Purification and primary structure of a lectin from *Buddleja coriacea* seeds

Purificación y estructura primaria de una lectina aislada de semillas de Buddleja coriácea

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ABSTRACT

A lectin from *Buddleja coriacea* R. ("colle negro", Buddlejaceae) seeds was purified by molecular exclusion chromatography and reverse phase high performance liquid chromatography (HPLC). Two dimensional SDS-PAGE analyses demonstrated that the purified lectin was homogeneous since it appeared as a single protein spot corresponding to ~12 kDa with an isoelectric point of 5.3. Its molecular weight was confirmed by mass spectrometry (MALDI-TOF) to be 12,630.1405 Da. Amino acid analysis revealed that the lectin from *Buddleja coriacea* R. (BCL) is acidic and highly hydrophobic (21% acid and 41% hydrophobic residues). The complete amino acid sequence showed that BCL contains 119 residues. Comparative studies with other lectins show that it has high similarity to lectins from *Cratylia mollis* (Leguminosae, 96,60% homology) and from *Cratylia argentea* (89,90% homology). According to a phylogenetic tree, BCL showed a sequence evolution of ~ 2000 nucleotides with the lectin extracted from *Cratylia mollis* seeds. BCL agglutinated "A", "B" and "AB" Rh (+) human blood groups with a minimum amount of hemagglutinating of 1,3 µg/mL and this activity was inhibited by D-lactose and D-mannose (0,048 and 0,097 mM, respectively) and by the chelating agents EDTA (25 mM) and EGTA (25 mM).

Key words: *Buddleja coriacea*, lectin, BCL, amino acid sequence, phylogenetic tree.

RESUMEN

Se purificó una lectina aislada de semillas de *Buddleja coriacea* R. ("colle negro", Buddlejaceae) mediante cromatografía de exclusión molecular y cromatografía líquida de alta eficiencia de fase reversa (HPLC). El análisis en SDS-PAGE de dos dimensiones demostró que la lectina purificada es homogénea porque aparece como un único spot proteico correspondiendo a ~12 kDa con un punto isoeléctrico de 5.3. Mediante espectrometría de masa (MALDI-TOF) se confirmó el peso molecular de la lectina mostrando una masa de 12,630.1405 Da. El análisis de aminoácidos reveló que la lectina de *Buddleja coriacea* R. (BCL) es de carácter ácido y altamente hidrofóbico (21% de residuos ácidos y 41% de hidrofóbicos). La secuencia completa de aminoácidos señaló que BCL contiene 119 residuos. El estudio comparativo con otras lectinas mostró que BCL tiene alta similaridad con lectinas de *Cratylia mollis* (Leguminosae, 96,60% de homología) y *Cratylia argentea* (89,90% de homología). Según el árbol filogenético, BCL presentó una secuencia evolutiva de ~2000 nucleótidos con respecto a la lectina extraída de semillas de *Cratylia mollis*. BCL exhibió aglutinación de los grupos sanguíneos humanos "A", "B" y "AB" Rh (+) con una concentración mínima de hemaglutinación de 1,3 µg/mL; la actividad hemaglutinante fue inhibida por la D-lactosa y D-manosa (0,048 y 0,097 mM, respectivamente) y por los agentes quelantes EDTA (25 mM) y EGTA (25 mM).

Palabras clave: *Buddleja coriacea*, lectina, BCL, secuencia de aminoácidos, árbol filogenético.

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INTRODUCTION

Plant lectins are a heterogeneous group of proteins classified on the basis of their ability to bind reversibly to well defined simple sugars and/or complex carbohydrates[14]. Lectins are found in many different species and in many different organs and tissues of plants, and perform several fundamental biological roles, including defense against insects, fungi, bacterial, etc. [12, 1, 6, 19]. Lectins may also have anti tumoral activity[3].

Buddleja coriacea (Buddlejaceae), known as "colle negro", grows throughout the Andean region of Peru, is a shrub resistant to attack of insects, livestock and wild animals[9]. Colle negro is a useful shrub, specially in the puna region, because it provides protection and shade to other species. In folklore medicine, its leaves are boiled together with leaves of "queñua" and are used for the treatment of rheumatism[4].

The present paper reports the purification and primary structure of a lectin from seeds of *B. coriacea* belonging to the family Buddlejaceae. In this respect, no data is currently available for other Buddlejaceae lectins.

MATERIALS AND METHODS

Plant material

Seeds of *B. coriacea* were obtained from a tree nursery at Potojani (Puno, Peru) belonging to the Programa Nacional de Manejo de Cuencas Hidrográficas y Conservación de Suelos (PRONAMACHCS-Perú).

Lectin purification

For purification of the lectin, seeds of B. coriacea were ground to a fine powder and extracted with 10% NaCl for 1 h with continuous stirring at room temperature. The slurry was then centrifuged at 3000 g for 20 min at 4 ºC and the clear supernatant was applied to a Sephadex G-75 column equilibrated with 580 ml buffer Tris-HCl 50 mM/150 mM NaCl, pH: 8.0. Fractions with hemagglutinant activity were pooled, freeze-dried and stored at 4ºC until use[16].

Separation of peaks with hemagglutinant activity was carried out using a HPLC-PDA 991 (Waters) system employing two pumps (model 510/B, Waters), an automatic injector for samples (model U6K) and a narrow bore µ-Bondapack C18 semipreparative column (0,78 x 30 cm). Solvent A was 0.1 % trifluoroacetic acid (TFA) (v/v) and solvent B was 66% acetonitrile (v/v). Fractions were monitored by absorbance at 280 nm[8].

Protein determination

Protein content was determined by the Biuret method [18]. To each 0.1 mL of sample was added 1 mL of 0.5 M NaOH and 4,0 mL of Biuret reagent. The samples were incubated at 37 ºC for 30 min and protein quantified by spectrophotometry at 280 nm.

Molecular mass determination

The samples were subjected to isoelectrofocusing (IEF) solubilized with 8 M urea, 4% CHAPS, 70 mM DTT, ampholine linear pH gradient 4-7 1.5% and 0.001% bromophenol blue. At this stage, 64 µg of protein sample were applied onto polyacrylamide dried strips, pH gradient 4-7 (immobilized pH gradient, IPG). The first dimension of two dimensional gel electrophoresis (2-DE) was performed on an Amersham Biosciences Electrophoresis System accumulating 96 kVh in the electrophoresis condition. Following isoelectric focusing, materials in the IPG strips were exposed for 8 min to an equilibration solution containing SDS/DTT and for 12 min with another solution containing SDS/iodoacetamide. The strips were covered with 0.5% agarose heated at 70 ºC and run in the second dimension using a SE-600 electrophoresis apparatus and 12.5% polyacrylamide gels. After staining with Coomassie Blue the gels were scanned and analyzed by Image Master 2D v3.1 Elite software (GE-Amersham Biosciences).

Mass Spectrometry (MS)

The molecular mass of the lectin from *B. coriacea* were determined by matrix-assisted laser desorption/ionization mass spectrometry using a Voyager DE PRO MALDI-TOF apparatus (Applied Biosystems, CA, USA). The lectin was purified and eluted with a matrix solution of 2 µL 2,5-dihydroxybenzoic acid (DHB) (Sigma), 60% acetonitrile and 0.1% (v/v) TFA [13]. The mass of the protein was analyzed in linear analysis mode using an acceleration voltage of 25 kV, with the laser in fixed 2890 µJ/cm^2 and a delay 300 ns.

Amino acid analysis

Amino acid analysis was performed on a Pico-Tag amino acid analyzer (Waters Systems) [10]. The purified protein (30 µg) was hydrolyzed at 105 ºC for 24 h in 6 M HCl containing 1% phenol (w/v). The hydrolyzates were reacted with 20 µL of derivatization solution (methanol: triethylamine: water: phenylisothiocyanate (PITC), 7:1:1:1, v/v) for 1 h at room temperature. Phenylthiohydantoin (PTH) amino acids were then identified and quantified by HPLC by the comparison of their retention times and peak areas with those of a standard amino acid mixture.

Primary structure

Trypsin was incubated with the lectin at 37ºC for 8h[5]. The SV8 protease of Staphylococcus aureus was incubated at 37ºC for 16h [11]. The digested products of these treatments were fractionated by reverse phase HPLC using a Waters PDA 991 system and a C18 µ-Bondapack column. The elution of peptide peaks was made using a linear gradient consisting of 0-100% of acetonitrile in 0.1 % trifluoroacetic acid (v/v). Sequencing of the lectin was conducted using a Procise automatic sequencer. The phenylthiohydantoin (PTH) amino acids were identified by comparing their retention times to the 20 PTH amino acid standards. The primary structure of the lectin was mainly built based on the purified peptides from the protein digested, trypsin and protease SV8.

Sequence alignment and phylogenetic tree

Alignment of the primary structure of BCL described here with others lectins was carried out using the DNASTAR software through the information of the amino acids sequence.

Hemagglutinating activity

For determination of the hemagglutinating activity, a 3% erythrocyte suspension (v/v) was used in microtiter plates (96 wells, Sigma, USA). Each microtiter well contained 50 µL of a suspension of erythrocytes and 50 µL of lectin solutions. Hemagglutinating, visible to the naked eye, was monitored after plates had been left for 2 h at room temperature. The minimum amount of lectin required for 100% agglutination was one hemagglutination unit (HAU). The specific activity of the lectin was calculated as HAU µg^{-1} protein[2].

Determination of sugar specificity

All the samples used for HA were also examined in a hemagglutinating inhibition assay. This included 2 h preincubation with different concentrations of 10 sugars (D-fructuose, D-glucose, D-galactose, D-lactose, D-maltose, D-mannose, N-acetylgalactosamine, N-acetyl glucosamine, sucrose and raffinose) (Sigma, USA) followed by a determination of HA titer. The microtiter plates were incubated for 2 h at room temperature. Inhibition by different sugars was indicated by the failure of the lectin to agglutinate the erythrocytes[2].

Effect of chelating agents

The purified lectin from B. coriacea seeds (BCL) was assayed for hemagglutinating in the presence of ethylenediamine tetraacetic acid (EDTA) and ethyleneglycol bistetraacetic (EGTA) (Sigma, USA), for which the BCL in TRIS buffered saline (TBS) was dialyzed extensively against various concentrations of EDTA and EGTA (from 50 to 0,10 mM). The microtiter plates were incubated for 2 h at room

temperature. After incubation We determined the minimum amount of chelating agent required for inhibition of hemagglutinating.

RESULTS AND DISCUSSION

Purified lectin was separated by Sephadex G-75 molecular exclusion chromatography and reverse phase high performance liquid chromatography (RP-HPLC). **Fig. 1** shows the elution profile of a lectin from seeds of *B. coriacea* on Sephadex G-75. Ten peaks were detected and the eluted fractions were monitored for hemagglutinating activity. Only peak nine showed hemagglutinating activity (**Fig. 1**). The fractions of peak 9 that showed hemagglutinating activity were pooled, dialyzed, lyophilized and applied to a reverse phase HPLC µ-Bondapack C18 column. Two peaks were obtained (peaks 9.1 and 9.2) and only peak 9.1 (elution time of 28 min) had hemagglutinating activity (**Fig. 2**).

The purified lectin (BCL) showed only one spot (**Fig. 3**) on 2D electrophoresis. This analysis demostrated that the mass of the purified lectin was homogeneous since it presented as a single protein spot corresponding to ~ 12 kDa with an isoelectric point of 5.3. The mass measured by MALDI-TOF analysis confirmed the homogeneity of the purified of lectin. A molecular mass of 12,630.1405 Da was determined by MALDI-TOF mass spectrometry (**Fig. 4**).

The amino acid composition showed that peak 9.1 (or BCL) had 18 Asp/Asn, 7 Glu/Gln, 18 Ser, 9 Gly, 3 His, 4 Arg, 7 Thr, 11 Ala, 6 Pro, 2 Tyr, 5 Val, 8 Ile, 9 Leu, 9 Phe and 3 Lys. No Cys is present in BCL.

The primary sequence of BCL was determined by treating it with trypsin and protease SV8 followed by sequencing of the resulting peptides. RP-HPLC of the peptide mixture obtained by digestion of BCL revealed the presence of 4 peptides (Trp-02, Trp-05, Trp-06 and Trp-07) and the digestion with protease SV8 resulted in 3 peptides (SV8-02, SV8-03 and SV8-07). The primary structure of the lectin was determined by comparing the sequences of the overlapping peptides purified from the above digests.

The B. coriacea lectin is built by 119 amino acid residues and showed high sequence homology with other lectins of the family Leguminosae **(Fig. 5)**. BCL showed a 96.6% of homology with a lectin isolated from seeds of Cratylia mollis (Leguminosae) and 89.9% with a lectin isolated from Cratylia argentea seeds **(Fig. 5)**. The lectin of C. mollis (Cramoll) [7] is taxonomically related with concanavalin A (Con A). Cramoll has 236 amino acid residues and is topologically similar to Con A. According to the phylogenetic tree obtained of the conversion of the amino acids sequence to nucleotides, BCL showed a sequence evolution of ca. 2000 nucleotides with the lectin extracted from Cratylia mollis seeds **(Fig. 6)**. This indicates a possible genetic divergence of BCL from the Leguminosae family in the past.

The BCL agglutinated trypsinized erythrocytes of human A, B, O blood groups. The minimum amount of hemagglutinating (CMH) of the A, AB and B blood types was 1.3 µg/mL while the CMH for O-type blood group was 5.2 µg/mL **(Table 1)**. The hemagglutinating activity of the lectin was inhibited by D-lactose and D-mannose **(Table 2)**, EDTA and EGTA (25 mM each), but the addition of Ca^{2+} recovered the hemagglutinating activity of the lectin. It has been described that metal ions are also essential for the activity of some lectins[17]. Other lactose-specific lectin is from Erythrina corallodendron (ECorL) [15]. Cramoll is specific to mannose/glucose.

In this study, a lectin has been purified and partially characterized from seeds of the Andean plant Buddleja coriacea. This lectin show high similarity with other seed lectins, suggesting that its biological properties are also similar. Cloning and further studies of this lectin will contribute

to a better description of its biological properties and eventual applications.

ACKNOWLEDGEMENTS

We thank Dr. Jose A. Casaretto (Universidad de Talca, Chile) for the revision of the manuscript.

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Table 1. Minimum amount of hemagglutinating activity of the *B. coriacea* lectin (CMH) for "A", "B", "AB" and "O" human blood groups.

Blood groups	Hemaglutination Titer (μg/mL)
A	1.3
B	1.3
AB	1.3
O	5.2

Carbohidrates	CMIc (mM)
D-Fructose	ND*
D-Galactose	ND
D-Glucose	ND
D-Lactose	0,048
D-Maltose	ND
D-Mannose	0,097
N-acetylgalactosamine	ND
N-acetylglucosamine	ND
Sucrose	ND
Raffinose	ND

Table 2. Inhibition of the hemagglutinating activity of the *B. coriacea* lectin by D-lactose and D-mannose. ND, not determined.

Fig. 1. Fractionation of the lectin from *B. coriacea* seeds by molecular exclusion chromatography on a Sephadex G-75 column at 4ºC. Fraction 9* was collected and tested for lectin activity.

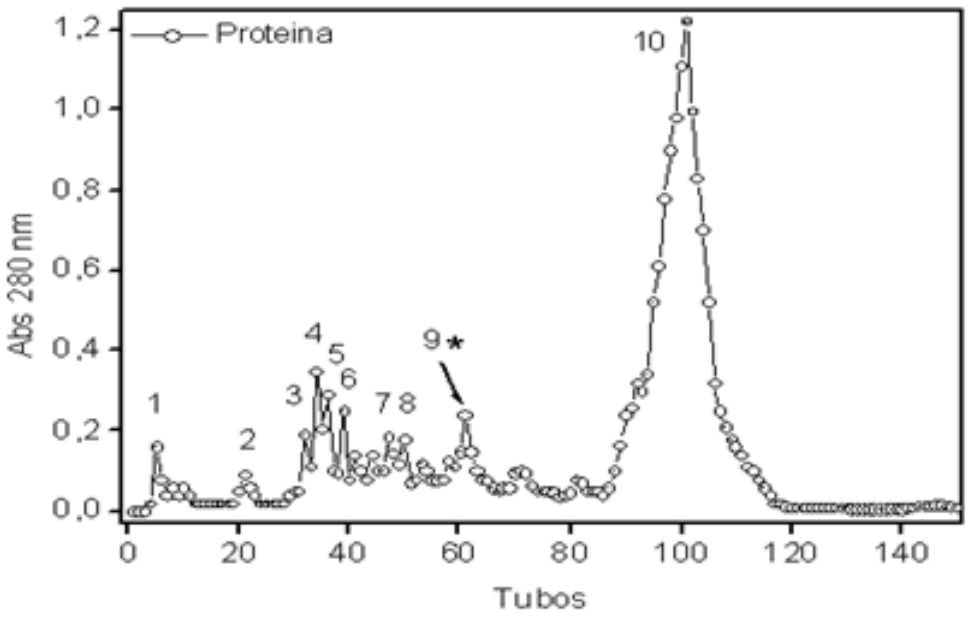


Fig. 2. Reverse phase HPLC of peak 9 on a preparative μ-Bondapack C18 column (0.78 x 30 cm). The proteins were eluted using a linear gradient of acetonitrile (buffer B). Fractions 9.1 and 9.2 was collected and tested for lectin activity. Only fraction 9.1* presented lectin activity.

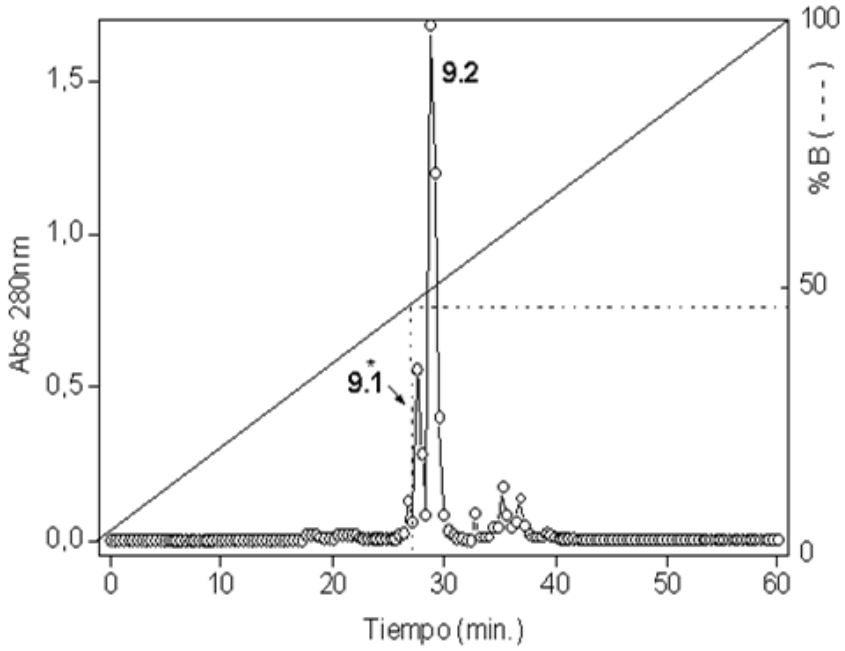


Fig. 3. 2D gel electrophoresis of the lectin purified from seeds of *B. coriacea*. The analysis demonstrated a single protein spot (arrow) corresponding to ~12 kDa with an isoelectric point of 5.3.

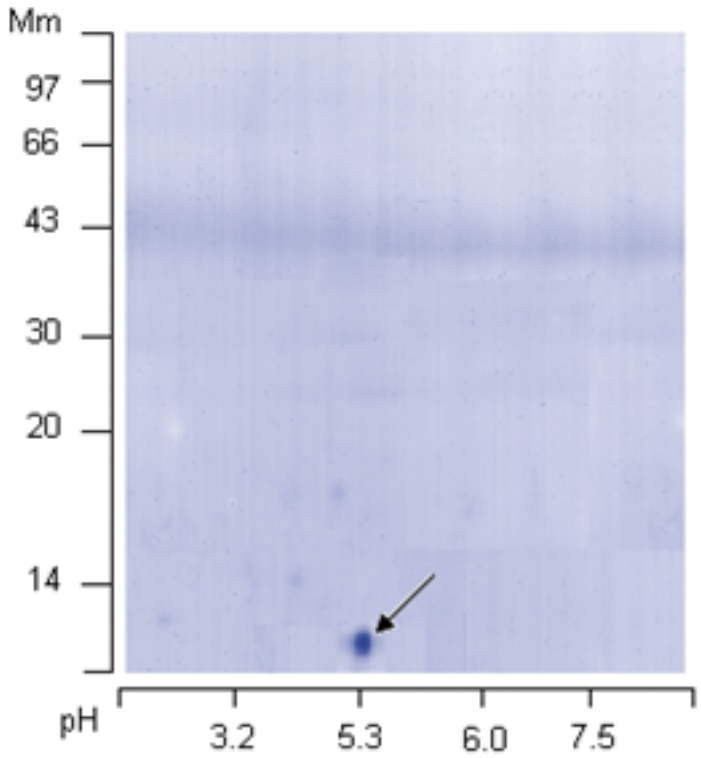


Fig. 4. Molecular mass of fraction 9.1 as determined by MALDI-TOF mass spectrometry.

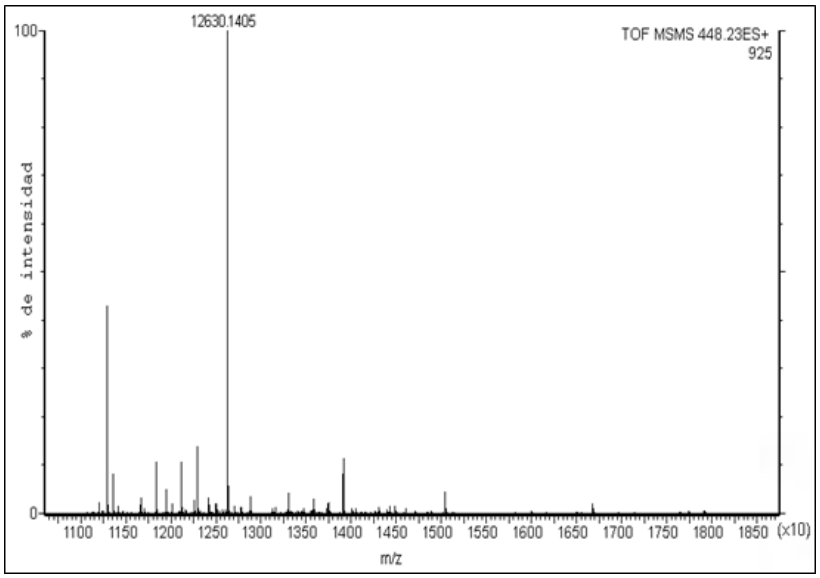
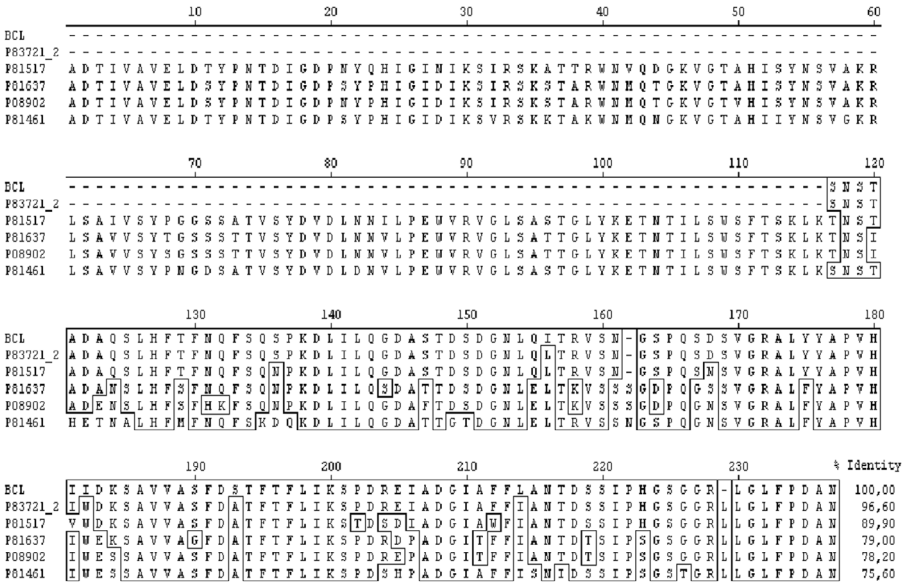


Fig. 5. Amino acid sequence alignment of *B. coriacea* lectin with lectin sequences obtained



using BLAST from GenBank database (PubMed). P83721_2, lectin isolated from *Cratylia mollis* seeds; P81517, lectin from *Cratylia argentea* seeds; P81637, lectin from *Dioclea guianensis* seeds; P08902, lectin from *Dioclea grandiflora* seeds; P81461, lectin from *Canavalia virosa* seeds. Gaps (-) have been inserted to maximize similarities.

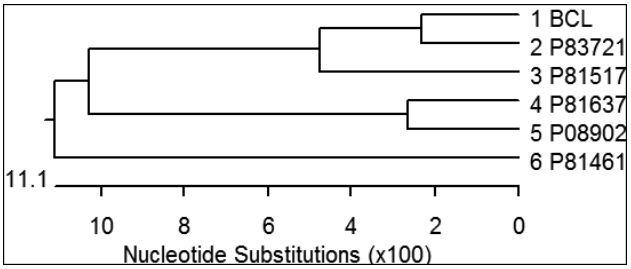


Fig. 6. Phylogenetic tree of *B. coriacea* lectin showing a evolutionary similarity of ca. 2000 nucleotides with a lectin extracted from *Cratylia mollis* seeds (P83721_2).