MOLECULAR IDENTIFICATION OF PARASITIC MISTLETOE (*ARCEUTHOBIUM OXYCEDRI)* OF JUNIPER ECOSYSTEM FROM DISTRICT ZIARAT, BALOCHISTAN, PAKISTAN

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خلاصہ

پودوں کو علیحدہ شناخت کرنے کے لیئے ڈی این اے (DNA) بار کوڈنگ ایک بہت عمدہ tool ہے۔ طوفیلہ پودے بہت سے علاقائی پودوں کی تباہی کا سبب بنے ہیں۔ آرسیتھوبیم آکسیدری (Witches Broom) زیارت میں جنونیپر کے جنگلات کی تباہی کا سبب بنا ہے۔ NCBI اور GENBANK میں پہلے اس پودے کا کوئی مولیکیولر ریکارڈ درج نہیں تھا۔ درج زیل تحقیق میں تین مارکرز Mat-k, RbcL اور ITS-2 مولیکیولر ریکارڈ کے استعمال میں کیئے گئے۔ آٹھ آرسیتھوبیم آکسیدری کے نمونوں سے CTAB طریقہ کار کے زریعے DNA حاصل کیا گیا، جو کہ زیادہ سسمامنا کے کچھ علاقوں سے اکٹھے کیے گئے تھے۔ RbcL کی PCR ایمپلیفیکیشن 100 فیصد جبکہ Mat-k اور ITS-2 کی صفر رہی۔ RbcL آرسیتھوبیم آکسیدی کو جینس کے درجے تک علیحدہ شناخت کر سکا کیونکہ GENBANK اور BOLD کے ریکارڈ میں کوئی مولیکیولر ریکارڈ درج نہیں تھا۔ گیارہ SNPs اور تین gaps پائے گئے جب ارسیتھوبیم آکسیدی کو آرسیتھوبیم آزورشم سے موازنی کیا گیا۔ اس تجرباتی مطالعے سے ہم اس نتیجے پر پہنچے ہیں کہ Mat-k, RbcL اور ITS-2 کے مقابلے میں ارسیتھوبیم آکسیدری کی شناخت کے لیئے ایک بہت موضوع پرائمر ہے۔

Abstract

DNA barcoding is very effective tool in discriminating plants. Parasitic plants are responsible for destruction of plant locality. *Arceuthobium oxycedri* is a major cause of decline of Juniper forest Ziarat. There was no record of molecular data regarding this species in NCBI and GENBANK. Three markers i.e. *RbcL*, *ITS-2* and *Mat-k* are used globally to obtain molecular data. CTAB method is used to extract DNA of eight *A.oxycedri* leaves from different areas of Sasnamanna, Ziarat. *RbcL* PCR amplification is 100% while *Mat-k* and *ITS-2* were not amplified. *RbcL* discriminated *Arceuthobium oxycedri* at genus level as there is no data available in GENBANK and BOLD. 11 SNPs and 3 gaps found when compare *A.oxycedri* with *Arceuthobium azoricum.* In this research we have found that *RbcL* is much better primer than *Mat-k* and *ITS-2* for identification of *Arceuthobium oxycedri.*

Keywords: CTAB, MEGA 7, *ITS-2*, *Mat-k*, SNPs and gaps

Introduction

Classical method of naming and classify plants were based on Carl Linnaeus system which was modified on the basis of genetic and molecular data. About 107 million species were identified on the basis of morphological so far. However, there are many morphological gradations that distinguish carefully allied species are so elaborate that almost all taxonomists specialize in a single team of closely related organisms. Therefore, a mess of taxonomic expertise could also be needed to identify specimens from a single biodiversity survey (Erickson *et al.*, 2008).

According to Chase *et al.* (2009), DNA barcoding is the multidisciplinary area. It includes a diverse mixture of taxonomy, genetics and computer science that implies the technique of acquiring proficient species identifications. The method is synonymous with Human Crook Forensic DNA Fingerprinting Systems a method that it makes use of common barcoding markers to establish unknown species in a suitable manner.

Arceuthobium is a parasitic plant which usually grows upon stems and branches of the other plants like apple and pines. It normally takes water and mineral nutrients from the host plant by utilizing a structure kenned as haustorium. It's commonly kenned as dwarf mistletoes. *Arceuthobium* (Viscaceae) is anindubitably defined

group of minutes (customarily less than 20 cm high), variously coloured (red, ebony, brown, or yellow) flowering plants that are midair parasites only on members of the Cupressaceae and Pinaceae. The dwarf mistletoes are commonly known as unwonted biological interest because they are the most evolutionarily specialized genus of the Viscaceae. Schuette (1992) was first to determine the interspecific relationship among 15 *Arceuthobium* species using ITS rDNA sequences.

According to Nickrent *et al.* (1994) *A.oxycedri* and *A.abietis* were remarkably similar to each other on the basis of inter specific distances (0 to 21.4%). New world species of *Arceuthobium* were compared to ITS region in the other plants shows up to 41% sequences divergence than other species. Interspecific distance places it in the heterogeneous group while minimum length trees supported *Arceuthobium* as a verticillate branched subgenus

Nickrent *et al.* (1995) reported that *RbcL* amplification is excellent in all studied samples and evolutionary tree of all 499 samples showed closely related species of *Arceuthobium*.

Old and new species of *Arceuthobium* were compared on the basis of *ITS* region. (Nickrent *et al.*, 2004) and 11 species of *Arceuthobium* phylogenetic tree were constructed. Similarly, in 2007 Amico and his coworkers conducted a research work in which phylogenetic *rbcl* and *trnL-F* primers (Amico *et al.*, 2007).

In May 2008, Vidal studied the origin of arial parasitism in Santalales (order of *Arceuthobium* species) by using nuclear ribosomal and chloroplast regions. For chloroplast region, they used *mat-k, rbcL* and *tranL-F* primers and used maximum parsimony and Bayesian inference methods for phylogenetic analysis. From this research they concluded that aerial parasites evolved from ancestors that were polymorphic for either root or stem parasitism. Amphiphagous term was used for parasitism. Conjunctions in morphological country side related with these plants species that have shown parasitism are due to structures, seed attachment, forfeiture of chlorophyll and unisexual flowers (Vidal-Russell *et al.*, 2008).

In 2013 a study about medicinal properties of parasitic plants (Viscaceae family) was conducted, they calculated the genetic distance between selected plants, to calculate this they used rbcL and psbA trn-H regions. They concluded that *Dendrophthoe* and *Viscum* plant species showed the genetic distance of 0.032 and 0.036 in rbcL spacer and 0.269 and 0.264 in psbA trn-H spacer region respectively (Kwanda *et al.*, 2013).

In this research studies our aim was to develop DNA barcode for *Arceuthobium oxycedri* by using *RbcL, Mat-k* and *ITS-2* and construct its phylogenetic and evolutionary tree to see how it is related to other plants species.

Material and Method

Chemicals and Materials: PCR Water, Tris-Boric-ETDA (TBE), Ethidium bromide (EtBr), Cetyl-Trimethyl-Ammonium-Bromide (CTAB), Ethanol (70%, 75% or 80%), Chloroform-Isoamyl-alcohol, Liquid nitrogen, Silica gel, Tris HCl, Ethylene Diamine Tetra Acetic Acid (EDTA), Sodium chloride (NaCl), Sodium Acetate, Isopropanol, Agarose powder, Proteinase-K, RNase

Sample Collection: 8 leaves of *Arceuthobium oxycedri* were collected on silica gel from collected sites Sasnamanna. Collection sites, are shown on the map in Figure 1

DNA extraction method: DNA was extracted from leaves by modifying some steps CTAB (Cetyl-Trimethyl-Ammonium-Bromide) method following by (Li *et al.*, 2013). After DNA extraction, estimation was done on 1% agarose gel.

Gene Amplification. In this study, the affectivity of two chloroplast loci (*RbcL & Mat-k*) and one nuclear locus (*ITS-2*) was once evaluated accompanied by way of detailed sets of primers. Table 1 shows evaluated loci used for amplification.

PCR products were sent for sequence analysis in alliance with Askar Co to China (Hong Kong). After sequencing samples were analysed for their phylogenetic trees and composite distance is calculated between Sample sequence and compared sequences. All this analysis was performed with the help of MEGA 7 Software

PCR program settings of ITS-2, mat-k and Rbcl are given in table 2

Table 1. Primers details used for PCR amplification

Genome	Gene	Barcode Name	Primers Sequence 5`-3`
Chloroplast region	Ribulose-1,5 Bisphosphate Carboxylase	Rbcl-α-F	ATGTCACCACAAACAGAGACTAAAGC'
		Rbcl-α-R	GTAAAATCAAGTCCACCRCG'
	Megakaryocyte-Associated Tyrosine Kinase	Mat-K-390 -F	CGATCTATTCATTCAATATTT C'
		Mat-K -1326-R	GTAAAATCAAGTCCACCRCG
Nuclear region	Internal Transcribed Spacer	ITS2-S2-F	ATGCGATACTTGGTGTGAAT
		ITS2-R	GACGCTTCTCCAGACTACAAT

Table 2. PCR Amplification programs (ITS-2, RbcL & Mat-k)

Marker name	**Number of cycles**	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
ITS-2	30	94^{o}C for 5 Minutes	94^{o}C for 45 Seconds	58^{o}C for 45 Seconds.	72^{o}C for 1 Minute.	72^{o}C for 5 Minutes
RbcL	30	94^{o}C for 5 Minutes	94^{o}C for 45 Seconds	61.5^{o}C for 45 Seconds.	72^{o}C for 1 Minute	72^{o}C for 4 Minutes
Mat-k	35	94^{o}C for 1 Minute	94^{o}C for 45 Seconds	52^{o}C for 45 Seconds.	72^{o}C for 1 Minute.	72^{o}C for 5 Minutes

Table 3. BLAST result of *A.oxycedri RbcL* sequence

Description	Query Cover %	Total Score	Identity %	E value	Accession No
Arceuthobium azoricum	99	917	97	0.0	HM849787.1
Arceuthobium campylopodum	99	859	95	0.0	MF963054.1
Notothixos subaureus	99	837	95	0.0	KF496485.1
Dendrophthora clavate	99	833	95	0.0	L26069.1
Arceuthobium verticilliflorum	99	828	94	0.0	L26067.1

Results and Discussion

Genomic DNA estimation: After DNA extraction of plants from the CTAB method (Li *et al.*, 2013). DNA extracted samples were run on 1 percent gel in gel electrophoresis compartment for 35 minutes on 110 volts. Gel image is given away in figure 2.

PCR Amplification: The PCR amplification was done for the samples, as described in materials and method by using *rbcL* and *mat-k* for chloroplast region and *ITS-2* for the nuclear region. PCR amplified products were run on 2 percent gel for 45 minutes on 110 volts. Results for primers are specified in table 6 and PCR amplification on the gel is shown in figure 3.

Taxonomic Identification of plant: The great and tiresome work of identification of *Arceuthobium oxycedri* (Ar 1 to Ar 8) was done by Assistant Professor Shazia Irfan, Botany Department, Sardar Bahadur Khan Women University, Quetta. *Arceuthobium oxycedri*. Plants were reconfirmed by the e-flora of Pakistan figure 4 shows the *Arceuthobium oxycedri* sample.

Sequence analysis: PCR products were sent for sequence analysis in alliance with Askar Co to China (Hong Kong). The results of sequencing for the *Arceuthobium oxycedri* was not very effective and show positive result only for *RbcL* loci, *Mat-k* and *ITS-2* did not show any result. The sequencing results are listed in figure 5

Basic Local Alignment Search Tool (BLAST) results: Sequencing results were arrived from China, from sequencing results it is identified that all the collected plant samples have the same species, for further analysis one sequence of *Arceuthobium oxycedri* was selected. BLAST was performed on sequence of *A. oxycedri*. The detail of BLAST results is as follows

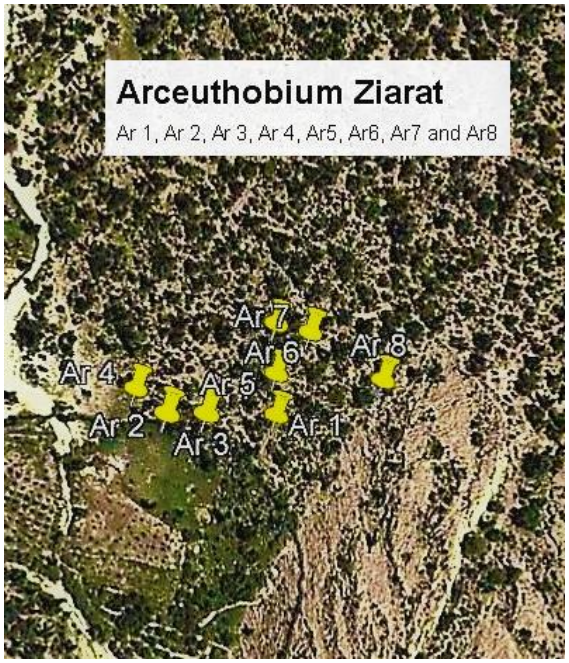

Fig.1. *A. oxycedri* Collection sites are marked at google earth map of (Sasnamanna) Ziarat

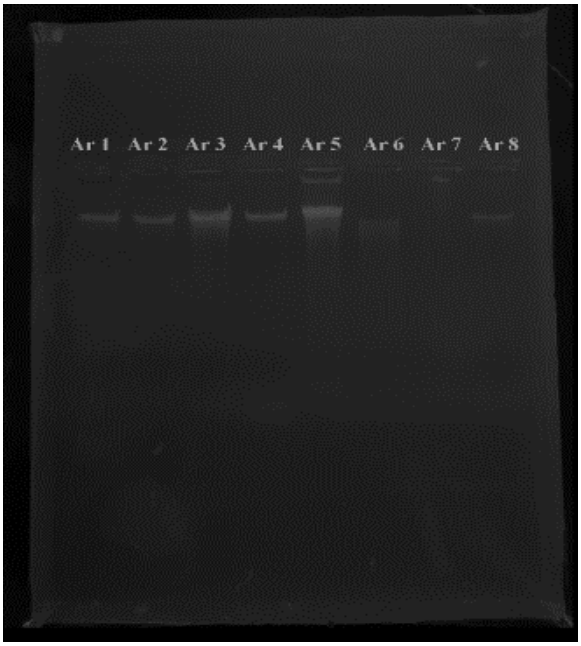

Fig.2. *A.oxycedri* samples DNA gel

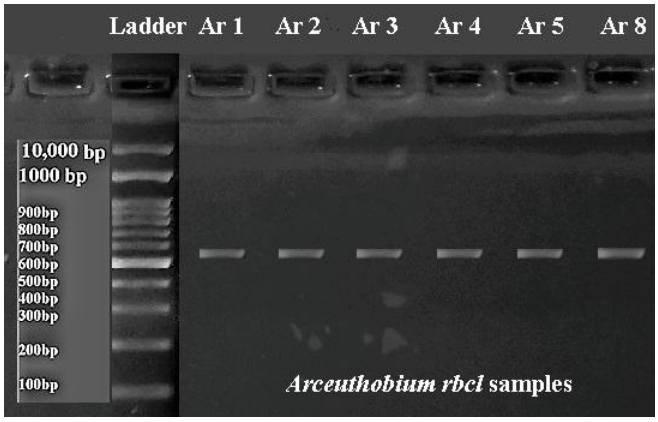

Fig.3. *A.oxycedri* samples *RbcL* Amplification gel result

Fig.4. *Arceuthobium.oxycedri* Plant

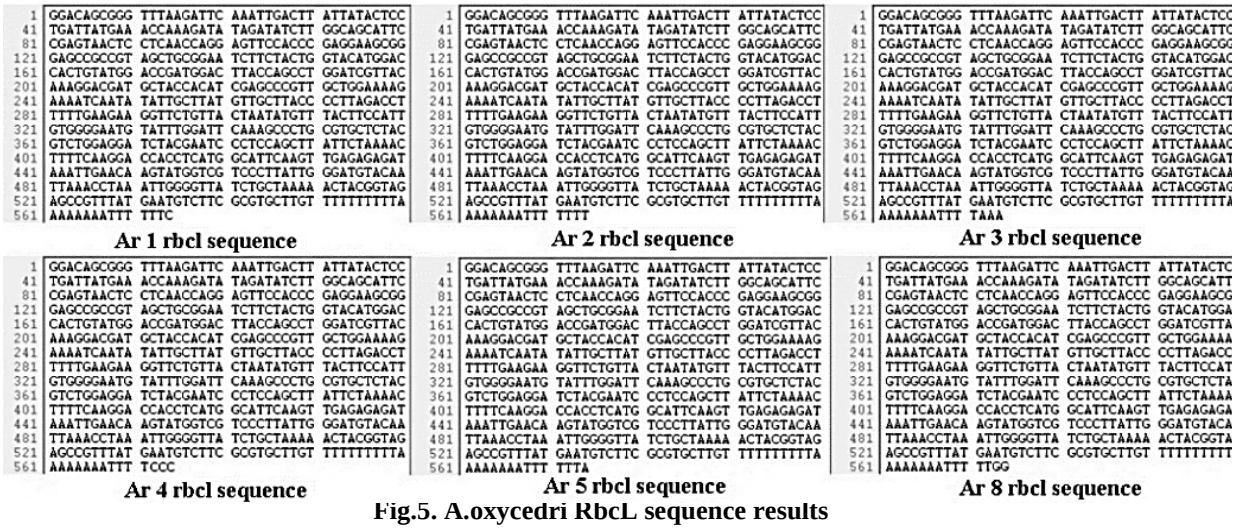

Fig.5. A.oxycedri RbcL sequence results

Fig. 6. *RbcL* sequence of *Arceuthobium oxycedri* compared with *RbcL* sequence of *Arceuthobium azoricum* total SNPs found are elaven and gaps are three

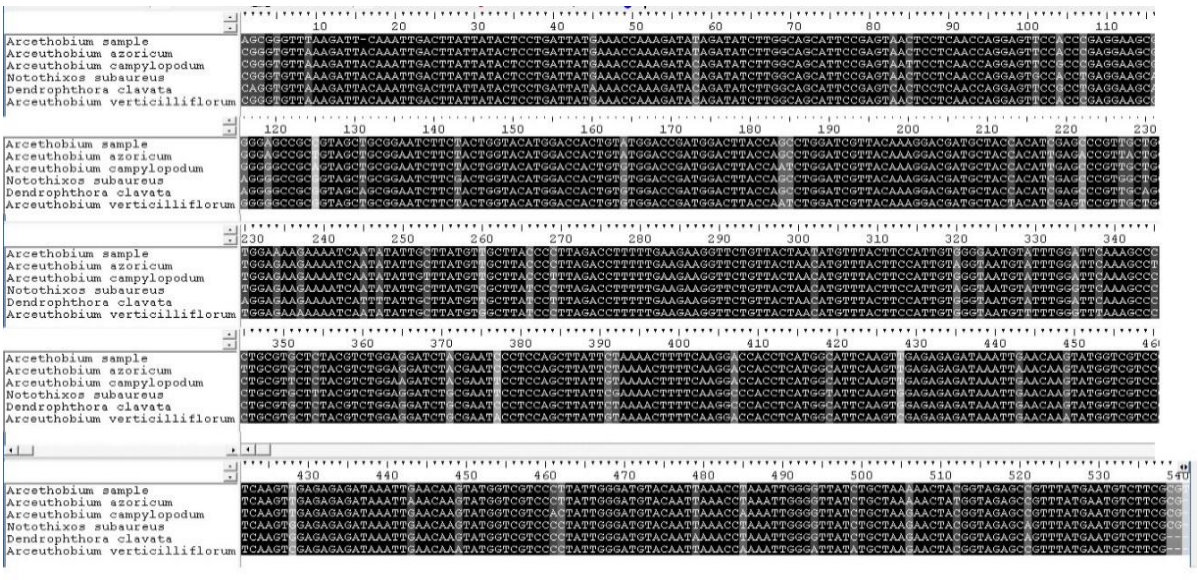

Fig.7. Multiple Sequence Alignment (MSA) between Arceuthobium sample and 5 matched NCBI database

Multiple Sequence Alignment (MSA): MSA of the sequenced sample was performed by comparing the sample sequence with NCBI database sequences. The software which was used for MSA was Bio-edit, in Bio-edit *Clustal-W* option was selected. The detail of MSA results are mentioned in figure 7 sequences

Phylogenetic Analysis: After multiple sequence alignment, phylogenetic trees are constructed of all the sample sequences and compared database sequences, the gene tracking of all the samples were also done by using the software Mega 7.

The phylogenetic analysis details of samples and detail of gene tracking of samples are in figure 8(a,b).The sequence of *A.oxycedri* under debate is submitted on NCBI website with GenBank accession number MH232031.

Website link (https://www.ncbi.nlm.nih.gov/nuccore/MH232031.1).

RbcL* Phylogenetic Trees for *Arceuthobium oxycedri

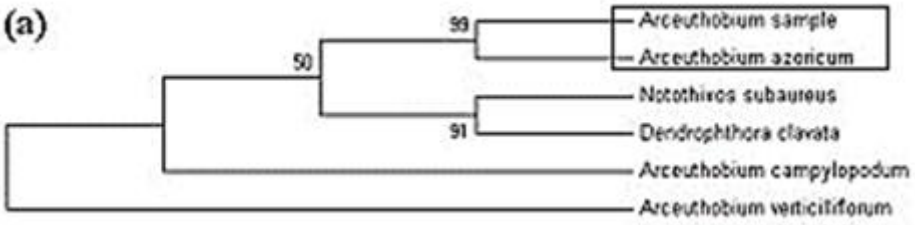

Fig. 8a. Displaying a phylogenetic tree of *RbcL* of *A.oxycedri* between the sample and 5 Database sequences. Neighbour-joining tree method is selected and the Model used in construction is Kimura 2. In the figure, an *A.oxycedri* sample shows similarity towards A. azoricum and less similar to *A.verticilliflorum*.

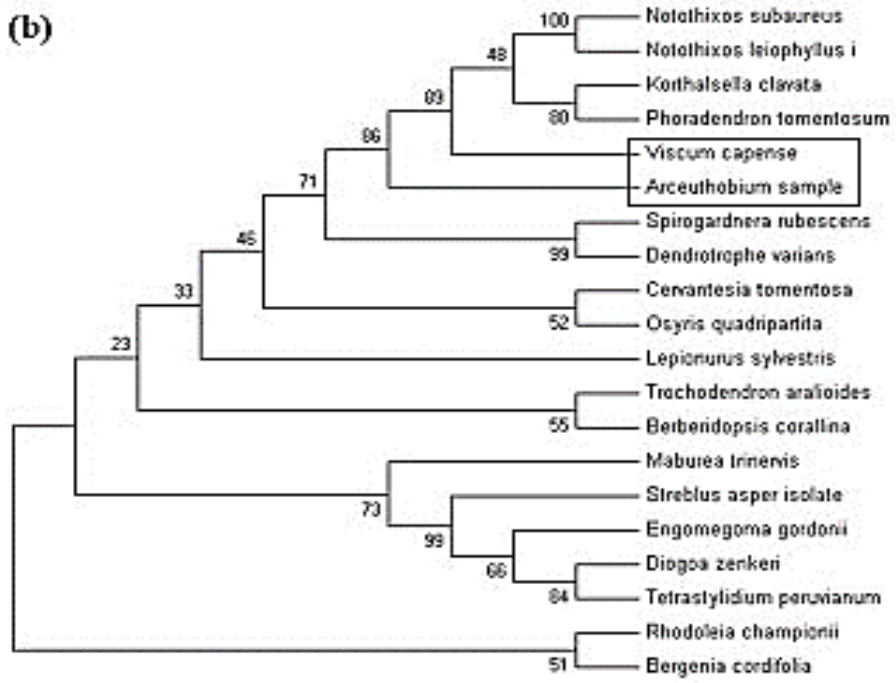

Fig.8b. Displaying Gene Tacking of *A.oxycedri RbcL* sample sequence with 19 NCBI database sequences here *A.oxycedri* sample is close to *Viscum capense*.

Characterization of plants through DNA barcoding starts after 2009. Before modern DNA extraction techniques and analysis of DNA sequences through bioinformatic tools barcoding of DNA of organisms were impossible. Thus, all these problems were solved by a combination of human efforts by removing all previous

errors and by internet which provides us all the necessary research data and computer tools for DNA barcoding, this has directed us to DNA barcoding in parasitic plants (Hollingsworth *et al.*, 2011).

Numerous issues were accountable for high fiasco rates for DNA extraction, one of the overbearing issues is the protocol used for the extraction of DNA. Methods like CTAB and SDS protocols for the extraction were used by research scientists. Conclusively CTAB protocol is the finest method used for parasitic plant DNA extraction, no results were found on SDS method. For extraction through a CTAB method is used by experts, so bearing in mind the explanations of preceding research in my research study I also used CTAB method for DNA extraction (Burr *et al.*, 2001; Xin *et al.*, 2012).

Arceuthobium oxycedri samples were extracted with CTAB protocol, the results were processed successfully by using bioinformatics tools. Particularly in plants, generally *ITS*-2 mark is used for nuclear region, *Mat-k* and *Rbcl* regions are used as the primers but rendering to the earlier stated work by the distinguished scientists, specified that *Mat-k* is not effective in *Arceuthobium* species and even ITS-2 region give low resolution results due to specific *ITS* marker on *A.oxycedri* as compared to the *RbcL*, signifying that Mat-k region is ineffective in *A.oxycedri* and *ITS* region is not that effective as compared to *RbcL*, even in this study, results also propose that *RbcL* is most effective region in the *A.oxycedri* as only rbcl marker show positive results.

Vijayan *et al.* (2010) DNA was obtained by using the CTAB protocol, the next step was the application of primers on samples to check resolution power. Indisputably the result of preceding scientific literature was similar. *RbcL* Marker's resolution power was up to the generic level. After PCR samples were amplified, to overwhelm the interest the PCR samples were referred to Hong Kong in association with ASKAR/co Lahore.

Debating on resolution power, codes (Ar 1, Ar 2, Ar 3, Ar 4, Ar 5 and Ar 8) were resolute till generic level and named as *Arceuthobium* but later taxonomic identification, it was called as *Arcethobium oxycedri.* After multiple sequence alignment and construction of phylogenetic tree, data clearly shows that *rbcl* resolution power is good for *Arceuthobium oxycedri* samples but only till generic level.

In our study ITS-2 and Mat-k marker show no discrimination in *Arceuthobium oxycedri* samples, after reading previous research papers and articles we find out that in *Arceuthobium* species-specific ITS primers are used as primers such as ITS-4(Nickrent *et al.*, 1994).

Mat-k also shows no discrimination in *Arceuthobium* sample in our study and from early research data we find out that none of them has used a *Mat-k* primer that shows discrimination completely in case of *Arceuthobium* species (Reif *et al.*, 2015).

RbcL loci show discrimination, after taxonomic identification and previous research data it showed that in this research no rbcl sequence of *A.oxycedri* is submitted in the database of barcoding (NCBI and BOLD) still date (Jeger *et al.*, 2017).

Similar research was done by Kress *et al.* (2007), BLAST is a bioinformatics application tool and it was used to discovery out the differences or similarities between the Query or Enquiry sequence and sequences that were previously existing in NCBI.

In the meantime, after the BLAST, multiple sequence alignment was done by another bioinformatic tool Bio-edit. These sequences were trimmed and edited in the software (Bortiri *et al.*, 2008). Additionally, Ford *et al.* (2009), followed the same outline during their research work as we likely to use as Bio informatics tools.

Cavalier-Smith (2003), used fascinating work of gene tracking with the help of bioinformatics tool named as MEGA 7; another software which aids in the manufacture of a Phylogenetic tree or gene track. The interest of researchers didn't end after gene tracking; exciting work was performed to construct evolutionary trees. The scheme in this study and also in the previous studies helped researchers to recognize that where from a specific species have changed genetically (Shufran *et al.*, 2011).

Conclusion

This study concludes that *RbcL* barcode show's better amplification power than *ITS-2* and *Mat-k* barcodes on *Arceuthobium oxycedri*; Also phylogenetic studies suggest that *RbcL* sequence of *A.oxycedri* is closely related to *A azoricum* and gene tracking suggest that *RbcL* sequence of *A.oxycedri* is closely related to *Viscum capense*

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