CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

INSTITUTE OF TROPICS AND SUBTROPICS

Methods of DNA extraction from selected Amazonian tree species

Diploma thesis

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2012

Declaration

I declare, that the thesis hereby submitted for the Master of Science degree at the Czech University of Life Sciences was done on my own. Every used material is cited in references.

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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DIPLOMA THESIS ASSIGNMENT

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Tropical Crop Management and Ecology

Thesis title

Metody extrakce DNA u vybraných druhů Amazonských dřevin

Objectives of thesis

The main objective of the study is evaluation of two methods of DNA extraction with three types of plant material to determine most suitable method of extraction for selected Amazonian tree species.

Methodology

For the study, leaves of eight Amazon tree species, from the greenhouse of the Institute of Tropics and Subtropics will be sampled first. For analysis, there will be evaluated with two methods extraction with the three types of plant material (fresh, dry by silica gel and lyophilized): Invisorb Spin Plant Mini Kit. Metohd and CTAB DNA extraction method. Determination of DNA quality will be done by electrophoresis. The putrity and DNA amount will be measured using Nanodrop1000 spectrophotometer.

Schedule for processing

Oct 2010 – Jan 2011 – literature review
Feb – May 2011 – methodology preparation
Jun – Oct 2011 – Getting data.
Nov 2011 – Jan 2012 – data evaluation
Feb – Mar 2012 – completing the thesis
Apr 2012 – finalizing the thesis

The proposed extent of the thesis
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Keywords
DNA extraction, quality, silica gel, tree species and plant material

Recommended information sources

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List of abbreviations

AFLP = Amplified Fragment Length Polymorphism
ANOVA = Analysis of Variance
AMOVA = Analysis of Molecular Variance
bp = Base Pairs
cpDNA = chloroplast DNA
CATIE = Centro Agronómico Tropical de Investigación y Enseñanza
CITES = Convention on International Trade in Endangered Species
CTAB = Cethyl Trimethyl Ammonium Bromide
CULS = Czech University of Life Sciences Prague
CV = Coefficient of Variation
DNA = Deoxyribonucleic acid
dsDNA = Double strand DNA
ESTs = Expressed Sequence Tags
ETDA = Ethylenediamine tetraacetic acid
FAO = Food and Agriculture Organization
HWE = Hardy Winberg Equilibrium
IAA = Isoamyl Alcohol
ICRAF = World Agroforestry Centre
IIAP = Instituto de Investigaciones de la Amazonia Peruana
INRENA = Instituto Nacional de Recursos Natural
INIA = Instituto Nacional de Innovacion Agraria
ITTO = International Tropical Timber Organization
IUCN = International Union for Conservation of Nature
ISSR = Inter Simple Sequence Repeat
ITS = Internal Transcribed Spacer
g DNA= Genomic DNA
m.a.s.l = Meters above sea level
MPTs = Multipurpose trees
P5CS = Pyrroline-5-Carboxylate Synthase
PCR = Polymerase Chain Reaction
RAPD = Random Amplification of Polymorphic DNA
RPM = Revolutions Per Minute
SE = Standard Error
SSR = Simple Sequence Repeat
TAE = Tris-acetate-EDTA
UNU = Universidad Nacional de Ucayali
UV = Ultraviolet
V = Voltage

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Abstract

For conservation of germplasm of native tropical trees and development of strategies for their breeding and domestication, it is firstly important to know their genetic diversity. Most of the current research use for that assessment fast developing molecular techniques, such as PCR method. However, the first step is an effective and successful DNA extraction from vegetative material. Objective of our study was to evaluate the most efficient conservation and extraction method of plant material collected from several native multipurpose trees (MPTs) used in the Peruvian Amazon. Our research tried to identify suitable method of DNA extraction for eight Amazon trees species. We evaluated three conservation methods of plant material (silicagel storage, lyophylization compared to fresh material) and two extraction methods (CTAB and KIT); their efficiency on DNA concentration, purity and quality. We also evaluated the cost-effectiveness of both extraction methods. DNA concentration and purity of was done by absorbance spectrophotometry using the Nanodrop1000 spectrophotometer, and the DNA quality was observed for its intactness by running the extracted DNA on 0.1% agarose gel. We found significant differences among conservation methods and also between the two extraction methods. For all species and also in total CTAB extraction method yielded significantly higher DNA concentration and purity, as well as the quality was better. The conservation of the leaf material using silica gel showed the best yield in terms of DNA concentration and purity. Our study confirmed that CTAB method of DNA extraction is more efficient in the sense of DNA concentration, purity, quality. Moreover, CTAB method is cheaper than using KIT. Based on our results we recommend to use silicagel for conservation of leaf material from tropical tree species and the use of CTAB method for DNA extraction in developing countries.

Keywords

DNA Concentration, DNA extraction, DNA quality, silica gel, tropical trees species and conservation material.

Abstrakt

Pro zachování genetického fondu tropických dřevin a vytvoření strategií pro jejich šlechtění a domestikaci je nezbytné nejdříve zjistit jejich genetickou rozmanitost. Většina současného výzkumu užívá pro tento účel rychle se rozvíjející molekulární technologie, jako např. PCR. Nicméně, prvním krokem je efektivní a úspěšná extrakce DNA. Cílem našeho výzkumu bylo zhodnocení metod konzervace rostlinného materiálu a následné extrakce DNA na vzorku víceúčelových dřevin používaných v peruánské Amazonii. Zhodnotili jsme tři metody konzervace rostlinného materiálu (silicagel, lyofilizace a čerstvý materiál), a dvě metody extrakce DNA (CTAB a KIT) a jejich vliv na koncentraci, čistotu a kvalitu extrahované DNA. Také jsme zhodnotili ekonomickou stránku věci, tedy efektivitu vynaložených nákladů na obě extrakční metody. Koncentrace DNA byla vyhodnocena pomocí absorpční spektrofotometrie (přístrojem Nanodrop 1000), její kvalita pak zhodnocena na základě vizuální integrity DNA nanesené na agarózní gel (koncentrace 0.1%). Mezi metodami extrakce a uchování materiálu jsme nalezli statisticky významné rozdíly. V případě všech zkoumaných druhů vykazovala metoda CTAB jednoznačně lepší výsledky: koncentrace, čistota a kvalita DNA byli u této metody vyšší. Konzervace rostlinného materiálu pomocí silicagelu se ukázala jako nejlepší s ohledem na koncentraci a čistotu DNA. Naše studie také potvrdila, že extrakce DNA pomocí CTAB je efektivnější s ohledem na čistotu, kvalitu a koncentraci výsledného materiálu. Tato metoda se navíc ukázala jako levnější řešení než KIT. Na základě našich výsledků doporučujeme užívání silicagelu pro konzervaci rostlinného materiálu tropických dřevin a také užívání metody CTAB v rozvojových zemích.

Klíčová slova

Koncentrace DNA, extrakce DNA, kvalita DNA, silikagel,tropické stromy a zachování materiálu.

Resumen

Para la conservación de germoplasma de especies de árboles tropicales nativos y el desarrollo de estrategias para su reproducción y domesticación es necesario primeramente conocer la diversidad genética. La mayoría de investigaciones actuales usa para su investigación técnicas moleculares de rápido desarrollo como el método PCR. Sin embargo el primer paso es un efectivo y exitoso método de extracción del material vegetal. El objetivo de nuestro estudio fue evaluar el método más adecuado de conservacion y extracción de ADN de material vegetal colectado de varios árboles multiusos nativos usados en la Amazonía del Perú. Nuestra investigación intentó identificar el metodo de extracción de ADN para ocho especies de árboles amazónicos. Evaluamos tres métodos de conservación de material vegetal (almacenado en gel sílice, liofilización comparados con material vegetal fresco) y dos métodos de extraccion (CTAB y KIT);su eficiencia en concentración, pureza y calidad de ADN. Tambien evaluamos el costo-efectivo de ambos métodos de extracción. La concentración y pureza del ADN fue por absorbancia espectrométrica usando el espectrómetro Nanodrop1000, y la calidad de ADN fue observada por su integridad en gel agarosa al 1 %. Encontramos diferencias significativas entre los métodos de conservación y también entre los dos métodos de extracción. Para todas las especies el método de extracción CTAB tuvo un rendimiento significativamente mayor en la concentración, pureza de ADN y calidad también fue mejor. La conservación de material vegetal usando gel sílice mostró el mejor rendimiento en términos de concentración y pureza. Nuestro estudio confirmó que el método de extracción de ADN, CTAB, es más eficiente en términos de concentración pureza y calidad de ADN. Además, el método CTAB es más económico que KIT. Basado en nuestro estudio recomendamos usar gel sílice para la conservación del material vegetal de especies tropicales y el uso de método CTAB para la extracción del ADN en países en desarrollo.

Palabras claves

Concentración de ADN, extracción de ADN, Calidad de ADN, gel sílice, especies de árboles tropicales y conservación de material.

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1. INTRODUCTION

Early studies and predictions about the genetic structure of tropical tree populations arose as part of efforts to explain the great diversity of tree species characteristic of neotropical forests. Such studies are essential for understanding ecological and evolutionary processes in natural populations (Nason, 2002; Jones & Hubbell, 2006). The knowledge of how genetic variation is partitioned among populations may have important implications not only in evolutionary biology and ecology, but also in conservation biology. Hence, reliable estimates of population differentiation are crucial to understand the connectivity among populations and represent important tools to develop conservation strategies. (Balloux & Lugon-Moulin, 2002)

It is important to know the genetic diversity of tropical species such as *Amburana cearensis, Calycophyllum spruceanum, Cederla odorata, Copaifera paupera, Guazuma crinita, Inga edulis, Schizolobium parahyba y Swietenia microphylla* as these are the main native multipurpose trees MPTs used in the Peruvian Amazon by many farmers in their agricultural systems (Hulm, 2011). In the agroforestry context, multipurpose trees (MPTs) are understood as (Nair, 1993) "those trees and shrubs which are deliberately kept and managed for more than one preferred use, product, and/or service; the retention or cultivation of these trees are usually economically motivated, in a multiple-output land-use system." Simply stated, the term "multipurpose" as applied to trees for agroforestry refers to their use for more than one service or production function in agroforestry system (Burley & Wood, 1991). Molecular biology techniques and in particular the use of molecular markers are able to characterize and estimate the genetic diversity and relationships among stakeholders. Studies have shown that there are different factors that affect the amplification profiles obtained by PCR-based molecular techniques. Some of the main factors affecting these amplification profiles are the quality, purity and quantity of DNA extracted (Molinari, 2001). Though several successful genomic DNA isolation protocols for high polyphenol and polysaccharide containing plant species have been developed, none of these are universally applicable to all plants (Varma *et al.*, 2007). Based on theses studies we can better conserve the existing germplasm of the tree species and also proposed best strategies for their domestication.

The first step in studying is genetic diversity is the proper DNA extraction from vegetative material. The objective of this study was to determine most suitable conservation method of plant material and DNA extraction method for important multipurpose Amazonian tree species, for obtaining DNA quality, purity and sufficient quantity for multiple end uses.

2. LITERATURE REVIEW

2.1 Amazonian tree species

Amazon basin is spread over the territory of nine countries, covering an area of 7 million km^2 of which 5.5 million km^2 is covered by rainforest. 60 % of the Amazon forest belongs to Brazil and 13% to Peru, making it the second most forested country in South America. Peru is historically a country of a great botanical interest since pioneering Russian geneticist Nikolai Ivanovich Vavilov recognized Peru as 'centre of diversity' in the 1930s (O'Neill *et al.*, 2010). Amazonian tropical rain forest is the biggest ecoregion in Peru. Its limit is 800m.a.s.l. (Egg, 2004). According to Köppen (1936) climate classification whole Amazonian area is laying in region A (Tropical), which is characterized by constant high temperature (at sea level and low elevations) — all twelve months of the year have average temperatures of 18 °C or higher. Hot tropical climate can be characterized as climate with very constant temperatures.

Average temperatures are between 24 and 26 °C, with minimum between 18 and 20 °C and maximums between 33 and 36 °C. The variation of temperature through the day oscillates between 5 and 8 °C, which is more than the annual 1 or 2 °C temperature oscillation. The relative humidity is generally high about 75 %. The optimal precipitation is around 2000 mm regularly distributed throughout the year. It does exist two or three with low precipitations between 50 and 100 mm, where can be seen some changes in vegetation, the forests looks like drier and some species may reduce its leaves (Egg, 2004).

The soils are very heterogeneous, but all of them have its fluvial origin. The sediments were drifted of years from the Andes through the transformation. This gave the origin to the different type of soils. Ultisols which are yellow red, acid with low natural fertility, deep, well drainaged and with content of clay, are the most common type of soil in areas of the Peruvian Amazon. The other type of soils occurred in this region are for example entisols, with the juvenile soil profiles, inceptisols, the soils with different covering areas with bed drainage (*aguajals*) (Egg, 2004).

The Amazon is made up of different forest types and assemblies made by different species in some way related to environmental factors such as relief, water, nutrient and drainage. There are approximately 250,000 of vascular plants. 30,000 can be indicated as edible and just 7,000 as cultural plants known as crops (Dotlačil, 1998). For us is important a set of usually wooden "agroforestry species", which have come to be known as "multipurpose trees" (MPTs). Trees play a crucial role in almost all terrestrial ecosystems and provide a range of products and services to rural and urban people (ICRAF, 2005).There are many valuable timber MPT species in the Peruvian Amazon, but there has been little research on genetic variation of commercial traits in these species. For example *Amburana cearensis* belongs to the Fabaceae family and is a medicinal plant used in northeastern Brazil in the treatment of respiratory diseases, inc1uding asthma (Braga, 1976). *Calycophyllum spruceanum* – capirona (13 articles) (*Rubiaceae* family) rank farmers as one of the best species for production of lumber, poles, firewood and charcoal in agroforestry systems and pure plantations (Sotelo Montes & Weber, 1997). Another very

important timber MPTs are *Cedrela odorata* – cedro and *Swietenia macrophylla* – caoba or mahogany (both *Meliaceae* family with three articles) with possible use in apiculture. *S. macrophylla* with medium growth rate has probably the most valued timber in the region. It can control erosion, helps to maintain organic matter in the soil and it's also used as a gene pool. Native *C. odorata* has medicinal properties and is suitable as overstory tree. In Peru, is now considered as threatened specie by national authorities (INRENA, 2004) Farmers also consider fast growing *Guazuma crinita* – bolaina blanca (one article) (*Sterculiacea*e family) as a priority timber specie for tree improvement programs in the Peruvian Amazon. It can be inter-cultivated with food crops because it has a small crown with thin branches, and the older branches naturally selfprune in the lower crown. It provides wood products at an early age, can be coppiced for successive harvests and contributes significantly to farmer's income (Labarta and Weber, 1998). Examples of medicinal species are: *Croton draconoides* and *Copaifera paupera.* (Lojka *et al.*, 2008). *Schizolobium amazonicum* grow fast and it is a big tree (20–30 m high and 1 m of diameter), occurring in dry land forest (Ducke, 1949). This legume tree has been replanted in agroforestry systems that were cleared from primary rain forest in the 1970s and 1980s in favor of pasture. Today it is an important source of wood to supply the local industry of wood sheet for the furniture industry in many countries. Probably the most important MPT specie is *Inga edulis* (occurs in eight and *Inga* sp. in nine scientific articles related to the Peruvian Amazon) (*Fabaceae* family), affording edible sweet pulp of pods and very useful overstory environment for perennial crops (mainly coffee and cacao). Duke (1983) also mentioned that the timber from *I. edulis* can be use for boxes, crates, furniture, general carpentry, and light construction, but farmers do not grow *Inga* for this purpose. Another possibility how to utilize guaba is to include it into the entomoforestry system as honey producing tree for apiculture. *I. edulis* is also very valuable for ability to improve fallows by maintaining of organic matter and fixing of nitrogen (Pennington, 1997; León, 1998). The genetic study as the beginning of the conservation of these species is important as these are the main native MPTs used in the Peruvian Amazon (Hulm, 2011)

2.1.1 *Amburana cearensis* (Allemão) A. C. Smith. (LEGUMINOSAE)- Ishpingo

Botanical description

Scientific name: *Amburana cearensis* (Allemão) A. C. Smith. Tree: is 80-150 cm in diameter and 20-35 m total height, with the shaft cylindrical, sometimes slightly swollen at the base, branching from the second third, the base of the shaft with small buttresses, up to 0.5 m high. Outer bark: smooth, greenish to reddish brown, whitish lenticels also dispersed in the plates papyraceous rhytidome irregular reddish-brown. Inner bark: granular, yellow-white, strong aromatic odor characteristic (Reynel *et al.*, 2003). Terminal twigs circular, light brown when dry, about 3-5 mm in diameter, smooth, sparsely lenticels and glabrous (Smith ,1940; López, 1987) describe the leaves as 6–20 cm in length, alternate and of 7–15 pinnate leaflets, each 2–5 cm long and 1.5–3.0 cm wide, normally distinctly ovate, alternate and petiolate with the lamina entire. Hermaphrodite flowers of 1.5-2 cm long, zygomorphic, with calyx and corolla

present, the pedicel 4-7 mm long, the calyx 2-3 mm long, the corolla consists of a single petal of 7-10 mm long, white with pink streaks, the androecium of 5-8 mm long, stamens 10, free, the gynoecium of 4-7 mm in length with a pestle and elongated supero ovary, stigma inconspicuous. Fruit: legumes 4-7 cm long and 1.5 cm wide, rounded at both ends, leathery, glossy surface, brown, open in two very flat except in the apical region, corresponding to the seeds, these 1-2 , about 5 mm long, broadly winged (Reynel *et al.*, 2003).

Distribution and habitat

In Amazon region it grows mostly below 700m.a.s.l. It is found in areas with high and constant rainfall, but also in areas with a marked dry season, is sciophyte specie present in primary forests, preferring loamy clay soils, fertile and well drained, with low to medium stoniness (Reynel *et al.*, 2003).

The species is found mostly in Brazil (Salomão and Leite, 1991). There are also reports of occurrences in Northern Argentina and Paraguay (Barbosa, 1983; López, 1987) and in Bolivia and Southeast Peru (Lewis, 1987). Information on Peruvian and, especially, Bolivian occurrences reported in the last 10 years was obtained from Missouri Botanical Garden's Tropics database.

Uses

Its wood is of excellent quality, semi-hard and semi-heavy, grain straight to wavy, coarse texture and yellow, fragrant. It is very durable, workable, valued in carpentry, joinery and decorative veneer (Reynel *et al.*, 2003). Folk medicine used seed and bark resin containing a fragrant volatile oil (Record & Hess, 1972). The seed is used as a basis for tea taken as a remedy for stomach and intestinal upsets, while a bark decoction is used against colds. Perfumery; seed is used for perfume and soap (Record & Hess, 1972) Medical investigation of Sampaio, Oliva, Tanaka, and Sampaio (1991) and Tanaka, Sampaio, Sampaio, and Oliva (1989) reported the inhibition of blood coagulation by factors in extracts from the seed of *A. cearensis*. Proteinase inhibitors are considered responsible for this effect.

Conservation status

The IUCN Red List of Threatened Species (Americas Regional Workshop, 1996) cites *A. cearensis* as being endangered. In Paraguay, the Conservation Data Centre regards the species as threatened (Cáceres, 1992). The FAO Panel of Experts lists *Torresia acreana*, a conspecific of *A. cearensis*, as high, global, regional and/or national priority species for programme conservation of forest genetic resources (FAO, 2002); Cenargen echoes this sentiment. The genetic variability existing in *A. cearensis* is expressed in a number of characters, which differ within its range. Unfortunately, it was not possible to pinpoint exact locations where such differences occurred. However, the simple fact of disagreements regarding taxonomic treatment is already a proof of variation in the species, as it is observed in the existence of southern Bahia State individuals bearing larger seeds than more northern locations (Lewis, 1992).

2.1.2 *Calycophyllum spruceanum* (Bentham) Hooker f. ex Schumann (RUBIACEAE) – Capirona

Botanical description

Scientific name *Calycophyllum spruceanum* (Bentham) Hooker f. ex Schumann origins of the name is from the greek kalyx (calyx) and phyllon (a leaf) refering to the calyx teeth. Botanical synonyms are *Calycophyllum spruceanum forma brasiliensis* K. Schum. *Calycophyllum spruceanum forma peruvianum* K. Schum., and *Eukylista spruceana* Benth. Indigenous names in Peru are Capirona, Capirona negra and Mulateiro. In Bolivia is *C. spruceanum* called Guayaboji, or Cojesche. Spanish name is Palo Amarillo (Quattrocchi, 2000). Indigenous name in Brazil is Pau-mulato-da-varzea (Almeida, 2004). It is the fast growing tree of up to 180 cm diameter and 20-35 m high with very straight, regular cylindrical stem (Almeida, 2004; Sears, 2003). In the nondense canopy in the last upper third may occur partial leaf abscission during the dry season (Sears, 2003). Outer bark is smooth green, very characteristic, homogeneous, shiny, giving the impression of a well polished pole, covered with red-brown papyraceous rhytidome which is separated in large plates, irregular, revealing the greenish surface of the cortex (Almeida, 2004; Reynel *et al.*, 2005). Homogeneous inner bark is creamy-green and1-2 mm thin (Reynel *et al.*, 2005). Branches have round or flat section in the terminal areas (diameter 5-6 mm), reddish brown when dry, smooth, shiny, with white lenticels. *C. spruceanum* has opposite decussate simple leave arrangement, with elliptic or oblong 5-10 cm long and 3-5 cm wide leaves. The non winged petiole (1.5-2.5 cm long) with grooved blades entire is provided with the apex acute coarsely acuminate, base obtuse. The venation is pinnate, secondary nerves (12-15 pairs) are lightly printed on upper and embossed on the underside, the axils of secondary veins with small domatia on the underside. Terminal inflorescences are 10-15 cm long, equipped with lots of hermaphrodite flowers with presence of calyx and corolla. Flowers (1-1.5 cm long) are entirely wrapped in a deciduous bract, which is normally removed from the central flower as the first. The pedicels are 2-3 mm long, calyx is 1 mm long, white tubular corolla is campanulate (5-6 lobed), with 5-6 equal stamens at the corners of the lobes. Anthers are fixed exserted dorsal. Pistil has inferior ovary, ellipsoid-truncated, filiform arranged and bifid opened stigma. Each flower matures in 2 to 3 years in open-grown conditions (Reynel *et al.*, 2005). Fruit is 4-lobed oblong capsule 8-11 mm long, densely pilose (Campbell, 1961). Capsules are arranged in modal units of three. Fruit is dehiscent, with many winged seeds (Sears, 2003; Gentry, 1992).

Distribution and habitat

The origin and distribution of *C. spruceanum* is in the Amazon Basin, up to the elevation of 1200m.a.s.l (Reynel *et al.*, 2005). It is a pioneer tree species that colonizes the floodplains, natural disturbed forests and slash-and-burn agriculture fallows (Linares *et al.*, 1992). It is common in secondary forest areas and naturally also in primary forests. *C.spruceanum* occurs in areas with high rainfall but also areas with notable dry season. It is stone tolerate tree prioritizing loamy to sandy alluvial fertile soils; *C. spruceanum* is heliophyte (Reynel *et al.*, 2005; Almeida, 2004) and hygrophyte species (Almeida, 2004). It is a typical

tree well adapted to growth in riparian forests temporarily flooded by clear water ("Várzeas"); (Linares *et al.,* 1992; Sears *et al.*, 2003; Reynel *et al.*, 2005; Wightman *et al.*, 2006; Almeida, 2004). Homogeneous natural and semi-natural stands of *C. spruceanum* can often be observed along riverbanks (Linares *et al.* 1992, De Jong, 2001). It is shade-tolerant species and it can survive beneath the fast growing trees (Díaz, 2009).

Uses

C. spruceanum can be incorporated in agroforestry plantations (Sotelo-Montes and Weber, 1997). The most used parts of the tree are wood and bark. The wood is widely used for constructions (*e.g.*, beams, poles), and further is suitable for production of firewood and charcoal because of the high calorific value and flame quality (Sotelo-Montes and Weber, 1997; Sears, 2003; Wightman *et al.*, 2006). It is smooth to the touch, hard, heavy, straight to curly grained with fine texture and excellent durability and woodworking qualities (Reynel *et al.*, 2005). *C. spruceannum* has diffuse porous wood, very dense when mature (0.65 g/cm^3). Luster is moderate, smell and taste imperceptible. Axial parenchyma is indistinct, with small very numerous, solitary and multiple empty pores. Visible, thin rays are not stratified. Growth rings are visible and demarcated by fibrous zones (Gomez *et al.,* 2006). Genetic correlations indicate that, in general, selection of faster growing trees and/or trees with denser wood would have little effect on wood color and its uniformity (Sotelo-Montes *et al*., 2008). *C. spruceanum* is noted for its ability to completely shed and regenerate its bark on a yearly basis, making harvesting the bark a totally renewable and sustainable enterprise. Indigenous people of the Amazon use a bark decoction internal and external, as an admixture in the *Ayahuasca* rituals, and in folk medicine and cosmetics. The decoction is ethnomedically used for treating abscesses, age spots, burns, cancer, cuts, diabetes, eye infections, fibromas, fungal infections, insect bites, liver problems, malaria, ovarian problems, pellegra, rashes, scabies, skin fungi, skin parasites, swelling, uterine cancer, wounds and wrinkles. The traditional remedy is 1 cup of decoction two to three times daily. It can be also applied externally directly to the affected area several times daily and allowed to dry before covering (Taylor, 2005).

Conservation status

An understanding of the level and partitioning of *C. spruceanum* is an important input into determining an appropriate and community-based genetic management strategy for the species within the Peruvian Amazon Basin. A study of Vos *et al.* (1995) employed amplified fragment length polymorphism (AFLP) analysis to assess genetic variation within and among nine populations of *C. spruceanum* systematically sampled across a wide range of the Peruvian Amazon Basin, along the Amazon River system. AFLP analysis has been applied to a range of genetics questions within plant species (Travis *et al.*1996; Beismann *et al.* 1997; Gaiotto *et al.* 1997; Paul *et al.*1997; Arents *et al.*1998; Winfield *et al.* 1998), and it is able to provide high levels of resolution to allow delineation of complex genetics structures (Powell *et*

*al.*1996), has proved to highly reproducible (Jones *et al.*1997). Altogether 13 scientific papers on *C. spruceanum* were published, all of them were done the on the same experimental plots near Pucallpa, Ucayali District, Peru. Up to now only one work aiming at DNA analysis was published. Russell et al. (1999) did AFLP analysis to partition genetic variation within and among nine natural populations of *C. spruceanum*. Analysis of molecular variance employed 65 AFLP markers and revealed the most variation among individuals within populations rather than among populations.

2.1.3 *Cedrela odorata* L (MELIACEAE) – Cedro.

Botanical description

Scientific name of cedro is *Cedrela odorata* L. with botanical synonym *C.longipetiolulata* Harms, C. Mexican M. Roemer. Tree of 50-100 cm in diameter and 20-30 m high, with cylindrical shaft, branched in the last third, the base of the stem straight or small buttresses. Outer crust cracked, light ash brown, cracks 2-5 cm apart from each other; the rhytidome plates almost rectangular shape about 2-5 x 8-15 cm. Cleavage inner bark plates irregularly in cream and pale pink, with faint characteristic odour of garlic. Terminal twigs circular, 4-8 mm in diameter, light brown when dry, whitish lenticels surface with 1 mm long, glabrous twigs. Paripinnate compound leaves also imparipinnate, alternate, spirally arranged, with a tendency to cluster at the ends of the twigs, 20-35 cm long, the rachis grooved, the leaflets 5-10 pairs, alternate or subopuestos, spaced about 3 -4 cm apart, oblong to oblong-lanceolate, about 9-12 cm long and 3.5-5 cm wide, apex acute, shortly acuminate, the acumen of from 0.4 to 0.8 cm long, acute to obtuse baseoften asymmetrical, the entire edge, the secondary veins 14-16 pairs, printed on the face, the leaflets glabrous (Reynel *et al.*, 2003). Inflorescence in terminal panicles, flowers unisexual, but with well developed vestiges of the opposite sex, actinomorphic, pentamerous, greenish-white, subsessile, 6-9 mm long, smelling of garlic. Calyx cupshaped, split on 1 side, shallowly to deeply toothed; petals free imbricate and adnate for 1/3 of their length, forming into a long, columnar androgynophore by a medium carina (therefore preventing their spreading in open flowers), white or cream tinged red near the margin. Stamens 5, free, but adnate to the androgynophore below; anthers dorsifixed, opening by longitudinal slits; ovary 5-locular, pubescent; each locule with 10-14 ovules; style short, stigma discoid. Fruit a pendulous, reddish-brown capsule with 5 thin, woody valves, oblong-ellipsoid, to obovoid (min. 1.5) 2-3.5 (max. 4) cm long. Seed a sharply angled or winged columella. Inflorescences: in long panicles of 35-60 cm in length. Flowers small and unisexual by atrophy of either sex, 8-10 mm long, with calyx and corolla present, pedicel 2 mm long, calyx cup-shaped, irregularly toothed, 2-3 mm in length, Sepals 5, glabrous, petals 5, free, 7-8 mm long, elliptic, glabrous or very sparsely pubescent on the back, the androecium with 5 stamens or staminodia basally androgynophore soldiers, filaments and anthers glabrous anterodios, ovary globose, glabrous, the style columnar and discoid stigma. Fruits capsules, ellipsoid, 3-4 (-5) cm long and 1.8-2.8 cm in diameter, light brown surface and covered with whitish lenticels, fruit

woody valves open at 5 and a central column has pinned many seeds winged, 2-3 cm long, light brown (Orwa *et al.*2009).

Distribution and habitat

Widely distributed from Central America to the Amazon region, mostly below 1600 m.a.s.l (Reynel *et al.*, 2003). In its natural area of distribution, *C. odorata* is found in both primary and secondary evergreen to semi-deciduous lowland or lower montane rainforest. It demands light and does not tolerate water logging or flooding. Native apparently throughout West Indies in Greater Antilles and Lesser Antilles to Trinidad and Tobago, the range spread by cultivation. Also native in continental tropical America from Mexico to Ecuador, Peru, Brazil and French Guyana (Orwa *et al.*2009).

Uses

The wood is of the highest workability and durability, although depending on site conditions can be somewhat variable attributes. It is soft and lightweight with straight grain and medium to coarse texture, light pink to reddish. It is extremely durable, valued for carpentry, joinery and recognized as one of the best woods Neotropical. (Reynel *et al.*, 2003). The timber is famous for its use in making cigar boxes and it is also used for musical instruments, light construction, veneer, and plywood. The insect repellent smell makes it suitable for wardrobes. In cocoa and coffee plantations it is planted for shade and as a windbreak (CATIE, 2000).

Conservation status

Cedrela odorata L., one of the most important neotropical timber species, is threatened by deforestation and unsustainable logging in many parts of its natural range. Information on patterns of genetic variation is useful in informing both reforestation and genetic conservation activities. However, to date, no such information is available in Peru or elsewhere in South America. Genetic diversity between and within nine Peruvian populations of the species, based on amplified fragment length polymorphism (AFLP) markers, is reported. Genetic and geographical distances were significantly correlated. The relatively strong genetic differences between populations may be related to the riparian, essentially one-dimensional spatial distribution pattern of the populations studied (De la Torre, *et al.*2008). One study describes 9 primers for amplification of microsatellite loci for the Neotropical tree *Cedrela odorata* L. (Meliaceae). Loci were isolated from an enriched library derived from a single DNA sample from a tree in Costa Rica. Levels of polymorphism were determined using samples from a large progeny trial. Across loci, the number of alleles ranged from 14 to 30. Observed heterozygosity levels ranged from 0.61 to 0.88. No linkage disequilibria were detected although some departures from Hardy- Weinberg equilibrium (HWE) were found, probably due to a Wahlund effect (Hernandez, *et al.*2008)

2.1.4 *Copaifera paupera* (Herzog) Dwyer (LEGUMINOSAE)- Copaiba

Botanical description

Scientific name of this tree is *Copaifera paupers* (Herzog) Dwyer, common names is "Copaiba" and used botanical synonyms, paupers Herzog Copaiba, *Copaifera reticulata* Ducke var. J.F. peruviana Macbride. Tree is of 50-150 cm in diameter and 20-35 m total height, with the cylindrical shaft, branching from the second third, the base of the stem straight. Outer bark lenticels, light brown to grayish flakes present rhytidome given off leaving footprints in isolation ("hammered bark"). Inner bark in two layers, an outer sandy yellow to pale orange and an inner thin and fibrous, aromatic bark. Terminal twigs circular, light brown when dry, about 3-5 mm in diameter, finely cracked and glabrous. Compound leaves odd-or paripinnate, alternate and spirally arranged, 15-20 cm long, the petiole 2-4 cm long, cylindrical, thin rachis, the leaflets 8-15, oblong-incurved, strongly asymmetric of 2.5-6 cm long and 1.5-2.5 cm wide, entire, the secondary veins 10-12 pairs, inconspicuous on both sides, and conspicuous tertiary nerves reticulate, apex of leaflets acute, acuminate, the base acute and strongly asymmetric, glabrous leaves. Inflorescences axillary clusters of spikes 10-20 cm long, the flowers subtended by bracts conspicuous, deciduous. Flowers small, hermaphrodite, zygomorphic, 5-7 mm long, sessile, with calyx and corolla present, the calyx 2-3 mm long, tubular corolla yellowish, 4-5 mm long, the stamens 5 - 6 mm in length, the numerous stamens, the pistil gynoecium elongated supero ovary, stigma inconspicuous. Discoid flattened fruits vegetables, brown, about 3-5 cm long, with smooth surface, glabrous, the seed only, black with a fleshy aril; yellow to orange. (Reynel *et al.*, 2003).

Distribution and habitat

This species is distributed in the Amazon region mostly below 700m.a.s.l (Reynel *et al.*, 2003). The genus Copaifera L. has 38 species distributed in Central America (4 species) and southern (29 species), Africa (4 species) and Asia (1 species). Of these, 26 species occur in Brazil. These species are distributed in America between Costa Rica – Antilles and northeastern Argentina - South of Brazil, occupying this range of latitude a portion of the African continent and Borneo (Malaysia) (Lewis, 1987).

Uses

The wood is of very good quality, medium hard to medium duty, off-white in the sapwood to heartwood yellowish red when dry, straight grain and medium to fine texture, also streaked with overlapping arcs and narrow longitudinal bands. It is workable, good durability, and with it made furniture, building structures such as beams, columns and beams, tongue and groove, parquet, plywood and laminates (INIA-ITTO, 1996). Piercing the tree obtain an oil discharge, copaiba balsam, which has medicinal properties and healing for skin lesions. This balm contains hydrocarbon sesquiterpenes, essential oils and resin acids, is also used in the manufacture of cosmetics and soaps (Trease & Evans, 1986).

Conservation status

An interesting tropical plant that deserves molecular investigation and lacks sequencing information is the genus *Copaifera* (Leguminosae), whose members include copaiba. Little molecular data (i.e., ESTs) have been published on this particular genus. With a better understanding of the genetic underpinnings of metabolic pathways in this species, we may be better able to understand the development and physiological conditions that lead to increased production and accumulation of the diesel-like resins (Calvin, 1980). Studies using molecular markers in *Copaifera* were conducted in *C. langsdorffii* Desf., a species with wide distribution in Brazil, based on allozymes (Oliveira *et al.* 2000) and AFLP (amplified fragment length polymorphism), microsatellite (simple sequence repeat - SSR) and sequences of plastids (cp DNA) (Ciampi & Grattapaglia 2001). In the Northeast of Brazil, samples of leaflets tissue were taken from 39 individual adults of *C. coriacea* and the molecular analysis revealed 224 *loci*. The AMOVA test revealed higher variability (57%) within populations than between them (46 %) (Silva *et al.* 2007).

2.1.5 *Guazuma crinita* Martius (STERCULIACEAE) – Bolaina

Botanical description

Guazuma crinita Martius known with common names as "Bolaina", "white Bolaina" botanical synonyms: *Guazuma* Poeppig rosea. Tree: of 25-80 cm in diameter and 15-30 m in total height with cylindrical stem, branching in the last third, the base of the stem straight. Smooth outer shell to finely cracked, light brown to grey. Inner bark fibrous tissue forming a finely reticulated, pale yellow to brown rapidly oxidized, it appears to be pulled into strips. Terminal twigs circular, dark when dry, about 3-4 mm in diameter, usually with ferruginous pubescence towards the apical parts, the bark peels off in strips fibrous to be pulled (Reynel *et al.*, 2003). Leaves simple, entire, chartaceous, margin strate, glabrous and shiny on the upper surface and little tomentose on the under surface, slightly discolored, 5–10 cm long by 3–6.5 cm wide, on petiole 5–15 mm long (Lorenzi, 2000). Inflorescences are axillary panicles about 8-12 x 3-6 cm flowers. Flowers are small, 8-12 mm long, hermaphrodite, with calyx and corolla present, the pedicels 4-8 mm long, the calyx 2-3 mm long, the corolla 6-12 mm long, pink with five petals, each spoon-shaped with two long appendages at the end, the androecium consists of five columns in the stem end bearing many anthers, gynoecium with ovary superior, ovoid, small. Fruits globose capsule about 4-8 mm in diameter with surface densely covered with long hair, about 3-4 cm in length (Reynel *et al.*, 2003).

Distribution and habitat

Very wide in the Neotropics from Central America to the Amazon region to southern Brazil and Bolivia, mostly up to 1500m.a.s.l.(Reynel *et al.*, 2003). *G. crinita* is a fast-growing pioneer species that colonizes the floodplain and disturbed secondary forests in the Amazon Basin of Peru, Ecuador and Brazil (Flores,

2000). Its distribution also occures inundating and noninundating Amazonian primary and secondary forests (Guerrero, 2004).

Uses

The wood is of good quality, but soft and light, white in the sapwood and brown heartwood very pale when dry, straight grain and medium texture (INIA ITTO, 1996). It has good durability. It is used in carpentry, making small tools and palettes lollipops, toothpicks, craft sticks matches and, in recent years is increasingly used in the plywood industry. The fibrous inner bark is used locally as tie material (Reynel *et al.*, 2003). *G. crinita* is an important timber-tree species for agroforestry plantations in the Peruvian Amazon Basin (Rochon, 2007).

Conservation status

An initial provenance test of *G. crinita* was established in farming communities in the Peruvian Amazon and the results suggested that the provenance from the ‘‘local’’ watershed would perform better, in general, than provenances from other watersheds in Peru (Sotelo Montes *et al*., 2000). The breeding system has not been studied, but is assumed to be primarily out-crossing like the majority of tropical trees (Bawa *et al*., 1990). Trees can begin flowering after 2–3 years in open-grown conditions (Weber and Sotelo Montes, 2001), and the lightweight, feathered capsules are dispersed by both wind and water. These reproductive characteristics probably result in extensive gene flow, which would produce high levels of genetic variation within populations and relatively low genetic differentiation among populations (Hamrick et al., 1992).

2.1.6 *Inga edulis* Mart. (LEGUMINOSAE)- Guaba

Botanical description

Tree to 40 m high and 65 cm in diameter (in ideal conditions), buttressed to 1 m high, and bole fluted to 2-3 m, cylindrical above (Pennington, 1997). Normally it reaches 16-30 m high (Macbride, 1943; Novoa, 1992; Lawrence, 1993). Bark smooth, pale grayish sometimes lenticellate and with hoop marks. Slash 3-5 mm thick, pink or pale brown, with clear exudates. Flowers sweetly scented with greyish-yellow perianth, white filaments, pale yellow anthers. Mature fruit greyish-green. Young shoots angular, pale lenticellate, puberulous. Stipules are 2-6 mm long, oblong to lanceolate, appressed, puberulous, caducous. Petiole 2-5 cm long, usually terete, rarely winged in the upper half, puberulous; rhachis 7-20 cm long, winged (to 1.6 cm wide), puberulous, appendix absent. Foliar nectaries sessile, 2-3 mm in diameter, aperture transversely compressed to reniform. Petiolule is 1.5-3 mm long. Leaflets 4-6 pairs; terminal pair 10-19 x 3,8-8,9 cm, elliptic to obovate, apex acute, obtusely cuspidate to narrowly attenuate, base rounded, slightly asymetrical; basal pair 3.8-7.5 x 1.9-4.3 cm, elliptic or ovate, apex obtusely cuspidate to narrowly attenuate, base rounded to to truncate; upper midrib puberulous, lamina minutely scabrid, lower lamina

scabrid to crisped puberulous, venation eucamptodromous to brochidodromous; secondary veins (12-)15-20 pairs, parallel to slightly convergent, slightl arcuate; intersecondaries short to moderate; tertiaries oblique. Inflorescence axillary, sometimes clustered at the shoot appex in the axils of undeveloped leaves, up to 6 in each axil, a congested or less frequently lax spike; peduncle 1-5 cm long, puberulous, floral rachis 1-4.5 cm long; bracts (3-)4-8(-10) mm long, caducous, flowers sessile. Calyx open in bud; tube 4-9 mm long, tubular, lobes 1-2 mm long; puberulous. Corolla tube is 0.9-1.9 cm long, lobes 2-4 mm long; sericeo-villose. Stamens 55-100, staminal tube 1-2 cm long, 1.5-2 mm in diameter, included or slightly exserted, free filaments, 1.5-3 cm long. Ovary of 1 carpel, glabrous, style slightly longer than stamens, style-head cup-shaped, ovules 20-30. Legume 30-100(-200) x 2-5 cm, cylindrical, straight or spirally twisted, apex acute to rostrate, base tapered, faces completely covered by expanded margins, margins longitudinally ribbed; puberulous. Seeds 2-3 x 1-1.5 cm.) (Pennington, 1997).

Distribution and habitat

Colombia and tropical South America east of the Andes, extending south to northwestern Argentina. Also present in Atlantic coastal of Brazil. Doubtfully native in Panama (Pennington, 1997). It grows in a hot and humid climate between 26°S and 10°N (Lawrence, 1993). The species is widely cultivated throughout its range in South America and has been introduced throughout Central America. *Inga edulis* is a light-demanding gap species of lowland rainforest, where it becomes a large tree, and it is also found in riparian situations. Its natural altitudinal range is mostly below 750 m, but it has been occasionally recorded as high as 1200 m in Roraima, Brazil. It is cultivated up to 1600 m (Pennington, 1997). The species requires at least 1200 mm of annual rainfall, but can tolerate short droughts. It is particularly tolerant to acid soils outgrowing many others leguminous trees in such conditions (Lawrence, 1993). This plant is able to adapt to all types of soils present in Amazonia, from the most fertile entisols, inceptisols, histosols and alfisols to most acid and non-fertile soils oxisols, ultisols and sandy espodosols (Paytan, 1997).

Uses

Inga edulis is widely cultivated for its greyish-green fruits (Pennington, 1997); inflorescence attracts the bees, so guaba may also be used in apiculture (Novoa, 1992; Paytan, 1997). Leaves and less quality fruit may be used as a fodder for cows, pigs, poultry and also for fish (Paytan, 1997) The species is very commonly used as a shade tree in small gardens due to its rapid growth and the broad spreading crown. It is sometimes found as a shade tree over coffee and cocoa in Central America (Novoa, 1992; Lawrence, 1993; Pennington, 1997; Paytan, 1997). The leaf litter protects the soil surface and the roots of other plants, helps retain nutrients in the topsoil, and (most importantly for farmers in the humid tropics) control weeds. Guaba is also used in improved fallows, alley cropping. The litter is high in nitrogen Its wood has a high calorific value with nearly no smoke, so it is a good source of fuel wood and possibly a charcoal (Lawrence, 1993).

Conservation status

To promote the conservation and sustainable use of tree species in the Peruvian Amazon, farmers are currently encouraged to undertake additional transfers of germplasm from natural stands into local cultivation (O'Neill *et al*. 2001; Weber *et al*. 2001). Observations of relatively high genetic differentiation between matched natural and planted stands of *I. edulis*, however, suggest that in this instance such transfer may not be the best means to promote onfarm conservation, because of possible productivity losses through interbreeding (out-breeding depression) and genetic dilution (Jamnadass *et al*. 2005). Instead, effort may be better focused on a strategy that involves maintaining the rather high level of genetic diversity already found within current planted stands. Practically, this would involve farmer-training exercises to maintain good collection practices of currently cultivated *I. edulis* germplasm as farmers ''regenerate'' diversity in subsequent rounds of onfarm propagation. In addition, coordinated exchange of *I.edulis* seed through improved network development among neighbouring farms may be appropriate, assuming that neighbouring farms obtained their material from similar sources. However, any strategy must be dynamic and also recognise that farmers have been introducing, and will continue to introduce, selected germplasm from other sources, notably from nearby markets (Brodie *et a*l. 1997). Cultivated *Inga edulis* trees in smallholders´ farms was genetically differentiated from local wild material, the origin of planted trees was unlike to be from the natural tree populations, while different planted populations had different genetic compositions from each other and suggest multiple external sources for cultivated germplasm (Dawson *et al*. 2007).

2.1.7 *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby (LEGUMINOSAE)- Pashaco

Botanical description

Scientific name of this tree is *Schizolobium parahyba* (Vellozo) Blake var.*amazonicum* (Huber ex Ducke) Barneby, common names are "Pino chuncho", "Pashaco". Botanical synonyms are Huber ex Ducke *amazonicum Schizolobium, S. excelsum* var. L. ex Ducke *amazonicum* Williams. Tree grows to 30-70 cm in diameter and 18-25 m total height, with the cylindrical shaft, branching in the last third, the base of the stem straight. A smooth outer shell cracked reddish brown to grey, with rectangular slabs rhytidome runs small, 1.5-4 cm wide. Inner bark smooth,whitish-yellow, scented vegetable. Terminal twigs with circular cross section, reddish brown to light brown when dry, about 5-10 mm in diameter, glabrous (Reynel *et al.*, 2003). Leaves bipinnate, large; pinnae 15-20 pairs, fernlike; leaflets small, elliptic, 10-20 pairs, stipules absent. Flowers golden yellow, large, profusely produced in axillary semi-erect racemes or terminal panicles; bracts minute; bracteoles absent; calyx tube obliquely turbinate; lobes 5, overlapping, reflexed at flowering; petals 5, clawed, subequal, overlapping, uppermost petal innermost; stamens 10, free, subdeclinate; filaments villous, basally rough; anthers uniform, longitudinally dehiscent; ovary subsessile affixed to 1 side of calyx tube, many-ovuled, style filiform; stigma minute, terminal. Pod flat, spoon or

tear-drop shaped, exocarp firm, leathery, tardily dehiscent. Seed large, oblong, compressed, located near apex (Orwa *et al.*2009). Fruit elongated and flat, oblanceolate, with the apex rounded, 8-10 cm long and 2.5-3.5 cm wide, smooth, glabrous, reddish brown or dark brown, single winged seed, shape and size similar to the fruit, with the side wing (Reynel *et al.*, 2003).

Distribution and habitat

S. parahybum is a widespread pioneer species from tropical and premontane forests zones of the American Atlantic coast, flourishing on well-drained moist soils on plains or hillsides (Orwa *et al.*2009). It was seen in areas with high and constant rainfall, but also in areas with a marked dry season, is a light-demanding species trend of rapid growth and is present in early and late secondary forests, is found in primary forest clearings; prefers loamy sandy soils of medium to high fertility necessarily well drained, with low to medium stoniness. This species is very sensitive to water logging and not tolerated, especially when it is a seedling (Reynel *et al.*, 2003).

Uses

The wood is very soft and very lightweight, straight grain interlocked, texture coarse and whitish It is used for drawers, local carpenter and wood, in Ecuador is an important source industry for the production of laminated plywood. (Reynel *et al.*, 2003).

Conservation status

Studies of the genetic diversity of tree species are very important to provide best practice policies for sourcing germplasm for reforestation within the range of degraded landscapes and for trees with a range of lifestyles that are key components of a diverse ecosystem composition. Presently, a restricted number of studies to Schizolobium have focused on molecular markers. Microsatellite loci have been isolated (Kamau *et al.*, 2003), an evolutionary analysis of the enzyme pyrroline-5-carboxylate synthase (P5CS) has been performed in S. parahyba and other Neotropical trees (Turchetto-Zolet *et al*., 2008), an analysis of the genetic structure of S. parahyba from the state of Rio de Janeiro (Brazil) using RAPD markes has been conduced (Freire *et al*., 2007), and the genetic relationship among ecotypes of S. parahyba from Ecuador and others countries were investigates using RAPD, AFLP and SSRs (Canchignia - Martines *et al.*, 2007).

2.1.8 *Swietenia macrophylla* G. King (MELIACEAE)- Caoba

Botanical description

Scientific name is *Swietenia macrophylla* G. King with common name "Mahogany". Botanical Synonym is *Swietenia tessmannii* Harms. Tree can reach 80-200 cm in diameter and 20-35 m high, with cylindrical shaft, branching from the second third, and the base of the stem usually with buttresses up to 1.5 m high. Outer crust cracked, light brown to reddish, with rhytidome that is apparent in elongated plates. Homogeneou inner bark is fibrous, pinkish white, bitter, astringent flavour. Terminal twigs circular, light brown when dry, finely lenticels, glabrous. It can occur every few scars congested stretch of falling leaves (Reynel *et al.*, 2003).

Leaves paripinnate, up to 60 cm long; leaflets 6-16, ovate, lanceolate, acuminate, slightly oblique, light green or reddish when young, dark green and shining when mature, up to 20 cm long, with 8-12 pale, secondary nerves. Flowers of 8 mm across, in narrow supra-axillary panicles about 8-13 cm long and fragrant; petals greenish-white, oblong, 4 mm long, rigidly pointed. The fruit is a woody capsule resembling a large inverted club, about 12.5 x 7.5cm and erect (Orwa *et al.*, 2009).

Distribution and habitat

S. macrohpylla is distributed from Mexico in Central America to the Amazon region to Bolivia mostly below 1200 m.a.s.l. Observations on the ecology of mahogany indicate that in its initial stages require plenty of light and protection from excessive shade. The competition control pioneer species for 2-3 years allowing them to grow rapidly among secondary vegetation (Pennington, 2002). *S. macrophylla* is found in all forest types, from the edge of the pine savannah to the climax rainforest, but mostly in mixed hardwood forest belts, along riverbanks, on deep alluvial soils of considerable fertility. It occurs scattered or in small groups, but densities of more than 4-8 trees/ha are rarely encountered. In tropical America, it is among the pioneer species reoccupying degraded agricultural land. It has been shown that teak is outcompeted by *S. macrophylla* in a mixed stand. In the Philippines, *S. macrophylla* is reported to be very firm in wind, resistant to cyclones (Orwa *et al.*, 2009).

Uses

The wood is of excellent quality, generally is considered the best wood Amazon. It has medium density, straight grain and medium texture, is pink to reddish. It has very high durability and workability, is prized for fine carpentry and joinery (Reynel *et al.*, 2003).

Conservation status

In recent years, the conservation status of *S. macrophylla* has been the subject of increasing concern (CITES 1996; Rodan and Campbell 1996). Mahogany is considered highly threatened throughout Central America and in some regions of Bolivia and Brazil (Collins 1990; Gullison et al. 1996; Verissimo *et al.*

199). The selective and intensive logging of *S. macrophylla* populations may compromise their viability as evolutionary units by reducing population sizes and levels of genetic polymorphism. Despite the economic importance of mahogany, studies on the extent and distribution of genetic variation in natural populations are scarce and have only been carried out in Central America. Gillies et al. (1999) used random amplified polymorphic DNA (RAPD) markers to investigate the patterns of genetic variation among *S. macrophylla* populations from Mexico south to Panama. In a study of a fragmented population of the congeneric *Swietenia humilis*, found only in Central America, White et al. (1999) used microsatellite markers to show the occurrence of extensive gene flow among populations, probably reflecting the species' continuous distribution prior to habitat destruction. Highly polymorphic microsatellites, also called simple sequence repeats (SSRs), are the most genetically informative class of molecular markers for studies of genetic variation in natural populations (Rafalski *et al. 1996*).

2.2 DNA extraction methods of tropical trees.

In the last decade, there has been an enormous increase worldwide in the use of molecular marker methods to assess genetic variation in trees. These approaches can provide significant insights into the defining features of different taxa and this information may be used to define appropriate management strategies for species. This is especially important when the total funds available for study of any particular species are limited, as is generally the case for tropical trees because there are so many taxa to research (Muchugi *et al.* 2008).

Tropical tree populations are increasingly being studied using molecular methods. Such studies require fresh or, more commonly given the often remote field sites, dried tissue samples for DNA extraction (Schierenbeck, 1994). Studies based on DNA markers require large amount of quality genomic DNA (gDNA), emphasizing the need for inexpensive, rapid and simple DNA extraction methods (Weishing *et al*., 1995). Most of the available protocols and commercial KITs are simple but not efficient for difficult material. Many protocols may provide gDNA when processing young leaves of herbaceous plants; however, they may be unsuitable for extracting gDNA from mature tree leaves. The quantity and quality of gDNA often varies among tree tissue types (Henry, 2001).

The problem of isolating high quality DNA from mature tree species is still an important issue in the field of plant molecular biology (Barzegari *et al*., 2010; Li *et al*., 2010; Smyth *et al*., 2010). Tree tissue often contains large amounts of polysaccharides and phenolic compounds that are difficult to separate from DNA. (Tibbits *et al*., 2006; Sharma *et al*., 2011), high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins in tree species usually interfere with DNA isolation (Mishra *et al*., 2008). Similarly, Diadema *et al*. (2003) observed that genomic DNA extraction from succulent plants is difficult. These authors reported that the difficulty could be attributed to small cell density in succulent tissues and high levels of contaminants (polyphenols or polysaccharides) that co-precipitate with DNA. Polyphenols released from the vacuoles during the cell lysis process are

oxidized by cellular oxidases and undergo irreversible interactions with nucleic acids causing browning of the DNA (Varma *et al.*, 2007). The presence of gelling polysaccharides prevents complete dissolution of nucleic acids and imparts a viscous constituency to the DNA making it stick to the wells during gel electrophoresis (Barnell *et al*., 1998; Diadema *et al*., 2003; Varma *et al*., 2007). Researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma *et al*., 2007). A good isolation protocol should be simple, rapid and efficient, yielding appreciable levels of high quality DNA suitable for molecular analysis.

Križman *et al*. (2006) were of the opinion that, among other factors, the amount of plant sample extracted could be critical in keeping an extraction procedure robust. The leaves from legume plants are particularly subject to the de novo formation of polyphenolics during the isolation procedures (Doyle, 1990). Different DNA isolation methods for PCR-based analysis of plants have been devised (Dellaporta *et al*., 1983; Murray et al., 1980; Doyle, 1990) they are time-consuming and/or problematic for some plant species such as woody plants, which accumulate a high amount of polyphenolic compounds and polysaccharides that make DNA isolation difficult. Based on the finding that DNA binds to silica matrix in the presence of a high concentration of chaotropic salt (Vogelstein, 1979), a variety of commercial DNA purification KITs have been developed. However, silica-based column KITs for plant genomic DNA generally are expensive and require many steps.

CTAB DNA purification methods extract high quantities of pure DNA from a variety of different plant tissues, e.g., cotton, blackcurrant, ferns, fruit trees, and conifers (Woodhead *et al*., 1998; Dempster *et al*., 1999 and Kim *et al.*, 1997). CTAB DNA isolation techniques for extracting the DNA from plant species has been widely used for PCR analysis (Doyle and Doyle, 1990; Kidwell and Osborn, 1992; Ferreira and Grattapaglia, 1995). Several different methods and technologies are available for the isolation of plant genomic DNA, however, the better results can be observed with those based in use of CTAB (Doyle and Doyle, 1990; Kidwell and Osborn, 1992; Ferreira and Grattapaglia, 1995; Dilworth and Frey, 2000; Shepherd *et al*., 2002; Kang and Yang, 2004; Narayanan *et al*., 2006).

In general, all methods consist of the following major steps: (1) grinding of samples, (2) phenol: chloroform: isoamyl alcohol extraction, and (3) DNA precipitation. The choice of a specific method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications, the time and the expense. Among the different extraction methods published so far, they have chosen a CTAB-based procedure because it is reliable (Roger *et al*., 1985 and Weising *et al*., 1991), time saving, reasonably safe and cheap. With this approach, DNA can be obtained from few milligram amounts of freeze-dried plant tissues (Roger *et al*., 1985). Alzate *et al*., 2009 reports some samples of DNA extraction yield from tropical trees such as: *Copaifera langsdorffii* Óleo de Copaíba 400 ng/µL, *Schizolobium parahyba* Guapuruvu 358ng/µL, *Acacia polyphylla* Monjoleiro 530ng/µL, *Caesalpinea ferrea* Pau Ferro 443ng/µL.

2.3 Methods for Determining DNA amount , purity and quality.

DNA yield can be assessed using three different methods: absorbance (optical density), agarose gel electrophoresis and fluorescent DNA-binding dyes. Each technique is described and includes information on necessary accessories (e.g., equipment). While all methods are useful, each has caveats to consider when choosing a quantization system (Glasel, 1997).

The most common technique to determine DNA yield and purity is also the easiest method—absorbance. All that is needed for measurement is a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are performed at 260nm (A_{260}) where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution. To ensure the numbers are useful, the A_{260} reading should be between 0.1–1.0 (Adams, 2003).

Evaluation DNA purity by spectrophotometry, measures absorbance from 230nm to 320nm in order to detect other possible contaminants present in the DNA solution (Manchester, 1995).The quality of DNA is analyzed in agarose gel electrophoresis of the purified DNA eliminates the issues associated with absorbance readings. To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are needed for quantitation. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity (Sambrook *et al.*1989).

3. OBJECTIVE OF THE THESIS

The first step as a pre-requisite for molecular techniques or any genetic study is the collection of plant material, its storage and the isolation of high molecular weight genomic DNA. It is hypothesized that storage of plant material in silica gel and then DNA extraction using classical methods will yield sufficient DNA for further analysis.

The main objective of the study is evaluation of two DNA extraction methods (CTAB and KIT) and three methods of plant material storage (silicagel, lyophylization and fresh material) for selected Amazonian tree species.

The specific objectives of this study are following:

- To assess if Invisorb Spin Plant mini KIT method, is more suitable than CTAB extraction method or on contrary.
- To assess if there is influence of plant material (mainly its storage) has influece on the amount and purity of DNA.
- Compare the financial cost of both methods.

4. MATERIALS AND METHODS

4.1 Plant material and leaves tissue sampling.

Seeds of 8 tropical tree species such as: *Amburana cearensis, Calycophyllum spruceanum, Cedrela odorata, Copaifera pauperea, Guazuma crinita, Inga edulis, Schizoloium parahyba* and *Swietenia macrophylla*, were brought from the Amazon of Ucayali-Peru, seven species were obtained from a seed bank of a commercial nursery, *I. edulis* was collected from a home garden . and then they were planted in the green house of the Botanical Garden of Institute of Tropics and Subtropics, CULS Prague (Czech Republic), for the study, were collected young leaves from seedlings of 8-10 months of age. The collected samples of above mentioned species were stored as follows:

- One tube with fresh leaves of each tested species, which were immediately analyzed.
- One tube with fresh leaves of each tested species filled by silica gel and fresh stored at environment temperature for a week and then analyzed.
- One tube with fresh leaves of each tested species, were lyophilized and were analyzed after that. They were stored in the same way described above.

4.2 DNA Extraction methods

To evaluate the best extraction method of DNA two methods were chosen - Invisorb ®Spin Plant Mini KIT method and CTAB method. For each one of the specie two DNA extraction methods with three types of plant material (fresh, dry by silica gel and lyophilized) were evaluated, with three repetitions per sample (Table 1).

Table 1 Organization of samples per specie

Specie	Extraction method	Conservation methods of Plant material	Repetition
Amburana cearensis, Calycophyllum spruceanum,	KIT	Fresh	3
Cedrela odorata,		Silica gel	3
Copaifera pauperea, Guazuma crinita,		Lyophilized	3
Inga edulis,	CTAB	Fresh	3
Schizoloium parahyba		Silica gel	3
Swietenia macrophylla		Lyophilized	3

DNA extraction by Invisorb ®Spin Plant Mini KIT

Starting material of each sample was weight 40 mg and than it was pulverized under liquid N2. Samples were placed briefly in liquid nitrogen and then grounded to get a fine powder. This material was used for the extraction. The KIT helped to process the lysis, adding 400 µl of Lysis Buffer P and 20 µl Proteinase K, than all the samples were vortexed briefly. After that, it was incubated at 65°C for 1 hour. The DNA binding took place adding 200 µl of Binding Buffer P and vortexed thoroughly. The samples were centrifuged at 12.000 rpm for 1 min. Two washings with 550 µl of Wash Buffer I and one with 550 µl of Wash Buffer II followed. After that, were discard the filtrates and the samples were centrifuged for 2 min at 12.000 rpm to remove residual ethanol. Finally it was added 100 µl the Elution Buffer. The extracted DNA was stored in controled conditions at -20ºC.

CTAB extraction method (Doyle 1991 Modified)

About 50-100 glass beads have been added to the tube containing plant tissue (20mg); then were frozen in liquid nitrogen and the tissues were grinded for 12 seconds with Silamat; 250 ul of DNA extraction buffer were added; samples were mixed thoroughly briefly and incubated at room temperature for 15-20 minutes; in the fume hood were added equal volume (250 ul) of chloroform / IAA (24:1) and mixed well. Spin for 10 minutes at 13000 RPM and were transferred aqueous layer to fresh tube (approx. 200 ul.) containing 0.7 transfer volumen of Iso-propanol (approx. 140 ul.); were left at room temperature for 5 minutes; were mixed, then were spin for 7 minutes at 13000 RPM and were discarded supernatant; were washed pellet with 1ml of 70% ethanol and spin for 7 minutes was done; were poured off ethanol; were removed remainder with pipette and vacuum dry pellet for 5-10 minutes. Finally dissolved in 50 ul of H2O and incubated at 55ºC for 5 minutes. DNA extracted was stored at -20ºC.

4.3 Determination of quality, purity and concentration of DNA extracted:

DNA purity was further tested by electrophoresing the extracted gDNA on 1% agarose gel at 80-100 V for 60 min in TAE (Tris-acetate-EDTA) buffer in a submerged agarose gel electrophoresis. The gel was stained with 0.2 µg/ml ethidium bromide, then visualized and photographed using the Gel Documentation System (Syngene Gel Doc, Syngene, Syn optics Ltd., UK).

The DNA was quantified by absorbance spectrophotometry at a wavelength of 260 nm (A260) using the Nanodrop1000 spectrophotometer, this method also gave an estimate of the purity of DNA by absorbance ratio (A260/A280nm). The most common purity calculation is determining the ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants presented. However, the best test of DNA quality is functionality in the application of interest (e.g., real-time PCR) (Manchester, 1995).

4.4 Statistical Evaluation and financial costs

Analysis of variance (ANOVA) and Tukey HSD test (Statistica software 7.0 CZ) were used to analyse the statistical significant differencies between DNA concentrations and DNA purity in four repetitions, in two exctraction methods, in three types of leaf materials and in tested species .

Statistically significant difference was set at the 5% level ($p \leq 0.05$)

We evaluated the cost of both methods according to the current catalog prices of the used chemical needed for DNA extraction.

5. RESULTS

5.1 DNA extracted of all tropical tree species

From the total DNA extracted content of whole investigated Amazonian species the best concentration and DNA purity were 235.73 ng/ul and A260/A280 = 1.98 respectively from CTAB method (Table 1). When considering three type of conservation and preparation of the leaf material (fresh, dried leaf material, because of storing in silicagel and lyophilized leaf material). When silicagel was used, it was measured the highest DNA concentration (173.65±37.47). The next two types showed lower DNA concentrations 95.99 ng/ul fresh and 150.84 ng/ul, when lyophilized.

According to Tukey HSD test, the two different extraction methods used in the experiment showed statistically significant differences at the 95% level of probability. The only statistical difference in case of type of leaf material was recognized between DNA concentrations. The best solution for the plant material seemed the storing in silicagel. The influence of plant species on quality of DNA extraction was significant just between Schizolobium parahyba which had the highest DNA concentration (310.18 ng/ul) and Cedrela odorata which had the lowest (37.95 ng/ul) DNA observed. The highest average of DNA purity was observe in Swietenia macrophylla (1.97) and Guazuma Crinita shows the lowest (1.64) DNA purity determined from the DNA extracted.

Table 2. Results of DNA extraction and the significant differences according the extraction methods, conservation of leaf material and species (Total)

		DNA Concent. ng/ul	**DNA Purity 260/280**
Extraction method	KIT	44.60±6.77^{a}	1.77±0.03^{a}
	CTAB	235.73±28.40^{b}	1.98±0.02^{b}
Conservation methods of leaf material	fresh	95.99±12.69^{a}	1.87±0.04^{a}
	silicagel	173.65±37.47^{b}	1.89±0.03^{a}
	lyophilized	150.84±29.69 ^{ab}	1.85±0.03^{a}
Species	*Schizolobium amazonico*	310.18±86.15^{d}	1.92±0.04^{a}
	Amburana cearensis	200.16±51.74^{bcd}	1.90±0.04^{a}
	Inga edulis	66.12±11.83^{ab}	1.88±0.04^{a}
	Copaifera reticulata	221.63±37.77^{cd}	1.90±0.02^{a}
	Calycophyllum spruceanum	64.15±24.80^{ab}	1.86±0.08^{a}
	Cedrela odorata	37.95±7.43^{a}	1.91±0.05^{a}
	Guazuma crinita	92.96±32.41^{abc}	1.64±0.07^{b}
	Swietenia macrophylla	128.14±37.86^{abc}	1.97±0.06^{a}

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

5.2 DNA extracted of *Amburana cearensis* - Ishpingo

Tthere was significant difference between the extraction methods conservation methods of leaf material for Amburana cearensis (Figure 1). The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT was 35.91ng/ul in case of fresh material. The CTAB method showed the highest DNA concentration 502.93ng/ul using the lyophilized material. Statistical analysis showed significant difference between the evaluated methods, but it does not show difference among the conservation methods of leaf samples (Table 3). CTAB method produced significantly highest concentration of DNA (358.66ng/ul).

Table 3. DNA concentration extracted and DNA purity from *Amburana cearensis*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation method of leaf material	Fresh	138.04±57.85a	2.02 ± 0.03 a
	Silica	193.03±57.85a	1.83 ± 0.03 b
	lyophilized	269.42±57.85a	1.86 ± 0.03 b
Extraction method	KIT	41.67±47.24a	1.79 ± 0.03 a
	CTAB	358.66±47.24b	2.1± 0.03 b

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at $p \leq 0.05$ Tukey test.

The highest average DNA purity determined from each methods were $A_{260}/A_{280} = 1.97$ and $A_{260}/A_{280} = 2.07$ respectively for fresh leaf material. In DNA purity there was found difference between the extracting methods and also among the conservation methods of the utilized leaf samples. It is evident that the methods of extraction and the different type of leaf material had significant influence on the DNA purity (Table 3). The obtained result suggested, that for the DNA extraction from *Amburana cearensis* it is better to use CTAB method and preferably fresh leaf material.

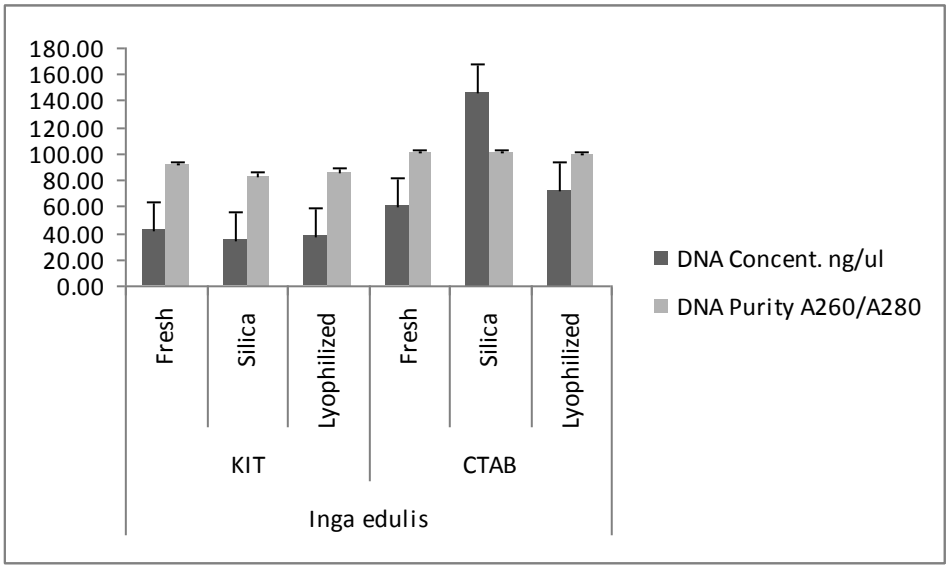

Figure 1. DNA concentration extracted and DNA purity from *Amburana cearensis*, obtained from three types of leaf material (fresh, silicate and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

5.3 DNA extraction of *Calycophyllum spruceanum* – Capirona

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 21.52ng/ul and 218.94ng/ul respectively for lyophilized leaf material. The figure 2 shows that there is difference between the extraction methods and the leaf material utilized. Statistical analysis showed significant difference between the evaluated methods but it does not show difference among the conservation methods of leaf samples (Table 4). CTAB method shows significantly highest concentration of DNA (112.31ng/ul).

Table 4. DNA concentration and DNA purity extracted from *Calycophyllum spruceanum*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation method of leaf material	fresh	138.04±57.85a	1.75± 0.15 a
	silica	193.03±57.85a	1.91± 0.15 a
	lyophilized	269.42±57.85a	1.92 ± 0.15 a
Extraction method	KIT	41.67±47.24a	1.88 ± 0.12 a
	CTAB	358.66±47.24b	1.83± 0.12 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

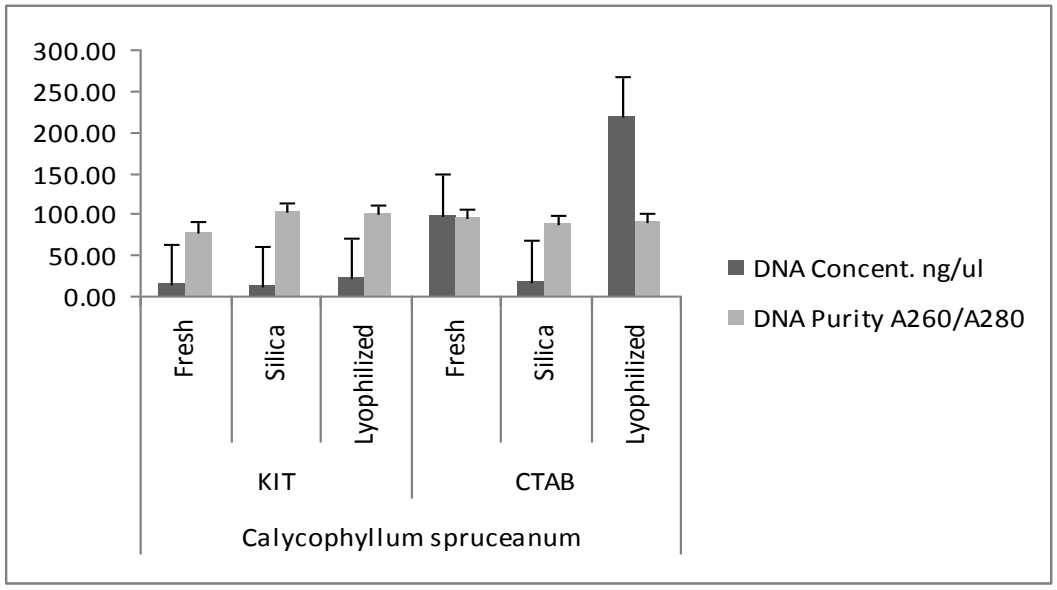

Figure 2. DNA concentration and DNA purity extracted from *Calycophyllum spruceanum***,** obtained from three types of leaf material (fresh, silicate and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at $p \leq 0.05$ Tukey test.

The highest average DNA purity determined from each methods were $A_{260}/A_{280} = 2.02$ and $A_{260}/A_{280} = 1.83$ respectively for lyophilized leaf material. There was not found difference between the extracted methods neither among the conservation methods of utilized leaf sample, it evidence that methods extraction and the conservation methods of leaf samples did not have influence in the purity of DNA determined for this specie (Table 4). The results obtained suggested that for the DNA extraction from *Calycophyllum spruceanum* is better to use CTAB method and preferably lyophilized leaf material.

5.4 DNA extracted of *Cedrela odorata* – Cedro.

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 15.72ng/ul and 80.97ng/ul respectively for fresh leaf material. The figure 3 shows that there is difference between the extraction methods and leaf material utilized. Statistical analysis showed significant difference between the evaluated methods but it does not among the conservation methods of leaf samples (Table 5). CTAB method processed significantly highest concentration of DNA (62.05 ng/ul).

Table 5. DNA concentration extracted and DNA purity from *Cedrela odorata*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation method of leaf material	fresh	48.35±8.05a	1.84 ± 0.07 a
	silica	32.21±8.05a	1.94 ± 0.07 a
	lyophilized	33.29±8.05a	1.96 ± 0.07 a
Extraction method	KIT	13.85±6.57a	1.80 ± 0.05 b
	CTAB	62.05±6.57b	2.03± 0.05 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

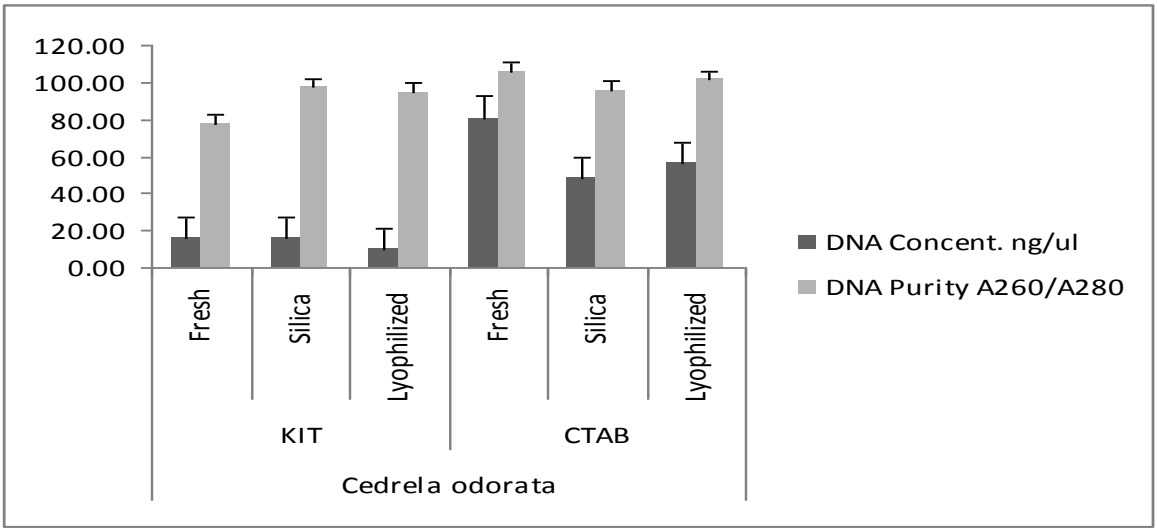

Figure 3. DNA concentration extracted and DNA purity from *Cedrela odorata*, obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

There was found difference between the extracted methods but not among the conservation methods of leaf sample utilized, it evidence that methods extraction had influence in the purity of DNA determined for this specie (Table 5). The highest average DNA purity extracted by each methods were A_{260}/A_{280} = 1.90 and A_{260}/A_{280} = 2.13 with lyophilized and fresh leaf material respectively. The results obtained suggested that for the DNA extraction from *Cedrela odorata* is better to use CTAB method and preferably fresh leaf material

5.5 DNA extraction of *Copaifera paupera* - Copaiba

Statistical analysis showed significant difference between the evaluated methods and among the conservation methods of leaf samples as well (Table 6). The figure 4 shows that there was difference between the extraction methods and leaf material utilized. The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 141.031ng/ul and 449.28ng/ul respectively for lyophilized leaf material. CTAB method shows significantly highest concentration of DNA (321.45 ng/ul).

Table 6. DNA concentration extracted and DNA purity from *Copaifera paupera*

		DNA Concent. ng/ul	**DNA Purity A260/A280**
Conservation method of leaf material	fresh	159±43.82a	1.95 ± 0.02 a
	silica	210.34±43.82 ab	1.86 ± 0.02 b
	lyophilized	295.16±43.82 b	1.87 ± 0.02 ab
Extraction method	KIT	121.81±35.78b	1.84 ± 0.01 b
	CTAB	321.45±35.78 a	1.97± 0.01 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

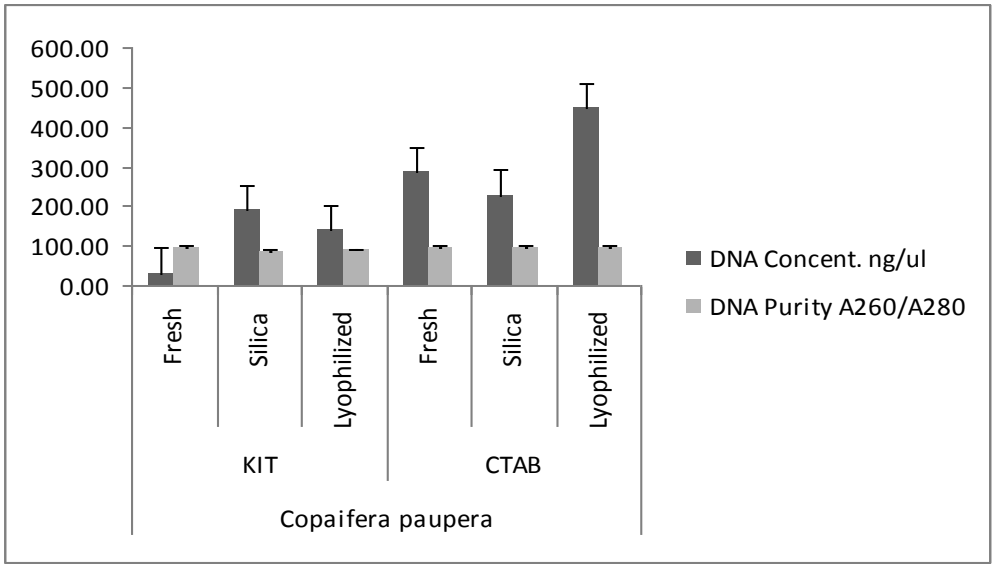

Figure 4. DNA concentration extracted and DNA purity from *Copaifera paupera*, obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

In purity DNA also there was found difference between the extracted methods and also among the conservation methods of utilized leaf sample, it evidence that methods extraction and conservation methods of leaf had influence in the DNA purity determined of this specie (Table 6).

The highest average DNA purity extracted by each methods were A_{260}/A_{280} = 1.96 and A_{260}/A_{280} = 1.96 respectively with fresh and silicate leaf samples. The results obtained suggest that for the DNA extraction from *Copaifera paupera* is better to use CTAB method and preferably lyophilized leaf material

5.6 DNA extracted of *Guazuma crinita* – Bolaina

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 36.39ng/ul and 289.86ng/ul for fresh and lyophilized leaf material respectively. The figure 5 shows that there is difference between the extraction methods and utilized leaf material. Statistical analysis showed significant difference between the evaluated methods but does not among the conservation methods of leaf samples (Table 7). CTAB method had significantly highest concentration of DNA (164.74ng/ul).

Table 7. DNA concentration and DNA purity extracted from *Guazuma crinita*

		DNA Concent. ng/ul	**DNA Purity A260/A280**
Conservation method of leaf material	Fresh	46.86±44.88 a	1.56 ± 0.08 a
	Silica	80.91±44.88 a	1.73± 0.08 a
	Lyophilized	151.13±44.88 a	1.64 ± 0.08 a
Extraction method	KIT	21.19±36.75 b	1.48 ± 0.01 b
	CTAB	164±74 a	1.81± 0.01 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at $p \leq 0.05$ Tukey test.

The highest average DNA purity determined from each methods were A_{260}/A_{280} = 1.73 and A_{260}/A_{280} = 1.87 with silicate and lyophilized leaf material respectively. In DNA purity was found difference between the extracted methods but not among the conservation methods of leaf sample utilized, it evidence that methods extraction had influence in the purity of DNA extracted independently of conservation methods of leaf (Table 7). The results obtained suggest that for the DNA extraction from *Guazuma crinita,* is better to use CTAB method and preferably lyophilized leaf

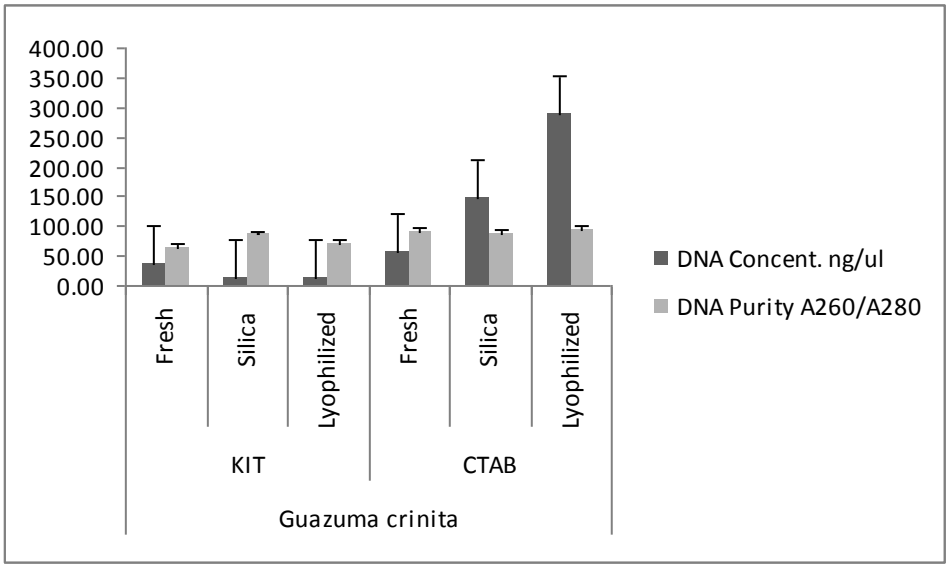

Figure 5. DNA concentration extracted and DNA purity from *Guazuma crinita*, obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

5.7 DNA extracted of *Inga edulis* – Guaba

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 42.64ng/ul and 147.04ng/ul for fresh and silicate leaf material respectively. The figure 6 shows that there is difference between the extraction methods and leaf material utilized. Statistical analysis showed significant difference between the evaluated methods but does not among the conservation methods of leaf samples (Table 8). CTAB method had significantly highest concentration of DNA (93.40ng/ul)

Table 8. DNA concentration extracted and DNA purity from *Inga edulis*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation method of leaf material	fresh	51.90±15.00 a	1.94 ± 0.03 a
	silica	91.22±15.00 a	1.85 ± 0.03 ab
	lyophilized	55.23±15.00 a	1.86 ± 0.03 b
Extraction method	KIT	38.83±12.25 b	1.75 ± 0.02 b
	CTAB	93.40±12.25 a	2.02 ± 0.02 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

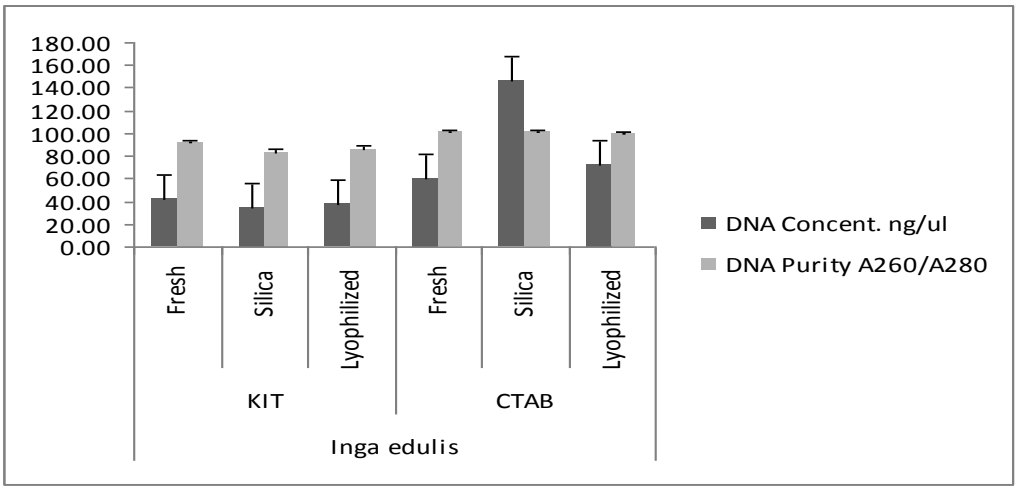

Figure 6. DNA concentration extracted and DNA purity from *Inga edulis*, obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at $p \leq 0.05$ Tukey test.

The highest average DNA purity extracted by each methods were 1.85 and A_{260}/A_{280} = 2.03 for fresh leaf material respectively. In purity there was found difference between the extracted methods and also among the conservation methods of leaf sample utilized (Table 8). The results obtained suggest that for the DNA extraction from *Inga edulis* is better to use CTAB method and preferably silicate leaf material

5.8 DNA extracted of *Schizolobium parahyba* - Pashaco

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 81.94ng/ul and 971.06ng/ul for silicate leaf material respectively. The figure 7 shows that there is difference between the extraction methods and leaf material utilized. Statistical analysis showed significant difference between the evaluated methods and among the conservation methods of leaf samples in DNA concentration extracted and DNA purity (Table 9). CTAB method showed significantly highest concentration of DNA (558.32ng/ul)

Table 9. DNA concentration extracted and DNA purity from *Schizolobium parahyba*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation method of leaf material	fresh	159.54 ±68.76 b	1.97 ± 0.02 b
	silica	526.5 ±68.76 a	1.91 ± 0.02 a
	lyophilized	244.51 ±68.76b	1.90 ± 0.02 b
Extraction method	KIT	62.05 ± 56.14b	1.7 ± 0.02 b
	CTAB	558.32 56.14a	2.8± 0.02 a

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

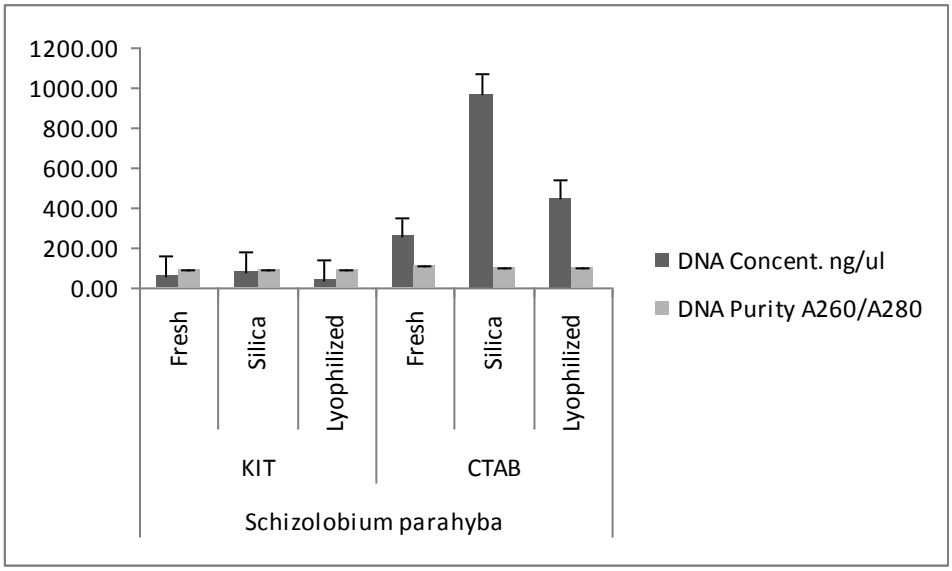

Figure 7. DNA concentration extracted and DNA purity from *Schizolobium parahyba,* obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

The highest average DNA purity extracted by each methods were 1.80 and A_{260}/A_{280} = 2.13 for fresh leaf material respectively. The results obtained suggested that for the DNA extraction from *Schizolobium parahyba* is better to use used CTAB method and preferably silicate leaf material

5.9 DNA extracted of *Swietenia macrophylla* - Caoba

The highest average of DNA concentration extracted by Invisorb ®Spin Plant Mini KIT and CTAB method were 77.42ng/ul and 439.54ng/ul for fresh and silicate leaf material respectively. Statistical analysis showed significant difference between the evaluated methods and among the conservation methods of leaf samples in DNA concentration but does not for DNA purity (Table 9). The figure 8 shows that there is difference between the extraction methods and leaf material utilized. CTAB method processed significantly highest concentration of DNA (214.89ng/ul).

Table 10. DNA concentration extracted and DNA purity from *Swietenia macrophylla*

		DNA Concent. ng/ul	DNA Purity A260/A280
Conservation methods of leaf material	fresh	107.08±29.25 b	1.94 ± 0.07a
	silica	239.57±29.25 a	2.14 ± 0.07a
	lyophilized	37.77±29.25 b	1.79 ± 0.07a
Extraction method	KIT	41.39±23.81 b	1.84 ± 0.06 b
	CTAB	214.89±23.81 a	2.09 ± 0.06 b

Each value represents the mean ± SE. Values within the column followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

The highest average DNA purity determined from each methods were A_{260}/A_{280} = 2.10 and A_{260}/A_{280} = 2.19 respectively for silicate leaf material. The results obtained suggested that for the DNA extraction from *Swietenia macrophylla* is better to use CTAB method and preferably silicate leaf material.

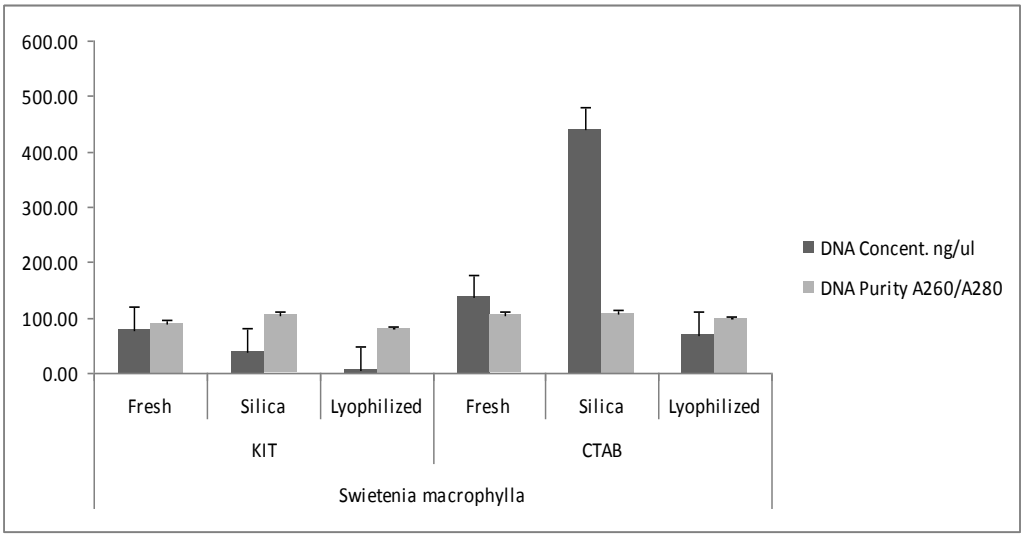

Figure 8. DNA concentration and extracted and DNA purity from *Swietenia macrophylla*, obtained from three types of leaf material (fresh, silica and lyophilized). Each value represents the mean ± SE. Bars followed by different lower-case letter are significantly different at p≤0.05 Tukey test.

5.10 Quality of DNA extracted

Considering the results of statistical analysis, we assessed the integrity of DNA obtained with the two extraction methods. Figure 9 and 10 shows that there is difference in the integrity of the DNA obtained from all species with the two methods evaluated in which the CTAB method shows better quality of DNA bands formed while the KIT method evidenced degraded DNA. Conservation methods of plant material showed difference as well (Appendix 1 and 2).

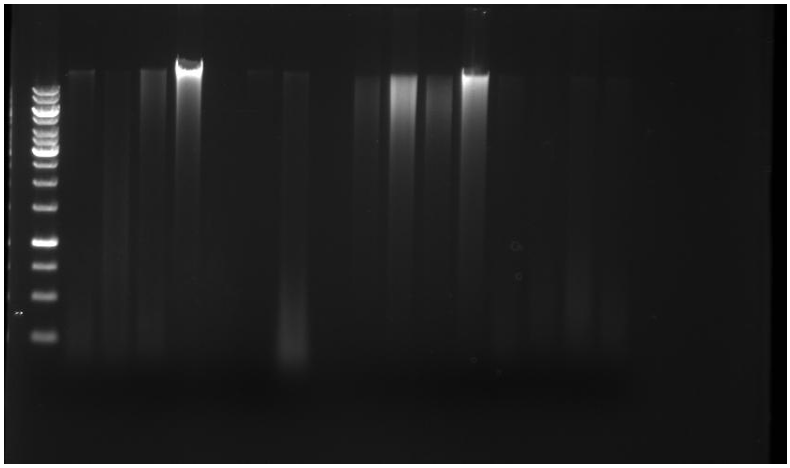

Figure 9. Agarose gel 1% used to observe the quality of DNA extracted by Invisorb ®Spin Plant Mini KIT from Amazon species

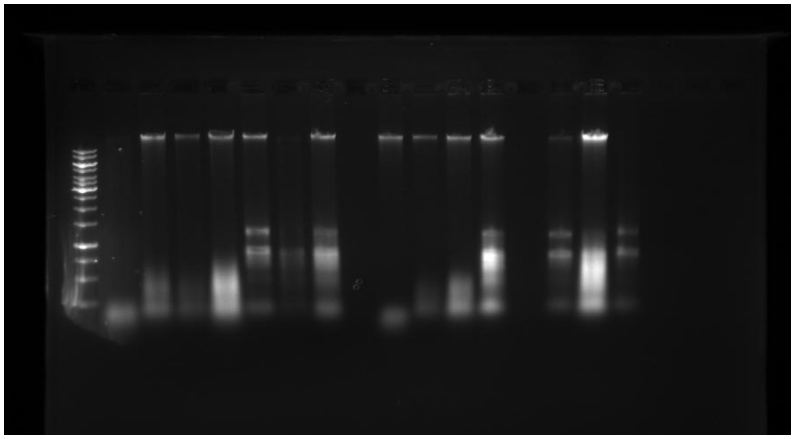

Figure 10. Agarose gel 1% used to observe the quality of DNA by CTAB methods extracted from Amazon species

5.11 Evaluation of costs

Using CTAB method is more economical and profitable, with 100 Euros we can extract DNA from 200 samples while with KIT just 40 samples, this shows that CTAB yields five times more comparing to KIT.

6. Discussion

The development of molecular techniques has led to the development of methodologies for DNA extraction simpler and more efficient as a first step and prerequisite for these techniques. The type or storage conditions of tissue used, variations in the efficiency of lysis and removing contaminants (proteins, polysaccharides and phenolic compounds, etc.). Influence the yield and purity of DNA affecting the results of molecular techniques such as PCR (Molinari, 2010).

Our study confirmed that that the CTAB method is more successful and more economical for DNA extraction. There was no problem with DNA isolation from tropical tree species. By help of the CTAB method we obtained higher DNA concentration and purity, than samples extracted by commercial KIT. The DNA quality was also better for all species, as bands on a gel were formed better that highlights the integrity of extracted DNA. In the present study the two extraction methods (Invisorb® Spin Plant Mini KIT and CTAB method) were variable that resulted statistically significant difference in the concentration and purity of DNA among all studied species. Different authors said that CTAB method showed better results for the evaluated variables (Doyle and Doyle, 1990; Kedwell and Osborn, 1992; Ferreira and Grattapaglia, 1995; Dilworth and Frey, 2000; Shepherd *et al*, 2002; Kang and Yan, 2004 and Narayanan *et al*, 2006). We found out significant differences in extracted DNA concentrations between *Copaifera paupera, Schizolobium parahyba* and *Swietenia microphylla* species as well. A significant difference existed in DNA purity between species *Amburana cearensis, Copaifera paupera*, *Inga edulis* and *Schizolobium parahyba*, belonging between Leguminosae. Doyle (1990) mentioned about difficulties with DNA isolations from plant material originated in Leguminosae family, because of its polyphenolic compounds, which makes problems during the isolation procedures. We observed that, DNA from all species mentioned in this study from the Leguminosae family were successfully isolated and did not influence in the obtained results.

Among the different extraction methods published so far, they have chosen a CTAB-based procedure because it is reliable (Roger *et al*., 1985 and Weising *et al*., 1991), time saving, reasonably safe and cheap. With this approach, DNA can be obtained from few milligram amounts of freeze-dried plant tissues (Roger *et al*., 1985) which result were similar compared with our results since with the same amount of money with CTAB method we get extraction five times more than KIT extraction.

We also found out that the DNA extraction with CTAB method is cheaper and the most suitable method especially with use in laboratories in developing tropical countries. The CTAB method is possible to consider as a time-saving method, because relatively continuous work during extraction and shorter time needed for incubation. The isolation process with the KIT needed more material for the isolation, the grinding was more difficult and the whole process was slower. One of the disadvantages of the CTAB method in comparing with the extraction using commercial KIT was, that the skills of the person working

in the laboratory, needs to be more advance. There is also the fact of biohazard, which was higher, when using CTAB, because of its chemical compounds used in the process.

The conservation of the leaf material using silica gel showed the best yield in terms of concentration and purity of DNA and therefore our hypothesis is accepted. For some species the material prepared with silica gel and also the fresh leaf material showed better results in terms of purity as it is published by Schierenbeck (1994).

7. Conclusion

The optimization of the DNA extraction method is very important step to introduce successfully the molecular techniques into the genetic and biodiversity studies of tropical plants. In this work we tried to found the best way how to do it more successfully in the conditions occurring in Peruvian Amazon. Based on our results we recommend the use of CTAB method for DNA extraction from tropical trees. The method is relatively easy and cheap, thus it can be used in developing countries and it also yields very good results concerning DNA quantity, purity and quality. Other important conclusion is, that the pre-preparation, drying and storing of the plant material in silicagel is simple and effective method, which does not decrease the quality of DNA. Our research is just the first basic study and following studies should focus on the evaluation of suitable molecular techniques, such as PCR methods that could be used to evaluate genetic diversity of Amazonian tree species. These studies could lead to better conservation of germaplasm of these species and development of strategies for their breeding and domestication.

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Appendix

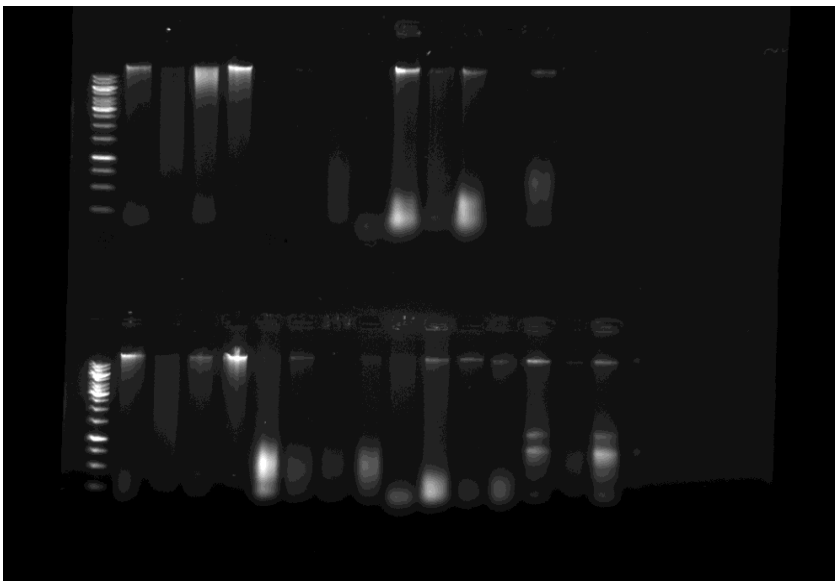

Figure 11. Agarose gel 1% used to observe the quality of DNA by Invisorb ®Spin Plant Mini KIT (up) CTAB methods (below) extracted from Amazon species. From silicate samples.

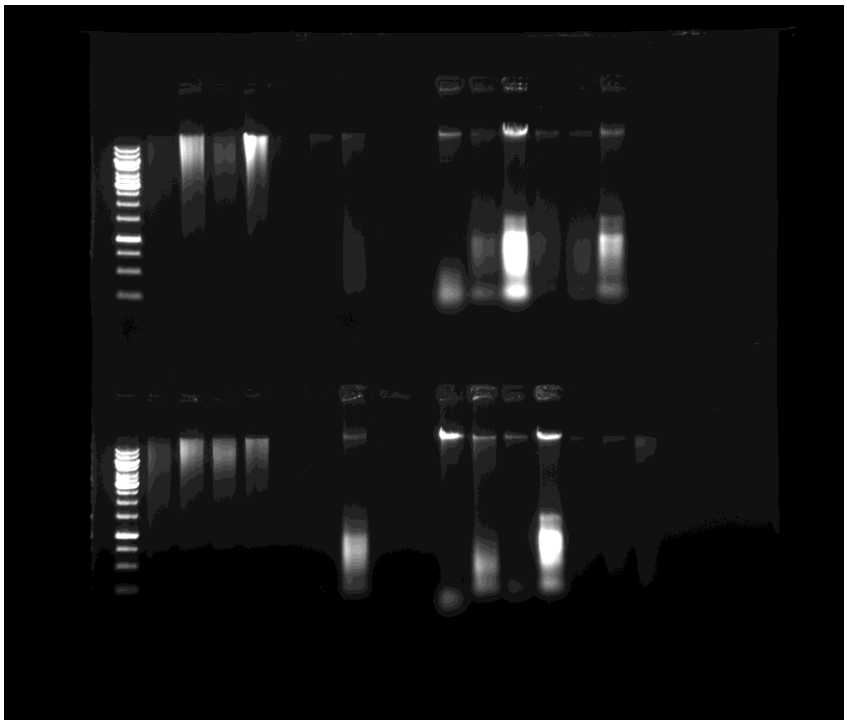

Figure 12. Agarose gel 1% used to observe the quality of DNA by Invisorb ®Spin Plant Mini KIT (right) CTAB methods (left) extracted from Amazon species from fresh samples.