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Use DNA Barcoding of *rbcL* Gene and Spectral Characters in a Taxonomy of some *Bougainvillea* Comm. ex Juss. Species Cultivated in Northern Iraq

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Abstract

The study aimed to barcode *rbcL* gene and to use spectral characteristics of leaf chlorophyll for the taxonomy of four species of genus *Bougainvillea* Comm. ex Juss. cultivated in Northern Iraq. The DNA of all species was extracted and *rbcL* gene was amplified, sequenced, and homology examined. The spectral characteristics were measured with a UV spectrophotometer at wavelengths between 400-800 nm. The amplification result showed that all species possessed the *rbcL* gene. The homology of the *rbcL* gene of sample 01 (OQ851473) at the size of 584 bp with *B. spectabilis* (100%). Sample 02 (OQ843469) was a new record of *B. x buttiana* that was recorded for the first time in GenBank. Whereas, sample 03 (OQ851470) with a sequence length of 552 bp matched with *B. glabra* (99.82% - 100%), and sample 04 (OQ851471) with a sequence length of 1229 bp matched with *B. peruviana* (99.59%-100%). The results of spectral characteristics of chlorophyll showed a clear variation between the species. The results of the current study can provide important information in making taxonomic decisions at the species level.

Keywords: DNA barcoding, *rbcL*, Spectral, Identification, Species, *Bougainvillea*, Iraq.

أستخدام ترميز DNA الشريطي لجين *rbcL* والصفات الطيفية في تصنيف بعض أنواع *Bougainvillea* Comm. ex Juss. المزروعة شمالي العراق

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الخلاصة

هدفت الدراسة إلى ترميز جين *rbcL* واستخدام الخصائص الطيفية لكلوروفيل الأوراق في تصنيف أربعة أنواع من جنس *Bougainvillea* Comm. ex Juss المزروعة شمالي العراق. تم استخلاص الحمض النووي لجميع الأنواع و تضخيم جين *rbcL* وتسلسله وفحص التماثل. تم قياس الخصائص الطيفية باستخدام مقياس الطيف الضوئي للأشعة فوق البنفسجية عند الأطوال الموجية بين 400-800 نانومتر. أظهرت نتيجة التضخيم أن جميع الأنواع تمتلك جين *rbcL*. تماثل جين *rbcL* للعينة 01 (OQ851473) بحجم 584 bp مع *B. spectabilis* (100%)، كانت العينة 02 (OQ843469) بمثابة تسجيل جديد لـ *B. x Buttiana* تم تسجيله لأول مرة في Gen Bank، بينما العينة 03 (OQ851470) بطول التسلسل 552 bp متطابقة مع *B. glabra* (99.82%-100%) والعينة 04 (OQ851471) التي يبلغ طول

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التسلسل bp 1229 متطابقة مع *B. peruviana* (99.59%-100%) أظهرت نتائج الخصائص الطيفية للكوروفيل تبايناً واضحاً بين الأنواع. يمكن أن توفر نتائج الدراسة الحالية معلومات مهمة في اتخاذ القرارات التصنيفية على مستوى النوع

1. Introduction

Bougainvillea is a very attractive shrub of ornamental plants that has a high value in landscape preparation, as its bracts are distinguished by wonderful colors [1, 2]. The bract's colour is due to the accumulation of betalain pigments [3].

The genus *Bougainvillea* Comm. ex Juss. belongs to Nyctaginaceae (Four- o'clock) family [4], and includes 18 species, including *B. glabra*, *B. buttiana* *B. spectabilis*, and *B. peruviana* species which are used for gardening purposes [5].

The species of the genus *Bougainvillea*, native to tropical and subtropical South America: only 1 exotic species (or possibly 2-3) is cultivated in Iraq, as *B. glabra* Choisy is sometimes cultivated in gardens on the alluvial plain, in the central and southern sectors of the desert regions of Iraq, while Lesser *Bougainvillea* (Am.), which is called GHANNAMIYA (for JAHANNAMIYA Ir. Baghdad, this species is less demanding than others of the genus and the easiest to grow. Two other species of *Bougainvillea* are widely cultivated in the tropics and subtropics, *B. buttiana* (*B. buttiana* Holttum and Standl) and *B. spectabilis* Willd. Researchers list both, and although we have seen no specimens of either from Iraq, they may well be cultivated in our country [6]. The species *B. glabra* is characterized by a weakly spiny sub scandent shrub, puberulous or glabrous in the younger parts; spines long, leaves petiolate, elliptic or elliptic lanceolate, broad, acuminate at the apex, puberulous or glabrous. Floral bracts ovate, broad, vividly, and variously coloured, usually magenta, purple, or white. Calyx-tube ± puberulous outside, pink; limb pale yellowish inside; and the species *B. spectabilis* is sometimes called Brazil *Bougainvillea* (Am.), is a more vigorous plant than *B. glabra* and requires more careful cultivation. It is distinguished by its stronger spines, pubescent stems and leaves, and densely pubescent calyx [6]. This species is noted for its hairy leaves and stems, the leaves are large and ovate, with rippling along the edges and hairs on the underside. The bracts are red, dark pink, or purple, while the small flowers are cream colored. Its thorns are large and may be curved; the species *B. peruviana*: is green bark, long, thin leaves that are strongly ovate and glabrous, the rounded bracts are magenta to pink and may have some distortion or wrinkling, and the flowers are yellow, the thorns are short and straight [7], *B. peruviana* is characterized with tricolor (white, magenta or white-magenta) inflorescence in the same plant [8]. Species *B. x buttiana* : leaves that are large and ovate or heart-shaped with slight hairiness on both the upper and lower sides. The bracts are usually rounded, red, dark pink [7], or yellow orang [9], and the thorns are straight and short. The small flowers are cream-colored with pink tones, although the floral tube may be the same color as the bract [7]; all the cultivars of the species *B. x buttiana* are characterized by rounded bracts (large or medium) with an apiculate apex [10]

DNA barcoding is a molecular tool for taxonomy and accurate identification at the species level [11-13]. Different research used ribulose 1,5- biphosphate carboxylase oxygenase *rbcL* gene sequence as a barcode for identifying species [14-16] or used to detecting phylogenetic variations [17], and discriminating among angiosperms [18], original region for species differentiation [19], as well as discovering diverse plant specimens [20].

Several studies at the molecular level have shown the great importance of the nucleotide sequence of the *rbcL* gene in accurate diagnosis and phylogenetic tree analysis between some different species [21-29].

The absorption spectral properties are important for the regulation of chloroplast energy and energy processes on a scale absorbed by accessory pigments [30].

The chlorophyll pigments are light-absorbing compounds found in the thylakoid membranes of chloroplasts [31], which absorb photon energy to carry out photosynthesis reactions of plants [32, 33], in which light energy is converted into chemical energy in the form of ATP and NADPH [34].

The values of λ_{max} , which represents the highest absorption, are among the physical constants that support taxonomic evidence in the classification of plants, and each value represents a special identity for each species that distinguishes it from other plant species and links the spectral study with the science of plant taxonomy will open a new door through which to find important new differences in isolating species of genus [35].

A study by Al-Badrany and Al- Mathidy [36] shed light on the use of the absorption spectrum of leaf chlorophyll and fruit pigments in the taxonomy of 6 cultivars of the species *Ficus carica* L. based on λ_{max} values, and the results showed that the spectral study has taxonomic importance in diagnosing, separating and isolating the studied cultivars and supporting other taxonomic evidence.

The current study aimed to use the DNA barcoding of the chloroplast gene (*rbcL*) by using PCR and spectral characteristics of leaf chlorophyll using UV spectrophotometer in the taxonomy of some species of the genus *Bougainvillea* Comm. ex Juss. cultivated in northern Iraq.

2. Materials and Methods

2.1 Study of the DNA barcoding of the *rbcL* gene

2.1.1 sampling species of *Bougainvillea*

Plant samples were collected from several regions of northern Iraq, including Nineveh and Duhok horticulture stations, as well as the nurseries and fields belonging to them.

2.1.2 Polymerase chain reaction (PCR)

Extraction of DNA

Following the manufacturer's instructions (Favorgen, Korea), *Bougainvillea* species (The FavorPrep Plant Genomic DNA Extraction kit was used to extract DNA).

Purity of genomic DNA

Using a nanophotometer (Implen / P360, Germany) at wavelengths 260/280 nm. Samples were considered to be of high purity if their reading was equal between 1.65 and 1.8, while samples that gave a reading higher than 1.8 or lower than 1.65 were discarded due to contamination with RNA or protein, respectively [37].

Primers and amplification

Specific primers (Macrogen Co., Korea) for amplification of *rbcL* gene were used (Table 1).

Table 1: Primer sequences used to amplify *rbcL* gene.

Primer	5' – 3'	Product	Ref.
<i>rbcL</i> -F	TGTCACCAAAAACAGAGACT	1350 bp	[38]
<i>rbcL</i> -R	TTCCATACTTCACAAGCAGC		

The PCR reaction mixture (20 μ l) was made with 10 μ l of 2X Taq Master Mix (AddBio, Korea), 1 μ l of each primer (10 pmol), 6 μ l of grade water, and 2 μ l (30 ng/ μ l) of DNA. A Bio-Rad thermocycler (Bio-Rad, USA) was used for the amplification, and the following settings were used: 95°C for 10 minutes was the first cycle, and then 35 cycles of 95°C for 45

seconds, 63°C for 45 seconds, and 72°C for 1 minute. For the last extension, a single cycle lasting five minutes at 72°C was set. The processes were finally cooled at 4°C.

Agarose gel electrophoresis

The amplified products were separated using 1.5% agarose gel electrophoresis in 1x TBE buffer (AddBio, Korea). 5 µl of each PCR product was placed into the agarose gel well. A power supply of 300mA and 80V was used for one hour. Using GeneDirex H3, Korea, a 100 bp DNA ladder in 5 µl was utilized as the standard molecular weight marker.

Gel documentation

Using a gel documentation system (Gel Doc EZ Image, Bio-Rad, USA), the gel was analyzed under UV light in order to document and identify the predicted bands.

2.1.3 Sequencing

Sequencing was performed using the Sanger sequencing method by Macrogen Co. (Korea). Using a basic local alignment tool (BLAST) at (blast.ncbi.nlm.nih.gov), the sequence homology of the *Bougainvillea* species was examined. Sequence alignment: In MEGA11 Clustal W algorithm was used to align nucleotide sequences. The phylogenetic tree was built using the Maximum Likelihood approach based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. The input data consisted of a partial *rbcL* gene sequence.

2.2 Spectral study

The spectroscopic study was carried out according to the method of Ciser [39], where an extract of chlorophyll was prepared by weighing 0.3 grams from the leaves of the species under study, then the leaves were cut into small pieces and placed in a ceramic mortar and crushed after adding 10 ml of absolute ethanol until small pieces remained. Then, 20 ml of pure ethanol was added, then the solution was filtered in a beaker through a separation funnel using filter paper, and the filtrate was placed in the quartz containers of a UV spectrophotometer (Shimadzu type, Japanese origin). The absorbances of chlorophyll were plotted after scanning the extract at wavelengths between 400 and 800 nm. The photon energy was calculated at the highest absorption λ_{max} according to the following equation: Energy photon (E) = Planck's constant (h) x speed of light (c)/wavelength (λ) [40].

3. Results and Discussion

The results showed the purity of the DNA extracted from the young leaves of the *Bougainvillea* species using a nanodrop device and electrophoresis on agarose gel was high purity between (1.73 and 1.80). The extracted DNA electrophoresis shown in Figure 1 was used to amplify *rbcL*, as shown in Figure 2. Later, the amplified DNA was sequenced. The partial *rbcL* sequences of *Bougainvillea*. species were deposited to the Gen Bank of NCBI and were assigned with the accession numbers, as illustrated in Table 4.

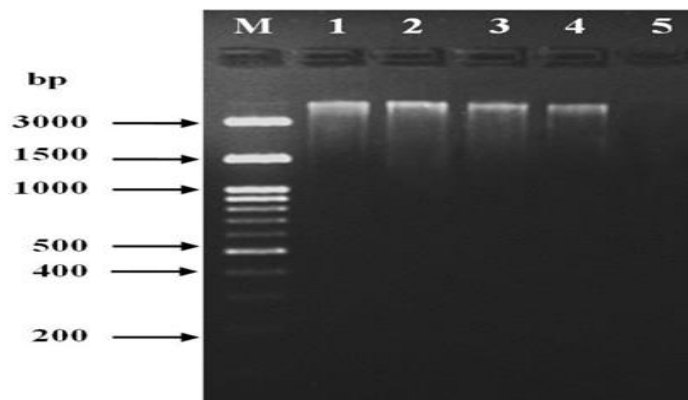


Figure 1: Total genomic DNA of *Bougainvillea* species M: DNA Ladder ;1- *B. spectabilis* ,2- *B. x buttiana*, 3- *B. glabra*,4- *B. peraviana*, 5-negative control.

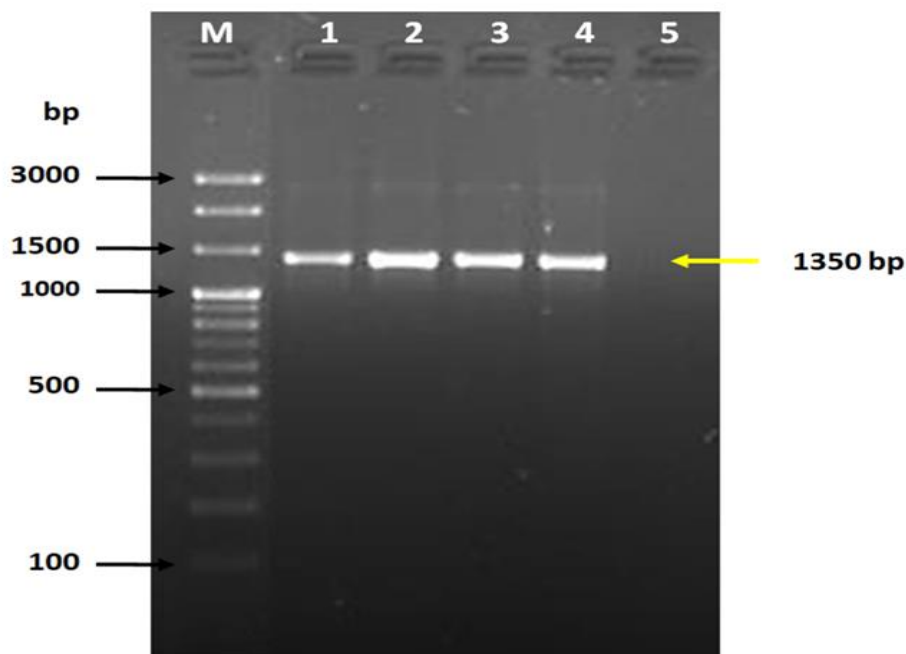


Figure 2: Amplified *rbcL* gene of the studied *Bougainvillea*. species. M: DNA ladder (100 bp). 1- *B. spectabilis* ,2- *B. x buttiana*, 3- *B. glabra*,4- *B. peraviana*, 5- negative control.

Table 2: A-ccession numbers of *rbcL* gene from *Bougainvillea*. species deposited to Gen Bank of NCBI.

No.	GenBank Accession No.	Plant
1.	OQ851473	<i>Bougainvillea spectabilis</i>
2.	OQ843469	<i>Bougainvillea x buttiana</i>
3.	OQ843470	<i>Bougainvillea glabra</i>
4.	OQ843471	<i>Bougainvillea peruviana</i>

3.1 A BLAST-based identification

The sequences of the studied *Bougainvillea* species samples were compared with the data with the Gen Bank using the BLAST in NCBI. The results of the BLAST analysis based on the *rbcL* gene sequence showed that sample (01) with code OQ851473 with a sequence

length of 584 bp matched with *B. spectabilis* by 100%, sample (02) code OQ843469 with length 584 bp was a new record of *B. x buttiana* that was recorded for the first time in the Gen Bank, sample (03) code OQ851470 with sequence length 552 bp matched with *B. glabra* by 99.82% - 100% and sample (04) with code OQ851471, sequence length 1229 bp, matched *B. peruviana* with a match rate ranging between 99.59%-100%, as shown in Table 5). These results agree with [14- 16, 20,41] that the barcoding of the *rbcL* gene is of importance in the accurate identification of plant species and in helping to diagnose and discover new species in some groups of plants.

Table 3: Percentage distribution of *Bougainvillea* species based on partial *rbcL* gene according to blast in GenBank of NCBI.

Sample Accession Number	Plant Identified	Identic Number %	GenBank Accession Number	Country Identification
OQ851473	<i>Bougainvillea spectabilis</i>	100	MN315508	China
		100	NC_041266	USA
		100	OM311950	India
		100	MW167297	China
OQ843469	<i>Bougainvillea x buttiana</i>	100	KF381106	India
			New Record	
OQ843470	<i>Bougainvillea glabra</i>	100	NC_046939	China
		100	JX571786	Egypt
		100	M88340	USA
		99.82	MG833637	Brazil
		99.82	JQ412331	South Africa
OQ843471	<i>Bougainvillea peruviana</i>	100	NC_049005	USA
		99.92	MW123901	Ecuador
		99.59	MW557550	China

3.2 Phylogenetic tree analysis

The molecular taxonomic relationship of the studied species phylogenetic tree (Figure 3) revealed from the barcoding of the *rbcL* gene from the chloroplast genome (cp DNA) showed distinction in the separation and isolation between the species, which presented the highest degrees of variation between species, where the phylogenetic tree supported the organization of species into two main groups. The two species *B. glabra* and *B. peruviana* met at the genetic distance (1.50) in the first group, and the two species *B. spectabilis* and *B. x buttiana*, at the genetic distance (0.50), in the second group,

Although *B. x buttiana* a hybrid of *B. glabra* and *B. peruviana*, it was closer to *B. spectabilis* and diverged from both species. Irregular meioses (abnormal chromosome number and disjunction of abnormal homologous chromosomes) because sterile might be one reason for this [42], where the offspring, especially those derived from interspecific hybridization of *Bougainvillea* species, are mostly sterile or partly sterile[43], and appears that the *B. x buttiana* and *specto-peruviana* hybrids are sterile and only the *specto-glabra* hybrids exhibit partial fertility [44].

These results agree with [45- 47] that the *rbcL* gene can be used as a tool for analyzing genetic relationships, distinguishing species, and taxonomy them at the molecular level. It also agrees with Yılmaz and Yeltekin [39] that the use of DNA barcoding is very important in

evaluating evolutionary relationships between species in addition to solving taxonomical problems, and that the preferred DNA region for analysis is the one that shows the diversity and good separation in some plant groups.

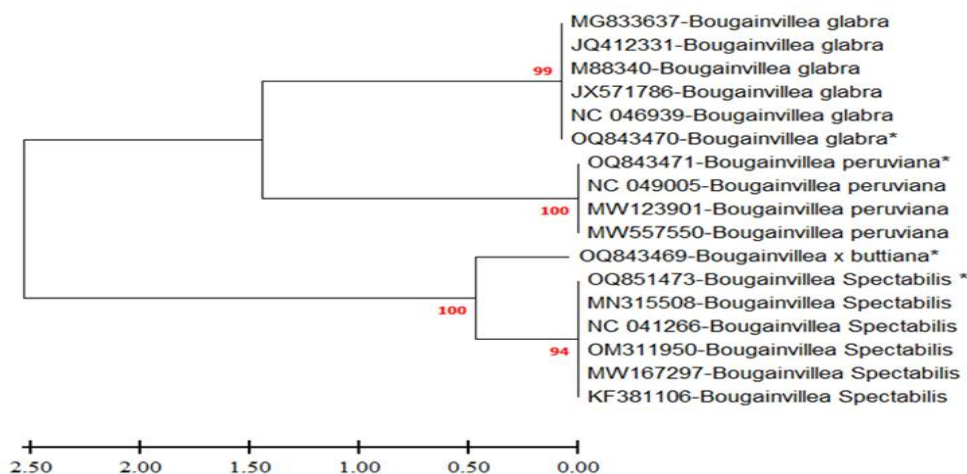


Figure 3: Phylogenetic tree of *Bougainvillea* species from Iraq. * represented current study species

3.3 Spectral study

The results of the study of spectral characteristics (Table 6, Figures 4,5) also showed differences in taxonomic importance between the studied species based on the values of λ_{max} and photon energy at the highest absorption of the alcoholic extract of chlorophyll plastids of the leaves of the studied species, where the species *B. x buttiana* recorded the highest absorption at wavelength 664 nm with an energy of 2.995×10^{-19} J per photon, and the species *B. spectabilis* recorded the highest absorption at the wavelength 438 nm with a photon energy of 4.541×10^{-19} J, the species *B. glabra* has a λ_{max} value of 436 nm and photon energy of 4.562×10^{-19} J, while *B. peruviana* recorded the lowest λ_{max} and photon energy with values of 434 nm and 4.583×10^{-19} J, respectively

Table 4: λ_{max} values, absorption, and Photon Energy for the chlorophyll leaves of *Bougainvillea* species

Species	λ_{max} (nm)	Absorption	Photon Energy at λ_{max} (J)
<i>B. glabra</i>	436	3.469	4.562×10^{-19}
<i>B. spectabilis</i>	438	3.874	4.541×10^{-19}
<i>B. peruviana</i>	434	2.229	4.583×10^{-19}
<i>B. x buttiana</i>	664	3.013	2.995×10^{-19}

These results agree with [36, 48-51] that the spectral study has an important and prominent role in attributing and supporting other taxonomic evidence because of the differences or variations it shows that can be used to enhance or support taxonomy in separating and isolating species and their varieties. It also agrees with Abdul-Fatah *et al.*, [52], that it is possible to rely on spectroscopic studies to qualitatively detect certain substances in a plant and compare it with another plant and know the difference by finding the highest wavelength that corresponds to the highest absorption. This result agrees with Mohammed and Abed [53] that spectroscopic methods are characterized by accuracy, ease, and low cost. The varied effects seen from change in the light spectrum result from several factors: different photoreceptors being excited, non-uniform efficiency of spectral

components in photosynthesis, and variable depth of penetration of spectral components in leaf foliage [54]. Leaf area, leaf angle distribution and leaf absorption coefficient play an important role in the amount of solar radiation absorbed by plant leaves [55]. The photon absorption of chloroplasts strongly depends on the distribution of pigment concentrations and its absorption spectra [30]. The influence of different parts of the lighting spectrum on the physiological processes in plants has been determined. The wavelength of 600–700 nm has a pronounced effect on photosynthesis, development, and regulation of processes, photomorphogenesis, and absorption of P660 phytochrome; 400–500 nm is necessary for photosynthesis and regulation of photoperiodic reaction, is absorbed by cryptochromes and phototropin [56].

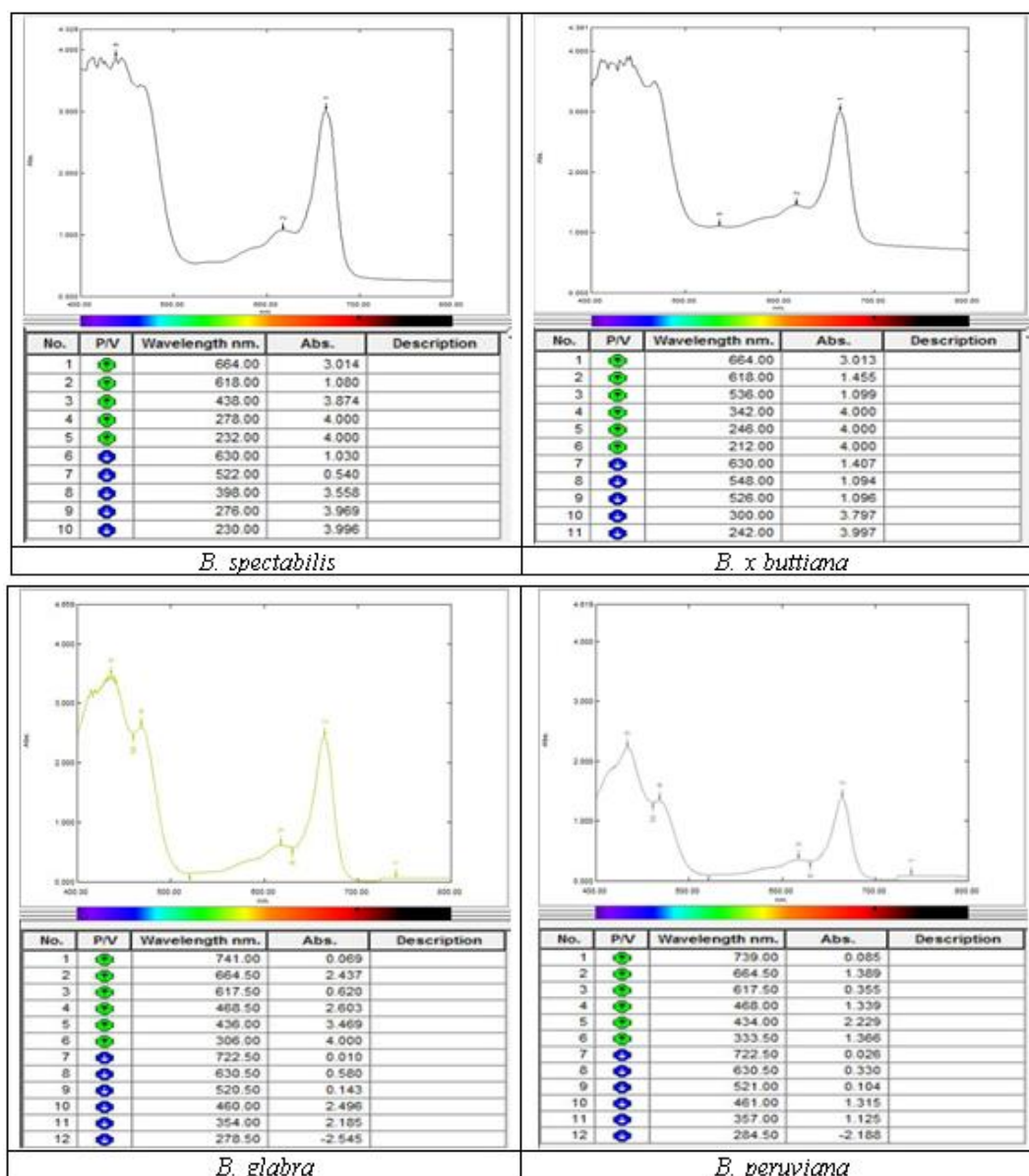


Figure 4: Absorbance spectrum and λ_{max} values for the chlorophyll leaves of the studied *Bougainvillea*. species

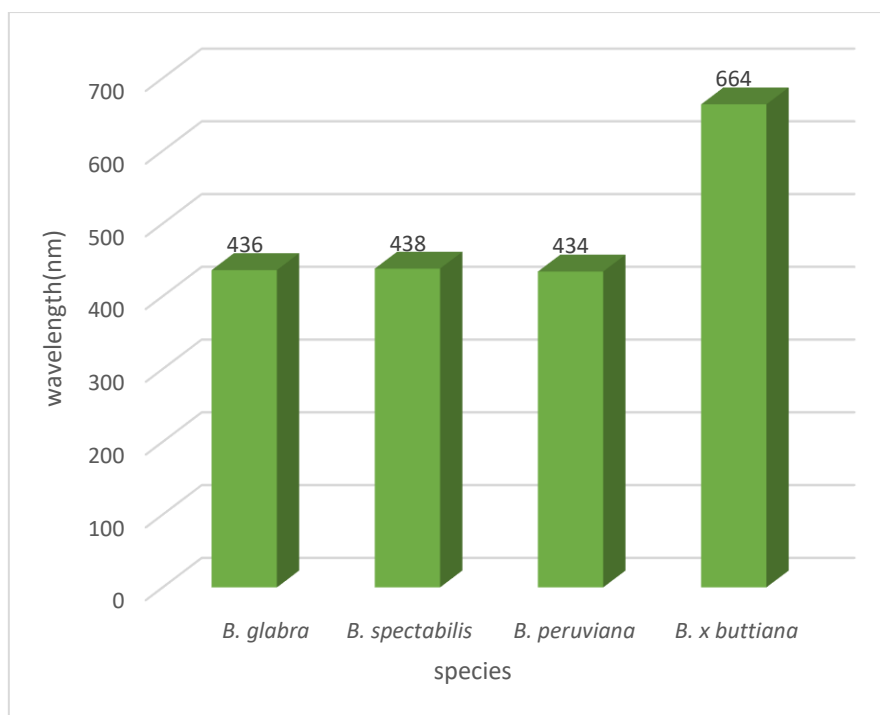


Figure 5: λ_{\max} values for the chlorophyll leaves of the studied *Bougainvillea*. species.

4. Conclusion

The barcoding of the *rbcL* gene and the spectral characteristics of leaf chlorophyll can provide important information in making taxonomic decisions at the genus level. This study will help in diagnosing and classifying the species of the *Bougainvillea* genus and provide a clear view of the genetic and evolutionary relationships within the genus.

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Disclosure and conflict of interest

The authors agree that there is no conflict of interest.

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