Sequencing Angiosperm Plastid Genomes Made Easy: A Complete Set of Universal Primers and a Case Study on the Phylogeny of Saxifragales

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Abstract

Plastid genomes are an invaluable resource for plant biological studies. However, the number of completely sequenced plant plastid genomes is still small compared with the vast number of species. To provide an alternative generalized approach, we designed a set of 138 pairs of universal primers for amplifying (termed “short-range PCR”) and sequencing the entire genomes of the angiosperm plastid genomes. The universality of the primers was tested by using species from the basal to asterid angiosperms. The polymerase chain reaction (PCR) success rate was higher than 96%. We sequenced the complete chloroplast genome of Liquidambar formosana as an example using this method and compared it to the genomes independently determined by long-range PCR (from 6.3 kb to 13.3 kb) and next-generation sequencing methods. The three genomes showed that they were completely identical. To test the phylogenetic efficiency of this method, we amplified and sequenced 18 chloroplast regions of 19 Saxifragales and Saxifragales-related taxa, as a case study, to reconstruct the phylogeny of all families of the order. Phylograms based on a combination of our data, together with those from GenBank, clearly indicate three family groups and three single families within the order. This set of universal primers is expected to accelerate the accumulation of angiosperm plastid genomes and to make faster mass data collection of plastid genomes for molecular systematics.

Key words: plastid genome, universal primers, Saxifragales.

Introduction

Complete plastid genomes have found extensive applications in sequence-based phylogeny. The systematically uncertain families, such as Vitaceae and Nelumbonaceae, have thus been pinpointed (Jansen et al. 2006). The plastid genome information has also proven very useful in studying evolution within families, for example, Pinaceae (Lin et al. 2010) and Pinus (Parks et al. 2009). Recently, plastid genomes have served as reservoirs of microsatellites (Scarchelli et al. 2011; Xue, Wang, et al. 2012) and high-resolution DNA barcodes (Kumar, Hahn, et al. 2009; Doorduin et al. 2011).

It has been shown that the reliability of a phylogeny is heavily dependent upon the number of informative characters. For instance, 10 kb of sequence is needed to resolve the familial relationship of Lamiales (Wortley et al. 2005). The best way to reach a convincing result is to add more genes to the data set. The rapid accumulation of the whole plastid genome sequences has made phylogenomics sensu O’Brien and Stanyon (1999). However, although the sequences of 269 plastid genomes of green plants have been deposited into the GenBank up to the end of 2012, this is still a very small number when compared with the 314,600 identified plant species (Mora et al. 2011). Plastid genome information is indispensable in biology, and easy and cheap methods for sequencing plastid genomes are urgently needed.

Three major strategies are usually used to sequence plastid genomes. The first approach is to isolate the plastids by the sucrose-gradient method. The plastid DNA is sometimes enriched by rolling circle amplification, long-range polymerase...
chain reaction (PCR), and bacterial artificial chromosome or fosmid library constructions. The long DNA is fragmented by endonucleases, ultrasonic devices, or other methods. The fragments are then cloned for sequencing (Goremykin et al. 2005; Wu et al. 2007; Cattolico et al. 2008; Lin et al. 2010; Wu, Lin, et al. 2011; Wu, Wang, et al. 2011). The second method is to first isolate total genomic DNA, construct a genomic DNA library, and sequence the library by the high-throughput next-generation sequencing (NGS) machines such as Illumina/Solexa, Roche/454, Danaher/Polonator, or ABI/SolID (Moore et al. 2006; Atherton et al. 2010; Lin et al. 2012). In the third method, genome-walking primers are designed for conserved regions, and the whole genome is sequenced by the Sanger process. The genomes of Amborella trichopoda (Goremykin et al. 2003a), fertilis Amborella trichopoda is sequenced by the Sanger process. The genomes of are designed for conserved regions, and the whole genome also demonstrate the application of the universal primers in ample, and compares the three genomes determined using range PCR, confirms their usefulness in sequencing the whole genomes, as they are not readily usable for such a purpose.

Isolation of chloroplast genomic DNA through the separation of chloroplasts appears to be reliable and was widely adopted in early studies. However, chloroplast isolation is time consuming and sometimes troublesome. Additionally, the resulting chloroplast DNA may not be pure, as it may be contaminated by material from other genomes. The determination of a plastid genome sequence through the NGS method is becoming increasingly popular because of the power of bioinformatics, the simplicity of the method, and the high throughput of data. The genome-walking method has attracted some attention. For example, partially universal primers have been proposed for the large single copy (LSC) region (Grivet et al. 2001) and for the inverted repeat (IR) sequence (Dhingra and Folta 2005). A chloroplast primer database has collected approximately 700 primers (Heinze 2007). Unfortunately, those primers are not intended for sequencing the whole plastid genomes, as they are not readily usable for such a purpose.

Many regions of the angiosperm plastid genomes are highly conserved. This conservativeness makes it possible to design universal primers for amplifying fragments covering almost the whole plastid genome, and the fragments can be sequenced using the Sanger method. We designed universal primers and utilized them on the species Sedum sarmentosum and Nelumbo spp. (Xue, Dong, et al. 2012; Xue, Wang, et al. 2012). These primers proved to be very useful. This article reports the development of those universal primers for short-range PCR, confirms their usefulness in sequencing the whole chloroplast genome using Liquidambar formosana as an example, and compares the three genomes determined using short-range PCR, long-range PCR, and the NGS methods. We also demonstrate the application of the universal primers in phylogenetic reconstruction of families in Saxifragales. We hope that these primers will accelerate the determination of the completely sequenced plastid genomes of flowering plants.

Materials and Methods

Genome Sequence Manipulation, Primer Design, and Universality Tests

Forty-eight complete plastid genomes of angiosperm species were downloaded from GenBank (supplementary table S1, Supplementary Material online). The coding genes (excepting tRNA) were extracted from the genome and were aligned with Clustal X ver. 2.0 (Larkin et al. 2007) and adjusted manually with Se-Al ver.2.0 (Rambaut 1996). Primer pairs were designed with Primer 5.0 using default settings to amplify fragments of approximately 1.5 kb. The overlap length was set to approximately 100 bp if possible. The primer pairs were synthesized by Sangon Biotech (Shanghai) Co. Ltd. (Beijing, China). Eight samples (supplementary table S2, Supplementary Material online) representing basal angiosperms, monocots, basal eudicots, Saxifragales, and rosid and asterid angiosperms were used to test the universality of the primers.

Confirmation of Single-Copy IR Boundary Regions

The IRa sequence is usually based on that of the IRb. The transition from LSC to IRb, IRb to small single copy (SSC), SSC to Ira, and Ira to LSC can be confirmed by the sequences of fragments amplified with region-specific primer pairs (fig. 1).

Determination of the Genome of L. formosana

Approximately 11 g of young, fresh leaves of L. formosana was homogenized in buffer A (Li et al. 2013), filtered into 50 μl centrifuge tubes, and centrifuged at 500 x g for 2 min. The supernatant was decanted to new tubes, and the precipitate was discarded to remove the nuclei. The new tubes were centrifuged at 1,000 x g to collect the chloroplasts, and the supernatant was discarded to remove the DNA and mitochondria. The precipitate (chloroplasts) was resuspended in buffer A and centrifuged to wash away the remaining DNA and mitochondria. DNA was extracted from the precipitate using the mCTAB method (Li et al. 2013) and purified using the Wizard DNA Clean-Up System (A7280, Promega Corporation, Madison, WI).

Two-thirds of the DNA was sent to the National Center for Gene Research, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, to sequence the genome on the Illumina/Solexa Genome Analyzer II. Before assembling the paired-end Illumina/Solexa reads, the script fastq_quality_filter included in the FASTAX-Toolkit version 0.0.13
Variability Tests and Suitability Evaluations of Genes for Phylogeny

The variability of all protein-coding genes was parameterized by the number of polymorphic sites (S), nucleotide diversity per site (π), average number of nucleotide differences (k), and number of parsimony-informative characters (Npi), which were calculated using DnaSP 5.0 (Librado and Rozas 2009).

Phylogenetic Application of Plastid Genome Information: A Case Study on Saxifragales

We selected Saxifragales as an example because there are some systematic uncertainties about the families that could be solved using the plastid genome sequence. Incomplete sampling of families can cause phylogenetic uncertainty. We sampled 20 species (supplementary table S3, Supplementary Material online) representing all Saxifragales-related families, including some families previously belonging to Hamamelidales and major lineages within some of these families. Because of the unavailability of materials for analysis, the sequences of six Saxifragales-related taxa were downloaded from GenBank (supplementary table S4, Supplementary Material online). Nineteen plastid genomes of eudicots from GenBank (supplementary table S4, Supplementary Material online) were used as outgroups. Taking the length of fragments, S, π, k, Npi, and the availability of sequences in GenBank into consideration, 18 genes were used to construct the phylogeny of Saxifragales.
The best substitution model for each of the 18 genes was selected by ModelTest version 3.7 (Posada and Crandall 1998) under the Akaike information criterion. Maximum parsimony (MP) analysis, maximum likelihood (ML) analysis, and Bayesian inference (BI) were conducted on the concatenate data set of 45 species. Heuristic searches with 10,000 random-addition replicates were performed for MP using PAUP* 4.0b10 (Swofford 2002) with TBR branch swapping and the Multrees options. Nonparametric bootstrap support was evaluated for 1,000 replicates, each with 100 random-addition replicates, using the same settings. The nucleotide sequences were converted into amino acid sequences, and we then conducted MP analyses using PAUP. ML analysis was run with RAxML version 7.2.6 (Stamatakis 2006), following the procedure outlined in Moore et al. (2010). ML analyses were conducted by first running 100 bootstrap replicates. Every tenth bootstrap tree was used as a starting tree for a full ML search. The best tree resulting from those searches was considered to be the ML tree. A nonparametric bootstrap of 1,000 replicates was conducted to assess the uncertainty of the ML trees. MrBayes 3.2 (Ronquist et al. 2012) was used for BI. Two parallel analyses of four chains each were run for 50,000,000 generations. A burn-in of 25% was used for both analyses. The ML and Bayesian analyses were performed on the freely available Bioportal server ([www.bioportal.uio.no](http://www.bioportal.uio.no)) (Kumar et al. 2009).

**Results**

**Primers and Their Universality**

A total of 138 pairs of primers (supplementary table S5, Supplementary Material online) were designed to cover the whole plastid genome (supplementary fig. S1, Supplementary Material online). Among the primer pairs used were 75 pairs of amplifying protein-coding genes, 43 pairs of amplifying intergenic spacers and RNA genes, and 20 pairs of amplifying introns. The PCR products were designed to be 0.8–1.5 kb, a length suitable for PCR amplification and sequencing. Long fragment amplifications were performed in case no desirable priming sites exist, such as around *trnT-psbD*, *rpoB-trnC*, *ycf1*, and *accD*. Approximately 100 bp of overlap was considered sufficient to generate a reliable assembly. The sequences of some tRNA genes are too short to allow consideration of overlaps, and small gaps therefore needed to be bridged (supplementary fig. S1, Supplementary Material online).

Most of the primer sets produced single strong bands on the eight test species representing major lineages of angiosperm (supplementary fig. S2, Supplementary Material online). The PCR success rate was greater than 96% (supplementary table S2, Supplementary Material online). Sixteen pairs of primers (supplementary table S6, Supplementary Material online) were used for long-range PCR. The lengths of the fragments showed that they were from 6,704 bp to 13,298 bp.

**Determination of Single-Copy IR Boundary Sites**

The transitions of LSC/IRb, IRb/SSC, SSC/IRa, and IRa/LSC (fig. 1) were confirmed by sequences amplified with the region-specific primers (supplementary table S7, Supplementary Material online). Some of the primer sequences were the same but annealed to the antisense strand. To avoid confusion, the primers were given new names (supplementary table S7, Supplementary Material online). Primer LSC-f (=[cp096F]) on *rps3* of LSC with primer IRb-r (=cp096R) on *rpl2* of IRb amplifies the single-copy IR boundary region fragment of LSC and IRb. Primer IRb-f2 (=[cp124F]) on the pseudogene *ycf1* of IRb and primer SSC-r (=[cp124R]) on *ndhF* of SSC determines the single-copy IR boundary region of IRb and SSC. If the pseudogene *ycf1* is lost in the taxon being tested, the combination of primer IRb-f1 (=[cp123F]) on *trnN* and primer SSC-r will work. The transitional area of SSC and IRa is more difficult to assess due to the long and variable *ycf1* sequence. A reaction including primer SSC-f (=[cp138F]) on *ndhF* of SSC with primer IRa-t2 (=[cp123F]) on *trnN* or primer IRa-t1on *ycf1* of IRa does not work efficiently due to the length of the fragment. Long-range PCR is usually necessary. Primer IRa-f (=[cp096R]) on *rpl2* of IRa and primer LSC-r on *psbA* of LSC amplify the transitional region of IRa and LSC.

**Complete Chloroplast Genome of *L. formosana***

The chloroplast genomes of *L. formosana* sequenced independently, using the three methods, were completely identical (KCS88388). A total of 311 fragments were used, covering the whole genome 1.966×. The chloroplast genome of *L. formosana* (fig. 2) had a length of 160,410 bp. The length of the LSC was 88,945 bp, the SSC was 18,917 bp, and the IRs were 26,274 bp. The IRa region terminates with the *rrn5* gene.

Relative Variability of Genes in Saxifragales

The variability of genes differs within the Saxifragales group (supplementary table S9, Supplementary Material online). The top 20 genes in terms of *S*, *K*, and *Npi* are *accD*, *atpA*, *atpB*, *ccsA*, *matK*, *ndhA*, *ndhD*, *ndhF*, *ndhH*, *petA*, *psaA*, *psaB*, *psbB*, *psbC*, *rbcL*, *rpoA*, *rpoB*, *rpoC1*, *rpoC2*, and *ycf1*. The top 10 candidates are *matK*, *ndhD*, *ndhF*, *rpoB*, *rpoC1*, *rpoC2*, and *ycf1*. These genes are usually longer than 1 kb. In terms of *π*, *accD*, *ccsA*, *matK*, *ndhA*, *ndhD*, *ndhF*, *rpoA*, *rpoC2*, and *ycf1* rank in the top 20, and *ccsA*, *matK*, *ndhD*, *ndhF*, and *ycf1* rank in the top 10. Therefore, *matK*, *ndhD*, *ndhF*, and *ycf1* are the most variable chloroplast genes in Saxifragales.
Phylogeny of Saxifragales

The combined data set of 18 plastid genes (supplementary table S10, Supplementary Material online) of all 45 accessions reached a length of 28,686 bp after the exclusion of rare insertions and some unreliably aligned sites. There are 6,578 parsimony-informative sites in the data set. MP, ML, and BI trees suggest that Gunneraceae belong to the basal eudicots, Hydrangeaceae belong to asterids, and Parnassiaceae belong to rosids. The monophyly of Saxifragales is highly supported (fig. 3, supplementary fig. S3, Supplementary Material online), and its close relationship with rosids is indicated. Within Saxifragales, there are six well-supported lineages: 1) Hamamelidaceae (including Altingiaceae, Cercidiphyllaceae, and Daphniphyllaceae); 2) Saxifragaceae (including Grossulariaceae, Iteaceae, and Pterostemonaceae); 3) Crassulaceae; 4)
Haloragaceae (including Aphanopetalaceae and Penthoraceae); (5) Paoniacae; and 6) Peridiscaceae.

**Discussion**

The PCR-based method (genome walking) had been used in the early attempts to sequence many plastid genomes such as *A. trichopoda* (Goremykin et al. 2003b), *C. fertilis* (Goremykin et al. 2003a), *N. alba* (Goremykin et al. 2004), *Oryza nivara* (Masood et al. 2004), and *Aco. calamus* (Goremykin et al. 2005). The field was silent for a few years due to concerns about incorporating extra fragments into the plastid genomes and producing incorrect genome structure because of plastid genome substructuring or high divergence (Jansen et al. 2005). Realizing that such mistakes are unlikely, the same strategy was soon used extensively on plants for which the reference genomes were available (Ibrahim et al. 2006; Mardanov et al. 2008; Wu et al. 2009). However, the same concern remains for the taxa without a reliable reference. There have been some attempts to solve the problem, for example, to determine the IRs of the chloroplast genomes of *Fragaria x ananassa* and *Prunus persica* with universal primers (Dhingra and Folta 2005). Similar to the logic of genome sequencing using NGS, the plastid genome de novo sequencing using either long-range or short-range PCR-based methods is theoretically reliable. The three identical genomes of *L. formosana*, determined by the three different methods used in this study, demonstrate the reliability of the three methods.

**Usefulness of the Universal Primer Set**

The primers given in supplementary table S5, Supplementary Material online, have been tested across all major groups of angiosperms (supplementary fig. S1, Supplementary Material online) and have proven to be efficient for most cases. In direct sequencing. Although there is only approximately 2× coverage, the Sanger sequencing method is the most robust technique in use, and an overlap of 50–100 bp is sufficient to exclude paralogs from assembling into contigs. The concerted evolution of the two copies of IR is complete, which makes direct sequencing possible without cloning. Conventional practice allows for the copying and pasting of one IR sequence into the correct position. The IR regions can also be sequenced separately by amplifying each of them using the long-range PCR method, as used in Wu, Lai, et al. (2009).

The transitions of LSC/IRb, IRb/SSC, SSC/IRa, and IRa/LSC (fig. 1) may be of concern. Although obtaining results is often not a problem, verifications are sometimes necessary because the IRa is usually based on IRb (Jansen et al. 2005). The primers for amplifying the single-copy IR boundary region (supplementary table S7, Supplementary Material online) sometimes do not work because of genome substructuring or too variable flanking sequences of the IR regions, for instance, within Araceae (Ahmed et al. 2012). Taxon-specific primers are preferred after the genome has been assembled. The direction of SSC is potentially erroneous if the *ycf1* and the *ycf1* pseudogene are misplaced. To ensure the correct orientation of SSC, amplification of the entire IR region is necessary, preferably amplifying the IRb for shorter length and more specific priming.

One concern in the use of this method is the possibility that exogenous fragments would be incorporated into the plastid genome or that the fragments of different loci would be assembled into the wrong loci. The alien can hardly be assembled into a plastid genome because it is usually very different or has unreadable peaks when the copies are different. To solve this problem, PCR using adjacent primer combinations would finally eliminate the nonplastid copies. Although repeats have been reported in several groups of plants (Jansen et al. 2005 and the references there in), long repeats outside the IR regions are very rare. Exceptions are found on the biparentally inherited plastid genomes, for instance, *Trachelium caeruleum* (Haberle et al. 2008) and Geraniaceae (Guisinger et al. 2011), which have extensive structural reorganizations. To sequence such genomes, the short-range PCR method will provide sequences for designing taxa-specific primers for long-range PCR.

PCR-based plastid genome sequencing was previously considered labor-intensive and time consuming. With this set of primers, only one and a half PCR plates are required for the whole genome. The efficiency is identical to that of conventional PCR direct sequencing.
addition to that of *L. formosana*, the chloroplast genomes of *Nelumbo lutea* (Xue, Dong, et al. 2012), *Penthorum chinense*, *S. sarmentosum*, and *Haloxylon* spp. (unpublished) have been determined largely using the primer set. The primers can also be very useful for studies such as Soltis et al. (2011) and Jian et al. (2008), in which large data sets are required but the exact plastid genomes are of less consequence.

The universal primer set significantly eases the determination of individual genomes for investigators who have specific interests in certain species. The sequences generated using the universal primer set serve as references for designing taxon-specific primers. The primer set is also useful for those interested in the genomes of remotely related species. In this case, the PCR products can be sequenced using a long-reading NGS.
machine (such as Roche 454) directly without tag labels. Sequencing many species simultaneously in one run is very convenient; the primers significantly reduce the cost and quicken the accumulation of genomes in GenBank.

Utility of the Primer Set in the Phylogenetic Reconstruction of Saxifragales

Recent phylogenetic studies based on multiple genes (Moore et al. 2011; Soltis et al. 2011) suggested a close relationship among the families of Saxifragales and the families formerly belonging to Hamamelidales. The morphology-based circum-scriptions of the two orders and their systematic positions are questionable from a molecular viewpoint. The systematic positions of several families formerly grouped in Hamamelidales and Saxifragales were no longer associated with these groups (fig. 3). Gunneraceae and Trochodendraceae are positioned among the basal eudicots rather than as close relatives of Haloragaceae and Hamamelidales, respectively. *Philadelphus* and *Parnassia*—formerly in Saxifragaceae—nest with asterids and rosids, respectively. Saxifragales can be treated as an order of the rosids. Considering short branches and relatively low bootstrap supports, it seems more natural to merge 1) Altingiaceae, Cercidiphyllaceae, and Daphniphyllaceae into Hamamelidaceae, 2) Grossulariaceae, Iteaceae, and Pterostemonaceae into Saxifragaceae, and 3) Aphanopetalaceae, Myriophyllaceae, Penthoraceae, and Tetracarpaceae into Haloragaceae.

Gaps of the Primer Set and Working Tips

Most of the primers used in this study are predicted to work for a majority of angiosperms. However, difficulties might arise from some primers located at rapidly evolving regions such as accD, matK, ndhF-rpl32, rpl20-clpP, rpoB, rps4, rps8, and ycf1. The ycf1 gene would require further analysis due to its long length and rapid evolution. For such regions, taxon-specific primers (Neubig and Abbott 2010; Franck et al. 2012) and long-range PCR are needed. Some PCR failures could result from the loss of genetic regions rather than primer problems. For example, ycf1 and ycf2 are absent in Poaceae. New combinations of forward and reverse primers at adjacent sites could rectify such problems. It is also strongly recommended to verify the IR regions with fragment sequences amplified with taxon-specific primers after the genome has been assembled.

Supplementary Material

Supplementary figures S1–S3 and tables S1–S10 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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