentiating among the diversity at a particular site or region in geographically focused studies, where various samples are not essentially closely linked and mostly where immature material and fragments of plants need identification ; (2) species in trade, in cases where the contest is often to differentiate between a set of objective species and mostly distantly associated possible substitutes or where to find the to identify members of greater taxonomic groups (e.g. genu, family) beside than specific species; and (3) in cases where the species identification becomes problematic due to unfamiliarity with a given species like that the handler have no idea even what family this specie belongs to. In these kind of circumstances, process of identification to a set of allied species is beneficial as it can constricted down the entire series of potential substitutions and also facilitate targeted usage of morphological sources or consultation with experts to get obtain a ultimate identification where ever it is needed. This kind of 'identification of group of species', trailed by consequent 'tie-breaker' studies is mostly likely to be useful in species rich situations and where the taxonomic expertise are in shortage.

One category of applications is in ecological forensics, here DNA barcoding is used for the identification of seedlings, cryptic life stages [e.g. fern gametophytes] and plant roots, likewise, DNA barcoding can be used for identifying where the material has been treated in which way, like investigating the nutrition of herbivores [49- 51] products of food [52], or the herbal medicine constituents [53]. For example, Baker and Little [54] used matK DNA barcodes to highpoint misidentified plant species in herbal medicines.

A new developing application of using DNA barcoding in plants is to identify the identification of endangered species in trade. About 29000 plant species are secure by CITES [Convention on International Trade in Endangered Species of Wild Fauna and Flora; http://www.cites.org/eng/disc/species.shtml) and also to devise effective approaches to separate CITES-listed plants from non CITES listed species. Ogden et al. [55] established a SNP based genotyping approach centered on matK DNA barcodes to differentiate between traded timber products of Ramin (Gonostylus) plant species that's are CITES protected and other con-familial species or structurally alike but distantly linked species, which are not.

To get full benefit of DNA barcoding the formation of a suitable reference library is a serious pre-requisite. This needs the generation of DNA barcode data from well recognized and vouchered samples. Various geographically and taxon based projects ongoing for the creation of this reference library.

Beside these developments, there is sequence information archived in GenBank. This is predominantly wide ranging for some of the barcode markers and provides a useful source for identifications.

Another comparatively undisputed feature of DNA barcoding is that it will also assist in the basic biodiversity records. Actually, from the evidences of molecular phylogenetics to gathering the tree of life [56, 57] DNA has proved useful in identifying clades and evolutionary relationships. Whether or not actual species

can be identified with DNA, the number of distinct DNA sequences in environmental sampling and reconstruction of phylogenetic trees to place these sequences into an evolutionary context have been used in several inventories of cryptic biodiversity e.g. soil bacteria or marine/freshwater micro-organisms. Initially referred to as DNA typing or profiling, the DNA barcoding initiative has taken this step forward and several taxa have now been surveyed in their natural habitats using this technique.

DNA barcodes have shown a valuable role in biosecurity, e.g. for the scrutiny of disease causing vectors [58] and invasive insects [59], as well as for primatology and law enforcement agencies [60]. Barcoding efforts have also recently received the attention of conservation agencies. For example, the UK Darwin Initiative for the Survival of Species has funded two projects this year that include DNA barcoding activities to support conservation priorities, capacity building and trade surveillance in meso-American orchids and cacti.

Challenges: A major challenge for barcoding comes in the isolation of high molecular weight DNA which is pure and can be used for processing. Especially in processed plant samples DNA is found to be degraded and accompanied by large quantities of polysaccharides, secondary metabolites, alkaloids, polyphenols etc. Modified traditional DNA protocols and commercial kits are available for extracting DNA from raw and processed plant samples like powdered plant material, capsules etc. [61, 62]. For damaged and fragmented DNA, repair reactions may be carried out which are available as commercial kits. For fragmented DNA, if the amplicon size is smaller, there are chances of successful PCR amplification [63, 64]. Fragmented DNA problems can be alleviated by practicing the next generation sequencing because NGS uses ligated DNA and not the fragmented DNA [63]. As compared to Sanger sequencing method, the Next Generation Sequencing can be more economical because it skips some of the steps like sorting specimens, clip tissues and place extracts into individual wells on a plate [65].

CONCLUSION

Morphology based characterization of plant taxa is always not sufficient to rightfully identify a specie and such cases are observed in morphologically similar species. If that is the case, DNA barcoding can be used for drawing distinct boundaries among them. One cannot negate the traditional taxonomical methods and the knowledge gained over the course of 250 years but integrating them with DNA sequencing and bioinformatics can contribute significantly to taxonomy and screening the biological diversity. The consensus on the potential DNA segments and best approaches is yet to be built. The taxonomic studies needed to be carried out on a broad level which extend behind the focal geographic regions to ensure that all sister taxa are evaluated.

DNA barcoding can be used as a very effective tool in the herbal industry for the identification of the medicinal plants and adulterants on the specie as well as genus level. Irrespective of the age of the plant, part of the plant and environmental factors barcoding gives reliable results in identification. Because of the increasing global demand and acceptance of herbal remedies, DNA barcoding for medicinal plants is inevitable.

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