METHODS AND TECHNIQUES

Keep the DNA rolling: Multiple Displacement Amplification of archival plant DNA extracts

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Insufficient reserves of genomic DNA can hamper molecular phylogenetic analysis. High-throughput genetic techniques that require relatively large amounts of DNA, the difficulty in obtaining samples of taxa from remote regions, and re-sampling of limited archival DNA by repeated phylogenetic surveys can often limit the DNA available for study. To provide a possible solution to this problem, we applied Multiple Displacement Amplification (MDA) to eight archival genomic DNA extracts. The performance of MDA-treated DNA versus untreated genomic extract was evaluated by PCR amplification of three common phylogenetic markers (*psbB*, *nad7*, ITS) across a dilution series. Generally, amplification of all three genetic markers from the MDA-treated DNA dilutions was greater than from equivalent dilutions of untreated genomic template. These results indicate that genes from all three plant genomes were amplified and that copies of the target genes *psbB*, *nad7*, and ITS were substantially increased during the MDA procedure. Sequencing of the *psbB*, *nad7*, and ITS PCR products from both the MDA-treated DNA and the untreated template was used to assess the fidelity of the MDA procedure. Sequences from the MDA-treated DNA and the untreated genomic template differed by 1.2×10^{-4}%, which is within the margin of *Taq* error. These findings emphasize the significance of Multiple Displacement Amplification for optimization of weak PCR, maintenance of depleted genetic stocks, increasing density of taxon sampling, and improving consistency between different phylogenetic analyses.

KEYWORDS: Caryophyllales, DNA bank, plant DNA extraction, Rolling Circle Amplification, RCA, whole genome amplification

INTRODUCTION

Many plant systematists work with taxa that are rare or difficult to collect, making it difficult or impossible to obtain samples for DNA isolation. Small fragments of silica-dried material may be the only source of material for hard-to-obtain taxa. Herbarium collections and DNA banks are therefore an invaluable source of material for phylogenetic studies and are increasingly important as a source of DNA for molecular analyses (Drabkova & al., 2002). The success of these molecular studies depends on DNA of adequate quantity and quality and yet extraction of sufficient amounts of high-quality DNA from herbarium specimens or silica-dried material can be challenging. Herbaria may limit destructive sampling of specimens in order to preserve collections relatively intact, specimens may be rare and unavailable for sampling, and the DNA of herbarium specimens may have degraded because of age or poor preservation, yielding low quantities of DNA. Furthermore, the DNA of hard-to-obtain samples is often used repeatedly in phylogenetic surveys (Chase & al., 1993; Soltis & al., 2000) and is ultimately depleted.

In recent years a number of methods has been developed for whole genome amplification that generate considerable amounts of DNA from minute quantities of starting material (Pinard & al., 2006). Rolling Circle Amplification (RCA), also known as Multiple Displacement Amplification (MDA), is a widely used method for whole genome amplification that employs a bacteriophage φ29 DNA polymerase together with exonuclease-resistant degenerate primers to amplify genomic DNA isothermally at 30ºC (Dean & al., 2002; Lage & al., 2003). MDA has a number of advantages over PCR-based methods of genome amplification. These include a less biased amplification of the genome, greater yields of DNA, and higher fidelity to the template DNA (Pinard & al., 2006).

The importance of genomic data in biological sciences often places considerable demand on supplies of DNA; therefore, there is interest in the use of MDA to augment stocks of low-abundance DNA (Lasken & Egholm, 2003). Several studies from a range of biological disciplines have

already demonstrated the utility of MDA in the molecular analysis of rare biological material. Examples of low-abundance biological samples that have been successfully amplified using MDA include: limited medical samples such as buccal swabs and single cell samples generated by laser capture microscopy (Lasken & Egholm, 2003), single microorganisms (Raghunathan & al., 2005), and single spores of abuscular mycorrhizal fungi (Gadkar & Rillig, 2005). Despite these successful applications of MDA, the technique does not seem to have been widely adopted by plant systematists, although supplies of DNA may often be limited.

The aim of this study was to assess the efficacy of MDA for amplification of plant DNA extractions by amplifying and sequencing common phylogenetic markers from MDA-treated DNA. We amplified genes from each of the three plant genomes: *psbB* (plastid genome), *nad7* (mitochondrial genome), and Internal Transcribed Spacer (ITS; nuclear genome) with the goal of investigating the PCR performance of MDA-treated DNA over its untreated template.

MATERIALS AND METHODS

DNA extractions from herbarium specimens. — DNA from eight different species (Appendix) belonging to the angiosperm order Caryophyllales was used as template for MDA. These DNA samples were extracted from materials of varying age and condition, which was reflected in the variable quality and quantity of the DNA extracted (Fig. 1)

Multiple Displacement Amplification. — DNA extracts from the eight species were amplified using the GenomiPhi kit (Amersham, Piscataway, New Jersey, U.S.A.) according to the manufacturer's instructions. Reactions were performed using crude DNA extract. In each case, 1 µl of crude DNA extract was combined with 9 µl of sample buffer, heated to 95°C for 3 min, and then chilled to 4°C. To each tube, 9 µl of reaction buffer and 1 µl of GenomiPhi enzyme mix were added, and the reactions incubated at 30°C for 16 hrs. A negative control was performed in which no input DNA was added. On completion of the MDA amplification, a 1 µl aliquot of the reaction was fractionated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. For comparison a 1 µl aliquot of the untreated DNA extraction was run along with its respective MDA-treated DNA.

PCR. — Untreated DNA extract and MDA-treated DNA were both serially diluted: 1 : 5, 1 : 10, 1 : 100, and 1 : 200. To assess the value of MDA-treated DNA versus untreated DNA extract, PCR was performed across this dilution series. Gene regions from the three plant genomes were amplified: *psbB*, *nad7*, and ITS. Amplification and sequencing was performed in accordance with previously published protocols (Soltis & al., 2000).

psbB was amplified according to previously published protocols (Soltis & al., 2000) (95°C for 3 min, followed by 30–35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with a final extension time of 7 min at 72°C) using primers 60F (ATG GGT TTG CCT TGG TAT CGT GTT CAT AC) and 66R (CCA AAA GTR AAC CAA CCC CTT GGA C) (Graham & Olmstead, 2000), which amplify 1,362 bp between positions 1 and 1,362 (inclusive) of *psbB*. *nad7* was amplified (95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with a final extension time of 7 min at 72°C) using primers nad7/2 (GCT TTA CCT TAT TCT GAT CG) and nad7/3 (TGT TCT TGG GCC ATC ATA GA) (Duminil & al., 2002) which amplify ~1,000 bp product. ITS was amplified (95°C for 2 min, followed

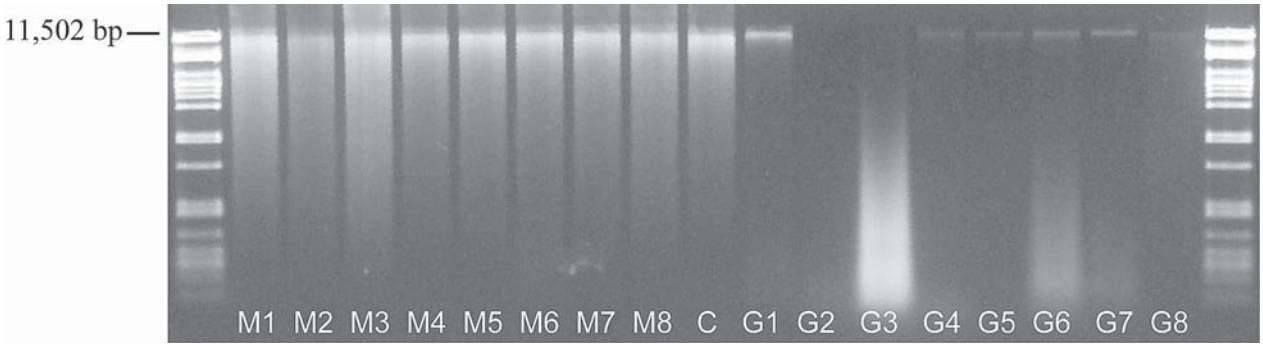

Fig 1. MDA-treated reaction products (M1–M8) and corresponding untreated genomic equivalent (G1–G8). M1 & G1, *Halophytum ameghinoi*; M2 & G2, *Mollugo verticillata*; M3 & G3, *Mirabilis jalapa*; M4 & G4, *Drosera capensis*; M5 & G5, *Limonium arborescens*; M6 & G6, *Talinum paniculatum*; M7 & G7, *Polygonum virginianum*; M8 & G8, *Rhabdodendron amazonicum*. C, the control MDA reaction to which no genomic template was added. DNA Ladder: λDNA/Pst1 (MBI Fermentas Inc, Hanover, New Hampshire, U.S.A)

by 5 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min with a step down to 28 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min) using primers ITSA (GGA AGG AGA AGT CGT AAC AAG G) and ITSB (CTT TTC CTC CGC TTA TTG ATA TG) (Blattner, 1999) which amplify a 650–700 bp product. The PCR products were fractionated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

PCR cleanup and sequencing. — To remove unused primers and nucleotides, PCR product was treated with ExoSAP-IT (USB Corporation, Cleveland, Ohio, U.S.A.). All PCR products were sequenced using the same primers as for the PCR with the exception of the *psbB* products, which were sequenced using nested primers B60F (CAT ACA GCT CTA GTT KCT GGT TGG) and B66R (CCC CTT GGA CTR CTA CGA AAA ACA CC) (Graham & Olmstead, 2000). Sequencing was performed on the Applied Biosystems 3730xl capillary DNA sequencer. Raw data signals were automatically base-called and subsequently manually checked using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.). Sequences were aligned using Se-Al (Department of Zoology, Oxford, U.K.).

The sequences generated by this study have been submitted to GenBank: EU410352–EU410375.

RESULTS

Multiple Displacement Amplification. — MDA on all eight DNA extracts resulted in an increase in high-molecular-weight DNA (10–11 Kb) relative to the template DNA (Fig. 1). This increase in high-molecular-weight DNA is especially apparent for *Mollugo verticillata* and *Mirabilis jalapa* from which either very little or highly degraded DNA was initially extracted. An amplification product also occurs in the negative control. This is expected when using the GenomiPhi kit and is most likely due to the non-specific amplification of degenerate hexamer primers rather than to DNA contamination (Amersham Biosciences, Protocol 74004229). The presence of this amplification product in the no-template control, however, prevents immediate evaluation of the success of MDA in specifically amplifying herbarium DNA. PCR was therefore performed to evaluate the utility of the MDA-treated DNA.

PCR. — Amplification of *psbB* was initially performed on a 1 : 25 dilution of the MDA-treated DNA. All eight MDA-treated DNAs generated a strong amplification product of the expected size (Fig. 3 in Taxon online issue). PCR performed on the no-template MDA control product did not generate the expected amplification product, demonstrating that the amplification products from PCR of MDA-treated DNA were not due to spurious amplification during the MDA procedure.

To assess the effectiveness of the MDA-treated DNA versus the original DNA template, amplification of *psbB*, *nad7*, and ITS was performed across a dilution series. Reactions contained 0.2, 0.1, 0.01, or 0.005 µl of either the untreated genomic template or its MDA-treated equivalent. As a general trend, for all species and at all dilutions, the MDA-treated product generated a similar or stronger PCR amplification than the corresponding genomic template (Fig. 3 in Taxon online issue). This is particularly apparent for the amplification of *psbB, nad7* and ITS from *M. verticillata* and *M. jalapa* (Fig. 2; Figs. 4–6 in Taxon online issue). Differences between dilutions for the amplification of ITS are less striking but still discernable. For all DNA regions however, it is most informative to compare the 1 : 5 and 1 : 10 MDA-treated dilution with the respective 1 : 100 and 1 : 200 dilutions of the untreated genomic template. This is an appropriate comparison because the MDA procedure effectively dilutes the initial genomic template by 1 : 20 (1 µl of template in a 20 µl MDA reaction) and thus following further dilution, the 1 : 5 (M1) and 1 : 100 (G3), and 1 : 10 (M2) and 1 : 200 (G4) represent equivalent dilutions of the initial genomic stock. Amplifications from the 1 : 5 and 1 : 10 dilutions of MDA-treated DNA are without exception considerably stronger than their respective 1 : 100 and 1 : 200 dilutions of genomic DNA across all eight species for all three genes (Fig. 2; and Figs. 4–6 in Taxon online issue).

Sequencing. — PCR products from the untreated DNA and corresponding MDA-treated DNA were sequenced to ensure that the amplifications were not due to spurious DNA amplified during the MDA procedure. In all cases, the sequences from untreated and MDA-treated DNA were nearly identical with a difference of only 1.2×10^{-4}%. These differences are within the margin of *Taq* error, demonstrating that no contamination had occurred during the MDA procedure and that replication of DNA in the MDA treatment had been of high fidelity.

DISCUSSION

Phylogenetic studies of plants are based on ever-increasing portions of the nuclear, mitochondrial, and plastid genomes and utilize high-throughput sequencing technology that can often exhaust reserves of DNA. Archived collections of DNA can gradually degrade with use and age and may be difficult to replenish or restore. Collaborative projects rely on the same samples for genetic analysis, yet limited stocks of archival DNA force participating laboratories to exchange minute quantities of DNA. Increased taxon sampling in molecular phylogenetics has created a demand for DNA from difficult-to-obtain species and

DNA from these hard-to-obtain species is often available in only limited amounts. For these reasons, DNA is often a limiting resource in molecular phylogenetic analyses.

Strong initial amplification of the *psbB* gene from MDA-treated DNA suggested that template DNA was successfully replicated during the MDA procedure (Fig. 3 in Taxon online issue). Comparisons of *psbB*, *nad7*, and ITS amplification across dilutions of untreated and MDA-treated DNA confirmed that amplification was generally greater from MDA-treated DNA than from the original template (Fig. 2; and Figs. 4–6 in Taxon online issue). This was particularly apparent in the amplification of the *psbB* and *nad7* genes; however, differences in the amplification of ITS from MDA-treated DNA versus genomic DNA, though present, were less distinct. This might be due to the efficiency of the ITS amplification protocol and to the high abundance of the 18S rDNA template in the genomic extract. Nonetheless, the generally increased amplification from MDA-treated DNA indicates that copies of the target genes *psbB*, *nad7*, and ITS were substantially increased during the MDA reaction as all three genomes were amplified during the MDA procedure. This enhanced amplification suggests that MDA could be a rapid and efficient procedure for the optimization of PCR for genes that weakly amplify from the original template. The comparisons of 1 : 5 (MDA-treated) with 1 : 100 (untreated genomic), and 1 : 10 (MDA-treated) with 1 : 200 (untreated genomic) dilutions revealed dramatic increases in amplification from MDA-treated DNA. A relatively small investment of 1 µl of genomic DNA generates 20 µl of MDA-treated DNA, and improved subsequent PCR of the genes of interest. This illustrates the value of MDA for the augmentation of depleted DNA stocks. Sequencing of the PCR products confirmed that MDA replicated the genomic template with a high degree of fidelity as expected from previous studies that have more rigorously assessed the fidelity of the ϕ29 DNA polymerase (Pinard & al., 2006).

These findings demonstrate that MDA is a useful procedure for pre-amplification of precious archival DNA prior to subsequent genetic analysis. Pretreatment by MDA enhanced amplification of all three molecular markers from the three plant genomes, while maintaining fidelity to the original template sequence. MDA therefore offers a way to alleviate problems of depleted DNA stocks and help to maintain DNA "standards" to ensure sampling consistency among phylogenetic studies. Multiple rounds of MDA may even be feasible ensuring almost "infinite" supplies of DNA (Sato & al., 2005).

It is important to note that all the DNA extracts utilized in this study were of sufficient quality to amplify the phylogenetic markers from the genomic extract prior to MDA treatment. Successful MDA requires the input of good quality DNA and thus it is not clear that this procedure would permit the augmentation of severely degraded DNA stocks. In our experience, poor quality DNA derived from very old or poorly preserved herbarium material may not be an appropriate substrate for MDA (data not shown). Furthermore, MDA enzymes, as in PCR, may be susceptible to inhibitory substances that can be present in extracted DNA. These limitations mean that the usefulness of MDA must be determined empirically, taking into account the quality of the extracted DNA and its performance in PCR prior to MDA treatment. We have observed that if successful PCR has been performed on a DNA extract, MDA is also likely to perform well. Despite these

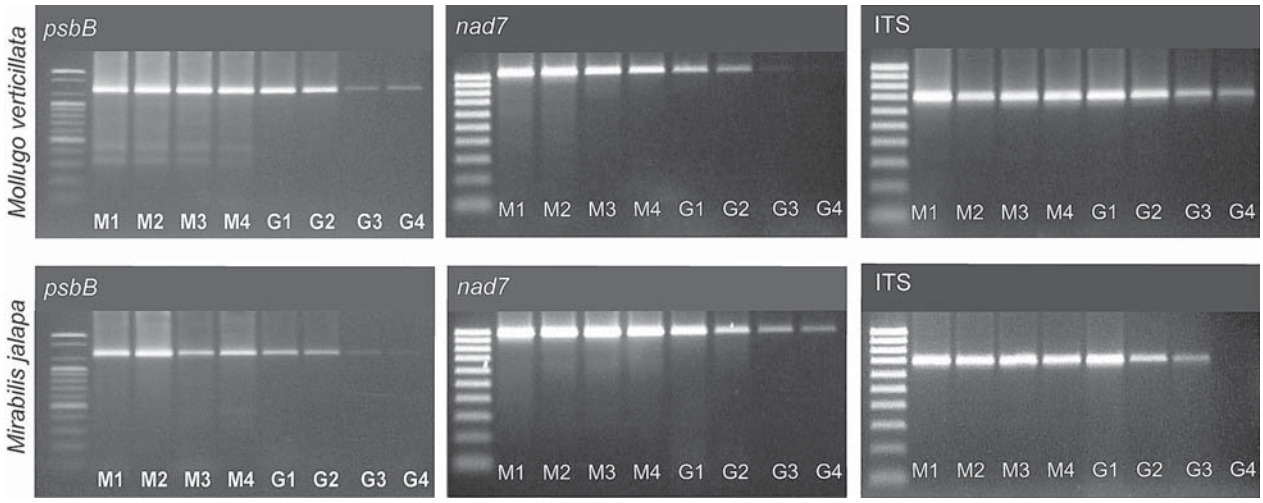

Fig. 2. Amplification of 1,363 bp fragment of *psbB*, fragment of *nad7* gene, fragment of ITS for two of the eight species, *Mollugo verticillata* and *Mirabilis jalapa*. Amplifications across dilution gradient of MDA-treated template (M1, 1 : 5; M2, 1 : 10; M3, 1 : 100; M4, 1 : 200) and untreated genomic template (G1, 1 : 5; G2, 1 : 10; G3, 1 : 100; G4, 1 : 200). 100 bp ladder (Bioneer, Alameda, California, U.S.A.).

final cautionary comments, this approach still provides a cheap and efficient method for enhancing and maintaining minute DNA stocks from numerous invaluable museum collections worldwide.

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LITERATURE CITED

Blattner, F.R. 1999. Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. *Biotechniques* 27: 1180–1186

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y.L., Kron, K.A, Rettig, J.H., Conti, E., Palmer, J.D., Manhart, J.R., Sytsma, K.J., Michaels, H.J., Kress, W.J., Karol, K.G., Clark, W.D., Hedren, M., Gaut, B.S., Jansen, R.K., Kim, K.J., Wimpee, C.F., Smith, J.F., Furnier, G.R., Strauss, S.H., Xiang, Q.Y., Plunkett, G.M., Soltis, P.S., Swensen, S.M., Williams, S.E., Gadek, P.A., Quinn, C.J., Eguiarte, L.E., Golenberg, E., Learn, G.H., Graham, S.W., Barrett, S.C.H., Dayanandan, S. & Albert, V.A. 1993. Phylogenetics of Seed plants—an analysis of nucleotide-sequences from the plastid gene *Rbcl*. *Ann. Missouri Bot. Gard.* 80: 528–580.

Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M. & Lasken, R.S. 2002. Comprehensive human genome amplification using multiple displacement amplification. *Proc. Natl. Acad. Sci. U.S.A.* 99: 5261–5266.

Drabkova, L., Kirschner J. & Vlcek, C. 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Juncaceae. *Pl. Molec. Biol. Rep.* 20: 161–175.

Duminil, J., Pemonge, M.H. & Petit, R.J. 2002. A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. *Molec. Ecol. Notes* 2: 428–430.

Gadkar, V. & Rillig, M.C. 2005. Application of Phi29 DNA polymerase mediated whole genome amplification on single spores of arbuscular mycorrhizal (AM) fungi. *F.E.M.S. Microbiol. Lett.* 242: 65–71.

Graham, S.W. & Olmstead, R.G. 2000. Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. *Amer. J. Bot.* 87: 1712–1730.

Lage, J.M., Leamon, J.H., Pejovic, T., Hamann, S., Lacey, M., Dillon, D., Segraves, R., Vossbrinck, B., Gonzalez, A., Pinkel, D., Albertson, D.G., Costa, J. & Lizardi, P.M. 2003. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. *Genome Res.* 13: 294–307.

Lasken, R.S. & Egholm, M. 2003. Whole genome amplification: abundant supplies of DNA from precious samples or clinical specimens. *Trends Biotechnol.* 21: 531–535.

Pinard, R., De Winter, A., Sarkis, G.J., Gerstein, M.B., Tartaro, K.R., Plant, R.N., Egholm, M., Rothberg, J.M. & Leamon, J.H. 2006. Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing: *BMC Genomics* 7: 216.

Raghunathan, A., Ferguson, H.R., Bornarth, C.J., Song, W., Driscoll, M. & Lasken, R.S. 2005. Genomic DNA amplification from a single bacterium. *Appl. Environ. Microbiol.* 71: 3342–3347.

Sato, M., Ohtsuka, M. & Ohmi, Y. 2005. Usefulness of repeated GenomiPhi, a phi29 DNA polymerase-based rolling circle amplification kit, for generation of large amounts of plasmid DNA. *Biomolec. Engin.* 22: 129–132.

Soltis, D.E., Soltis P.S., Chase, M.W., Mort, M.E., Albach, D.C., Zanis, M., Savolainen, V., Hahn, W.H., Hoot, S.B., Fay, M.F, Axtell, M., Swensen, S.M., Prince, L.M., Kress, W.J., Nixon, K.C. & Farris, J. S. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. *Bot. J. Linn. Soc.* 133: 381–461.

Appendix. Species of Caryophyllales used in this study, family, source, collection date, accession number and DNA extraction method.

Species, family, voucher, date of collection, DNA extraction method
Halophytum ameghinoi, Halophytaceae, *Tortosa & al. s.n* K, 1997, CTAB; *Mollugo verticillata*, Molluginaceae, *Moore 321* FLAS, 2006, DNAeasy; *Mirabilis jalapa*, Nyctaginaceae, *Soltis 2638* FLAS, 2004, CTAB; *Drosera capensis*, Droseraceae, *Moore 267* FLAS, 2006, DNAeasy; *Limonium arborescens*, Plumbaginaceae, *Chase 1649* K, Uncertain , CTAB; *Talinum paniculatum*, Portulaccaceae, *Soltis 2646* FLAS, 2004, CTAB; *Polygonum virginianum*, Polygonaceae, *Soltis 2656* FLAS, 2005, DNAeasy; *Rhabdodendron amazonicum*, Rhabdodendraceae, *E. Ribéiro* 1187 K, 1993, CTAB.