REVIEW

Chloroplast-specific universal primers and their uses in plant studies

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Abstract

Universal (consensus) primers are those primers that have the ability to amplify the targeted region of DNA across a broad range of individuals in a certain group of organisms. In plants, such universal primers have been designed to target regions in the nuclear, mitochondrial or chloroplast genome. Among these three genomes, the chloroplast genome is the most suited for the design of consensus primers due to the lower rate of evolution and hence conservation of gene order and sequence of the genome among the different plant species compared to the other two genomes. Several molecular studies in plants have developed and used chloroplast-specific universal primers. In this review, I present some examples of the nuclear DNA-specific universal primers and discuss the features of the chloroplast DNA that make it the most suited for the design of such primers. I then refer to all chloroplast-specific primers developed so far and provide some examples of molecular studies and applications that made use of them.

Additional key words: chloroplast genome, consensus primers, molecular systematics.

Introduction

Several molecular systematic studies have made use of primers that are described as being ‘universal’. In such instances, the term ‘universal’ was generally used to describe the ability of those primers to amplify a target DNA sequence efficiently across a range of individuals belonging to a particular group of organisms (Haider 2003). The taxonomic rank at which the primers apply varies with different sets of primers reported to have different levels of universality (taxonomic spread). For example, primers designed by Taberlet *et al*. (1991) are apparently able to amplify products using template DNA from species across the whole plant kingdom. Such primers have proved valuable for molecular systematic studies since they save time, effort and cost that would otherwise be required to identify and sequence a suitable locus across all target organisms.

Nuclear DNA-based universal primers

The internal transcribed sequence (ITS) forms a part of the nuclear genome that contains multiple copies of coding regions for rDNA genes that are arranged in several tandem arrays (Appels and Honeycutt 1986). The ITS region has been commonly used as a target for the design and use of universal primers in plant research. Indeed, there is now a set of seven ITS universal primers (White *et al*. 1990, Sun *et al*. 1994) that are widely applied for diagnostic (Linder *et al.* 2000) and phylogenetic studies in many angiosperm families (Baldwin *et al*. 1995). In 2005, Moore and Field were able to identify the roots of different plant species in mixed samples using sequences of the nuclear ITS region. Sequencing of the same region proved useful for DNA barcoding of members of the *Cycadales* (Sass *et al*. 2007).

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Abbreviations: cp - chloroplast; ETS - external transcribed spacer; GM - genetically modified; IGS - intergenic spacer; ITS - internal transcribed sequence, NTS - nontranscribed spacer; SSR - simple sequence repeats or microsatellites; STS - sequence-tagged site.

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The intergenic spacer (IGS), which constitutes of a nontranscribed spacer (NTS) and an external transcribed spacer (ETS) between 26S and 18S rDNA genes, of the nuclear genome has been similarly targeted for the design of universal primers, although these primers have been generally less widely used. Baldwin and Markos (1998) designed two universal primers (18S-IGS and 26S-IGS) for the amplification of the entire IGS region. In the same study, an internal primer within the ETS was developed and used with the primer 18S-IGS for universal amplification of the ETS region in the tribe *Heliantheae* (*Compositae)* and phylogeny reconstruction of *Calycadenia* (*Compositae*). Likewise, the NTS between repeated 5S-ribosomal RNA genes has also been used for the construction of universal primers such as those described by Cox *et al*. (1992), who reported the efficiency of four primers they designed when amplifying target DNA from several distantly related monocotyledonous plants.

Taylor *et al*. (2001) designed primers that target single locus coding regions to generate sequence-tagged site (STS) markers in cereals that could also amplify in *Lolium perenne* for use in comparative mapping. The use of such nuclear universal primers, also allows the acquisition of unknown sequence adjacent to known sequences, both in the upstream and downstream directions (Weber *et al*. 1998). Recently, a set of universal primers has been developed by Prado *et al.* (2004) to clone conserved domains of the acetolactate synthase (ALS) gene in monocotyledonous and dicotyledonous plants. More recently, McIntosh *et al*. (2005) designed three pairs of grass-specific universal primers that amplify the nuclear gene GBSS1. These primers proved to have utility in identification of some grass species and may be also useful in the distinction of species from other plant families as the authors revealed.

Cytoplasmic DNA-based universal primers

The cytoplasm of plants contains two genomes, namely the chloroplast and mitochondrial genomes. The chloroplast genome contrasts strikingly with the nuclear genome. The inheritance of chloroplast DNA (cpDNA) is most commonly clonal (through maternal parent in 80 % of angiosperms; Matsushima *et al*. 2008). It is also not influenced by polyploidy, gene duplication and recombination that are widespread features of the nuclear genomes of plants (Tanksley and Pichersky 1988, Harris and Ingram 1991). Therefore, cpDNA varies little among angiosperms in terms of size, structure and gene content (Curtis and Clegg 1984).

The mitochondrial DNA (mtDNA) bears contrasting features to those of the chloroplast genome that influence their utility for the design and application of universal primers. The mtDNA is large in plants, has a slow nucleotides substitution rate, extensive level of intramolecular recombination and in turn, non-conserved order of genes. Consequently, universal primers that target sequences of mitochondrial loci are more difficult to design and generate fewer polymorphisms. There are therefore only a very limited number of mtDNA-based universal primers that have been developed (Demesure *et al.* 1995, Dumolin-Lapegue *et al*. 1997a). In contrast, the cpDNA features stated above make it ideal for the design of the majority of universal primers available for the molecular analysis of plants. The increasing number of published sequences of the entire chloroplast genome from several diverse plant species (reached 122 in 2010, http://megasun.bch.umontreal.ca/ogmp/projects/other/cp_list.html) that are freely available in DNA sequence databases facilitated the design of such primers. Amplification of target DNA using these primers is usually followed by direct sequencing (*e.g.* Meudt and Bayly 2008) or electrophoresis of either entire or restricted amplified fragments (*e.g*. Gillespie and Boles 2001).

Universal primers that target the chloroplast simple sequence repeats (cpSSRs): The slower rate of mutation in the chloroplast genome compared to the nuclear genome means that the primers that target the mononucleotide cpSSRs are usually more universal and can often amplify across related species (Provan *et al.* 2001). For instance, Vendramin *et al.* (1996) designed universal primers for the amplification of 20 SSRs (microsatellites) in *Pinaceae*. Similarly, five out of seven cpSSR primer pairs that were designed using the complete chloroplast genome of *Orysa sativa* amplified efficiently across other wild and cultivated rice species (Provan *et al*. 1997). Weising and Gardner (1999) likewise designed a set of ten pairs of universal primers to detect mononucleotide repeat variation in cpDNA of dicotyledonous angiosperms, and used the resultant amplicons to reveal intraspecific and interspecific polymorphism within the genera *Nicotiana*, *Lycopersicon* (both *Solanaceae*) and *Actinidia* (*Actinidiaceae*). Such universal primers that can amplify across a broad range of plant families have been also reported. For instance, Cato and Richardson (1996) designed five pairs of cpSSR primers that amplified successfully in eight families of higher plants. In 2003, Chung and Staub designed 23 pairs of cpSSRs consensus primers that amplify targeted regions in a diverse array of plant species. Recently, Provan *et al.* (2004a) developed five pairs of universal primers for the amplification of cpSSRs in grasses (*Poaceae*). More recently, Cheng *et al.* (2006) developed a set of universal cpSSR primers to explore cpDNA diversity among sub-tropical and tropical fruit crops, where cpDNA sequences are unknown.

Universal primers that target the chloroplast ITS (cpITS): The ITS region of chloroplast rDNA have been similarly targeted for development of universal primers. For example, Goremykin *et al*. (1996) designed two primers to amplify the region spanning from the 3' terminus of the 23S rRNA gene to the 5' terminus of the 5S rRNA gene (located in chloroplast inverted repeats (IRs)). These primers amplified successfully the targeted DNA region that includes the 4.5S rRNA gene and the two chloroplast ITS regions (cpITS2 and cpITS3) in 20 angiosperms, seven gymnosperms and 16 ferns. Samigullin *et al*. (1998) showed that these primers as well as the primer pair designed by the authors to target the chloroplast ITS4 (chloroplast 5S rRNA-tRNA (ACG) spacer) to amplify efficiently across 26 bryophytes.

Universal primers that target the chloroplast noncoding regions: Some chloroplast-encoded genes are interrupted by introns (Clegg 1993). These introns and other noncoding cpDNA regions (intergenic spacers) are variable but separate highly conserved regions. Their variability can be exploited in systematic studies in plants even at lower taxonomic levels and so such regions have been targeted for the design of universal primers. The dispersed distribution of transfer RNAs (*trns*) genes over the chloroplast genome, together with their high level of sequence conservation make them ideal targets for the design of universal primers that bind to conserved regions (Petit *et al.* 1996) that flank more variable regions (noncoding regions). Chloroplast-specific universal primers designed so far have various degrees of "universality" varying from family level such as those of Tsumura *et al*. (1996) that amplify 11 different chloroplast genes across *Dipterocarpacea*, through to those of Taberlet *et al.* (1991) that target noncoding regions of *trnT* (UGU)-*trnL* (UAA) 5'exon (primers a and b), *trnL* (UAA) intron (primers c and d) and *trnL* (UAA) 3'exon-*trnF* (GAA) (primers e and f) and amplify products in the majority of plant species including algae, bryophytes, pteridophytes, gymnosperms and angiosperm. Primers designed by Taberlet *et al.* (1991) to amplify the *trnL* intron have been shown to amplify the targeted intron in many angiosperms (*e.g*. King and Ferris 1998, Horres *et al.* 2000, Zuber and Widmer 2000) and gymnosperm species (*e.g*. Sperisen *et al.* 2000, Terry *et al*. 2000).

Several other universal primers have been reported that amplify noncoding spacers of the chloroplast genome in several angiosperms and gymnosperms. For instance, Fofana *et al.* (1997) designed three pairs of universal primers that target the intergenic spacers of *atpB-rbcL, rpsl4-psaB* and *petA-psbE*. Chiang *et al*. (1998) produced universal primers to amplify and sequence a chloroplast noncoding spacer between the *atpB* and *rbcL* genes. The authors suggested that analysis of this spacer would be useful for molecular systematics at the generic, specific, and even subspecific levels to study population genetics in some plant species. Furthermore, Demesure *et al*. (1995) and Dumolin-Lapegue *et al*. (1997a) designed nine and 16 universal primer pairs, to amplify spacers in the chloroplast and mitochondrial genomes, respectively. In 2001, Grivet *et al.* designed 20 conserved cpDNA primer pairs that target chloroplast intergenic spacers that were tested on 20 plant species belonging to 13 families. Primers developed by Demesure *et al*. (1995) have been proved to amplify in many plant species. For instance, Ziegenhagen and Fladung (1997) were able to PCR-amplify the chloroplast intergenic spacer *trnS-psbC* (Demesure *et al.* 1995) in 62 woody plant species that represented a broad systematic range in both gymnosperms and dicotyledonous angiosperms. Moreover, Sun (2002) indicated that published universal primers by Demesure *et al*. (1995) for amplification of *trnK* (exon 1)-*trnK* (exon 2) successfully amplified the respective regions in *Elymus* species.

Added to those consensus primers mentioned above, Hamilton (1999) developed four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. In 2000, Graham and Olmstead developed primers for 14 slowly evolving chloroplast genes that revealed to be efficient in amplifying and sequencing across the seed plants. These primers proved to be a useful tool for inferring the relationships of basal angiosperms and other land plants. In the same year, Nishizawa and Watano (2000) developed universal primer pairs that are suitable for PCR-SSCP analysis of cpDNA in angiosperms. Similarly, Provan *et al.* (2004b) have developed two sets of universal primers for the amplification of chloroplast coding and non-coding regions in green (*Chlorophyta,* eight pairs) and red (*Rhodophyta*, nine pairs) algae. In another study, Lee and Wen (2004) designed universal primers that successfully amplified the entire chloroplast *trnC-trnD* region in different groups of flowering plants. Recently, Watts *et al.* (2008) designed universal primers that amplify and sequence the large Domain IV (D4) loop of the cpDNA in several angiosperm introns. Haider and Wilkinson (in preparation) developed 84 potential universal primers to target 42 regions of the chloroplast genome, and investigated the level of variation in regions flanked by primers designed.

It is worth noting that the design of the striking number of cpDNA-specific universal primers mentioned above was facilitated by the comprehensive DNA sequence information available on the NCBI database (http/:www3.ncbi.nlm.nih.gov/Entrez/nucleotide.html) for the entire chloroplast genomes of many plant species and more importantly, the availability of nucleotide sequences of chloroplast loci targeted in several plant species representing monocotyledons, dicotyledons and gymnosperms for some loci. When Haider and Wilkinson (in preparation) developed their enlarged set of universal primers, homologous sequences recovered from the NCBI database for each locus targeted by those primes

exhibited, generally, a high level of sequence conservation among plant species. This made the alignment of homologous sequences relatively simple exercise for each locus, and in turn allowed easy detection of the most highly conserved regions that were selected as primer binding sites.

Uses of chloroplast-specific universal primers in plant molecular studies

The majority of chloroplast universal primers described above and in particular those of Taberlet *et al.* (1991) and Demesure *et al.* (1995) have been used for various taxonomic studies in plants such as:

Evolution and origin of plants: The maternal mode of inheritance of cpDNA is a particular advantage in studying the origin and maternal parent of the hybrid and polyploid species (Palmer 1987). CpDNA, therefore, has been useful for the study of plants origins and evolution. For instance*, Allium fistulosum* was found to have originated monophytically from its close wild relative *A. altaicum* as revealed by RFLP analysis of five noncoding cpDNA regions and RAPD analysis of nuclear DNA (Friesen *et al.* 1999). Fofana *et al*. (1999) also examined an overview on the evolution of the genus *Phaseolus* on the basis of cpDNA diversity using PCR-RFLP. Based on both nuclear DNA and cpDNA data, Friesen *et al.* (2000) unambiguously placed *A. altaicum* within *Allium* subgenus *Rhizirideum.* In another study, comparison of data based on nuclear genome and sequences of the chloroplast regions *trnL, trnL-F* and *trnT-L* suggested that *Raphanus sativus* is a hybrid between the *Brassica nigra* and the *B. rapa* × *B. olercacea* lineages with the latter as the maternal parent (Yang *et al.* 2002). Other examples of using universal chloroplast primers to study origin of plants is the study of origin of *Spiranthes diluvialis* (Szalanski et al. 2001), *Armeria villosa* ssp. *carratracensis* (Feliner *et al*. 2002), the hybrid *Potamogeton* × *sudermanicus* Hagstr. (Fant *et al.* 2003), some species of kiwifruit (*Actinidia deliciosa, Actinidiaceae*) (Chat *et al.* 2004), and the diversity and the evolution of several other legumes (Angioi *et al.* 2009).

Phylogenetics: Molecular analysis offers a useful way to measure the amount of genetic variability in cultivated species and their wild relatives. Evaluation of genetic diversity in turn allows the clarification of phylogenetic relationships among those species and may provide a rationale for choosing strategies for germplasm collection, conservation and use of genetic resources (Buso *et al*. 2001). Most importantly, such information also has value for the improvement of cultivated species, for which the wild relatives are of considerable value because they possess extensive genetic variability. Vakhitov *et al*. (2003) argued that it is important to understand the phylogenetic relationships among cultivated wheat species and their wild relatives for the genetic improvement of these crops.

Palmer (1987) argued that parallelisms and convergences occur so rarely at the species level that it is often possible to build unambiguous phylogenetic using cladistic approaches. Therefore, cpDNA has been widely used for the phylogenetic reconstruction of crop plant species (Olmstead and Palmer 1994). For instance, Lakshmi *et al*. (2002) constructed molecular phylogeny for species of the Indian mangrove tribe *Rhizophoreae* based on sequences of *rbcL.* Chloroplast universal primers available enhance the capacity of evolutionary scientists to determine phylogenetic relationships of both closely or distantly related plant species most commonly using sequence analysis. Noncoding regions for which universal primers are published have also been targeted for phylogenetic studies in 39 species of the tribe *Rubieae* (Natali *et al*. 1995), common beech (*Fagus sylvatica*) (Demesure *et al*. 1996) and eight white oak species (Dumolin-Lapègue *et al.* 1997b). Other examples on using chloroplast universal primers to study phylogenetic relationships are those on the genus *Leonardoxa* (*Leguminosae*: *Caesalpinioideae*) (Brouat *et al*. 2001), *Phaseolus* (Fofana *et al.* 1997), *Hordeum* (Nishikawa *et al*. 2002), *Hypericum* (Pilepic 2002), *Crinum* (*Amaryllidaceae*) (Meerow *et al*. 2003), *Panax* (Lee and Wen 2004), *Poa* (Gillespie and Soreng 2005), and some *Diospyros* spp. (*Ebenaceae*) including *Diospyros kaki* (Hu *et al*. 2008).

Mode of inheritance: Determination of the mode of the cpDNA inheritance for a group of plant species is useful for studies of species origins, hybridisation, introgression and population genetics (Yang *et al*. 2000). For example, Hollingsworth *et al*. (1999) established the direction of hybridisation and the mode of chloroplast inheritance to investigate spread of invasive weeds belonging to the genus *Fallopia.* Recently, the mode of cpDNA inheritance has been determined in intergeneric hybrids between *Carica papaya* and four different *Vasconcellea* species (Van Droogenbroeck *et al*. 2005).

Feliner *et al*. (2002) similarly provided evidence for a maternal inheritance of cpDNA in *Armeria* based on sequences from two chloroplast regions, namely *trnL-trnF* and *trnD-trnT* that were amplified using published universal primers. In a study carried out by Collins *et al*. (2003), species-specific chloroplast markers developed by analysing restriction digestions of PCR-amplified *trnL-F* fragments confirmed the direction of crosses that generated putative hybrids of three species of *Taxus* (*Taxaceae*), namely *T. baccata, T. canadensis* and *T. cuspidate*. Likewise, Panda *et al.* (2003) developed

PCR-RFLP markers that differentiated clearly between sweet cherry (*Prunus avium*) and sour cherry (*P. cerasus*) using six chloroplast universal primer pairs. These markers allowed the authors to understand the maternal inheritance of chloroplast genome in *P. avium* and indicated that the latter is not the maternal ancestral species of *P. cerasus.*

Exclusively uniparental paternal inheritance of cpDNA has been reported to be the commonest in gymnosperms (cited by Rebound and Zeyl 1994) such as red and black spruce (Bobola *et al*. 1996), but is rare amongst angiosperms. Paternal inheritance of the cpDNA has been observed in some angiosperm plant species perhaps slightly compromising the value of analysis of cpDNA. For instance, strict paternal inheritance of cpDNA was found in progeny of interspecific controlled crosses in the genus *Actinidia* (Testolin and Cipriani 1997) based on PCR-RFLP on *trnT-L* and *trnL-F*. Paternal transmission of chloroplast genomes allowed the detection of the paternal genetic lineages of species in the genus. Restriction analysis of three cpDNA regions (*trnT-L, trnL-F* and *trnD-T*) that were amplified using universal primers also indicated that the cpDNA inheritance is uniparental and paternal in the genus *Larrea* (*Zygophyllaceae*), which was the fifth genus to have paternal inheritance in angiosperms beside *Medicago, Turnera, Pharbitis* and *Actinidia* as stated by Yang *et al*. (2000). In a recent study, Matsushima *et al*. (2008) used cpDNA-specific universal primers to determine cpDNA inheritance in *Medicago truncatula.* Based on sequences of the chloroplast *trnL-F*, Zhang *et al*. (2009) were also able to infer the maternal donor of species in the genus *Kengyilia*.

Forensic botany: Compared to the one nucleus within each plant cell, chloroplasts are found in large numbers and each of them is protected by a protein membrane that reduce bacterial breakdown of the cpDNA (Wilkinson and Linacre 2000). The latter believe that this gives the DNA test based on chloroplast genome advantages over tests based on nDNA for forensic purposes. Although, forensic botany can provide significant evidence during criminal investigations, its most common application still, however, limited to identifying specific and suspected illegal plants (Ferri *et al.* 2009).

To date, very few studies, however, have used cpDNA-based evidence in forensic investigations. For instance, nested PCR using universal primers c-d (Taberlet *et al*. 1991) and 'H' primer specific to *Cannabis sativa* (Linacre and Thorpe 1998) allowed Wilkinson and Linacre (2000) to detect the presence of *C. sativa* DNA on the hands of subjects who have recently handled *Cannabis* material and then performed a number of tasks such as rubbing hands on trousers or placing hands in pockets. Bever *et al.* (on-line reference) used DNA sequence variation in *rbcL* to identify the individual plant components found in botanical trace evidence from as little as a 1 mm punch of dried plant tissue or from five grains of fresh pine pollen. Generally, studies of the identification of species in relation to forensic sciences have used DNA obtained from individual plant specimens of a known species (Linacre and Thorpe 1998). More recently, *rbcL* locus was amplified with universal primers and sequenced across all the elements in a DNA mixture of unknown plant composition (Bever and Cimino 2001). Sequences generated enabled the authors to identify each of the plants as part of the sample. It seems also promising that cpDNA-specific universal primers available may provide a valuable means to generate species-specific markers that can be used in crime investigations such as murder, distribution of drugs, kidnapping, acts of terrorism and sexual assault (Bever *et al.* on-line reference). For example, Wesselink and Kuiper (2008) made a successful attempt for species identification of botanical trace evidence using molecular markers that were generated using cpDNA-specific universal primers. Similarly, Ferri *et al.* (2009) investigated the forensic use of two non-coding chloroplast regions, *psbA-trnH* and *trnL-trnF*, to create a multimarker system for species identification that could be useful throughout the plant kingdom.

Seed dispersal: Seed dispersal has a profound effect on vegetation structure and its dynamics, colonization of new habitats and maintenance of diversity (Wang and Smith 2002). The authors believe that the analysis of molecular markers allows matching dispersed seeds and seedlings with parent plants for an easier detection of long distance dispersal events.

Since cpDNA is maternally transmitted in the majority of angiosperms and thus usually reflects only seed dispersal, using chloroplast markers specific to plant species can therefore provide useful information for the detection of seed dispersal of a plant species to the cultivation area of another species. For example, using universal primers for cpDNA analysis, Petit *et al*. (1997) observed interspecific sharing of the maternal genome of two oak species: *Quercus petraea* and *Q. robur* that strongly suggests that long-distance seed dispersal events followed by interspecific exchanges were involved at the time of colonization of oak species in Europe. In another study, Potgieter and Albert (2001) analysed sequences of the *trnL* intron and *trnL-F* spacer in *Apocynaceae* to resolve phylogenetic relationships in the family that were used as a basis to investigate trends in seed dispersal in *Apocynaceae*. In the same year, the spatial distribution of maternally inherited cpDNA markers over the French part of the range of *Sorbus torminalis* was carried out by Oddou-Muratorio *et al.* (2001) to quantify the relative importance of seed and pollen dispersal. Similarly, using chloroplast universal primers for cleaved amplified polymorphic sequence (CAPS), the spatial mixing of haplotypes in *Quercus robur, Q. petraea* and *Q. pubescens* throughout the Swiss Alps and adjacent

regions as a consequence of seed dispersal was low (Matyas and Sperisen 2001). In a recent study, Griffin and Barrett (2004) indicated that seed dispersal between populations of *Trillium grandiflorum* is less likely than pollen flow. Oliver *et al.* (2006) studied seed dispersal of the arctic-montane species *Saxifraga hirculus* (*Saxifragaceae*).

Interspecific variation: Polymorphism that may be detected among different plant species using chloroplast markers can be exploited for the study of interspecific variation that can describe the history of these species. Published chloroplast universal primers have proved useful for the detection of interspecific variation among several plant groups. For instance, PCR-RFLP using universal primers allowed the detection of interspecific variation in various plant genera such as *Leguminosae* (Fofana *et al.* 1997), *Abies* (Parducci and Szmidt 1999) and *Elymus* (Sun 2002). Using universal chloroplast primers referred to as AS, CD and DT (Demesure *et al.* 1995) for PCR and then digestion of amplification products, Pilepic (2002) revealed considerable degree of interspecific polymorphism between *Hypericum perforatum* and *H. maculatum*. In 2004, interspecific differences in the genus *Citrullus* were resolved by Dane *et al*. Genetic variation and relationships among *Ulex* (*Fabaceae*) species in Southern Spain and Northern Morocco were similarly assessed by cpSSR markers (Cubas *et al*. 2005). Using three universal primer pairs, Bouhadida (2007) analyzed cpDNA diversity and revealed genetic relationships in the genus *Prunus*. Recently, Basha and Sujatha (2009) investigated interspecific variation among *Jatropha* species using consensus cpSSR markers.

Somatic hybridisation: Sexual barriers (ploidy and/or endosperm balance number) that prevent interspecific crosses between cultivated species and their wild relatives may restrict exploitation of wild germplasm in breeding programs. Somatic hybridization (fusion of somatic cells in culture) has made many wild species accessible for gene transfer to their cultivated relatives (Bastia *et al.* 2001). The inheritance of chloroplasts in somatic hybrids is uncertain and can vary between experiments or species combinations. Chloroplast-specific universal primers can be used as an easy tool to follow the fate of chloroplasts after somatic fusion of cells from two species. Bastia *et al*. (2000) developed somatic hybrids *via* protoplast fusion between *Solanum tuberusum* and *S. commersonii* and used cpRFLP to characterize chloroplast transmission into the hybrids. Similarly, Bastia *et al.* (2001) used universal primers to amplify intronic and intergenic regions to determine the chloroplast genome composition of interspecific somatic hybrids in the genera *Solanum* and *Brassica*. Cheng *et al.* (2003) also analysed cpDNA inheritance of 10-year-old intergeneric somatic hybrids trees between *Citrus sinensis* and *Fortunella crassifolia* using CAPS that implied universal primers. In another study, PCR-RFLP using universal primers has been applied for the analysis of the cytoplasmic constitution of *Aurantioideae* intergeneric somatic hybrids (Lotfy *et al.* 2003). Fu *et al.* (2004) analysed the cytoplasmic genomes of intergeneric somatic hybrid combining Goutou sour orange and *Poncirus trifoliate* using universal primers. Recently, Olivares-Fuster *et al.* (2007) used cpDNA universal primers to characterize somatic hybrids and cybrids obtained by fusion of *Citrus sinensis* and *C. excelsa* protoplasts.

Conservation: The generation of species-specific markers using chloroplast universal primers may be useful for studies of the maintenance of germplasm collections. Such repositories are valuable for the conservation and use of plant genetic resources for the improvement of agricultural and forestry crops. In this context, the primers can be used to: *1*) ensure identity and integrity of accessions held within a germplasm collection in a genebank, *2*) characterisation of seeds entering a genebank, *3*) checking seeds held in genebanks if they are contaminated with seed of other species and *4*) detect any introgressive hybridization in *ex situ* collections. They can be also exploited to protect wild species of crop species in their natural habitats (*in situ* conservation). For example, sequencing of the chloroplast intron *trnL* and the intergenic spacer *trnL-F* were used to investigate the genetic variability in *Milicia* from three West African countries to design conservation strategies for species in this genus (Ofori *et al.* 2001). In 2004, Honjo *et al.* carried out a study in Japan on the intraspecific phylogeography of the endangered species *Primula sieboldii* to help design conservation programs. Similarly, Aoki *et al.* (2006) illustrated how sequencing of noncoding regions of cpDNA that were amplified using universal primers for phytogeographic studies could provide a basis for identifying suitable units for conservation. Recently, using cpDNA-specific universal primers, De Lange *et al.* (2008) carried out a case study with two enigmatic and uncommon species of *Crassula* from New Zealand for biosystematics and conservation purposes.

Spontaneous hybridisation: Hybridization is an important evolutionary phenomenon, and therefore a better understanding of the dynamics of interspecific gene flow and resulting morphological and genetic patterns has been of widespread interest (Luhová *et al*. 2007).

Interspecific hybridisation and introgression have been reported to be common phenomena in many plant species, *e.g. Chaenomeles cathayensis* and *C. speciosa* (Bartish *et al*. 2000) or even genera, *e.g. Brassica napus* and *Raphanus raphanistrum* (Eber *et al*. 1994). Hence, natural hybridisation can occur between cultivated plants and their wild relatives when they come into contact (Ellstrand *et al*. 1999).

Numerous studies have attempted to characterise or quantify the occurrence of spontaneous hybrids in natural habitats (*e.g*. Scott and Wilkinson 1998). In works of this kind, it is clearly important to be able to distinguish both parental species from the hybrid and also from each other. Bartish *et al.* (2000) was able to detect spontaneous hybridisation between *Chaenomeles cathayensis* and *C. speciosa*. Interest in the quantification of interspecific hybridisation has increased dramatically with the commercial cultivation of genetically modified (GM) crops, where attention is focussed on the ecological consequences of transgene recruitment by wild relatives of GM crops by gene flow (Lavigne *et al*. 2002).

Amplification and sequencing of the *trnL-trnF* region (Taberlet *et al.* 1991) of cpDNA provided an evidence of hybridization between two polyploid *Cardamine* (*Brassicaceae*) species in North-western Spain (Luhová *et al*. 2007). Based on sequence information generated from the same chloroplast region, a natural hybrid between *Ligularia paradoxa* and *L. duciformis* (*Asteraceae*, *Senecioneae*) from Yunnan, China was recovered (Pan *et al*. 2008). In 2009, Csencsicsa *et al.* carried out a large scale survey of *Populus nigra* presence and genetic introgression from non-native poplars in Switzerland based on molecular identification using universal primers for the analysis of cpDNA. Similarly, Haider *et al.* (2009) used chloroplast CAPS markers that differentiate between the chloroplasts of *B*. *napus* and *B*. *rapa* to survey wild and weedy populations of *B*. *rapa* for the capture of *B*. *napus* chloroplasts.

Intraspecific variation: Intraspecific diversity in plant cpDNA has been believed to be the result of mutations, genetic drift, selection by man for particular cpDNA-encoded characters (herbicide resistance), or the introgression of a foreign cpDNA (chloroplast capture) from related species during the early domestication of the taxon and even in wild taxa (Harris and Ingram 1991). Such introgression has been reported to be common for many plant species (*e.g*. in *Iris* species; Arnold *et al.* 1992). Systematics and phylogenetic implications of intraspecific variation that may be detected in cpDNA of plant species are reviewed by Soltis *et al.* (1992).

Prentice *et al.* (2003) implied chloroplast universal primers to study variation in cultivated and wild *Prunus avium.* Other examples for the use of universal primers to assess intraspecific variation are those carried out on *Argania spinosa* (*Sapotaceae*) of Morocco (El Mousadik and Petit 1996), *Primula cuneifolia* (*Primulaceae*) (Fujii *et al*. 1999), *Cannabis sativa* (Kohjyouma *et al.* 2000), the rare Spanish endemic, *Silene hifacensis* (Prentice *et al*. 2003), *Sagittaria latifolia* (Dorken and Barrett 2004), and *Oryza sativa* (Ou *et al*. 2009).

Identification of plants: There are several features of the chloroplast genome that make it well suited for species diagnosis. First, the low evolutionary rate of cpDNA below the species level, as was revealed by several studies (*e.g*. Jordan *et al.* 1996, Szalanski *et al*. 2001) means that sequence variation discovered between species is unlikely to be confounded by infraspecific variation at the same site. Second, the level of genetic variation in the chloroplast genome has been reported to be sufficient (Nissen *et al.* 1995, Lakshmi *et al.* 2002) to allow discrimination of closely related plant species. Such variation was even found to be numerous in some groups such as the *Senecio* complex (Bain and Jansen 1996), and species belonging to the *Solanaceae* (Bryan *et al.* 1999).

Linacre and Thorpe (1998) used DNA-specific primers for the nucleotide sequences between the *trnL* and *trnF* genes in the chloroplast genome for the unambiguous identification of *Cannabis sativa.* Similarly, Ohyama *et al.* (1999) used sequences of *trnL-trnF* and *trnT-trnL* to distinguish *Quercus gilva* from five other species in the genus *Quercus*. Brunner *et al.* (2001) was also able to identify fine roots of tree species of the Alps. Other examples are provided by Parani *et al*. (2001), who distinguished seven small millet species using PCR-RFLP on amplicons generated using universal primers that amplify the chloroplast *trnS-psbC* region (Demesure *et al.* 1995). Further, Fineschi *et al.* (2002), used *trnL* (and ITS2) sequences to differentiate between *Zelkova abelicea* and *Z. sicula* (*Ulmaceae*), and Haider and Nabulsi (2008), were able to identify *Aegilops* species based on restriction and sequencing of chloroplast loci, which they amplified using universal primers. More recently, universal primers have been proved to be of a great value for the DNA-barcoding (analysis of a short DNA sequence from a uniform target gene to enable species identification) of plant species (Kress and Erickson 2007, Ford *et al.* 2009).

Examples on other applications of universal primers in molecular studies on plant species are *1*) analysis of phyto-geographic structure in plant species such as those of *Lolium* (Balfourier *et al.* 2000) and *Dryas integrifolia* (Tremblay and Schoen 1999), *2*) investigation of the postglacial colonization history the plant species as the oak species *Quercus robur* and *Q. petraea* (Ferris *et al.* 1998), and *3*) to determine the nucleotide sequence of a chloroplast region in a species of interest when that sequence is not available on public databases. For instance, Linacre and Thorpe (1998) used *trnL-F* universal primers (Taberlet *et al.* 1991) to recover the nucleotide sequence of this region in *Cannabis sativa.*

Conclusions

Universal primers have been developed to target the different three genomes (nuclear, mitochondrial and the chloroplastic) in plants. The most useful among these genomes, however, for the design of such primers has been the chloroplast genome. In certain occasions, and for some parts of the chloroplast genome, however, the design of chloroplast-specific universal premiers is troublesome. This difficulty is experienced for chloroplast regions that have a higher rate of sequence evolution (intergenic spacers and introns), and when genes order rearrangements such as inversions, insertions, deletions, inverted repeats expansion or contraction or loss, and transpositions occur especially above the genus level in some plant groups.

Variation that can be detected among plant species using cpDNA-specific universal primers can be useful for many applications in plant science. Among these, systematics and evolutionary relationships studies at the different taxonomic levels (*e.g*. interfamilial, intergeneric, interspecific, intraspecific and interpopulational level) have been by far the most prevalent. Examples of other studies that made use of such primers have been reported in this review.

The availability of such primers facilitates especially those studies that are usually carried out on a large set of different plant species (such as identification or DNA-barcoding studies). The availability of universal primers for molecular systematic studies on plants can save time, effort and cost that would otherwise be required to identify and sequence a suitable locus across all target plants.

Since the same genes in cpDNA may have different levels of variation among different groups of plant species and different levels of variation can be observed among the different genes of cpDNA within the same group, designing more universal primers that target both coding and non-coding regions would ease the choice of the chloroplast region to be targeted in molecular studies on a particular group, for a particular application and a particular taxonomic level.

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