Original Research Paper

MORPHOLOGICAL AND GENETIC DIVERSITY OF *Calycophyllum spruceanum* (BENTH) K. SCHUM (Rubiaceae) IN PERUVIAN AMAZON

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Abstract

Calycophyllum spruceanum is an important timber tree species in Peruvian Amazon Basin. Diversity was observed with reference to both morphological traits and genetic variance. Variance analysis showed significant differences in locality (site of collection) (p < 0.05). Provenance and progenies showed lower variation than locality. Genetic diversity was assessed using two Internal Transcribed Spacer (ITS) primers ITS1 and ITS4. Fragment sizes ranged between 600 and 700 bp. UPGMA analysis separated provenances heterogeneously. The resulting similarity matrix revealed values ranging between 0.672 and 0.977, with an average of 0.823. PCA analysis was unable to separate provenances. Our results suggest that morphological diversity is higher than the genetic one. The discovered genetic diversity under introduced analyses proved the outcrossing reproduction cycle and population genetics of *C. spruceanum*. Cleary distinguishable characteristics for each provenance were not found. Environment factor had a higher impact on phenotype on these studied provenances and localities. Our results are in line with statements of previous studies on *C. spruceanum*, suggesting a higher variation within provenances than among provenances. Acquired data will be used for Inter Simple Sequence Repeat (ISSR) assessment, giving more precise view on genetic diversity of *C. spruceanum* in Peruvian Amazon.

Keywords: Calycophyllum spruceanum; genetic variation; Internal Transcribed Spacer; morphological description, provenances.

INTRODUCTION

Calycophyllum spruceanum (Benth) K. Schum has origin in Amazon Basin and has widespread distribution from Bolivia, Peru and Colombia through to the lower Amazon in Brazil (Sears et al., 2003). It is also commonly known as Capirona, Pao mulato, Polo camaron, Brasil Zitroneholz, Citronnier bresilien. *C. spruceanum* is a multi-purpose canopy tree, belonging to the family Rubiaceae (Taylor, 2005). It is a rainforest hardwood tree which is logged in the Amazon and exported around the world for high density wood, durable lumber and building materials, but also as a medicinal plant. It has recently evoked interest in scientific community and formulators of natural body care products in South America for its beneficial effect to the skin (use in pharmaceutical industry) (Taylor, 2005).

To our knowledge, up to this day only one work aiming at DNA analysis was done. Russell et al. (1999) made an Amplified Fragment Length Polymorphism (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. Nevertheless, variation among populations was also significant. Sotelo-Montes et al. (2003 – 2008) studied morphological diversity on both natural and artificial stands. At first, they made the provenance/progeny test and discovered that density of wood had a higher heritability than growth traits (height, diameter at breast height) and these variables were positively correlated at both genetic and phenotypic level (Sotelo-Montes et al., 2006). The correlation between tree growth and wood properties varied among provenances, species and planting environments (Dawson et al., 2009).

The ITS region of 18S-28S nuclear ribosomal DNA (nrDNA) proved to be a useful source of characters for phylogenetic studies in many angiosperms families. The two spacers (ITS1, ITS2) of this region can be readily amplified by PCR and sequenced using universal primers (Baldwin, 1992; Baldwin et al., 1995). The aim of this study was to detect genetic variability in a model collection of selected samples of *C. spruceanum*. This variability was assessed on the basis of orientation on morphological description of selected features and the level of DNA polymorphism assessed by ITS non-specific primers. The basic purpose of this study was to

characterize the plant material of *C. spruceanum* from Pucallpa, Ucayali Department, Peruvian Amazon.

MATERIALS AND METHODS

Experimental plots

The research took place in an area near Pucallpa, Ucayali Department, Peruvian Amazon. The study site is here represented by hot humid tropical climate (lowland tropical rain forest) with a mean annual temperature of 25.7 °C (maximum 31 °C, minimum 19.5 °C), mean annual relative humidity reaching 80% and rainfall ranging from 1500 to 2100 mm/year (average 1546 mm/year) (MINAG, 2002; Vicha, 2008). Morphological features, fresh leafy material (2 repetitions from each individual) and fresh wood samples were obtained from total of 46 individuals of C. spruceanum coming from 8 provenances (Table 1) from experimental plots of World Agroforestry centre (ICRAF). Our design included collection of individuals from two experimental plots - 23 individuals from San Alejandro (SA), 23 from Curimana (CU), and in addition 9 individuals were chosen randomly from parcel of natural regeneration on road to Curimana. It served as control for main samples and as comparison factor for differences between natural and artificial stands. Individuals on experimental plots were grown into plantation 2×2 m. On these two experimental plots, individuals were gathered from all over the Ucayali Region. For every provenance, two digit codes were given according to its location of origin (provenance) and families within the provenances (Table 1).

Morphological features

Morphological features were measured using a defined descriptor. Height and diameter at breast height (dbh)

was measured using inclinometer and meter stick (with help of ICRAF) in the years 2009 and 2010. Additional characteristics such as colour of trunk, shape of trunk, smoothness of trunk, excurvation of trunk and shape of crown were measured. For these features, except for the colour of trunk, 4 code evaluation was used (0 = regular, 1 = slightly irregular, 2 = moderately irregular, 3 = irregular). We also measured swirl (stem appearance like *Carpinus betulus*) and presence of bark. For these features 2 code evaluation was used (0 = absence, 1 = presence). For bark colour evaluation RHS colour chart of fifth edition (Royal Horticulture Society, 2007) was used.

Plant tissue samples

Collected leafy material of each individual tree was immediately stored in silica gel (P-Lab, Czech Republic), in order to dry and preserve the samples. Samples were then brought to Czech University of Life Sciences Prague (CULS), for further DNA analysis using ITS PCR method.

DNA isolation and PCR analysis

DNA from leaves (80 mg of each sample) was isolated using Invisorb Spin Plant Minikit (Invitek, Germany) and stored in the dark under -20 °C. For PCR reaction two non-specific primers ITS 1 and ITS 4 (Sigma Aldrich, USA) were used. 25 μ l of isolated DNA mixture (12.5 μ l MasterMix, 11.5 μ l dH2O, 0.25 μ l ITS1, 0.25 μ l ITS4 +1 μ l tested DNA), was then submitted to PCR using Quanta Biotech Thermal Cycler (USA). Amplification was carried out with one initial cycle of denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 62.4 °C for 3 min and elongation for 72 °C for 1 min. The last cycle was followed by an additional extension at 72 °C for 10 min. PCR products were

Table 1: Provenances of C. spruceanum, codes and number of samples

Provenance	Code of provenance	Number of samples	Samples		
Nueva Requena-Rio (NR)	1	6	1-3, 1-8, 1-10, 1-11, 1-18, 1-34		
(CFB.Km.72) (QT)	3	1	3-1		
Puerto Inca (PI)	7	1	7-1		
Von Humboldt (VH)	8	4	8-2, 8-3, 8-4, 8-10		
Macuya (MA)	9	2	9-12, 9-39		
San Alejandro (SA)	10	7	10-4, 10-9, 10-14, 10-18, 10-22, 10-25, 10-26		
Road to Curimana (RC)	11	1	11-2		
Road to Tournavista (RT)	14	1	14-1		

Descriptors	Features	Rate Curimana (%)	Rate San Alejandro (%)	Total Rate (%)
Trunk colour	 1-very bright green, 2-bright green, 3-bright grey green, 4-green yellow, 5-green grey, 6-green brown, 7- green, 8-dark green, 9-bright grey, 10-grey green, 11-grey, 12-dark grey, 13-grey brown, 14-brown 	0:9:0:0:30:4:0:0:22:4:22:4: 4:0	$4:13:4:4:26:22:4:9:0:9:0:0\\0:4$): 2:11:2:2:28:13:2:4:11:7:11:2 :2:2
Trunk shape	0-regular, 1-slightly irregular, 2- moderately irregular, 3-irregular	52:0:26:22	52:4:44:0	52:2:35:11
Crown shape	0-regular, 1-slightly irregular, 2- moderately irregular, 3-irregular	26:26:35:13	17:35:39:9	22: 30 : 37 : 11
Trunk smoothness	0-smooth, 1-slightly rough, 2- moderately rough, 3-rough	22:57:22:0	13:74:13:0	17:66:17:0
Excurvation	0-straight, 1-slightly curved, 2- moderately curved, 3- curved	43:43:9:0	48:30:22:0	48:37:15:0
Swirl	0-absent, 1-presence	83:17	91:9	87:13
Bark presence	0-absent, 1-presence	87:13	78:22	83:17

separated on 1% (80 mg agarose, 80 ml 1 \times TBE and 1 µl SYBR green (Invitrogen,USA)) and 2.5% (250 mg agarose, $100.1 \times \text{TBE}$ and 2 µl SYBR green) agarose gels at 100V, 100mA for 50 min in electrophoresis (Power supply - GE EPS 301, USA; Main body - OmniBio, Czech Republic). For polymorphism detection DNA ladder was used into the first and last slot in agarose gels. DNA fragments were analyzed using UV illuminator ECX-26-MX (Schoeller, Germany) and photographed using CSLMicrodoc System (Cleaver Scientific Ltd., UK).

Statistical analysis

Statistical analysis on morphological features (height, dbh) was done using ANOVA and statistical significant differences were verified using Tukey HSD test in STATISTICA CZ 7.0 software (Statsoft Inc., USA). A data matrix of ITS profiles for fragments of similar molecular weight were scored as 1 for present or 0 when absent. Morphological and genetic features were analyzed using MVSP 3.1 software (Kovach Computing Services, Great Britain). UPGMA Cluster Analysis using Gower General Similarity Coefficient and PCA analysis was conducted.

RESULTS

With the RHS colour chart of total of 14 colour types were established. From the total rate of colour of trunk the most abundant colour was green-grey (138C) (28%), followed by green-brown (141B) (13%), bright grey (189D) (11%), bright green (143D) (11%), grey (188A) (11%) and grey-green (188C) (7%) (Table 2). On both experimental plots the most abundant colour was green-grey (SA 26%, CU 30%). However, green brown abundant in SA (22%) was nearly absent in CU (4%). On the other hand, bright grey was absent in SA (0%), but abundant on CU (22%). SA experimental plot showed higher diversity in colour types (4 colour types absent) and composing of rare colour types (bright green, brown N199C) (Table 2). CU was absent on 6 types of colours. The majority of individuals from both plots tended to have regular trunk

Locality Curimana	Mean ±SE	Range	CV (%)
Height (m)	16.73 ± 0.41	13.8-20.4	16
Dbh (m)	0.14 ± 0.01	0.05-0.19	21
Locality San Alejandro	Mean±SE	Range	CV (%)
Height (m)	19.10 ± 1.85	10.7-24.3	17
Dbh (m)	0.18 ± 0.01	0.09-0.24	21

Table 3: ANOVA of morphological features (locality).

Figure 1: Dendrogram of 55 individuals of C. spruceanum covering 8 provenances



shape but moderately irregular crown shape. Most individuals had slightly rough trunk and straight or slight excurvation. It was apparent, that individuals were rougher in SA but more inflexed in CU plot. Only few individuals showed stem swirl (13%), more in CU plot (17%) than SA (9%). Interesting is that individuals with swirl tended to have brighter colour (Table 2).

According to Tukey HSD test, differences according to cultivation locality showed the most statistical significance (p = 0.05) (Table 3). Progeny and provenance were also statistical significant, progeny only in dbh, provenance in both height and dbh. The year of measurement showed no statistical significance. Heights and dbh of trees were relatively greater in SA than in CU. For SA and CU mean height 19.10 m and 16.73 m with mean dbh 0.18 m and 0.14 m, respectively. CV was higher for dbh than height in both experimental plots. Other CVs were very similar. Values showed same CVs for middle zone (CU) and for higher zone (SA). These results show that environmental impact on morphological variation was relatively low (0-30%).

Fragment sizes from PCR ranged approximately between 600-700 bp. From a total of 101 fragments (55 from 1% gel and 46 from 2.5% gel) 34 (33.66%) showed polymorphic bands. Similarity matrix from UPGMA Cluster analysis revealed values between 0.672-0.977, while the average was 0.823. The generated dendrogram (Fig. 1) of 55 accessions showed 8 clusters. Distribution of samples was heterogeneous and no evidence was found of dependence on provenances. First cluster (cluster A) had two individuals, one from natural regeneration (cluster F) and one from SA provenance. This cluster differed greatly from other clusters. Most diverse provenance seemed to be SA group, which was interspersed in almost every cluster (except for cluster D and G). Cluster D was entirely formed by individuals from natural regeneration. NR provenance was distributed mainly in cluster H, while VH provenance was distributed mainly in cluster F. Also VH provenance seemed to be closely related to SA provenance. Other provenance distribution was not significant. Cluster H joined together individuals with best growth traits for selection factors in agroforestry



Figure 2: PCA of morphogenetic variance

practices, that is, height, dbh, straight trunk and ideal shape of trunk.

Another accession to diversity evaluation was the use of PCA. PCA was conducted to show whether it is possible to group the populations into different clusters. As it can be seen (Fig. 2), no clustering was possible. Eigenvalues indicated that the first two components accounted for 82.37% of variance.

DISCUSSION

Statistical analysis of growth traits (diameter and dbh) revealed that our results were very similar to those described by Sotelo-Montes et al. (2006). From all statistical criteria (progeny, provenance, locality, year of measurement) only differences by year were not confirmed as statistically significant. This is in line with findings with Sotelo-Montes et al. (2006), where progeny, provenance and locality were also statistically significant. Moreover, they found that lower zone had higher CV and therefore higher variability in growth than middle and higher zones. In our study, lower CVs for height and dbh were found across zones than those in the study of Sotelo-Montes et al. (2006). Moreover, CVs were the same in middle and upper zones. These findings may be affected by relatively low number of samples and absence of lower zone site in our study. Sotelo-Montes et al. (2006) suggest that this variability across zones could reflect phenotypic variation due drought-tolerance mechanism. Leaf abscission for example, would be more expected in lower and middle zones.

It was observed that in the upper zone leaf abscission rate was much lower than in middle or lower zone (Sotelo-Montes et al., 2006). Since leaf abscission reduces photosynthetic surface, variation in leaf abscission among neighbouring individuals would produce greater variations in growth rates (Dvorak et al., 1998). The results from cluster analysis show that distribution of individuals in dendrogram was due the site of collection and morphological traits. This is in line with our results showing that a statistically significant parameter was the site of collection and two morphological traits (diameter and dbh). Individuals from natural regeneration behaved unexpectedly and therefore their significance in this study was unreliable. It must be mentioned, that ITS primers are not primarily designed for provenance/progeny tests (Baldwin, 1992; Baldwin et al., 1995; Alvarez and Wendel, 2003) and therefore in this study the genetic diversity could be hidden. Nevertheless, a relatively low level of genetic diversity of C. spruceanum in natural stands was discovered by AFLP by Russel et al. (1999). It was also attempted to divide provenances with PCA. But with our measured morphological traits, it was impossible to separate the provenances. The morphogenetic distances between provenances from UPGMA analysis were fairly uniform. Similar results were reported by Masumbuko et al. (2003) in Tanzanian provenances of Arabica coffee (Coffea arabica), suggesting a limited variation. Masumbuko et al. (2003) suggested that uniform distances could be due to reproductive biology of particular species. C. spruceanum is outcrossing which would on the other hand explain a heterogeneous distribution of

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our provenances in the dendrogram (Russel et al., 1999). Provenance San Alejandro (SA) was the most diverse while Nueva Requena (NR) showed to have the best growth traits. The recommendations are to use these two provenances for agroforestry practices in the Peruvian Amazon. Also the San Alejandro experimental plot was more suitable than Curimana for production of *C. spruceanum*.

CONCLUSIONS

The discovered genetic diversity under introduced analyses proved the outcrossing reproduction cycle and related population genetics of *C. spruceanum*. Monitored provenances were behaving as if they were one entire population. Clearly distinguishable characteristics for each provenance were not found, therefore it seems that environment factor has a higher impact on phenotype on these studied provenances and localities. Also there is a high variation within provenances, phenotypic response across provenances is very similar, and consequently variability among provenances is low. Data from this study will be used for ISSR assessment, giving more precise view on genetic diversity of *C. spruceanum* in Peruvian Amazon.

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REFERENCES

- ALVAREZ I., WENDEL J.F. (2003): Ribosomal ITS sequences and plant phylogenetic inference. Molecular Phylogenetics and Evolution 29: 417-434
- BALDWIN B.G. (1992): Phylogenetic utility of the Internal Transcribed Spacers of nuclear ribosomal DNA in Plants: An example from the *Compositae*. Molecular Phylogenetics and Evolution 1: 3-16
- BALDWIN B.G., SANDERSON M.J, PORTER J.M., WOJCIECHOWSKI M.F., CAMBELL C.S., DONOGHUE M.J. (1995): The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperms

phylogeny. Annals of the Missouri Botanical Garden 82: 247-277

- DAWSON I.K., LENGKEEK A., WEBER J.C., JAMANDASS R. (2009): Managing genetic variation in tropical trees: Linking knowledge with action in agroforestry ecosystems for improved conservation and enhanced livelihoods. Biodiversity Conservation 18: 969-986
- DVORAK W.S., URUENA H., MORENO L.A., GOFORTH J. (1998): Provenance and family variation in *Sterculia apetala* in Colombia. Forest Ecology and Management 111: 127-135
- MASUMBUKO L.I., BRYNGELSSON T., MNENEY E.E., SALAMON B. (2003): Genetic diversity in Tanzanian Arabica coffee using random amplified polymorphic DNA (RAPD) markers. Hereditas 139: 56-63
- MINAG (MINISTERIO DE AGRICULTURA UCAYALI) (2002): Datos Climatologicos de la Región Ucayali. Dirección del Información Agraria, Pucallpa, Peru
- SEARS R. (2003): New forestry on the floodplain: The ecology and management of *Calycophyllum spruceanum (Rubiaceae)* on the Amazon landscape. Ph.D. Thesis, Columbia University, pp. 52-62
- SOTELO-MONTES C., VIDDAURRE H., WEBER J.C. (2003): Variation in stem-growth and branch-wood traits among provenances of *Calycophyllum spruceanum* Benth. from the Peruvian Amazon. New Forest 26: 1-16.
- SOTELO-MONTES C., HERNANDEZ R.E., BEAULIEU J., WEBER J.C. (2006): Genetic variation and correlations between growth and wood density of *Calycophyllum spruceanum* at an early age in the Peruvian Amazon. Silvae Genetica 55: 217-228.
- SOTELO-MONTES C., HERNANDEZ R.E., BEAULIEU J. (2007a): Radial variation in wood density and correlations with growth of *Calycophyllum spruceanum* at an early age in the Peruvian Amazon. Wood and Fiber Science 39: 377-387.
- SOTELO-MONTES C., BEAULIEU J., HERNANDEZ R.E. (2007b): Genetic variation in wood shrinkage and its correlations with tree growth and wood density of *Calycophyllum spruceanum* at an early age in the Peruvian Amazon. Canadian Journal of Forest Resources 37: 966-976.
- SOTELO-MONTES C., HERNANDEZ R.E., BEAULIEU J., WEBER J.C. (2008): Genetic variation in wood color and its correlations with tree growth and wood density of *Calycophyllum spruceanum* at an early age in the Peruvian Amazon. New Forests 35: 57-73.
- TAYLOR L. (2005): The healing power of rainforest herbs: A guide to understanding and using herbal medicinals, Square One Publishers, New York, pp. 357-359.
- THE ROYAL HORTICULTURE SOCIETY. (2007): RHS color chart: 5th edition, Royal Horticulture Society, London

- VICHA J. (2008): Local climate changes around the city of Pucallpa (Peruvian Amazon), Diploma thesis, Czech University of Life Sciences Prague, Prague
- WEBER J.C., SOTELO-MONTES C. (2005): Variation and correlations among stem growth and wood traits of

Calycophyllum spruceanum Benth. from the Peruvian Amazon. Silvae Genetica 54: 31-41.

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