Successful DNA amplification of a more than 200-year-old herbarium specimen: recovering genetic material from the Linnaean era

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The limit for successful DNA extraction was tested by amplification and sequencing of an over 200-year-old herbarium specimen collected by Adam Afzelius, a student of Carl Linnaeus. We amplified and sequenced a 800-bp region between 16S ribosomal DNA and the 3′ part of the *trnI* gene (16S-*trnI*) in the chloroplast genome of *Phaulopsis talbotii* S. Moore (Acanthaceae). To test the replicability and to control for contamination the procedure was performed in sealed vials and with negative PCR controls. The procedure was also repeated in a separate laboratory. In addition, the chloroplast *rpl16* intron was successfully amplified and sequenced and the *rps16* intron amplified. Sequences of taxa closely related to Acanthaceae were found to be most similar to the produced sequences. The results suggest that molecular investigations of other 18th century botanical collections are feasible and that molecular methods could be employed for comparative studies to extant plant collections. An important application would be to identify descendants or clones of Linnaean lectotypes by comparing DNA from these with potentially remnant plants from Linnaeus' cultivations.

KEYWORDS: 16S-*trnI*, chloroplast DNA, DNA amplification, herbarium specimens, *Phaulopsis*, *rpl16*

INTRODUCTION

Museum specimens constitute the basic source of biodiversity information not only for morphological studies, but also for studies in molecular evolution, due to the recent rapid development of molecular methods, especially PCR (polymerase chain reaction; Mullis & Faloona, 1987). There are several examples of studies of plants, fungi, and animals where DNA has been successfully recovered from minute amounts of material from museum collections (e.g., Rogers & Benedich, 1985; Pääbo, 1989; Savolainen & al., 1995; De Castro & Menale, 2004; Jankowiak & al., 2005; Cota-Sánchez & al., 2006). This has opened up the possibility to answer evolutionary questions that otherwise would remain unanswered.

The age of herbarium material from which DNA has been successfully amplified has varied in different studies. The length of the amplified fragment that has been recovered has also varied and should be correlated to the degree of degradation of the DNA. DNA extracted from two *Pinus* collections from 1811 (De Castro & Menale, 2004) was amplified and sequenced, although the fragments were short (less than 150 basepairs [bp]). Liverwort material collected in 1905 was successfully amplified for a fragment of about 450 bp (but not sequenced; Jankowiak & al., 2005). In flowering plants, Savolainen & al. (1995) tested several old specimens and succeeded in amplifying a 369-bp long fragment of the chloroplast *atpB-rbcL* spacer in a 109-year-old collection of *Cardiopteris*. Although they did not attempt to obtain a sequence Cota-Sánchez & al. (2006) got weak amplifications of a 1,000-bp long chloroplast region from a 62-year-old herbarium specimen. We have also succeeded in sequencing DNA from 80 and 100-year-old material (Andreasen, unpub. data; Razafimandimbison, unpub. data). Consensus from these studies is that the preservation of DNA varies depending on the type of material and how rapidly the material was dried is more important than the actual age of the specimen (Savolainen & al., 1995; Drábková & al.,2002; Jankowiak & al., 2005).

Our study was initiated to investigate the possibility of identifying the remnant plants from Carl Linnaeus' cultivations in his country estate Hammarby outside Uppsala in Sweden (Manktelow, 2001). This could be accomplished by comparing DNA from extant living plants with specimens selected as lectotypes in the Linnaean collections of the Linnean Society of London (Jarvis, 2007). If we could show that successful amplification of 18th century historical specimens was feasible, then sampling of the invaluable Linnaeus material could be warranted for comparative analysis aimed at identifying descendants or clones of Linnaean lectotypes. As the 18th century was an intensive period of research in plant systematics and horticulture, this could also help to identify extant living collections as well as herbarium specimens from other

contemporary, important botanists (see e.g., De Castro & Menale, 2004).

Based on the great potential of molecular studies including material from the century that saw the dawn of modern taxonomy, the aim with this study was to (1) test if herbarium material over 200 years old could successfully be extracted, amplified and yield readable sequences and (2) try to extend the length of the amplified fragment in comparison to earlier attempts with historic material.

MATERIALS AND METHODS

Plant accession. — Adam Afzelius (1750–1837) was one of the later students of Carl Linnaeus. He took his doctor's degree in 1776, only two years before Linnaeus died. As the last of Linnaeus' students to travel abroad, he was recommended by Sir Joseph Banks to join the Sierra Leone Company for two expeditions to Sierra Leone in 1792–1793 and 1794–1796. His resulting West African herbarium was later included in the collections of Uppsala University, and one of the species he collected was *Phaulopsis talbotii* S. Moore (Acanthaceae). The genus *Phaulopsis* is well known to us (Manktelow, 1996), and Afzelius' specimens are well preserved with leaves that are still green. One inflorescence bract (ca. 0.5 cm^2) was removed from *Afzelius s.n.* (UPS loan number 3843/17) for our molecular investigation.

DNA extraction. — Total DNA was extracted following the CTAB protocol (Saghai-Maroof & al., 1984; Doyle & Doyle, 1987), with ethanol precipitation. A tube with plant material, buffer and beads (2.5 mm zirconia/silica) was shaken at 5,000 rpm for 40 s in a mini-Beadbeater (BioSpec Products). The total DNA was cleaned using Glass Milk (Gene Clean, Bio 101) following the protocol of the manufacturer and visualized on a 1% agarose gel before subsequent PCR.

The extraction of the plant material, and PCR and sequencing of the 16S-*trnI* region were carried out at the Department of Systematic Botany, Uppsala University. To test the replicability and control for contamination the procedure was also performed in a different laboratory (Bergius Foundation, Department of Botany, Stockholm University) as follows. Total DNA was isolated following the miniprep procedure of Saghai-Maroof & al. (1984) as modified by Doyle & Doyle (1987). We grinded ca. 0.02 g of the leaf material with a mini-Beadbeater (BioSpec Products) set at 5,000 rpm for 30 s. The purified total DNA was directly used for subsequent PCR amplification.

PCR amplification and sequencing. — We amplified a region between 16S ribosomal DNA and the 3′ part of the *trnI* gene in the chloroplast genome. Primers used for amplification and sequencing were trnI-1F (5′-GACT GGAGTGAAGTCGTAACAAGG-3′) which starts 61 bp upstream from the 3′ end of 16S ribosomal DNA, and trnI-4R (5′-GCTCGTAGTTCTTGGTCTGTG-3′) which attaches in the middle of the *trnI* intron (369 bp downstream from the 5′ part of the *trnI* gene in *Mentha* × *piperita* DQ001743). We also amplified the *rpl16* intron in the chloroplast using the primer pair 1067F (designed by Conny Asmussen, pers. comm.) and L16 exon1 (Downie & al., 2000).

The PCR reactions were performed using an Eppendorf Mastercycler Gradient thermal cycler with an initial 95°C denaturation (2 min) and 35 cycles of 95°C denaturation (35 s), 55°C annealing (1 min), and 72°C extension (2 min). The 25-µl reactions consisted of 0.1 µl Thermostable DNA Polymerase (Advanced Biotechnologies), 2.5 µl each of reaction buffer, 25 mM $MgCl_2$, 10 mM dNTP, and 0.1 M TMACL, 1.25 µl each of 10 µM forward and reverse primer, and 12.5 µl of diluted DNA (1 : 50). A negative control with all the mentioned ingredients but with water instead of DNA was included to check for contamination of the PCR chemicals. The resulting product was visualized on a 1% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen). The DNA concentration was determined spectrophotometrically on a Gene Quant II (Pharmacia Biotech).

One microliter of the cleaned PCR product was included in a 10-µl reaction and sequenced with the primers trnI-1F and trnI-4R and 1067F and L16 exon1, respectively, using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech) on an Eppendorf Mastercycler Gradient thermal cycler using the specifications included in the kit. The samples were purified by ethanol precipitation and run on a MegaBACE 1000 DNA Analysis System (Amersham Pharmacia Biotech).

At the Department of Botany, Stockholm University, two 50-µl PCR reactions were run on another Eppendorf Mastercycler Gradient thermal cycler using the same conditions outlined above. One reaction contained 0.25 µl Thermostable DNA Polymerase (Advanced Biotechnologies), 5 µl each of reaction buffer, 25 mM $MgCl_2$, and 0.1 M TMACL, 4 µl of 10 mM dNTP, 0.5 µl each of 20 µM of the forward and reverse primers, 1.5 µl non-diluted DNA template, and 28.25 µl sterilized water. A negative control with water instead of DNA was included. The *rps16* intron region of the chloroplast genome was also amplified using the primer pair F/R2 (Oxelman & al., 1997).

We prepared 10 µl sequencing reactions containing the same primer pairs as for the PCR and 1 µl of the purified PCR product. The sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Sequencing kit and subsequently analyzed with the 3100 Genetic Analyzer (Applied Biosystems).

The two *trnI* sequences (EMBL accession number FM210468) produced by the different laboratories were

compared to each other and checked for sequencing/PCR artefacts. They were also blasted at http://ncbi.nlm.nih.gov/blast/Blast.cgi for similar sequences, as was the *rpl16* sequence (EMBL accession number FM210469).

RESULTS

The PCR reactions resulted in bright bands of approximately 800 bp for the 16S-*trnI* region (Fig. 1). There were no visible bands in the negative controls. The DNA concentration after amplification was 30 ng/µl as measured on a spectrophotometer. The two sequences produced by the different laboratories were compared to each other and they were found to be nearly identical (2 bp differed at the beginning of the sequence). The BLAST search showed that the taxa with highest match scores (96% similarity) were *Solanum tuberosum* (GenBank accession number DQ386163), *Jasminum nudiflorum* (DQ673255) and *Mentha* × *piperita* (DQ001743).

The amplification and sequencing of the *rpl16* intron resulted in a sequence of 823 bp that BLASTed to *Mimulus* spp. (e.g., DQ090905). The amplification of the *rps16* chloroplast region resulted in a ca. 700-bp fragment.

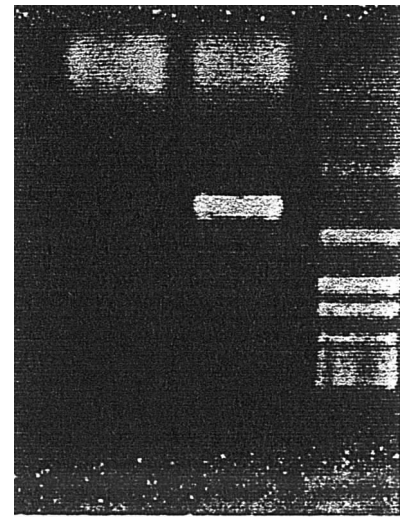

Fig. 1. The PCR product of the approximately 800-bp region between 16S ribosomal DNA and the 3′ part of the *trnI* gene in the chloroplast genome loaded on a 1% agarose gel. Lane one: negative control, lane two: *Phaulopsis talbotii*, lane three: 1 kbp ladder.

DISCUSSION

Our 16S-*trnI* sequences produced in two different laboratories were identical except for two positions at the beginning of the sequence, which are likely due to PCR or sequencing artefacts. Both 16S-*trnI* sequences as well as the *rpl16* intron BLASTed to taxa (*Solanum*, Solanaceae; *Jasminum*, Oleaceae; *Mentha*, Lamiaceae; and *Mimulus*, Phrymaceae) belonging to the Lamiids as do *Phaulopsis* (Acanthaceae; Angiosperm Phylogeny Group, 2003). As there are no published complete chloroplast genome sequences from the family Acanthaceae, nor any published 16S-*trnI* or *rpl16* sequences, the result with taxa closely related to Acanthaceae turning up in our BLAST query was expected.

Many researchers have emphasized the risk of contamination, especially when trying to amplify old material with low DNA concentration (e.g., Taylor & Swann, 1994; Savolainen & al., 1995). In order to get an amplification double-PCR (when a second round of PCR is performed with the PCR product from the first round) and/or more cycles are often attempted. Savolainen & al. (1995) reported on contamination in several of their PCR attempts unless the chemicals were exposed to UV light before adding enzyme and template. In the present study we avoided using double-PCR and the number of cycles was not increased. In addition to reproducing our results in a different laboratory we aimed at avoiding contamination by carrying out the extraction in a sealed tube using a bead-beater instead of an open vial. We included negative controls when performing the PCR reactions to be able to detect possible contamination of the PCR chemicals. Since there were no other Acanthaceae extractions carried out at the same time as we did our extractions, and also since we were able to reproduce the 16S-*trnI* sequence in a different laboratory, we regard our sequences as originating from the Afzelius material and not from contamination.

Recently, Ames & Spooner (2008) amplified and sequenced ca. 440-bp DNA fragments from potato material from the early 17th century. This is the oldest published flowering plant material known to us from which DNA has been successfully amplified and sequenced. In addition, Savolainen & al. (1995) sequenced 369 bp from a 109-year-old collection of *Cardiopteris* (Cardiopteridaceae, Aquifoliales). Our results extend the fragment length of successful PCR amplification of plant material from the 17th century and together with the recent results of Ames & Spooner (2008) suggest that even older herbarium material than earlier thought may produce amplifiable DNA. This is due to the fact that, as indicated in earlier studies (e.g., Savolainen & al., 1995; Jankowiak & al., 2005), the speed and method of drying is likely more important than the age of the sample.

From the Afzelius material we amplified PCR fragments of three different chloroplast regions from ca. 700 to over 800 bp. This is about twice the length of fragments from herbarium material of *Cardiopteris* and potato (Savolainen & al., 1995; Ames & Spooner, 2008), which further supports the idea that DNA from old material not

necessarily is more fragmented or degraded compared to younger material. The drying method as well as presence of PCR-inhibiting substances affect the outcome of PCR (Savolainen & al., 1995; Jansen & al., 1999). The Afzelius specimens are well preserved with leaves that are still green, suggesting a short desiccation process as well as continuously dry herbarium storage. In addition, the amounts of the amplified PCR product (30 ng/µl) suggest that the Afzelius material contain relatively large amounts and high grade DNA. As a comparison, Jankowiak & al. (2005) obtained 40 ng/µl from herbarium material only a few years old.

The results presented here could potentially enable us to identify and preserve the descendants of Linnaean lectotypes still growing where Linnaeus once planted them in Hammarby, Uppsala. Furthermore, it opens up the possibility for historical gardens around the world to use molecular methods in similar plant identification projects. This increased availability of 18th century plant material could enable us to find answers on scientific questions that earlier was considered to be beyond our reach.

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