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Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus *Dalbergia*

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ABSTRACT. Five published DNA extraction protocols were compared for their ability to produce good quality DNA from fresh and herbarium leaves of several species of the genus *Dalbergia*. The leaves of these species contain high amounts of secondary metabolites, which make it difficult to perform a clean DNA extraction and thereby interfering with subsequent PCR amplification. The protocol that produced the best DNA quality in most of the *Dalbergia* species analyzed, utilizes polyvinylpyrrolidone to bind the phenolic compounds, a high molar concentration of NaCl to inhibit co-precipitation of polysaccharides and DNA, and LiCl for removing RNA by selective precipitation. The DNA quality of herbarium specimens was worse than that for fresh leaves, due to collecting conditions and preservation of samples. We analyzed 54 herbarium specimens, but the recovered DNA allowed successful PCR amplification in only eight. For the genus *Dalbergia*, the herbarium is an important source of material for phylogenetic and evolutionary studies; due to the occurrence of the different species in various geographical regions

in Brazil, it is difficult to obtain fresh material in nature. Our results demonstrated that for *Dalbergia* species the methods used for the collection and preservation of herbarium specimens have a mayor influence on DNA quality and in the success of phylogenetic studies of the species.

Key words: *Dalbergia* species, DNA extraction, Herbarium specimens, PCR amplification

INTRODUCTION

The genus *Dalbergia* (Fabaceae - Papilionoideae) comprises more than 100 species of trees, shrubs and lianas distributed pantropically (Polhill, 1981), of which 41 species occur in Brazil (Carvalho, 1997). Many of these species are economically important due to their attractive and valuable timber; however, over-exploitation and habitat fragmentation have caused them to be in danger of extinction in the tropics, such as *D. nigra* (Brazilian rosewood; Ribeiro et al., 2005). Carvalho (1989) performed a systematic study of the genus *Dalbergia* in Brazil, combining reviews of taxonomic history and biogeography, and data on vegetative and reproductive morphology, comparative anatomy of leaf and fruits, as well as of leaf flavonoids and pollen morphology. The genus *Dalbergia* is recognized as having five sections defined by inflorescence and fruit characters: sects. *Dalbergia* L.f., *Triptolemea* (Mart. ex Benth.) Benth., *Selenolobium* Benth., *Pseudecastaphyllum* A.M. de Carvalho, and *Ecastaphyllum* (P.Browne) Ducke (Carvalho, 1997). Such a classification reflects the phylogenetic relationships among *Dalbergia* species (Carvalho, 1989), but DNA sequence data are necessary to corroborate the relationships.

The recent advent of techniques of DNA analysis by polymerase chain reaction (PCR) and DNA sequencing has increased interest in plant systematics by using DNA sequence data to study the relationships among species, thereby complementing the enormous amount of morphological data available mainly for legume taxa (e.g., Lavin et al., 2001; Gervais and Bruneau, 2002; Wojciechowski, 2003). However, to obtain accurate DNA sequence information, it is necessary to isolate good quality DNA that is relatively free from the many contaminants found in plant cells (Jobes et al., 1995). Many plant species contain characteristically high amounts of polysaccharides, polyphenols and other secondary metabolites, substances known for binding firmly to nucleic acids during DNA extraction and interfering with subsequent reactions (Pirttilä et al., 2001).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Numerous protocols for DNA extraction from plants have been published (e.g., Doyle and Doyle, 1990; Scott and Playford, 1996; Csaikl et al., 1998; Sharma et al., 2000; Li et al., 2001; Pirttilä et al., 2001; Drabkova et al., 2002; Shepherd et al., 2002; Mogg and Bond, 2003;

Haymes et al., 2004). Because plants contain high amounts of many different substances, it is unlikely that just one DNA extraction protocol is suitable for all plants (Loomis, 1974). To obtain good quality DNA, the utilization of fresh and young leaf tissue is ideal (Sytsma et al., 1993). However, DNA has been widely isolated from desiccated leaves and material stored in silica gel or dehydrated and stored in a herbarium. In these cases, the extraction protocols frequently produce low DNA yield and quality. Satisfactory DNA quality from herbarium specimens is essential for the success of further studies using DNA sequences. For Brazilian *Dalbergia* species, herbaria are an important source of material for phylogenetic and evolutionary studies, since the species occur in different geographical regions comprising all Brazilian biomes, making it difficult to obtain fresh material in nature.

This study is the first part of a phylogenetic analysis of *Dalbergia* and related genera from the Fabaceae family for which it was necessary to select satisfactory methods of DNA extraction for amplification and sequencing of both chloroplast DNA (cpDNA - *trnL* intron) and nuclear ribosomal DNA (nrDNA - ITS region). Five published DNA extraction protocols were compared for their ability to produce good quality DNA from fresh and herbarium leaves of several *Dalbergia* species from four herbaria in Brazil.

MATERIAL AND METHODS

Plant material

Seventy-three samples of fresh leaves and herbarium specimens from 33 species pertaining to five sections of the genus *Dalbergia* were analyzed (Table 1, Appendix A). Fresh leaves were collected from species of different geographical regions in Brazil and stored frozen at -20°C until DNA extraction. The dried leaves used came from herbarium specimens catalogued in four herbaria in Brazil: BHCB (Departamento de Botânica, Universidade Federal de Minas Gerais, Belo Horizonte, MG), INPA (Instituto Nacional de Pesquisas da Amazônia, Manaus, AM), CEPEC (Centro de Pesquisas do Cacau, CEPLAC, Itabuna, BA) and SP (Instituto de Botânica de São Paulo, São Paulo, SP). Herbarium codes are according to Holmgren et al. (1981).

Testing DNA extraction protocols

The DNA extraction protocols were analyzed in three groups of data: 1) fresh leaves from five species, 2) fresh leaves and herbarium specimens from four species, and 3) several herbarium specimens of 24 species (Table 1, Appendix A). To facilitate the distinction between fresh and herbarium specimens the letter "H" was added to its abbreviation. Total genomic DNA was extracted from fresh leaves by the protocols A to D, whereas herbarium specimens by protocols A to E (including the fresh leaves from four species in group 2). In all DNA extraction protocols 0.1 g of leaf tissue was utilized from each sample, ground in liquid nitrogen immediately prior to the procedure. Equipment and materials used in all protocols were: 1) mortar and pestle; 2) 1.5- and 2.0-mL microcentrifuge tubes; 3) liquid nitrogen; 4) water bath (65°C); 5) centrifuge and rotor capable of 14,000 rpm (= 17,746 *g*) and 2-mL holding tubes (e.g., centrifuge A14 Thermo Electron Corporation, United States). All centrifugation steps were performed at 14,000 rpm.

Table 1. Description of samples from fresh leaves and herbarium specimens analyzed for *Dalbergia* species.

N°	Species	Fresh	Herbarium specimens			
			BHCB	INPA	CEPEC	SP
Group 1						
1	*D. decipularis*	DD	-	-	-	-
2	*D. elegans*	DEL	-	-	-	-
3	*D. frutescens*	DF	-	-	-	-
4	*D. miscolobium*	DM	-	-	-	-
5	*D. nigra*	DN	-	-	-	-
Group 2						
6	*D. brasiliensis*	DB	DBH	-	DBH2	-
7	*D. ecastaphyllum*	DE	DEH	-	DEH2	DEH3
8	*D. monetaria*	DMO	-	DMOH	DMOH2	DMOH3
9	*D. villosa*	DVI	DVIH	DVIH2	DVIH3	-
Group3						
10	*D. acuta*	-	DAH	-	DAH2	-
11	*D. amazonica*	-	-	-	-	DAMH
12	*D. catingicola*	-	-	DCAH	-	-
13	*D. cearensis*	-	DCH	DCH2	DCH3	DCH4
14	*D. cuiabensis*	-	-	-	-	DCUH
15	*D. foliolosa*	-	DFOH	-	-	DFOH2
16	*D. foliosa*	-	-	-	DFLH	-
17	*D. glandulosa*	-	-	-	-	DGAH
18	*D. glaziovii*	-	-	-	DGIH	DGIH2
19	*D. gracilis*	-	DGRH	DGRH2	DGRH3	-
20	*D. guttembergii*	-	-	-	DGUH	-
21	*D. hiemalis*	-	-	-	-	DHIH
22	*D. hortensis*	-	-	-	DHH	DHH2
23	*D. hygrophila*	-	-	-	-	DHYH
24	*D. intermedia*	-	-	DTH	DTH2	DTH3
25	*D. inundata*	-	-	DIH	DIH2	DIH3
26	*D. iquitosensis*	-	-	-	DIQH	-
27	*D. lateriflora*	-	-	-	DLAH	-
28	*D. revoluta*	-	-	DREH	-	-
29	*D. riedelii*	-	-	DRIH	DRIH2	DRIH3
30	*D. riparia*	-	-	DRH	DRH2	DRH3
31	*D. sampaioana*	-	-	-	DSAH	-
32	*D. spruceana*	-	-	DSPH	DSPH2	DSPH3
33	*D. subcymosa*	-	-	DSUH	-	-

DNA extraction protocol A (modified - Doyle and Doyle, 1990)

As a “classical” method, we used a modified cetyl trimethyl ammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1990), which has been utilized with success in many plant species. This protocol is based on lysis and purification with CTAB that selectively precipitates DNA while maintaining the solubility of many polysaccharides.

Chemicals

- Extraction buffer: 2% CTAB, 100 mM Tris/HCl, pH 7.5, 1.4 M NaCl, 2% polyvinylpyrrolidone (PVP)-40, 20 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0. Add 20 µL/mL β-mercaptoethanol immediately prior to use.
- Chroloform:isoamyl alcohol 24:1 (CIA).
- Isopropanol, 70% ethanol.
- TE-RNase solution: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 10 mg/mL RNase.

Protocol

1. To each ground leaf sample, add 1 mL extraction buffer and incubate samples for 1 h at 60°C with occasional swirling.
2. Cool samples at room temperature, add 600 µL CIA and mix gently for 5 min.
3. Centrifuge for 15 min. Transfer the supernatant to a new tube and add equal volume of isopropanol. Mix gently and incubate at -20°C overnight.
4. Centrifuge for 15 min. Wash the pellet with 70% ethanol and centrifuge for 10 min. Repeat this wash one or two times.
5. Dry the DNA and dissolve the pellet in 20-50 µL (according to your quantity) TE-RNase solution. Incubate for 1 h at 37°C.

DNA extraction protocol B (Jobes et al., 1995)

This protocol utilizes PVP to bind the phenolic compounds, a high molar concentration of sodium chloride to inhibit co-precipitation of the polysaccharides and DNA, and an improved method for removing RNA by selective precipitation with lithium chloride.

Chemicals

- Extraction buffer: 100 mM sodium acetate, pH 4.8, 100 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM dithiothreitol (DTT), 2% PVP, pH 5.5. Add 100 µg/mL proteinase K immediately prior to use.
- 20% sodium dodecyl sulfate (SDS) solution.
- 5 M potassium acetate, 8 M LiCl, 5 M NaCl.
- Isopropanol, absolute and 70% ethanol.
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Protocol

1. To each ground leaf sample, add 1 mL extraction buffer and incubate sample for 1 h at 55°C with occasional swirling.
2. Add SDS to each tube (final concentration 1.5%). Mix gently and incubate for 1 h at 55°C with occasional swirling.
3. Centrifuge for 10 min. Transfer the supernatant to a new tube and add 1/3 volume of 5 M potassium acetate. Mix gently and incubate for 30 min at -20°C.

4. Centrifuge for 10 min. Transfer the supernatant to new tube and add 0.6 volume of isopropanol. Mix gently and incubate at -20°C overnight.
5. Centrifuge for 10 min and carefully pour off the supernatant.
6. Dissolve the pellet in autoclaved deionized water. Add 0.5 volume of 5 M NaCl and mix well. Add two volumes of cold absolute ethanol and incubate for 30 min at -20°C.
7. Centrifuge for 10 min. Dissolve the pellet in autoclaved deionized water. Precipitate RNA with 1/3 volume of cold 8 M LiCl (final concentration 2 M) and incubate at -20°C for at least 1 h.
8. Recover RNA by centrifugation for 15 min and carefully transfer supernatant to a new tube. Precipitate DNA by adding 0.6 volume of isopropanol and incubate for 1 h at -20°C.
9. Centrifuge for 10 min. Wash the pellet with 70% ethanol and centrifuge for 5 min. Pour off ethanol and briefly dry the pellet. Dissolve the pellet in 20-50 µL TE buffer.

DNA extraction protocol C (Dellaporta et al., 1983)

This protocol utilizes SDS as detergent and the addition of potassium acetate resulted in the removal of some proteins and polysaccharides as a complex with the potassium-SDS precipitate.

Chemicals

- Extraction buffer: 10% SDS, 50 mM Tris/HCl, 100 mM NaCl, 10 mM EDTA, pH 8.0. Add 20 µL/mL β-mercaptoethanol and approximately 0.01 g PVP-40 immediately prior to use.
- 5 M potassium acetate, 3 M sodium acetate.
- Isopropanol, absolute and 70% ethanol.
- TE-RNase solution, TE buffer.

Protocol

1. To each ground leaf sample, add 1 mL extraction buffer and incubate sample for 45 min at 65°C with occasional swirling.
2. Add 300 µL 5 M potassium acetate and mix gently. Incubate sample for 20 min on ice.
3. Centrifuge for 10 min. Transfer the supernatant to a new tube and add equal volume of isopropanol. Mix gently and incubate for 1 h at -20°C.
4. Centrifuge for 15 min. Wash the pellet with 70% ethanol and centrifuge for 5 min.
5. Dry the DNA and dissolve the pellet in 20-50 µL TE-RNase solution. Incubate for 1 h at 37°C.
6. Precipitate the DNA by adding 10% volume of 3 M sodium acetate and two volumes of absolute ethanol. Incubate at -20°C overnight. Repeat steps 5 and 6 twice.
7. Dry the DNA and dissolve the pellet in 20-50 µL TE buffer.

DNA extraction protocol D (modified - Scott and Playford, 1996)

This protocol modifies the CTAB method by isolating membrane-bound organelles in order to remove polysaccharides and secondary metabolites. It should be suitable for a wide range of species, with particular applicability to rainforest plants with high polysaccharide or secondary metabolite content.

Chemicals

- Extraction buffer: 50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 0.35 M sorbitol, 0.1% bovine serum albumin, 10% PVP-6000.
- Wash buffer: 50 mM Tris/HCl, pH 8.0, 25 mM EDTA, 0.35 M sorbitol.
- CTAB buffer: 0.05 M CTAB, 1 M Tris-HCl, pH 8.0, 0.5 M EDTA, 5 M NaCl.
- 3 M sodium acetate, 7.5 M ammonium acetate, 5% sarkosyl.
- CIA, absolute ethanol.
- TE-RNase solution, TE buffer.

Protocol

1. To each ground leaf sample, add 1 mL extraction buffer at room temperature.
2. Centrifuge for 5 min. Discard the supernatant and dissolve the pellet in 400 µL wash buffer followed by 100 µL 5% sarkosyl and leave incubating for 15 min at room temperature.
3. Add 1 mL CTAB buffer, mix gently and incubate at 55°C for 30 min.
4. Centrifuge for 5 min. Transfer the supernatant to a new tube and add equal volume of CIA. Mix gently.
5. Centrifuge for 10 min. Transfer the supernatant to a new tube and add TE-RNase solution. Incubate for 15 min at 37°C.
6. Precipitate the DNA by adding 10% volume of 7.5 M ammonium acetate and equal volume of absolute ethanol. Incubate at -20°C for 30 min.
7. Centrifuge for 15 min. Dry the DNA and dissolve the pellet in 20-50 µL TE buffer.

DNA extraction protocol E (Qiagen DNeasy Mini Plant Kit)

This method uses silica-gel-membrane technology and simple spin procedures to isolate high-quality DNA. Total DNA was extracted strictly following the instructions of the manufacturer.

Comparison of the extraction protocols for efficacy

The presence and quality of DNA obtained by these protocols were determined by electrophoresis on a 1% TBE agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA extract was classified according to visual inspection of coloration which indicated oxidation of the samples (colorless, yellowish or dark). The quality of DNA obtained was estimated by measuring the A260/280 absorbance ratio using a spectrophotometer (Hitachi

U-200). DNA samples were assessed for successful PCR amplification of *trnL* intron of cpDNA (~470 bp) and of ITS region of nrDNA (<700 bp). The *trnL* intron and ITS region were amplified using primers and PCR conditions described by Taberlet et al. (1991); Beyra and Lavin (1999) and Delgado-Salinas et al. (1999), respectively.

RESULTS

In group 1, the fresh leaves of *Dalbergia* species showed good DNA quality, with low degradation, by means of the protocols A to D. The DNA solutions from some of the samples were colored (yellowish or dark) when extracted by means of protocols A, C and D, mainly due to fast oxidation of the extract. For species bearing a high content of secondary metabolites and/or polysaccharides, protocol B was the best choice. The results for species *D. nigra*, *D. miscolobium* and *D. frutescens* were compared by electrophoresis on agarose gels (Figure 1). In group 2, fresh leaves of *D. brasiliensis*, *D. ecastaphyllum*, *D. monetaria*, and *D. villosa* exhibited better DNA quality than herbarium specimens for all tested DNA extraction protocols. Figure 2 shows some of these comparisons obtained mainly through the use of protocol B (samples 1-10). Protocol E produced DNA of very good quality and no degradation from fresh leaves (Figure 2; sample 1); however, it did not produce DNA from herbarium specimens (Figure 2, sample 3). In group 3, most of the herbarium specimens yielded no DNA or DNA with a high level of degradation. DNA extraction from herbarium specimens was only achievable using protocol B (Figure 2, samples 11-17).

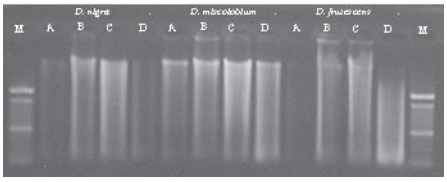

Figure 1. Electrophoretic analysis of total DNA from fresh leaves of *Darbergia nigra*, *D. miscolobium* and *D. frutescens* extracted by the following four different protocols: **A.** Doyle and Doyle, 1990; **B.** Jobes et al., 1995; **C.** Dellaporta et al., 1983; **D.** Scott and Playford, 1996. M = 100-bp ladder.

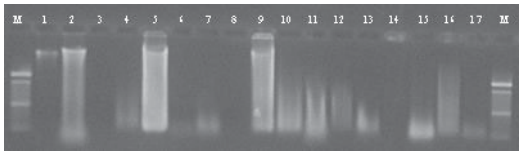

Figure 2. Electrophoretic analysis of total DNA from fresh and herbarium samples of *Dalbergia* species extracted by the following protocols: 1, 3. Protocol E (Qiagen); 2, 4-17. Protocol B (Jobes et al., 1995). Samples: 1 and 2. DVI; 3 and 4. DVIH; 5. DMO; 6. DMOH; 7. DMOH2; 8. DMOH3; 9. DE; 10. DEH; 11. DRH; 12. DRH2; 13. DRH3; 14. DGRH2; 15. DGRH3; 16. DFOH; 17. DFOH2. M = 100-bp ladder.

Of the 73 samples analyzed, only 38 yielded DNA in at least one tested protocol. The samples obtained by means of protocol B were evaluated according to DNA quality, color, spectral absorbance ratio, final concentration (ng/µL), and PCR amplification (Table 2). Most of

Table 2. Evaluation of samples extracted using protocol B (Jobes et al., 1995) according to DNA quality, color, spectral absorbance ratio (A260/280), final concentration (ng/µL), and PCR amplification.

Species	Sample	DNA quality[1]	Color[2]	$A_{260/280}$[3]	Cc (ng/µL)[4]	PCR[5]
Group 1						
D. decipularis	DD	1	1	1	1	1
D. elegans	DEL	1	1	1	1	1
D. frutescens	DF	1	1	1	1	1
D. nigra	DN	1	1	1	1	1
D. miscolobium	DM	1	1	1	1	1
Group 2						
D. brasiliensis	DB	1	1	1	1	1
	DBH	2	2	2	2	1
	DBH2	2	3	2	2	2
D. ecastaphyllum	DE	1	1	1	1	1
	DEH	2	1	2	2	1
D. monetaria	DMO	1	1	1	1	1
	DMOH	2	1	2	2	1
	DMOH2	3	3	2	2	2
	DMOH3	2	2	2	2	1
D. villosa	DVI	1	1	1	1	1
	DVIH	2	1	2	2	1
Group 3						
D. acuta	DAH	2	2	2	2	1
	DAH2	3	3	2	2	2
D. cuiabensis	DCUH	2	1	2	2	1
D. foliosa	DFLH	2	2	2	2	2
D. foliolosa	DFOH	2	2	2	2	1
	DFOH2	3	3	2	2	2
D. glaziovii	DGIH2	3	3	2	2	2
D. gracilis	DGRH	2	1	2	2	2
	DGRH3	3	3	2	2	2
D. hortensis	DHH2	3	3	2	2	2
D. inundata	DIH2	3	3	2	2	2
D. iquitosensis	DIQH	2	3	2	2	2
D. riparia	DRH	2	3	2	2	2
	DRH2	2	3	2	2	2
	DRH3	3	3	2	2	2
D. riedelii	DRIH	3	3	2	2	2
D. spruceana	DSPH	3	3	2	2	2
	DSPH2	3	3	2	2	2
D. tomentosa	DTH	2	3	2	2	2
	DTH2	3	3	2	2	2
D. variabilis	DVH2	3	3	2	2	2
	DVH3	3	3	2	2	2

[1](1) High-molecular weight DNA, low degradation - good quality DNA; (2) medium degradation - medium quality DNA; (3) highly degraded DNA - poor quality DNA.
[2](1) transparent; (2) colored (yellowish); (3) brown (dark).
[3](1) $1.8 \leq A_{260/280} \leq 2.0$; (2) $A_{260/280} \leq 1.5$
[4](1) 500 ng/µL ≤ Cc ≤ 100 ng/µL; (2) Cc ≤ 50 ng/µL.
[5]PCR amplification of *trnL* intron and ITS region: (1) exhibited specific amplification; (2) no amplification.

the herbarium specimens exhibited moderate to high degradation of DNA, yellowish or dark extract, low A260/A280 ratios and low final concentrations. In group 1, specific cpDNA amplification of the *trnL* intron was successful for all samples of fresh leaves obtained by means of protocols A to D (Figure 3, samples 1-8). However, only eight herbarium specimens exhibited successful amplification of this region (e.g., Figure 3, samples 10-14). All these samples also exhibited successful amplification of ITS region (Table 1). PCR amplifications were not successful with any of the herbarium samples that exhibited highly degraded DNA and dark extracts. Only part of herbarium samples that showed moderate DNA degradation and colorless or yellowish extract were suitable for PCR amplification.

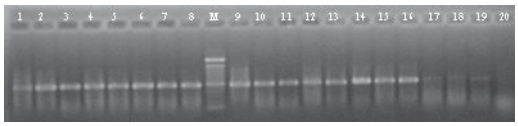

Figure 3. Amplification of *trnL* region of cpDNA from fresh and herbarium samples of *Dalbergia* species extracted by the following protocols: 1, 5, 9-14, 16, 18-20. Protocol A (Doyle and Doyle, 1990); 2, 6. B (Jobes et al., 1995); 3, 7. C (Dellaporta et al., 1983); 4, 8. D (Scott and Playford, 1996); 15, 17. E (Qiagen). Samples: 1-4. DN; 5-8. DEL; 9. DE; 10. DEH; 11. DFOH; 12. DCUH; 13. DBH; 14. DMOH; 15, 16. DVI; 17. DVIH; 18. DRH3; 19. DGRH; 20. DVH3. M = 100-bp ladder.

DISCUSSION

DNA extraction from leaves of *Dalbergia* species was complicated probably by the abundance of secondary metabolites. In a comparison of the four protocols analyzed with fresh and dried leaves, protocol B, described by Jobes et al. (1995), was labor intensive because several solutions needed to be prepared, demanded a great deal of time for extraction of the samples, and required a great quantity of microcentrifuge tubes. However, this protocol produced the best DNA quality in most of the *Dalbergia* species examined, both in fresh leaves and herbarium specimens. The DNA quality for herbarium specimens was worse than for fresh leaves, due to their preservation status. According to Jobes et al. (1995), in the presence of PVP, phenolics adhere to DNA in solution forming a colored extract around the DNA that becomes cleaner after the addition of the detergent SDS. The addition of high molar concentration of NaCl increases the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of the polysaccharides and DNA (Fang et al., 1992). Finally, the addition of LiCl selectively precipitates large RNA molecules reducing the amount of RNA present in the final DNA solution (Sambrook et al., 1989). Selective precipitation has an advantage over RNAse treatment in that the RNA is removed and not simply degraded into smaller units (Storts, 1993). Protocol B also exhibited satisfactory results with *Acer rubrum*, *Magnolia grandiflora*, *Pinus* sp (Jobes et al., 1995), and *Dimorphandra mollis* (Viana HA, unpublished results).

Protocols A and C demonstrated reasonable results for *Dalbergia* species, as the DNA obtained was yellowish in most of the samples. Protocol D successfully extracted DNA from many genera including *Schefflera* (Araliaceae), *Macadamia* (Proteaceae), *Dysoxylum* (Meliaceae), *Flindersia* (Rutaceae), *Sarcopteryx* (Sapindaceae), *Acacia* (Mimosaceae), and *Melicope* (Rutaceae) (Scott and Playford, 1996). However, this protocol did not exhibit satis-

factory results for *Dalbergia* species, maybe due to the modification introduced in the protocol, as the samples were ground with liquid nitrogen instead of with sand. Protocol E, Qiagen DNeasy Mini Plant Kit, produced high-quality DNA and no degradation in fresh leaves; however, for herbarium specimens it did not yield DNA, and consequently there was no PCR amplification, probably due to high degradation of the samples. However, considering the disadvantage of the high per-sample cost of any commercial kit, protocol E cannot be adequate for studies involving many samples of fresh leaves.

Herbarium specimens were supplied by four different herbaria for DNA analysis and complementary phylogenetic study. Each herbarium had a way of preserving the samples, e.g., the INPA herbarium informed us that the plant samples collected in the field are often immersed in alcohol due to high humidity in the Amazon rainforest. The CEPEC and SP herbaria use several chemicals in regular disinfections, whereas the BHCB herbarium stocked the samples in an enclosed space with air-conditioning (± 20°C). We analyzed 54 herbarium specimens, but only eight yielded DNA which was suitable for successful PCR amplification, five of them pertaining to the BHCB herbarium. In fact, success in the extraction of DNA depends on methods of sampling in the field and preservation of the samples in the laboratory (see Drabkova et al., 2002; Feres et al., 2005). If samples are air-dried at up to 42ºC, they probably will yield high-quality DNA. Air-drying is considered to be better than the preservation of tissues in silica gel or anhydrous $CaSO_4$ (Taylor and Swann, 1994). In general, leaves that are immersed in chemical solutions show seriously degraded DNA (Drabkova et al., 2002). *Dalbergia* leaves exhibited oxidation when left under humid conditions for some time after collection, which means that it is necessary to dry or freeze them as fast as possible to better preserve DNA. Another important factor is the disinfestation methods of the herbarium collections. The material stocked at medium to low temperature and free of chemical preservatives has the best chance of yielding good quality DNA (Taylor and Swann, 1994), as in the BHCB preservation method. The satisfactory quality of DNA from herbarium specimens is essential for the success of further phylogenetic studies using DNA sequences in *Dalbergia*. Our results proved that, at least for the *Dalbergia* species tested, the methods for the collection and long-term preservation of herbarium specimens have a major influence on DNA quality and in the success of molecular phylogenetic studies.

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APPENDIX A. Species and sections from herbarium specimens. Each entry includes species, locality and voucher specimen.

Section Dalbergia

Dalbergia acuta Benth.; Brazil, Minas Gerais, Januária; A. Salino & J.A. Lombardi 1692 (BHCB).
Dalbergia acuta Benth.; Brazil, Bahia, Caetité; A.M. de Carvalho 3688 (CEPEC).
Dalbergia cuiabensis Benth.; Brazil, Mato Grosso; H.S. Irwin 15942 (SP).
Dalbergia foliolosa Benth.; Brazil, Minas Gerais; F.R. Couto 244 (BHCB).
Dalbergia foliolosa Benth.; Brazil, Rio de Janeiro, Petrópolis; A.M. de Carvalho & Martini (SP).
Dalbergia glandulosa Benth.; Brazil, Mato Grosso; A.M. Carvalho & Lewis (SP).
Dalbergia gracilis Benth.; Brazil, Minas Gerais, Caratinga; H.C. Lima 7601 (BHCB).
Dalbergia gracilis Benth.; Brazil, Rondônia; W.R. Anderson et al. 12220 (INPA).
Dalbergia gracilis Benth.; Brazil, Acre, Serra Madureira; D.C. Daly et al. 8146 (CEPEC).
Dalbergia hiemalis Malme; Brazil, Mato Grosso; A.M. de Carvalho & G.P. Lewis (SP).
Dalbergia lateriflora Benth.; Brazil, Paraná, Guaratuba; J.M. Silva 1057 (CEPEC).
Dalbergia spruceana (Benth.) Benth.; Brazil, Acre; C.A. Ferreira 10132 (INPA).
Dalbergia spruceana (Benth.) Benth.; Brazil, Pará; A.P. Duarte 7264 (CEPEC).
Dalbergia spruceana (Benth.) Benth.; Brazil, Pará, Santarém; A.M. de Carvalho (SP).
Dalbergia villosa (Benth.) Benth.; Brazil, Minas Gerais, Itabirito; A.M. de Carvalho (BHCB).
Dalbergia villosa (Benth.) Benth.; Brazil, São Paulo; H.F. Leitão 19878 & D.B. Azevedo (INPA).
Dalbergia villosa (Benth.) Benth.; Brazil, Minas Gerais, Lavras; G.P. Heringer 01153 (CEPEC).

Section Triptolemea

Dalbergia brasiliensis Vogel; Brazil, Paraná; A.O.S. Vieira (BHCB).
Dalbergia brasiliensis Vogel; Brazil, Paraná, Ponta Grossa; A.M. de Carvalho et al. 2339 (CEPEC).
Dalbergia catingicola Harms.; Brazil, Bahia, Jequié; G.P. Lewis & A.M. de Carvalho 980 (CEPEC).
Dalbergia cearensis Vogel (*D. variabilis*); Brazil, Brasília (BHCB).
Dalbergia cearensis Vogel (*D. variabilis*); Brazil, Rio de Janeiro, Petrópolis; G. Martinelli et al. 124 (INPA).
Dalbergia cearensis Ducke (*D. variabilis*); Brazil, Bahia; B.B. Klitgaard et al. 73 (CEPEC).
Dalbergia cearensis Ducke (*D. variabilis*); Brazil, A. Mattos Filho (SP).
Dalbergia glaziovii Harms.; Brazil, Rio de Janeiro, Nova Friburgo; A.M. de Carvalho et al. 2334 (CEPEC).
Dalbergia glaziovii Harms.; Brazil, Rio de Janeiro, Nova Friburgo; A.M. de Carvalho & H.C. Lima (SP).
Dalbergia hortensis Heringer, Rizzini & Mattos; Brazil, Minas Gerais, Juiz de Fora; P.L. Krieger 11981 (CEPEC).
Dalbergia hortensis Heringer, Rizz & Mattos; Brazil, Minas Gerais; A.M. de Carvalho (SP).

Dalbergia iquitosensis Harms.; Brazil, Acre, Porto Velho; J.U. Santos et al. 106 (CEPEC).
Dalbergia riparia (Marth.) Benth.; Brazil, Amazonas, Manacapuru; F.M.M. Magalhães 54 (INPA).
Dalbergia riparia (Marth.) Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho et al. 2125 (CEPEC).
Dalbergia riparia (Marth.) Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho (SP).
Dalbergia sampaioana Kuhlmann & Hoehne; Brazil, Rio Janeiro; R.B. Antenor 136212 (CEPEC).

Section Selenolobium

Dalbergia foliosa Benth.; Brazil, São Paulo; J.Y. Tamasleiro et al. 88 (CEPEC).
Dalbergia inundata Spruce ex Benth.; Brazil, Amazonas; L. Augusto 661 (INPA).
Dalbergia inundata Spruce ex Benth.; Brazil, Amazonas, Manaus; A.M. de Carvalho et al. 2121 (CEPEC).
Dalbergia inundata Spruce ex Benth.; Brazil, Amazonas; A.M. de Carvalho (SP).
Dalbergia revoluta Ducke; Brazil, Acre, Cruzeiro do Sul; O.P. Monteiro 300 & C.D.A. Mota (INPA).

Section Pseudecastaphyllum

Dalbergia intermedia Vogel (*Dalbergia tomentosa* Benth.); Venezuela, Atures; O. Huber 1501 & J. Cerda (INPA).
Dalbergia intermedia Vogel (*Dalbergia tomentosa* Benth.); Brazil, Pará, Santarém; A.M. de Carvalho 2131 (CEPEC).
Dalbergia intermedia Vogel (*Dalbergia tomentosa* Benth.); Brazil, Pará, Santarém; A.M. de Carvalho (SP).

Section Ecastaphyllum

Dalbergia amazonica (Radlk) Ducke; Ex. Herb Museu Paraensis 16971 (A. Ducke) (SP).
Dalbergia ecastaphyllum (L.) Taub.; Brazil, Santa Catarina, Itapema; A.C. Cervi 2569 (BHCB).
Dalbergia ecastaphyllum (L.) Taub.; Brazil, Bahia, Belmonte; A.M. de Carvalho et al. 477 (CEPEC).
Dalbergia ecastaphyllum (L.) Taub.; Brazil, São Paulo, Peruíbe; Bianchini 352 (SP).
Dalbergia guttembergii A.M. de Carvalho; Brazil, Amazonas; A. Ducke 35513 (CEPEC).
Dalbergia hydrophila (Mart. Ex Benth) Hoehne; A. Ducke (SP).
Dalbergia monetaria L.f.; Brazil, Amazonas, Humaitá; L.O.A. Teixeira et al. 1250 (INPA).
Dalbergia monetaria L.f.; Brazil, Pará, Belém; A.M. de Carvalho et al. 2132 (CEPEC).
Dalbergia monetaria L.f.; Brazil, Pará, Abaetatuba; A.S.L. Silva (SP).
Dalbergia riedelii (Radlk.) Sandw.; Brazil, Amazonas; F. Magalhães 331 (INPA).
Dalbergia riedelii (Radlk.) Sandw.; Brazil, Amazonas, Rio Negro; S. Mori et al. 21849 (CEPEC).
Dalbergia riedelii (Radlk.) Sandw.; Brazil, Pará, Conceição do Araguaia; A.M. Carvalho (SP).
Dalbergia subcymosa Ducke; Brazil, Amazonas, Autaz-Mirim; A. Loureiro, O.P. Monteiro & A. Miranda (INPA).