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DNA Barcoding and Biochemical Profiling of Medical Plants of Northern and Desert Areas of Pakistan to Improve Rural Living Standards

Amer Jamil and Muhammad Ashfaq

THE PAKISTAN STRATEGY SUPPORT PROGRAM (PSSP) WOKRING PAPERS

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EXECUTIVE SUMMARY

Pakistan is a country with a varied climate and hosts a large number of medicinal plant species. Most of the medicinal plants are collected in the wild by local communities. These plants are an important source of livelihood for rural economies. However, no systematic documentation has been undertaken to assist in proving ownership of the plant resources. This project focused on the conservation of natural plant resources by using modern molecular techniques and creating awareness for determining the active ingredients of the plants through biochemical profiling. Further objectives of the study were to identify marketing channels for medicinal plants, costs and margins of stakeholders involved in the marketing of medicinal plants, and factors responsible for the poor trade and decreasing population of these plants in the two study areas of Swat Valley and Cholistan Desert.

Biochemical profiling of twelve selected plants, followed by their comparison with marketed products, revealed that they contain highly valuable compounds with possible commercial applications. After their isolation, DNA barcoding was done on many of the medicinal plant species, for most of which the barcode sequences had not been reported earlier, and the sequences were deposited in GenBank and BOLD. Four DNA barcoding markers were evaluated for their amplification, sequencing, and species identification capacity. TaxonID trees were generated for barcode sequence validation. Phylogenetic analysis did not infer meaningful interpretation due to a small and incomplete dataset. This provided the insight that a barcode library was needed for all the medicinal species. DNA barcoding was successfully applied in testing for adulteration in the medicinal plants which could have a tremendous impact in checking the purity of traditional medicines.

Structured questionnaires were used to collect descriptive statistics and cost related data from stakeholders in the medicinal plants trade. A total of 120 respondents, including 80 collectors (40 from each area) and 40 assemblers/shopkeepers (20 from each area), were selected for this purpose. Margin analysis was used to measure the revenues of collectors and assemblers/shopkeepers. A majority (30%) of the respondents were illiterate, and most (66%) reported that the population of medicinal plants had been decreasing in both areas. Collectors, assemblers/shopkeepers, pansaries (grocery shops), and hakims (traditional healers) were the main stakeholders involved in the marketing of medicinal plant products. Medicinal plants were an important source of income for locals, contributing about 32% and 29% in assemblers and collector's income in Swat, respectively. Cholistani assemblers and collectors were getting about 25% and 18% of their per month income from medicinal plants, respectively. Collectors in Swat were selling on average 337 kg of plants in the market per month, as compared to 114 kg of plant parts sold per month by collectors in Cholistan. Assemblers had 2 to 7 times higher margins than collectors. Despite this, collection of medicinal plants was an important income generating activity for collectors.

The main reasons reported for the declining plant population in both areas were overgrazing, increasing human population, unavailability of seeds, poor collection techniques, lack of awareness among people, removal of roots, deforestation, and rapidly increasing demand for medicinal plants. The main marketing problems faced by stakeholders were a lack of awareness regarding the importance of medicinal plants, no pricing policy, a monopoly of a few big dealers, and high transportation costs. The medicinal plants markets were well developed in Swat, as compared to Cholistan which did not have any market structure.

Recommendations are made on the basis of the project outcomes and translated into policy implications for the establishment of well-structured markets along with conservation measures to make full use of the medicinal plants' products and to raise the living standard of the people residing around this valuable resource.

INTRODUCTION

Pakistan is home to a huge variety of plants, animals, birds, and insects. From the desolate deserts of Thar to the forested valleys of Dir and Kohistan and the great mountain masses Karakoram, Himalaya, and Hindukash, all are ripe with diverse natural resources. The northern and desert regions have become important assets for the eco-tourism industry, and much of the credit goes to the plant biodiversity. People come from far and wide to admire the colors and patterns of the mountain ranges - features which are themselves products of biodiversity. Unfortunately no proper identification and documentation system is available to prove ownership of this heritage. This allows for the illegal trade and adulteration of processed plant materials.

Medicinal plants contain active ingredients, and are used to cure disease or relieve pain (Okigbo *et al.*, 2008). Man has been using different parts of medicinal plants to cure ailments since ancient times. The indigenous systems of medicine, namely ayurvedic, siddha, and unani have existed for several centuries. These systems of medicine are prevalent in Pakistan, India, Korea, China, Singapore, Western Asia, and in many other countries and demand medicinal plants as raw material. Plants also occupy a significant station as raw material for many modern medicines. Although synthetic drugs and antibiotics brought about a revolution in medical science, still millions of people living in remote areas have no access to these synthetic drugs. These individuals mostly depend on traditional healers whom they trust. Deadly diseases that have long defied synthetic drugs can be cured by judicious use of medicinal plants (Bhattacharjee, 1998).

Species Identification

Fast and accurate identification of plant species is indispensable in almost all kinds of plant research including biodiversity studies. Taxonomy and species identification is the main discipline in which development of new systematic tools can contribute significantly to conservation and biodiversity assessment (Steele and Pires, 2011). In the studies undertaken to understand biodiversity, species is the most commonly used word by scientists and the public. Defining the boundaries of medicinal plant species would be of great help to conservation planners and government agencies (Primack, 2008).

Species discrimination based on an organisms' morphological attributes is the typical way of identification by taxonomists. Other than morphological characteristics, habitats, ecological niche, life history, traits, and geographic distribution are also important tools to describe species (Steele and Pires, 2011). Taxonomists identify a species of plant on the basis of flower and leaf shapes, colors, etc. But if environmental samples are dry or damaged, or only seeds are available, then classical taxonomic methods are not sufficient, and there is a need to supplement older taxonomic techniques with the modern techniques of molecular biology (Steele *et al*., 2010).

The technique of using small, globally agreed upon, standardized genomic portions for the purpose of species identification is termed as DNA barcoding. By now, this technique has contributed to many scientific investigations. Although data based on molecular studies can't take the place of physical characterization, they can contribute by providing supporting evidence in taxonomic decisions (Lowenstein *et al*., 2009).

A DNA barcode must possess certain characteristics for global acceptance, including easily amplifiable regions throughout the taxa by standardized primers, simple and shortly amplified and sequenced, and they must be variable enough for species level identification (Chase, 2007). Several coding and non-coding nuclear and plastid genomic regions such as ITS, *matK*, *rbcL*, *rpoC1*, *rpoB*, *trnH-psbA*, and *atpF-atpH* fulfilled these barcode standards and have emerged as an agreed upon barcode loci by a number of scientists (Sucher *et al.,* 2012).

Another important aspect is that, although many of the plants have been used in folk medicine for centuries, the information regarding the active ingredients is not known explicitly except in a very few cases. Biochemical profile of the plants is imperative to know if we want to get maximum benefit from the medicinal potential at a large scale. Determining the active ingredients in the plants is therefore highly necessary for developing pharmaceuticals.

Socio-economic Factors Relating to Medicinal Plants

The agricultural sector is an essential component of Pakistan's economy, contributing about 21% to Gross Domestic Product (GDP). Agriculture contributes significantly to overall growth and the wellbeing of people both directly and indirectly. The contribution of horticultural crops to GDP is about 6% and is 22%of national food production (Government of Pakistan, 2013). Horticultural crops such as fruits, vegetables, ornamental, medicinal, and others are grown in Pakistan because the agricultural land is suitable for a large range of crops due to its rich topographic and climatic conditions and variations in soil. This sector has the potential to provide opportunities for increasing incomes and decreasing socio-economic problems (Alam and Mujtaba, 2002).

Medicinal plants play a crucial role in local economies and contribute significantly to healthcare activities as well as improving socio-economic status. For a long time these plants have provided livelihood opportunities to many rural communities, and their importance has begun to be acknowledged over the last decade (Okigbo *et al.*, 2008). Pakistan has about 6,000 species of higher plants. It has been reported that 600 to 700 species are used for medicinal purposes. However, production in Pakistan does not meet total demand for herbal medicines and significant portions are imported from Nepal, Sri Lanka, India, China, and Kenya. Out of the 600 species used as medicines, only 300 species are available in local markets (Shinwari, 2010).

It has also been estimated that 70% of the total species are uni-regional and only about 30% are bi- or pluri-regional. The country has four phytogeographical regions: (i) Irano-Turanian (45% of species); (ii) Sino-Himalayan (10%); (iii) Saharo-Sindian (9.5%); and (iv) Indian element (6%). Despite the Saharo-Sindian Region being the biggest in area, the diversity of species confined to this area is lowest for any phytogeographical region (Ali and Qaiser, 1986).

There are hundreds of plant species which possess medicinal, industrial, and economic potential. Unfortunately, the fact is that little attention is paid to the conservation, proper usage, and sustainable flow of these vulnerable species. A wide variety of ecological, sociological, and economic issues are contributing to the extremely serious threat to the population of these medicinal plants. As these plant species are treated as public goods, personal benefits are maximized with no regard given to conservation.

A majority of the population in these areas is unaware of the importance of the medicinal plants found near their houses and places of work. Lack of education is the main reason. High valued plants are used as fodder, fuel, and animal grazing by the local community. These species are raw materials for many important synthetic drugs, but in spite of this fact, non-productive uses are increasing with the increase in population. These plants are vital components of biodiversity. Any change causing the population of these plant species to fall below the sustainable level will also have an undesirable effect on biodiversity, leaving future generations to bear the cost in terms of land degradation, loss of natural habitat, and loss of vulnerable plant and animal species.

The most important aspect of these medicinal plants is that they are a source of livelihood and income for many rural families, but the people residing in those areas have very low per capita incomes. These plants can play an important role in developing and strengthening the rural economies.

Objectives

Because of the economic importance of medicinal plants to rural livelihoods, and the lack of significant study regarding their DNA identification and barcoding, this study has set out with the following objectives:

- To assess the status of the population of medicinal plants in the study area, and to authenticate DNA barcoding based identification of plant species.
- Biochemical profiling of selected medicinal plants.
- Identification of the medicinal plants' marketing channels in the study area and estimation of the costs, margins, and profits of stakeholders involved in the marketing of medicinal plants.
- To suggest remedial measures for improving the marketing system and conservation of medicinal plants in the study area.

STUDY AREA

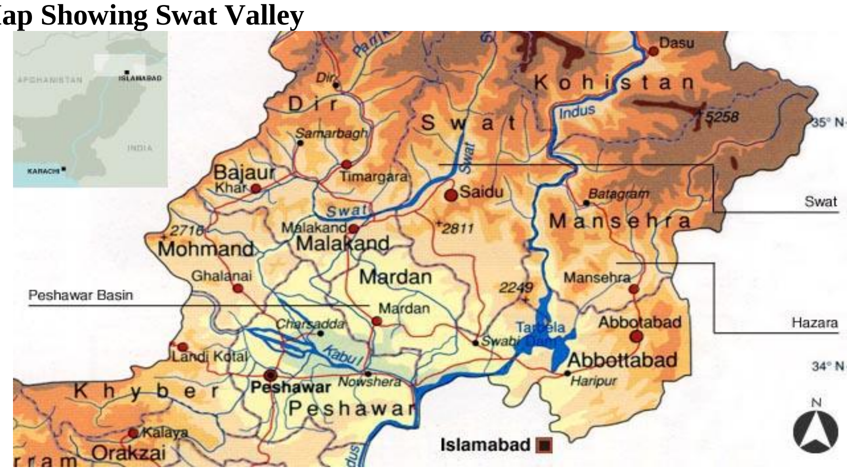
The Swat valley and Cholistan desert regions were selected for the study. The areas were selected as they host a large number of medicinal plants species having both economic and medicinal values. Local community members of these areas are largely dependent on the use of medicinal plants because of the absence of proper primary healthcare facilities.

These areas were also selected on the basis of previous studies on medicinal plants. Swat valley was chosen due to the presence of diverse types of medicinal plant species being used locally and exported. The Swat valley has also been investigated to study different aspects of medicinal and aromatic plants because of the large variety present (Sher *et al*., 2014). Cholistan desert was selected due to the presence of high potential, but under-explored, medicinal plant species.

Swat Valley

The Swat Valley is situated in the north of Khyber Pakhtunkhwa (Fig. 1), between latitudes 35° north and 72° east and 30° east longitude and is enclosed by mountains. The regions of Chitral and Gilgit–Baltistan are situated in the north, Dir in the west, and Mardan in the south. The Indus River separates it from Hazarain the east. Swat valley, the land of fruits and flowers and that of springs and streams, is situated to the northeast of Peshawar. Its main commercial centre is Mingora City, which lies 120km from Peshawar. It is divided into two parts: Swat Kohistān and proper Swat. The mountainous area above the village of Ayin near Madyan is called Swat Kohistān, whereas from Ayin down to Kalangai in the west is proper Swat. Visits to Swat valley were made in October 2012 and May 2013 for collecting data about socio-economic variables and plant collections. Swat is very mountainous, and its highest peak, Falaksair, measures 6,098 meters in height. The district occupies the floristically rich Southern extension of Hindu Kush Raj of the Hindu Kush mountain range. On the basis of altitude, climate, and vegetation, the area can be divided into many climatic and vegetation zones that vary from sub-tropical chirr pine forest to alpine pastures and meadows.

Figure 1: Map Showing Swat Valley

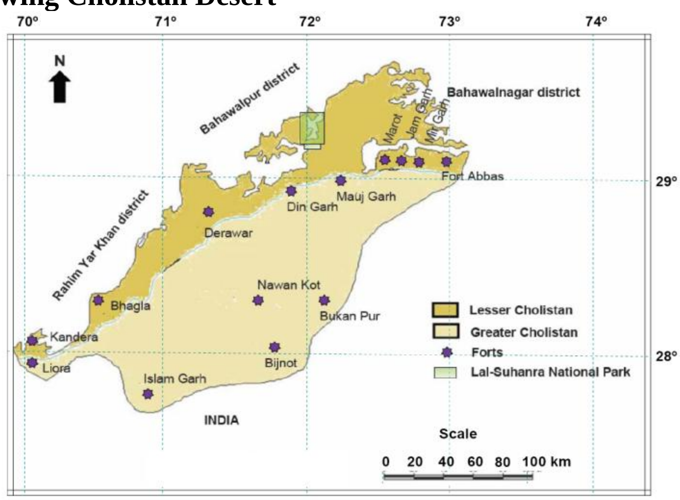


Source: http://www.nccr-pakistan.org/research_mapSwat.html

Cholistan Desert

The Cholistan desert is a part of the world's seventh largest desert, the Great Desert, which stretches along the south border of Punjab province (Fig. 2), Pakistan (Rao *et al*., 1989). The total area of the Cholistan desert is about 26,000 km^2; and it lies between27º 42' and 29º 45' north and 69º 52' and 75º 24' east at an altitude of about 112 m above sea level (Ali, 2009). Topography, soil type and texture, and vegetation structure divide this desert into two distinct regions: the northern region (Lesser Cholistan) covers about 7,770 km^{2,} and the southern region (Greater Cholistan) about 18,130 km^2. One of the important geological features of Cholistan is the old Hakra River, which dried out about 600 years ago. The Hakra riverbed forms the dividing line between the two eco-regions of the desert. Greater Cholistan extends from the most recent course of the extinct Hakra River to the border with India. A gradual change in climate caused a shift in monsoon winds away from the area, resulting in a decline in precipitation, and ultimately converting the area into a desert. Two visits were made in Cholistan desert, one in January 2013, and another in April 2013, for collecting data regarding socio-economic conditions and plant collections.

Figure 2: Map Showing Cholistan Desert



Source: Hameed *et al.* (2011)

METHODOLOGY

The methodologies used in this study are aimed at exploring the DNA profile of medicinal plants and the market situation for medicinal plants in the desert and northern areas of Pakistan. First we will describe the methods used for DNA barcoding analysis and biochemical profiling of selected medicinal plants from these regions, followed by the methods for the socio-economic analysis.

Plant Collection

According to the criteria suggested by Barcode of Life Database (BOLD), at least three populations of several medicinal plant species were collected in this study during the two visits to each region (Swat: October 2012 and May 2013; Cholistan: November 2012 and April 2013). Whole plant (if small) or a branch of plant showing maximum characters for morphological identification was picked for making vouchers, while a few leaves of each sample were preserved in plastic bags containing silica gel for laboratory analysis. All the samples were given specific Field IDs.

Front End Processing

The preliminary requirements of DNA barcoding involve following steps:

MORPHOLOGICAL IDENTIFICATION

Morphological identification of collected plant materials was done with the help of taxonomists in the Department of Botany, University of Agriculture Faisalabad. Plants' names and taxonomical information were confirmed using the Flora of Pakistan Database (http://www.tropicos.org/Project/Pakistan).

PREPARATION OF VOUCHERS

Vouchers of collected plants were made after drying. Plants consisting of leaves, stem, buds, and roots were dried by pressing those between layers of blotting paper and then pasted on 11.5 in x 16.5 in herbarium sheets. Each sheet was labeled with the plants' scientific and common names as well as the date and precise location of collection. Seeds of many of the medicinal plants were also collected and preserved for future reference.

SCANNING OF VOUCHERS

For a reliable and comprehensive DNA barcoding library, all the vouchered specimens were scanned. The scanned pictures were edited according to BOLD format so they could be made available for future reference. Primary data was then recorded using the BOLD systems.

DNA Barcoding

DNA barcoding provides authentic identification of plants on a molecular basis. It involves the isolation of DNA followed by PCR and DNA sequencing of the amplified products.

DNA EXTRACTION

DNA was extracted from plants using two methods:

- **FavorPrep™ plant genomic DNA extraction mini kit:** Plant genomic DNA was extracted by using buffers of FavorPrep™plant genomic DNA extraction mini kit following the manufacturer's protocol.

- **Plant genomic DNA extraction using CTAB:** CTAB method was applied following Doyle (1991).

DNA QUALITY CONFIRMATION

The DNA quality was assessed by running the samples on 0.8% agarose gel by electrophoresis at 80 V for 40 min. The gel was stained in ethidium bromide followed by analysis on Gel Documentation and Analysis System (Syngene, UK).

PRIMER SELECTION

The PCR primers selected for DNA barcoding study are listed in Table 1.

Table 1: Gene Regions and Primer Sequences for DNA Barcoding of Medicinal Plants

Gene and Region	Name of Primer	Primer Sequence 5′-3′	Reference
matK	KIM_3F	CGTACAGTACTTTTGTGTTTACGAG	CBOL Plant Working Group, 2009
	KIM_1R	ACCCAGTCCATCTGGAAATCTTGGTTC	CBOL Plant Working Group, 2009
rbcL	1f	ATGTCACCACAAACAGAAAC	Fay *et al*., 1997
	724r	TCGCATGTACCTGCAGTAGC	Fay *et al*., 1997
trnH-psbA	*trnHf*_05	CGCGCATGGTGGATTCACAATCC	Kress *et al*., 2005
	*psbA*3′f	GTTATGCATGAACGTAATGCTC	Kress *et al*., 2005
ITS	ITS 5F	GGAAGTAAAAGTCGTAACAAGG	White *et al*., 1990
	ITS 4R	TCCTCCGCTTATTGATATGC	White *et al*., 1990

ITS, Internal Transcribed Spacer

AMPLIFICATION METHODS

Polymerase chain reaction (PCR) amplification of the four candidate DNA barcodes was carried out in Bio-Rad Thermal Cycler by the following two methods:

Thermo Scientific Phire Plant Direct PCR Kit

According to the kit's enclosed protocol, a few samples were processed by direct protocol and others by dilution protocol. In direct protocol, a small plant sample (approximately the size of this dot ·) was cut and placed directly in the PCR reaction mixture. In dilution protocol, a small piece of the sample was cut and placed in a 20 µL dilution buffer (provided in the kit). The tube was vortexed briefly and incubated at room temperature for 3 minutes. Following that the sample was centrifuged for 5 minutes and supernatant was used in PCR reaction mixture. The PCR reactions and cycling protocols for amplification were optimized (Table 2).

Thermo-Fermentas PCR reagents and Taq DNA polymerase

The PCR conditions for amplification using Thermo-Fermentas PCR reagents and *Taq* DNA polymerase are given in Table 3.

Table 2: PCR Conditions Optimized for Plant Direct PCR Kit

Pipetting Volumes		**Cycling Protocol**			
Component	25 µL reaction	Cycle step	3 Step Protocol		Cycles
			Temp.	Time	
H_2O	9.5 µL	Initial Denat.	98 ˚C	5 min	1
Phire Plant PCR Buffer (Includes dNTPs and MgCl2)	12.5 µL	Denaturation	98˚C	5 sec	30
		Annealing	X ˚C	1 min	
		Extension	72 ˚C	1 min	
Primer F	1.3 µL	Final Extension	72 ˚C	7 min	1
Primer R	1.3 µL				
Hot Start DNA Polymerase	0.5 µL				
Sample	0.5 mm Punch or 1 µL Diluted Sample				

X=52 ˚C for *mat*K, 56 ˚C for *rbcL*, 55 ˚C for *trnH-psbA*, and 51 ˚C for ITS

Table 3: Optimization of PCR Conditions Using *Taq* DNA Polymerase

Pipetting Volume		Cycling Protocol			
Component	25 µL Reaction	Cycle Step	3 Step Protocol		Cycles
			Temp.	Time	
H_2O	14.4 µL	Initial Denat.	94 ˚C	1 min	1
10X PCR Buffer	2.5 µL	Denaturation	94 ˚C	30 sec	30
2 mm dNTPs Mix	2.5 µL	Annealing	X ˚C	20 min	
25 mm MgCl2	2.5 µL	Extension	72 ˚C	50 sec	
Primer F (15 µm)	0.8 µL	Final Extension	72 ˚C	5 min	1
Primer R (15 µm)	0.8 µL				
DNA Polymerase	0.5 µL				
Seed Sample	1 µL Extracted DNA				

X=55˚C for *mat*K

DNA QUALITY CONFIRMATION

Amplified products were checked in 1% agarose gel by following the procedure given previously.

PURIFICATION OF AMPLICONS

Amplified products were cleaned and purified of primer dimers and other reaction impurities by using FavorPrep™ PCR Clean-Up Mini kit and FavorPrep™ Gel Purification Mini Kit by following the manufacturer's protocols.

SEQUENCING

Purified PCR products were sequenced at the DNA sequencing facility at CAMB (Centre for Applied Molecular Biology), Lahore and the 1st BASE sequencing facilities in Singapore.

SEQUENCE ANALYSIS

Editing sequence traces: Obtained sequences were imported in CodonCode, trimmed, and checked for ambiguous nucleotides. Contigs were obtained by joining a forward and reverse sequence of each plant, and then the consensus sequence was exported from CodonCode for further analysis. Sequence editing was done using the trial version of CodonCode (www.codoncode.com/aligner/).

Database search using BLAST: All the consensus sequences obtained from CodonCode editing were queried in BLAST, one by one, against the sequence database available on the NCBI website.

Sequence uploading on BOLD: All of the barcode sequences that showed more than 98% sequence similarity using BLAST were uploaded on BOLD to join them with their respective preliminary data.
Validation of barcode sequences: BOLD workbench provides several bioinformatics tools for its users to address various research questions. We obtained TaxonID trees separately for *matK*, *rbcL*, and ITS by taking our own sequence library of medicinal plants of Pakistan. Taxon ID trees helped us in validating that our barcode sequences are free from contamination or misidentification. Taxon ID trees were obtained using the Kimura 2 Parameter distance model.

Biochemical profiling

Biochemical profiling of selected plant species was done by LC-MS analysis. Sample preparation before applying the sample to LC-MS was made as follows:

A representative amount (1 g) of the plant was dried and powdered. Following this, 100 mL of methanol was added to the powdered sample in a conical flask and left at room temperature for 36 hours. The extracted solution was filtered with Whattman filter paper. Next, 5 g of Na_2HSO_4 was added into the solution and left for 10 hours at room temperature to absorb water from the solution, and the methanol was evaporated using a rotary evaporator. The solid sample was dissolved in 1 mL of methanol and transferred into a pre-weighed empty glass bottle. The sample was air-dried and the bottle weighed again to obtain the weight of the dried sample. The final concentration of the sample was prepared to be 1 mg/mL.

The sample was filtered into a LC-MS vial with a 0.2 µm filter assembly using 10 µL of filtered sample for LC-MS analysis (Fraser *et al.*, 2012). LC-MS analysis was conducted at the University of California at San Diego, USA.

Socio-Economic Survey

A socio-economic survey of the designated areas was carried out via questionnaire. Research teams, comprising researchers from economics, biochemistry/molecular biology, and botany were sent to Swat Valley and Cholistan Desert for the survey at time intervals already discussed.

SAMPLE SELECTION

The selected areas host many medicinal plant species which have economic, as well as medicinal, importance and provide income generating opportunities to local communities. A representative sample of plant collectors, assemblers/shopkeepers, dealers, and wholesalers was drawn. Stratified random sampling technique was used to select the sample. For the purpose of sample selection and making it representative, locals were asked about main assemblers of medicinal plants in their respective areas (markets) during pre-testing of the questionnaire. Then assemblers (most frequently reported by locals) were interviewed using the questionnaire in each respective area. For the selection of collectors, assemblers interviewed were asked about those collectors who visited them most. Then those most frequently reported collectors by assemblers were interviewed using the questionnaire in that area.

MARKETING CHANNELS

In the study area, the marketing of medicinal plants is carried out by collectors (individuals), assemblers/shopkeepers, and exporters (wholesalers) who are the principal market agents. The existing medicinal plant marketing channels have been identified and their brief functions are as follows:

Collectors

As a majority of the medicinal plants are found in their natural habitat, plant collectors are the primary stakeholders of the marketing channels in both areas. In Swat valley these plants are found at high altitudes (in forests located at the top of mountains), and in Cholistan desert they are found in deep desert areas. Collectors are individuals, or groups of individuals, who collect various species of medicinal plants in the wild and sell them to earn money to support their family. Because of the lack of job opportunities and geographic location of the areas, many people are engaged in the collection of medicinal plants. A total of 80 plant collectors were accounted for in the data collection, including 40 from Swat valley and 40 from Cholistan desert.

Assemblers/Shopkeepers

An assembler/shopkeeper usually buys medicinal plants from local collectors and other selling agents. There are some assemblers in Swat and Cholistan who purchase different species of medicinal plants from collectors and sell them further down to other dealers and wholesalers. A total of 40 assemblers were surveyed, 20 from each area.

Dealers

These are small-scale traders who normally are not involved in supplying inputs or credit. They assemble small amounts of medicinal plants which they sell directly to wholesalers or processing factories. Usually they buy from small assemblers and collectors. They do not themselves arrange for transport from the farm; this is done by their suppliers. However, they do arrange transport from their site to the wholesaler/processing factory.

Wholesalers

Wholesalers buy and sell large quantities of plant products. Usually they perform their business in wholesale markets. They deal in several commodities such as fruits, vegetables, and other agricultural produce within inter-regional markets and also supply produce to processing industries, exporters, and retailers according to their demand. A wholesaler usually purchases medicinal plants from dealers and assemblers and sells in smaller quantities to the retailers (*pansaries*, small retailers of medicinal plants), hakims (traditional healers), and consumers.

DATA COLLECTION

Pre-tested questionnaires were used to collect the data from selected respondents through personal interviews. For this purpose, different questionnaires were designed for different market actors. Data was collected in intervals from October 2012- May 2013. After data collection, the interview schedules were properly checked to make sure that all the responses had been recorded accurately. After editing the interview schedules, data was transferred from the questionnaires to computerized spreadsheets.

STATISTICAL TECHNIQUES FOR DATA ANALYSIS

Following collection, data was edited, analyzed, and interpreted following the statistical techniques detailed below.

Marketing Margin

For calculating the margins of different stakeholders, we used the following formula:

$$MM = Ps / Sp * 100$$

Where; MM = Marketing Margin, Ps = Price spread, Sp = Sale price, and Price spread = Sale price – Purchase price

Gross Margin

Gross margins are calculated as value of output (revenue) less variable costs.

The profit margins of traders, assemblers, and wholesalers in the supply-chain are assessed by using the partial budgeting approach (Vodouhe *et al*., 2008):

$$GM_1 = \text{Selling Price} - \text{Buying Price}$$

Where GM_1 is the actor's (traders, assemblers, and wholesalers) unit gross margin.

The Gross margins (GM_2) of collectors are estimated as:

$$GM_2 = \text{Selling Price} - \text{Collection costs}$$

Collectors usually harvest medicinal plant parts directly and do not pay any royalties. The collection costs cover equipment and labour costs. Wage rates for farm activities were taken as the labor opportunity cost. Therefore, the collection costs are

$$\text{Collection Cost} = (\text{Total man days involved} * \text{Wage rate} + \text{cost of equipment}) / \text{Total quantity sold.}$$

Net Margin

$$NM = GM - TC$$

Where; NM = Net Margin, GM = Gross Margin, and TC = Total cost

The Total Costs for collectors is TC1. Where TC1 = Marketing Cost + Transportation Cost

The Total Costs for Assemblers is TC2. Where TC2 = Transportation cost + labor cost + utility bills payment + rent paid + market fee paid

Marketing Costs

Marketing costs are defined as:

$$MC = AS \times QH$$

Where 'MC' stands for the marketing cost of a specific unit quantity, 'AS' for actual amount spent, and 'QH' represents quantity handled.

Field Experience

Researchers spent several weeks in the field for data collection in both regions and faced several problems in conducting interviews. In Pakistan, where few people are familiar with social research and its utility, the major problem faced by the researchers was explaining the purpose of their research and also convincing the respondents of the confidentiality of the information provided by them. Another problem faced by the researchers was obtaining information regarding income, as most of the respondents were hesitant to answer these questions. Some of the respondents believed the researchers to be tax collectors. However, these misunderstandings were alleviated through appropriate explanations and by winning the confidence of respondents.

RESULTS AND DISCUSSION

Plant Collection

In two different seasons, 36 plant species (shown in Table 4) were collected from Swat valley, and 55 plant species (shown in Table 5) were collected from Cholistan desert. The plant species were taxonomically identified by the Department of Botany, University of Agriculture Faisalabad.

Morphological identification revealed that 163 plant samples collected from Cholistan desert belonged to 55 species, 47 genera, 25 families, and 14 orders. The 48 plant samples collected from Swat represented 36 medicinal species belonging to 35 genera, 27 families, and 17 orders. Two vouchers were prepared for each species, one was deposited in Herbarium, Department of Botany, University of Agriculture Faisalabad and the other is kept in Molecular Biochemistry Lab, Department of Biochemistry University of Agriculture Faisalabad.

Table 4: Medicinal Plant Species Collected from Swat Valley

Sr. No.	Plant Name	Family	Order
1	*Achyranthes aspera*	Amaranthaceae	Caryophyllales
2	*Adiantum incisum*	Pteridaceae	Polypodiales
3	*Adiantum venustum*	Pteridaceae	Polypodiales
4	*Amaranthus hybridus*	Amaranthaceae	Caryophyllales
5	*Anethum graveolens*	Apiaceae	Apiales
6	*Artemisia sp.*	Asteraceae	Asterales
7	*Asparagus adscendens*	Asparagaceae	Asparagales
8	*Berberis lycium*	Berberidaceae	Ranunculales
9	*Bergenia ciliata*	Saxifragaceae	Saxifragales
10	*Cannabis sativa*	Cannabaceae	Rosales
11	*Cedrus deodara*	Pinaceae	Pinales
12	*Convolvulus arvensis*	Convolvulaceae	Solanales
13	*Dioscorea bulbifera*	Dioscoreaceae	Dioscoreales
14	*Dryopteris ramosum*	Dryopteridaceae	Polypodiales
15	*Euphorbia helioscopia*	Euphorbiaceae	Malpighiales
16	*Ficus palmata*	Moraceae	Rosales
17	*Foeniculum vulgare*	Apiaceae	Apiales
18	*Fragaria sp.*	Rosaceae	Rosales
19	*Indigofera heteantha*	Fabaceae	Fabales
20	*Inula grandiflora*	Asteraceae	Asterales
21	*Juglans regia*	Juglandaceae	Fagales
22	*Lantana camara*	Verbenaceae	Lamiales
23	*Mentharoyleana*	Lamiaceae	Lamiales
24	*Morus alba*	Moraceae	Rosales
25	*Oxalis corniculata*	Oxalidaceae	Oxalidales
26	*Pinus wallichiana*	Pinaceae	Pinales
27	*Plantago lanceolata*	Plantaginaceae	Lamiales
28	*Quercus incana*	Fagaceae	Fagales
29	*Rumex nepalensis*	Polygonaceae	Caryophyllales
30	*Salix tetrasperma*	Salicaceae	Malpighiales
31	*Salvia moorcroftiana*	Lamiaceae	Lamiales
32	*Sarcococcas aligna*	Buxaceae	Buxales
33	*Silene apetala*	Caryophyllaceae	Caryophyllales
34	*Urtica dioica*	Urticaceae	Rosales
35	*Urtica dioica*	Urticaceae	Rosales
36	*Daphne sp.*	Thymelaeaceae	Malvales

Table 5: Medicinal Plant Species Collected from Cholistan Desert

S. No.	Plant name	Family	Order
1	*Abutilon muticum*	Malvaceae	Malvales
2	*Acacia jacquemontii*	Fabaceae	Fabales
3	*Acacia modesta*	Fabaceae	Fabales
4	*Acacia nilotica*	Fabaceae	Fabales
5	*Achyranthes aspera*	Amaranthaceae	Caryophyllales
6	*Aerva javanica*	Amaranthaceae	Caryophyllales
7	*Aizoon canariense*	Aizoaceae	Caryophyllales
8	*Aristida sp.*	Poaceae	Poales
9	*Asphodelus tenuifolius*	Xanthorrhoeaceae	Asparagales
10	*Calligonum polygonoides*	Polygonaceae	Caryophyllales
11	*Calotropis procera*	Apocynaceae	Gentianales
12	*Capparis aphylla*	Capparaceae	Brassicales
13	*Cenchrus ciliaris*	Poaceae	Poales
14	*Chenopodium album*	Amaranthaceae	Caryophyllales
15	*Cirsium arvense*	Asteraceae	Asterales
16	*Citrullus colocynthis*	Cucurbitaceae	Cucurbitales
17	*Conyza bonariensis*	Asteraceae	Asterales
18	*Corchorus depressus*	Malvaceae	Malvales
19	*Cressa cretica*	Convolvulaceae	Solanales
20	*Crotalaria burhia*	Fabaceae	Fabales
21	*Cymbopogon jwarancusa*	Poaceae	Poales
22	*Cyperus conglomeratus*	Cyperaceae	Poales
23	*Dipterygium glaucum*	Capparaceae	Brassicales
24	*Euphorbia prostrata*	Euphorbiaceae	Malpighiales
25	*Fagonia indica*	Zygophyllaceae	Zygophyllales
26	*Farsetia hamiltonii*	Brassicaceae	Brassicales
27	*Gisekia pharnacioides*	Gisekiaceae	Caryophyllales
28	*Haloxylon solicornicum*	Amaranthaceae	Caryophyllales
29	*Haloxylon stocksii*	Amaranthaceae	Caryophyllales
30	*Heliotropium europaeum*	Boraginaceae	Gentianales
31	*Heliotropium strigosum*	Boraginaceae	Gentianales
32	*Indigofera hochstetteri*	Fabaceae	Fabales
33	*Lasiurus scindicus*	Poaceae	Poales
34	*Launaea nudicaulis*	Asteraceae	Asterales
35	*Leptadenia pyrotechnica*	Apocynaceae	Gentianales
36	*Limeum indicum*	Limeaceae	Caryophyllales
37	*Neurada procumbens*	Neuradaceae	Malvales
38	*Oligochaeta ramosa*	Asteraceae	Asterales
39	*Peganum harmala*	Nitrariaceae	Sapindales
40	*Prosopis cineraria*	Fabaceae	Fabales
41	*Pulicaria crispa*	Asteraceae	Asterales
42	*Salsola imbricata*	Amaranthaceae	Caryophyllales
43	*Salvadora sp.*	Salvadoraceae	Brassicales
44	*Solanum nigrum*	Solanaceae	Solanales
45	*Solanum virginianum*	Solanaceae	Solanales
46	*Sonchus arvensis*	Asteraceae	Asterales
47	*Suaeda fruticosa*	Amaranthaceae	Caryophyllales
48	*Tamarix aphylla*	Tamaricaceae	Caryophyllales
49	*Tamarix dioica*	Tamaricaceae	Caryophyllales
50	*Tribulus longipetalous*	Zygophyllaceae	Zygophyllales
51	*Tribulus pentandrus*	Zygophyllaceae	Zygophyllales
52	*Tribulus terrestris*	Zygophyllaceae	Zygophyllales
53	*Withania somnifera*	Solanaceae	Solanales

54	*Zaleya pentandra*	Aizoaceae	Caryophyllales
55	*Zizyphus nummularia*	Rhamnaceae	Rosales

INITIATION OF PROJECT ON BOLD

The spreadsheet obtained from the BOLD website was completed with all available metadata such as taxonomic information, habit and habitat of medicinal species, and GPS coordinates of collection sites. Photos of all vouchers were also edited according to BOLD requirements. The spreadsheet and photos were uploaded on BOLD under the project name 'DNA Barcoding of Medicinal Plants of Pakistan (DBMPP). Thus the first step of creating the DNA barcode library of medicinal plants of Pakistan was completed. Collection data can be accessed on www.boldsystems.org/. Process IDs allotted by BOLD for species with uploaded sequences are shown further on in Table 6.

DNA Barcoding

DNA barcoding is an efficient technique in the tool box of taxonomists. However, it is an emerging technique in Pakistan, and therefore had to be optimized for the local plant species. After front end processing of collected medicinal species, a series of experiments were conducted for this purpose.

DNA EXTRACTION

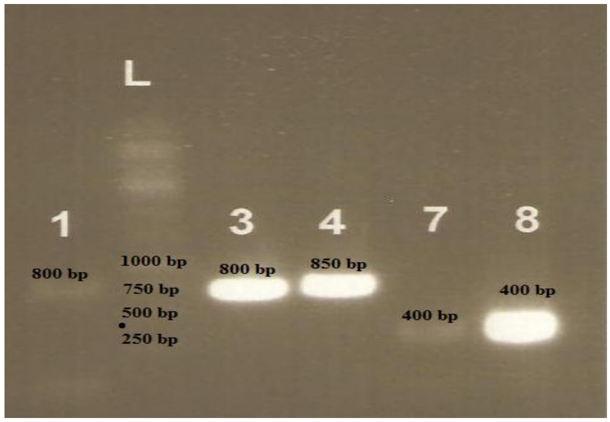
Isolation of DNA is an important step in this technique, which is quite time consuming. We used an alternative strategy, Plant Direct PCR, where this step was omitted. The species for which Direct Plant PCR repeatedly failed, we optimized both methods and achieved successful results i.e. amplification of the targeted DNA sequences. However, in order to obtain a complete picture of the collected medicinal plants, more time was needed. The results obtained during the report period are discussed below.

PCR AMPLIFICATION OF SELECTED BARCODE REGIONS

Lab analyses were initiated from Direct plant PCR which gave successful amplification of only 30% plants with different markers. Amplicons obtained from this method were clean and of good quality (Fig. 3 and 4).

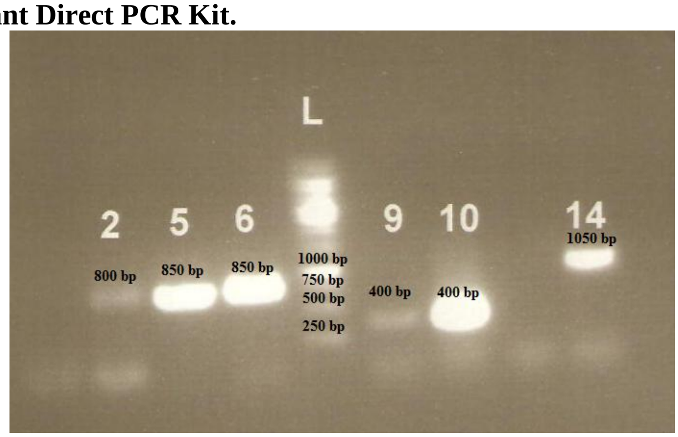
Plant samples which were not successful with Direct PCR kit were processed for DNA extraction with other DNA extraction protocols (Fig. 5). After obtaining a plant's maximum DNA, PCR amplification was done by using *Taq* polymerase method (Fig. 6). Out of 211 samples, the *matK* gene was amplified (850-950 bp) for 60 plant specimens made up of 58 species. *rbcL* of 140 plants (76 species) was successfully amplified (650-750 bp long). ITS of 162 plant samples (81 species) was successfully amplified giving 250-450 bp amplicons. While *trnH-pbA* gave the least species amplification, so we omitted from our further experimentation because multiple trials for amplification showed that it requires species specific primers which were beyond the budget of the project.

Figure 3: Representative PCR for DNA Barcode Regions of Some Medicinal Plants with Phire Plant Direct PCR Kit.



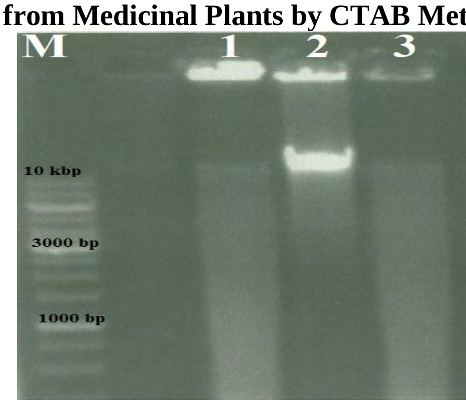
1=ITS of *Jatropha gossypifolia*, L=1kb DNA ladder, 3=rbcL of *Ocimumbasilicum*, 4=rbcL of *Jatropha gossypifolia*, 7=trnH-psbA of *Linumusitatissimum*, 8=ITS of *Asteracanthalingifolia*

Figure 4: Representative PCR for DNA Barcode Regions of Some Medicinal Plants with Phire Plant Direct PCR Kit.



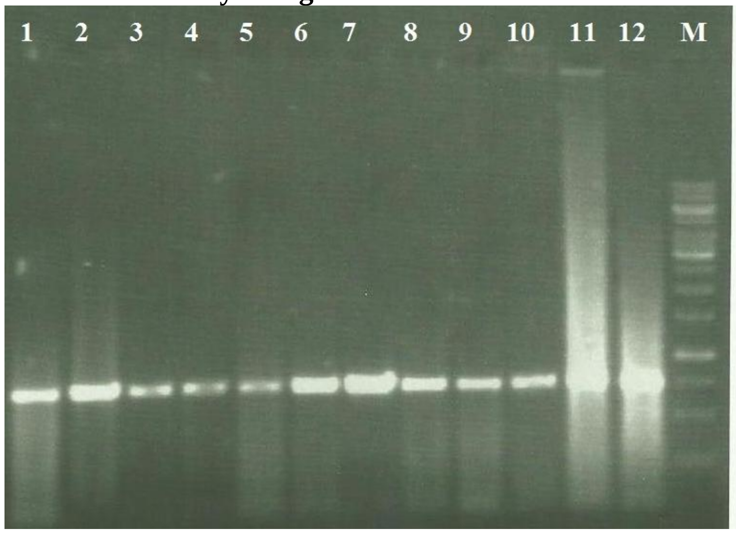
2=rbcL of *Mimosa pudica*, L=1kb DNA ladder, 5&6=matK of *Jatropha gossypi folia* &*Achyranthes aspera*, 9=trnH-psbA of *Jatropha gossypi foli*, 10=ITS of *Achyranthes aspera*, 14=*matK* of *Withani acoagulans*

Figure 5: DNA Extraction from Medicinal Plants by CTAB Method



M=1kb DNA ladder, 1= *Cichorium intybus*, 2= *Plantago ovate*, 3= *Foeniculum vulgare*

Figure 6: Amplification of *matK* by Using Extracted DNA of Different Plant Species



SEQUENCING OF AMPLICONS

Successfully amplified samples were sent for sequencing with both forward and reverse primers. After CodonCode editing and BLAST analysis, sequence data was uploaded on BOLD (Appendix-I). This study contributed almost 200 barcode compliant sequences (covering 90 species) to the database with different barcoding regions (Table 6).

Table 6: Summary of Barcoded Medicinal Plant Species Investigated Under the Study

Locality	No. of Medicinal Plants Species Processed for Sequencing	No. of Species Successfully Barcoded			No. of Species with Barcode Compliance Sequences with Either Marker
		matK	rbcL	ITS	
Cholistan desert	54	40	48	48	42
Swat valley	34	15	23	22	16

BLAST ANALYSIS AND SEQUENCE UPLOADING ON BOLD

Nucleotide sequences obtained after the sequencing of amplified regions were trimmed and cleaned from ambiguous nucleotides in CodonCode. *matK* and *rbcL* were also checked for stop codons as both of these are coding regions. A few of the plant species' amplicons encountered sequencing problems, and the data was not worth analyzing. Then all the sequences were searched for their matches against GenBank database using BLAST.

The study revealed that ITS has maximum species resolution ability while *rbcL* identified most of the taxa (60%) up to either genus or family level. *matK* sequences were found to be difficult while editing due to presence of stop codons that appeared to be a sequencing problem, but this region also gave better species resolution for the species which were present in database.

Although *matK*, *rbcL*, and ITS showed 97%-99% sequence similarity, they identified most specimens up to family or genus level. The sequences that showed better species resolution with any of the three regions were submitted in GenBank. For instance *matK* gene sequence of *E. sativa* showed 100% similarity with the same species. Eighteen sequences of *matK*, 6 of *rbcL*, and 3 of ITS were submitted in GenBank, and their accession numbers are as follows KF803250 (*Nigella sativa*), KF803251 (*Nepeta cataria*), and KF551972 (*Jatropha gossypifolia*). Other sequences, specifically of *matK* and *rbcL*, need detailed treatment prior to submission which is currently being done. Moreover, *rbcL* sequences of six taxa were also successfully submitted and are online now with GenBank with accessions KJ008939 (*Eruca sativa*), KJ008940 (*Jatropha gossypifolia*), KJ008941 (*Mimosa pudica*), KJ008942 (*Nepetacataria*), KJ008943 (*Ocimum basilicum*), and KJ008944 (*Salvia virgata*). Other sequences are uploaded on BOLD and kept on hold to be submitted in GenBank until research publication. Out of the total 91 medicinal species from both regions, sequences of 58 species with *matK*, 76 species with *rbcL*, and 81 species with ITS are on BOLD. This study showed that chloroplast gene *matK* was relatively difficult to amplify, but its identification capacity is comparatively remarkable.

In order to generate the maximum number of barcodes for the DNA barcode library of medicinal plants of Pakistan, we made multiple attempts to optimize amplification protocols for *matK* and the other two regions. *trnH-psbA*, which is an intergenic region of chloroplast DNA, showed a low PCR amplification rate and generated variable amplicon lengths for different taxa, hence we concluded that for the *trnH-psbA*, a single primer pair may not be applicable to all land plants. Chloroplast genomic region, *rbcL*, was found to be the most appropriate region for DNA barcoding because its amplification and sequencing was found easier and to be more reproducible, but *rbcL* alone was not found suitable for species level resolution, so another region like ITS should be used as well for accurate results. The PCR success rate of *rbcL* was 100% in different medicinal species with a single primer pair and under the same conditions. Similarly, although the nuclear ITS region didn't show a significant success rate in PCR amplification, the taxa, for which it was amplified, were identified up to the species level.

Therefore, according to this study, *matK*, *rbcL* and ITS appeared appropriate DNA markers for medicinal plant identification and phylogenetic studies. *trnH-psbA* and *matK* require species to species optimization of all steps in the identification process. When we discuss the species discrimination capacity of DNA markers for identification up to the species level, we must generate reference data prior to using this technique for authenticated species discrimination, only then we can make best use of this tool.

VALIDATION OF BARCODES USING TAXON-ID TREE:

Taxon ID trees were obtained separately for *matK*, *rbcL*, and ITS using sequence analysis tools on BOLD. There was only one option of tree i.e. Neighbour Joining. After aligning sequences in MUSCLE (Multiple Sequence Comparison by Log-Expectation), the K2P was chosen as a distance model. Braches of the trees were labeled with region of collection, order, and family along with species name so that the cladding pattern could be read thoroughly. Specimens belonging to the same family were colored alike. Taxon ID trees for *matK*, *rbcL*, and ITS are shown in Appendix II, Figures A.1, A.2, and A.3 respectively. Taxon ID trees were not for phylogenetic analysis but to identify misidentified or contaminated samples.

Fifty eight *matK* sequences of 58 species and 30 families were present in our project which was included in this analysis. It was observed that *matK* sequences showed cladding patterns in accordance with their taxonomy. As the dataset was so brief, it simply pointed out one plant with morphological identification as *Heliotropium strigosum* (order: Genitales) grouped with a species belonging to order Solanales, and the same pattern was observed in the ITS tree, so it was concluded as contamination after comparing the voucher photos. The *matK* tree also showed sequence quality issues with *Aizoon canariense* and *Zaleya pentandra*.

Several other sequences were found problematic by analyzing *rbcL* (127 sequences) and ITS (156 sequences) taxon ID trees such as *Cressa cretica*, *Plantago lanceolata*, all the species of order Rosales, Malvales, and Fabales. *Salix tetrasperma* and *Urtica dioica* were placed in irrelevant clades. Similarly, *Heliotrpium strigosum and Euphorbia prostrate* in *rbcL* were found to be in the wrong clades. All doubtful specimens are under consideration and ambiguities will be resolved and updated on BOLD.

IDENTIFYING ADULTERATION USING DNA BARCODES

The use of medicinal plants for healthcare and herbal medicine is increasing worldwide evidenced by the growing international market for medicinal plants. The higher the demand for herbal materials, the higher the chance of adulteration in their recipes. Adulteration is common, whether it be the materials have indistinguishable morphological attributes, the plant materials have the same common names, or intentional mixing of expensive materials with invaluable herbs. By using traditional identification methods, dried, processed, or damaged medicinal plant materials are almost impossible to identify. The latest molecular biological methods are a reliable alternative tool for species discrimination, and the generation of DNA barcodes is the most recent move to explore the identification of herbal mixtures.

A case study was performed to probe the species discrimination capacity of DNA barcodes. An equal amount of morphologically identified samples of the plant species *Eruca sativa* and *Jatropha gossypifolia* were ground together and processed for sequencing by amplifying an 800 bp portion of *matK* gene of chloroplast genome. The barcode of *E. sativa* was already submitted in GenBank in the beginning of project, but that of *J. gossypifolia* was not submitted. Keeping all the parameters under consideration, BLAST analysis of the 10 cloned amplicons' sequences obtained from the mixture of the plant samples revealed 100% identity with the *Eruca vesicaria* sub sp. sativus which was originally present in the mixture and whose barcode was also submitted to the GenBank database for comparison. Although this sequence was also matched with other plants, the similarity was below 99%, and the sequence of none of the clone was matched with the *Jatropha gossypifolia*. The reason for this is the unavailability of its barcode in the database. Another mixture was analyzed, and similar results were obtained. After which it was concluded that DNA barcoding was an accurate and authentic method to identify adulteration in herbal mixtures, but there is a need to generate a comprehensive barcode library of medicinal plants' for identification and confirmation of species.

CONCLUDING REMARKS ABOUT DNA BARCODING

Rapid and accurate methods of species discrimination is a common issue in ethnobotany. Plant parts which are indistinguishable are almost impossible to be assigned to any species because classical taxonomy identification methods demand specific physical characters (Kritpetcharat*et al*., 2011). Conservation planners need such a tool in their toolbox which could help them in identifying and preserving plant species even when an expert taxonomist is not available. Using molecular markers for managing large numbers of plant species in a bio diverse region, and for the prevention of the extinction of a species, are new concepts for facing several old challenges (Valentini*et al*., 2008).

In this regard, one major issue is establishing a reference library of DNA sequences (Schori and Schowalter, 2011). The selection of standard genome loci possessing three main qualities i.e. (i) universality, (ii) sequence quality, and (iii) discriminatory power can help in generating comparative data covering all potential markers (Hollingsworth *et al*., 2011). Improvements in DNA sequencing technology have resulted in generating sequence data for barcode markers in publically accessible databases (Valentini*et al*., 2008). However, at times the quality of the data appears to be substandard due to errors in sequencing or taxonomic problem etc. (Harris, 2003). In this study, globally studied regions (*matK*, *rbcL*, *trnH-psbA*, and ITS) were selected to generate reference data of the medicinal plants.

Available universal primers for *matK* (KIM_3F/KIM_1R) resulted in 99% successful PCR and sequencing in contrast to the previous studies of Hollingsworth *et al*. (2011). Still, modifications are underway to be made to existing universal primers for designing clade-specific primers to enhance their success rate for angiosperms and land plants (Wicke and Quandt, 2009). In other studies, the unpredictable nature of the *matK* region requires separate primer design for each clade. Primer infidelity has also been observed in the case of *matK* (Schori and Schowalter, 2011). The *matK* region has high inter and intraspecific variability, so medicinal materials belonging to various geographical origins were successfully identified by using *matK* regions (Yan *et al*., 2008).

According to the CBOL Plant Working Group (2009), another protein coding region of chloroplast genome *rbcL* has a successful amplification rate of PCR in higher altitude plants. Because of less interspecific variation in this region, it has low discriminatory power to identify species, but it can work well if combined with the *trnH-psbA* intergenic region (Kress and Erickson, 2007). Here, the CBOL Plant Working Group's proposed primers for *rbcL* resulted in successful amplifications in six species of seeds.

Detailed study and trimming is needed before making obtained data available to the public database after sequencing.

The *trnH-psbA* intergenic spacer region did not show effective success in PCR amplifications. Multiple efforts were made to optimize the PCR conditions, and ultimately eight species of plants gave 450-650bp long PCR products. This region has the highest discrimination rate among all loci. The nuclear ITS region is the most frequently sequenced in plant phylogenetic studies (Kress *et al*., 2005). According to another study, ITS has the highest discriminatory power and is used to identify adulterants in herbal mixtures. Fourteen *Hedyotis sp.* were successfully differentiated by amplifying and sequencing the ITS region (Karehed *et al*., 2008; Li *et al*., 2010). Keeping these advantages in mind, DNA barcoding has obtained greater importance in the field of conservation and biodiversity in recent years.

Another challenge in the application of DNA barcoding is the authenticity of barcode sequences. DNA extraction may be a difficult step if dry or damaged samples are collected for identification. It is recommended that once a barcode region has been obtained, it should be evaluated before using it for species identification. According to Wang *et al*. (2001), currently available barcodes are poor in differentiating among species belonging to different families, which is why they are not universally applicable. Bioinformatics tools provide support for barcoding data analysis and management. Pursuing the goal of making DNA barcoding an influential tool for identification of plant specimens, it is suggested to establish multiple voucher sequences for each different barcode, especially intraspecific variable barcoding regions. The research goes on to describe that no matter what part of plant is used it should be compared successfully to reference sequences in the database (Schori and Schowalter, 2011).

Outcomes of the current research will contribute significantly in the establishment of DNA barcoding analysis of medicinal plant species in Pakistan. Checking for adulteration in medicinal plants would be quite useful in providing high quality herbal medicine and in monitoring the plant markets.

Biochemical Profiling

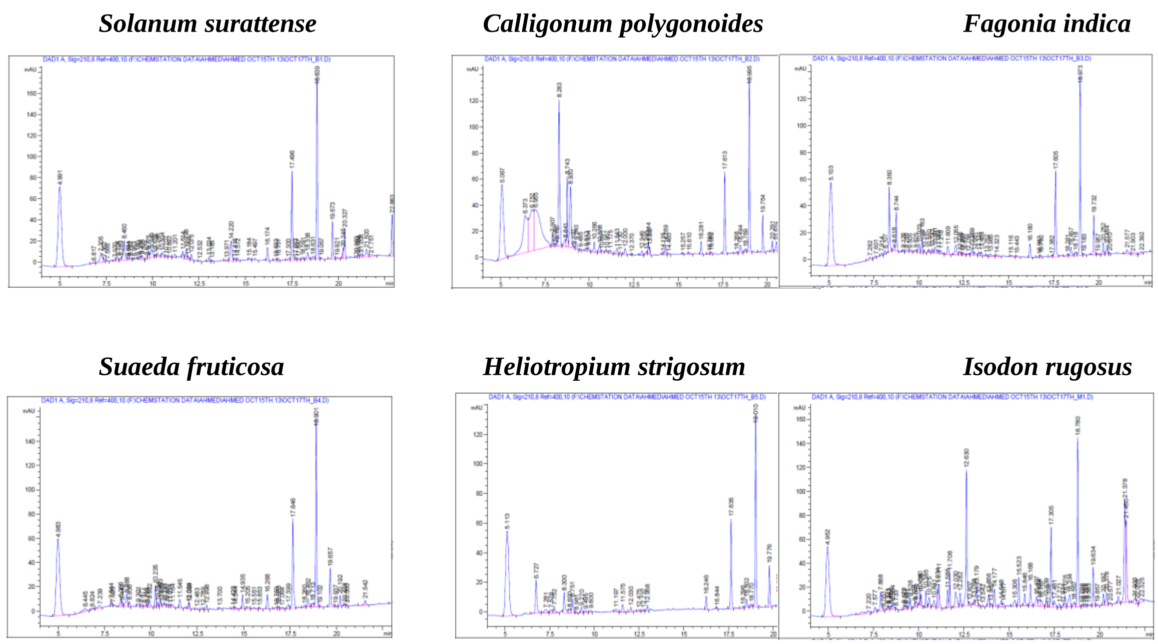
Biochemical profiling of twelve selected medicinal plant species (Table 6) was carried out. Representative chromatograms for LCMS analysis of the selected medicinal plants are also shown in Fig. 7. Selected medicinal plants for LCMS were those which had bioactivities against a wide range of diseases according to the local people of northern and desert areas. Due to the presence of potent bioactivity, these are also plants which are exported.

Table 6: Medicinal Plants for Biochemical Profiling

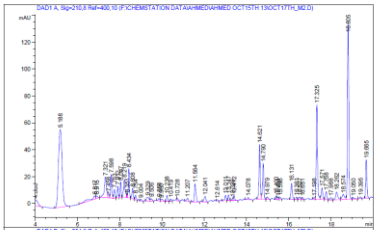
Sr. No.	Botanical Name	Locality
1	*Dryopteris ramosa*	Swat Valley
2	*Bergenia ciliata*	Swat Valley
3	*Quercus baloot*	Swat Valley
4	*Isodon rugosus*	Swat Valley
5	*Fragria bucharia*	Swat Valley
6	*Valeriana jatamansi*	Swat Valley
7	*Trillium govanianum*	Swat Valley
8	*Solanum surattense*	Cholistan Desert
9	*Calligonum polygonoides*	Cholistan Desert
10	*Fagonia indica*	Cholistan Desert
11	*Suaeda fruticosa*	Cholistan Desert
12	*Heliotropium strigosum*	Cholistan Desert

The biochemical profiling revealed that most of the tested plant species had bioactive compounds that could be used as an anticancer, anti-inflammatory, antimicrobial, antiviral, etc. The greatest number of compounds were detected in *Quercus baloot* and *Dryopteris ramosa*. A library search was also conducted to investigate active ingredients, and the results are presented in Table A.2 in the Appendix III. The most potent compounds were compared with those reported in literature. LCMS analysis revealed that the plants in question had reactions against a broad range of diseases. A literature review was conducted for each of the compounds detected in the medicinal plants for its potential use as medicine. It was found that the selected medicinal plant species had great potential to be used against the reported diseases.

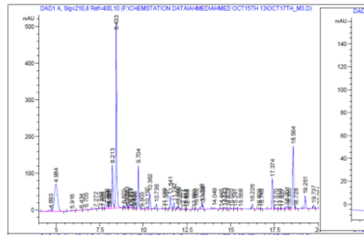
Figure 7: Representative Chromatograms of Medicinal Plants Obtained after LC-MS



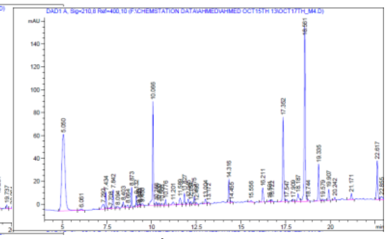
Fragria bucharia



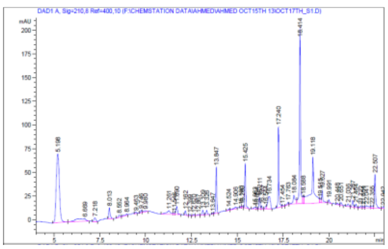
Valeriana jatamansi



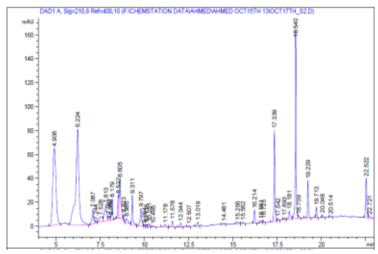
Trillium govanianum



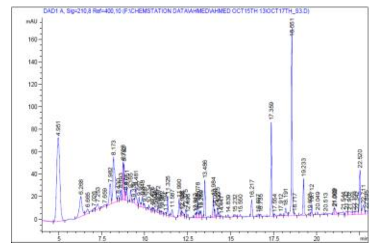
Dryopteris ramosa



Bergenia ciliata



Quercus baloot



Further research is needed to explore the possibility of isolating such active ingredients from the plant species. Due to scarcity of funds and time during the current project, it was not possible to perform biochemical profiling of all the collected medicinal plant species, but if accomplished, it would generate useful information for herbal medicine. Biochemical profiling will help establish information regarding the active ingredients in medicinal plants which could contribute to the export of the plant species at competitive costs rather than selling at the currently low prices.

Socio-economic Analysis

The social and economic variables obtained from the survey of respondents and field visits are discussed in detail in the following sections. These are broken down by demographic variables, stakeholders and their perceptions, stakeholder margins, and a brief market analysis of the medicinal plant marketing chain.[1]

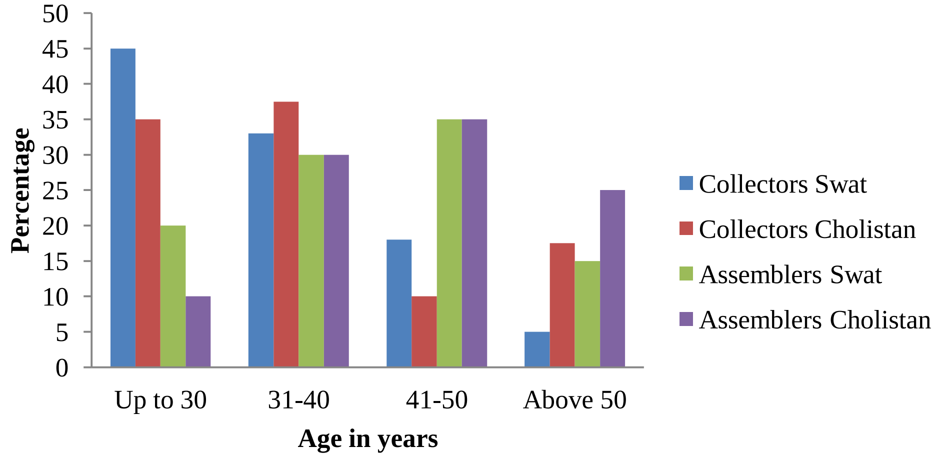
DEMOGRAPHIC VARIABLES

Socio-economic and demographic variables are important in social science research as these are the basis for dividing up the market actors whose roles in the buying and selling decisions are vital. The important demographic variables such as age, relevant field experience, family size, family type, income, and education were collected and studied.

The age distribution of respondents (both from Swat and Cholistan) is given in Fig. 8 which shows that the majority of stakeholders were reasonably mature. In Swat valley relatively more young people, being up to 30 years old, were involved in the collection business.

[1]Two awareness workshops were also held, one in Swat and one in Cholistan. Information about these workshops, and pictures from the workshops, can be found in Appendix IV.

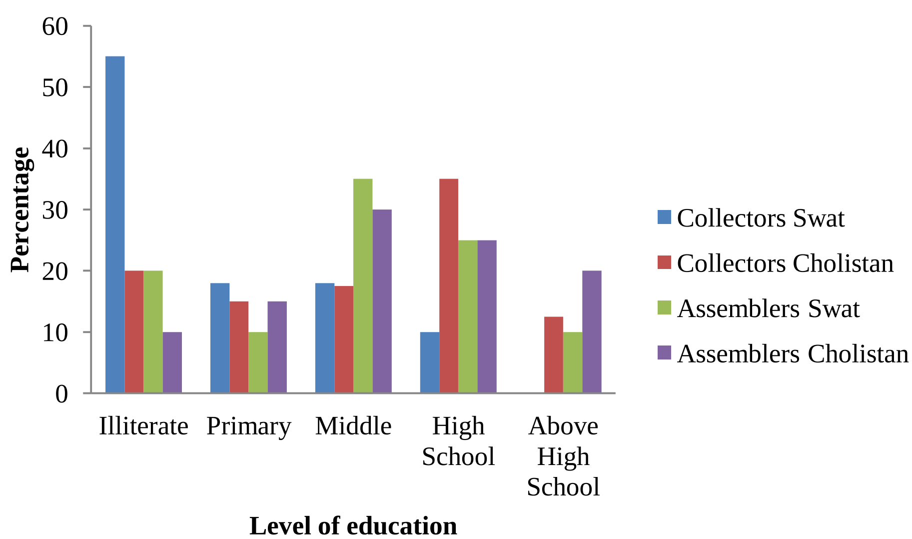
Figure 8: Respondents' Age



The data comprises of 20 assemblers and 40 collectors from each region.

The results regarding education level showed that 30% of the respondents were illiterate and had never gone to school (Fig. 9). This might be because of the fact that education has always been given less importance in areas like Swat and Cholistan. Interestingly, the Cholistan collectors were found to be more educated as compared to the collectors from Swat; a high proportion of the Cholistan collectors had some high school education, whereas a large fraction of Swat collectors were found to be illiterate. The differences were much less noticeable for assemblers, but again Swat had more illiterate assemblers and Cholistan had more assemblers with post-high school educations. Educated persons are more likely to adopt new technologies and thus may use better collection and marketing practices to get the premium price for their medicinal plants. This is normally expected as education increases. However, just this difference in education between the two regions did not make a large difference with reference to medicinal plants. This is due to the absence of marketing channels for medicinal plants in Cholistan desert, as discussed later in this report.

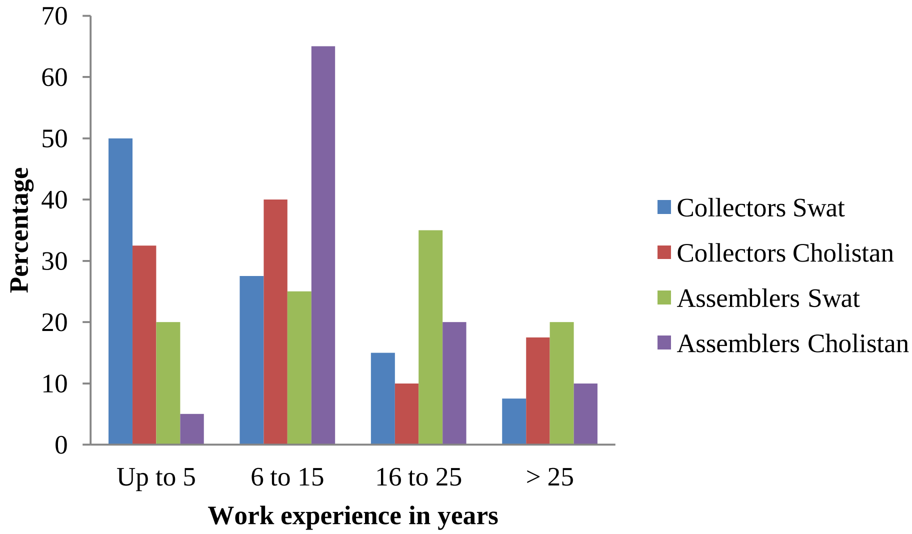
Figure 9: Respondents' Education



The data comprises of 20 assemblers and 40 collectors from each region.

With reference to work experience, 37.5% of the respondents had work experience ranging from 6-15 years (Fig. 10), meaning the marketing and collection of medicinal plants was being carried out by quite experienced persons. A more experienced individual is more likely to engage in collection and marketing activities with a greater degree of ease and efficiency and can earn more income from the same work as compared to less experienced worker.

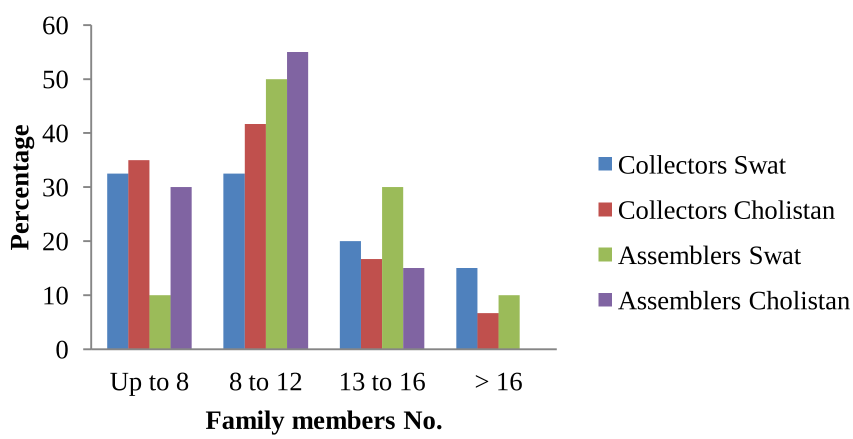
Figure 10: Respondents' Work Experience



The data comprises of 20 assemblers and 40 collectors from each region.

The majority of the respondents had a family size ranging from 8-12 members (Fig. 11). The number of family members affects income level and working hours of an individual. Generally, a person with more family members has to work more in the field to support the family. With a fixed level of income, increases in family size causes the per capita income of the family to decline.

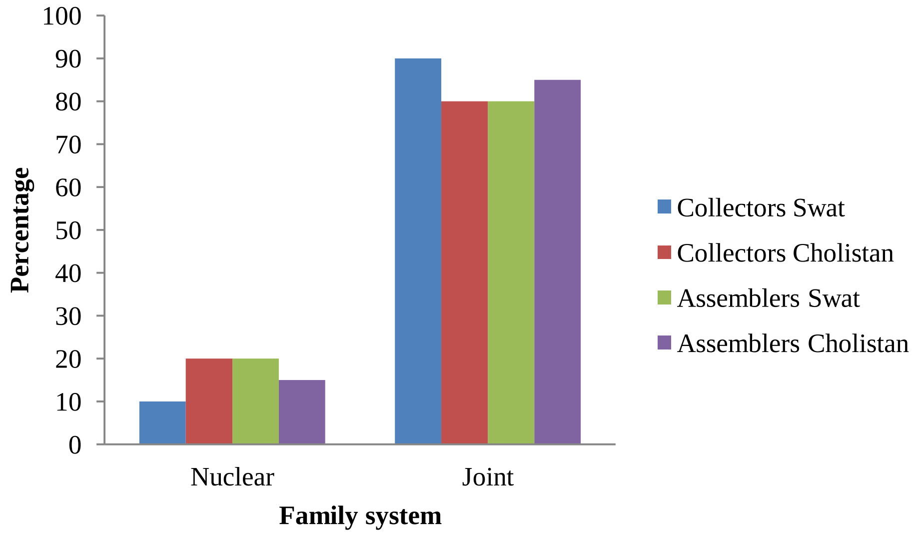
Figure 11: Respondents' Number of Family Members



The data comprises of 20 assemblers and 40 collectors from each region.

It was found that the majority (82.5%) of the respondents were living under a joint family system (Fig. 12). This is due to the fact that the joint family system is a part of the culture and traditions of both the regions, where the children look after their elderly parents. Both joint and nuclear family systems have certain advantages and disadvantages that directly affect the social life of an individual. There is a certain degree of correlation between social life and income level. Comparing of the two regions, with reference to family system, revealed that the Cholistan collectors had a slightly higher ratio of nuclear family systems as compared to the Swat collectors. This might be due to the nomadic versus stationary lifestyle in the two regions, respectively.

Figure 12: Respondents' Family System

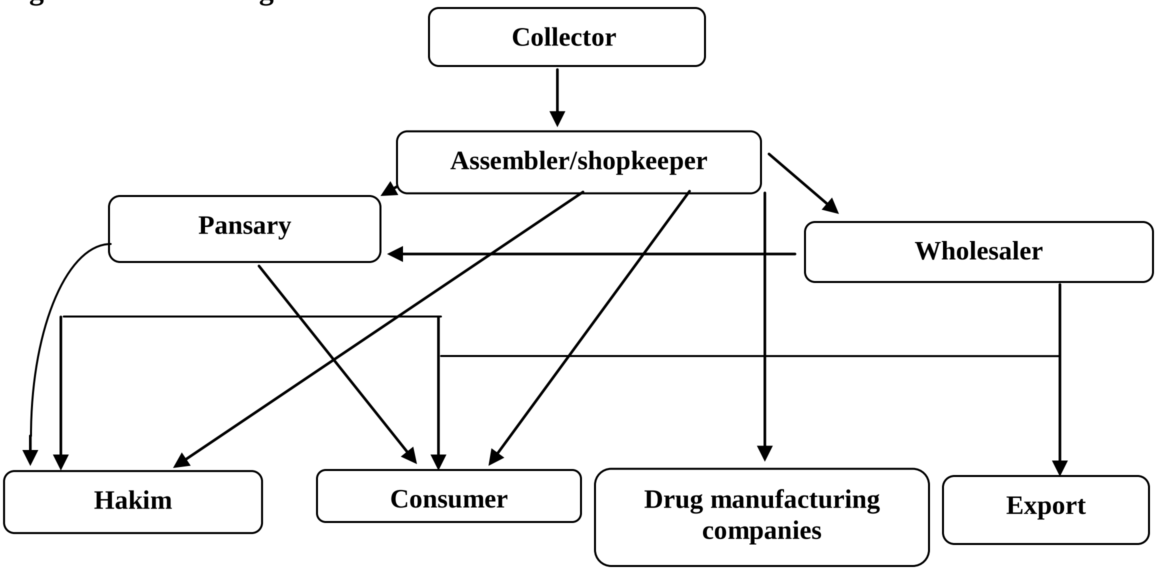


The data comprises of 20 assemblers and 40 collectors from each region.

MARKETING CHANNELS OF MEDICINAL PLANTS

Marketing channels refer to the outlets or routes through which medicinal plants reach the final consumers. The marketing channels of the medicinal plants found in Swat valley and Cholistan desert are shown in Figure 13 and discussed below. To investigate these channels, in addition to our survey results and field visits to the respective areas, we also visited the medicinal plants market in Lahore. Lahore is the central market for selling/purchase/export of plants including those from the northern and desert areas. During this market visit, observations were made about the stakeholders and their actions at the market. These observations provided us with further information about the latter stages of the marketing chain not taking place directly in the study areas. The findings of socio-economic analysis (including marketing channels) have come from the combination of field visit interviews and survey responses. Sher (2013) and Sher *et al.* (2014) also provides analysis of the marketing channels for Swat valley.

Figure 13: Marketing Channels of Medicinal Plants in Swat and Cholistan Desert



Marketing channels of medicinal plants in Swat

During our field visits, we identified five primary market channels in Swat valley. These are listed, and elaborated on, below.

Channel 1: Collector → Assembler (shopkeeper) → Pansary → Consumer

Channel 2: Collector → Assembler (shopkeeper) → Wholesaler → Consumer

Channel 3: Collector → Assembler (shopkeeper) → Wholesaler → Pansary → Consumer

Channel 4: Collector → Assembler (shopkeeper) → Consumer

Channel 5: Collector → Assembler (shopkeeper) → Hakim

Collectors were gathering different species of medicinal plants from the forests atop hills in the wild. After collection, these plants were sun dried and sold to assemblers (shopkeepers) in the local markets of Madyan, Mangora, Bahrain, Kalam, and Miandam.

Assemblers/Shopkeepers create a purchasing point for medicinal plants and were found usually in the local markets of Madyan, Bahrain, Kalam, and Mangora. They were purchasing plants from collectors in nearby areas and villages, and some of them were also purchasing from other small scale assemblers. The majority of collectors were coming directly to their shops to sell their daily and weekly collection of medicinal plants. After purchasing from collectors, assemblers were selling these plants to consumers, hakims (traditional healers), pansaries, and wholesalers in other markets of the country such as Lahore (akbari mandi), Rawalpindi, and Peshawar.

Wholesalers were purchasing different species of medicinal plants from various small scale assemblers/shopkeepers across the country. There were some wholesalers in Mangora city, but the main market of wholesalers was akbari market (mandi) in Lahore, along with Rawalpindi and Peshawar. These wholesalers were selling plants in larger, bulk quantities to mostly drug manufacturing companies. Some of these wholesalers were exporting to other parts of the world. The majority of *pansaries* and *dawakhanas* (small retailers of medicinal plants) were also purchasing plants from wholesalers. Pansaries and dawakhanas were purchasing plants from wholesalers and selling them to hakims and final consumers both in raw and processed forms.

Weak linkages in channels

Strong and smoothly performing marketing channels are vital for the sustainable flow of plant products to the final consumer. Therefore any weak linkage between, or within, marketing channels should be identified and strengthened.

The weakest linkage observed was between small assemblers and wholesalers. This was because of the market power (monopoly) of some large dealers which compelled small assemblers and dealers to sell at low price instead of selling directly to wholesalers. Transport and trade permit problems, and a complex (lengthy) system of duty payments, were also factors causing problems for small scale assemblers.

Marketing channels of medicinal plants in Cholistan desert

As compared to Swat, only four types of channels were identified in Cholistan:

Channel 1: Collector → Assembler (shopkeeper) → Wholesaler → Consumer

Channel 2: Collector → Assembler (shopkeeper) → Wholesaler→ Pansary → Consumer

Channel 3: Collector → Assembler (shopkeeper) → Consumer

Channel 4: Collector → Assembler (shopkeeper) → Hakim

Collectors were the locals who were collecting different species of medicinal plants from the desert. These plants were dried under the sun after collection. They were selling these plants to assemblers/shopkeepers in the local markets of Mandi Yazman, and Bahawalpur. The majority of the collectors were less skilled and unaware of the true economic value of plants.

Similar to Swat, these assemblers create a purchasing point, and were found usually in local markets near desert areas. They were purchasing plants from collectors of nearby villages, and some were also purchasing from other small scale assemblers. The majority of collectors were coming to their shops to sell their weekly collection of medicinal plants. After purchasing from collectors, assemblers in Cholistan were also selling these plants to final consumers, hakims (traditional healers) and wholesalers in other markets of country such as Lahore (Akbari mandi).

There were no wholesalers of medicinal plants in Cholistan, but the main market of wholesalers was akbari mandi in Lahore. These wholesalers were selling plants in larger, bulk quantities to herbal medicine companies. Some of these wholesalers were exporting, as they were also in Swat. Most pansaries and dawakhanas were also purchasing plants from wholesalers.

Pansaries and dawakhanas were the same as in Swat: purchasing plants from wholesalers and selling them to hakims and final consumers, both raw and after processing.

Weak linkages in Channels

The weakest linkage observed in the marketing channels was again the link between collectors and assemblers. This is because of the fact that collectors were collecting 26 different plant species from various part of desert, but only 9 of these plant species were entering in the market for further sale and exchange. The reasons were that a majority of the collectors were less educated, unaware of the true economic value of the plants, and had no access to plant markets where they could sell.

The conclusions drawn from the visit to the Lahore market were quite informative. To supplement these, the survey respondents were also asked about the marketing channels for medicinal plants. These results are presented below.

Mode of purchase

Assemblers purchase these medicinal plants from collectors as well as from other small assemblers. Table 7 shows the distribution of assemblers according to mode of purchase. According to the findings, all 20 assemblers in Swat purchased from local collectors. The reason purchases are only from collectors is that cultivation of medicinal plants is not carried out due to the unavailability of seeds. Out of 20 assemblers, 3 were also purchasing medicinal plants from other small assemblers along with collectors.

In contrast, out of 20 assemblers in Cholistan, 13 assemblers reported that they were purchasing medicinal plants from collectors, making up 65% of the assemblers. Seven of the assemblers said collectors were not the mode of purchase, constituting 35% of assemblers. Nine of them reported that they were purchasing medicinal plants from small assemblers, constituting 45%, while 11 reported that small assemblers were not a mode of purchase for them, constituting 55%.

These results are somewhat supported by Vodouhe *et al.* (2008) who found that collectors harvest plants from forests and sell them directly to retailers in nearby markets. Hamilton (2004) reported that many people generated incomes through the collection and marketing of medicinal plants.

Table 7: Assemblers Mode of Purchase

		Swat	Cholistan	Total
Collector	Yes	20	13	33
	No	0	7	7
Small Assemblers	Yes	3	9	12
	No	17	11	28

Shop ownership

The distribution of assemblers according to shop ownership is reported in Table 8. In Swat, 55% of the assemblers were carrying out their business in their own shops while 45% were paying monthly rents on a shop. In Cholistan, the ratio of assemblers with their own shops to those renting was 40% to 60% respectively.

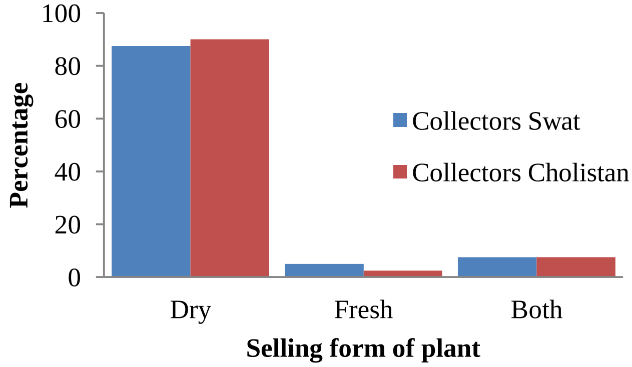
Table 8: Assemblers Shop Ownership

	Owned			
	Yes	%	No	%
Swat	11	55	9	45
Cholistan	8	40	12	60
Total	19	47.5	21	52.5

Selling form of medicinal plants

Medicinal plants collected from the wild are sold in the market either fresh or after sun drying. Fig. 14 shows the distribution of respondents according to the selling form of plants. It was found that plants were mostly sold in the dry form by collectors with a very small percentage selling fresh plants. Assemblers in both study areas sold medicinal plants in dried form and none reported selling the plants in fresh form.

Figure 14: Selling Form of Medicinal Plants



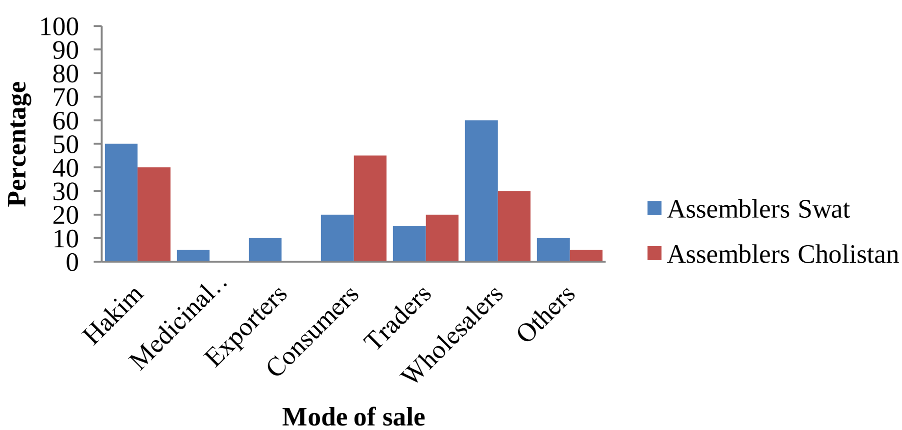
The data comprises of 40 collectors from each region. All the assemblers in both the regions sell the medicinal plants in dry form.

Mode of sale

It was found that assemblers all had more than one mode of sale (Fig. 15). This fact means that they were selling plants to different individuals including *hakims* (traditional healers), dealers, consumers, and wholesalers simultaneously. It was also found that most of the medicinal plant species were sold to traditional healers and wholesalers in both the regions with higher percentages in Swat as compared to Cholistan. In Cholistan, most of the assemblers sold the plants directly to the consumer at a low cost. The consumers use the medicinal plants either in some herbal medicine, for animal fodder, or as timber. Only a small percentage of the assemblers had access to medicinal companies and exporters in the Swat region; no such facility was found in Cholistan. It was concluded from this observation that a market existed, to some extent, in Swat valley for the sale of medicinal plants and export at the industrial level but did not exist in Cholistan. Sher *et al.* (2014) also provides analysis of the mode of sale for Swat valley by reporting that the medicinal and aromatic plant species were mainly collected from the forest areas of Swat district, and the collectors sell the material fresh or in semi-dried form

The results are the similar with Cenitkaya (2010) who found that retailers of medicinal plants in Swat were selling plants to consumers, medicine companies, and trading dealers. Iqbal *et al.* (2011) found that many plant species were scarce globally, but because of the unique geographical location of Pakistan, were found in these regions. These species were highly prized and had huge export potential.

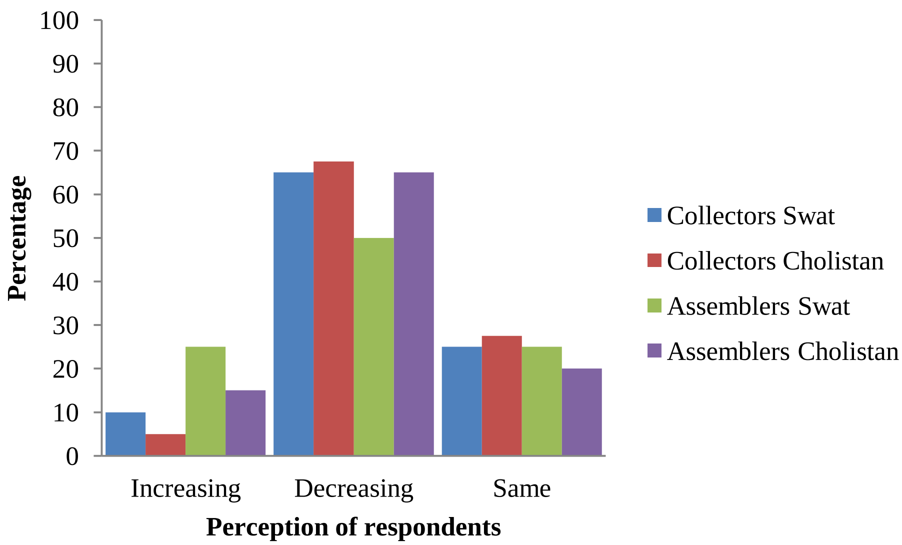
Figure 15: Mode of Sale of Medicinal Plants



PERCEPTION OF STAKEHOLDERS ABOUT MEDICINAL PLANT POPULATION

A sustainable population of medicinal plant species is necessary for many rural communities and economies. Respondents were asked what they thought about plant populations in their area over time. The majority (63%) of the respondents reported that the population of important medicinal plants was decreasing (Fig. 16). This could be a result of the plant species were exploited for maximum personal benefit and income generation, leading to the destruction of biodiversity, with little importance given to plant conservation for future use. Chaudhary and Karmacharya (2001) also found that overexploitation and deforestation was causing the destruction of biodiversity and declining populations of medicinal plant species. Declining plant populations in district Battagram (Pakistan) was reported by Haq *et al*. (2011). Hussain *et al*. (2012) found that only 8% of medicinal plants in Kurram agency (Pakistan) were secure, while the rest of the important species were critically endangered and vulnerable. We also found similar results, and concluded that both the regions were losing plant diversity.

Figure 16: Perception of Medicinal Plants Population



The data comprises of 20 assemblers and 40 collectors from each region.

To further explore this question, the survey respondents were also asked about the reasons they believe medicinal plant populations were decreasing. The results are reported in Table 9. Overgrazing by animals was most often said to be the most important issue regarding the conservation of medicinal plants. This could be because those living in Swat are very much dependent on livestock for meat, milk, and other purposes. The same overgrazing is true in Cholistan but for different reasons. The majority of the population living in deep desert areas lead a semi-nomadic life in which they move in search of water and food carrying their livestock.

Lack of awareness was reported second most often as the most important issue affecting the conservation of medicinal plants. This issue of awareness has been discussed frequently in other areas of this study and clearly represents an important aspect of conservation. It is a more difficult aspect to isolate completely has it relates so closely to other issues of conservation (overgrazing, poor collection techniques, lack of knowledge, etc…), so the fact that it was reported so frequently, and that it will come up in other categories, is perhaps not surprising.

Increasing human population was also reported as a factor affecting conservation. Larger human populations cause the destruction of natural habitat, deforestation, and various other calamities that ultimately result in decreasing plant populations. In this vein, the population of some important species of medicinal plants have been negatively affected by the loss of natural habitats.

Over-collection combined with poor collection techniques were also causing plant population decrease. As a majority of the collectors in both the areas were illiterate or less qualified, they had very little knowledge about proper sustainable techniques regarding the collection of medicinal plants. Root removal, collection at an immature stage, and digging of land to collect plants were some of the issues that were causing the loss of biodiversity.

The majority of the medicinal plants in both areas have economic as well as medicinal importance, but unfortunately, non-productive uses of these plants were very common as many were being used as fodder, fuel, food, and for other domestic purposes. This was because the majority of the population in those areas were unaware of the true economic and medicinal value of these vulnerable species, causing over-exploitation of plants. Some other reasons causing lower plants populations reported by respondents were deficiencies of water, unavailability of seeds for cultivation, increasing demand for plants, and adverse weather conditions.

Conclusively, economic, demographic, and natural factors are posing a serious threat to the existence of important plant species (Sher and Aldodari, 2012). Non-productive uses of medicinal plants are also posing a threat to their population (Awan *et al*., 2011).

Table 9: Perceptions Regarding Decreasing Plant Population

Reason for Decreasing Population	Swat		Cholistan		Total
	Collectors	Assemblers	Collectors	Assemblers	
Overgrazing	3	11	18	5	37
Lack of Awareness	6	3	15	11	35
Increase in Population	9	3	6	7	25
Non Availability of Seeds	8	3	8	4	23
Over Collection/Poor Collection Techniques	6	3	8	5	22
Picked Once Does Not Grow Again	3	-	6	4	13
Lack of Water (No Rain No Plants)	3	2	1	3	9
Removal of Roots	5	1	2	-	8
Increasing Demand	-	2	2	4	8
Climate Variability	1	-	4	3	8

MEDICINAL USES OF PLANTS

As established earlier, some plants possess high medicinal value as they provide the raw material for various synthetic drugs. These medicinal plants are directly and indirectly used for treatment of various diseases and ailments by the local communities of Swat and Cholistan. These plants are also a source of many medicinally important chemical compounds like terpenes, steroids, gums and resins, antioxidants, and fatty acids. Because of the fact that allopathic medicines are expensive, are not easily available in desert and hilly areas, and have side effects associated with their use, local inhabitants of Swat and Cholistan are very much dependent on medicinal plants found where they live for the treatment of diseases. Tables 10 and 11 list the most common medicinal plants found in Swat valley and Cholistan desert, respectively, along with their uses.

Table 10: Most Common Medicinal Plants from Swat Valley

Sr. No.	Plant's Name (Local)	Botanical Name	Use
1	Konjay		Treatment of Flu & Fever
2	Sumbal	*Adiantum incisum*	Stomach disorder
3	Mushkebala		To cure liver disorder
4	Zakhm-e-Hayat	*Bergenia ciliata*	To cure external wounds and healing
5	Anjbar	*Bistorta amplexicaulis*	Treatment of chest infection
6	Guchi	*Morchella esculenta*	
7	Mameekh	*Paeonia emodi*	Treatment of wounds
8	Kakora	*Podophyllum emodi*	To cure joint pain
9	Matar Jarri	*Trillium govanianum*	Treatment of weak children
10	Banafsha	*Viola serpens*	Treatment of cough & Flu

Table 11: Most Common Medicinal Plants from Cholistan Desert

Sr. No.	Plant Name	Botanical name	Use
1	Canghe booti	Abutilon muticum	Used against leprosy, used for dialysis.
2	Phagosia	Calligonum polygonoides	Used against foot related diseases
3	Akk	Calotropis procera	To cure fever and wounds
4	Kaurr tuma	Citrullus colocynthis	Helpful against diabetes and useful in dialysis
5	Dhamasa	Fagonia indica	To cure liver disorder
6	Fareed booti	Farsetia hamiltonii	To cure joint pains
7	Gorakh pan	Heliotropium strigosum	Used for mental disorder, used for fever and jaundice
8	Chapri booti	Neurada procumbens	To cure sexual diseases
9	Bhakra	Tribulus terrestris	Used for strengthening of muscles, useful for nervous system, acts as diuretics, used for treatment of urinary tract infections (UTI).
10	Asgand	Withania somnifera	To cure skin diseases, used for energy (power), acts as diuretics

MARKETING MARGINS OF STAKEHOLDERS

Marketing margin, or price spread, is a commonly used measure of the performance of a marketing system. It can be a useful descriptive statistic to show how the consumers' expenditure is divided among market participants at different levels of the marketing systems. A large number of studies have analyzed the marketing margins for different types of commodities to examine the marketing performance of agricultural products. A review of the margins and market channels studies for various food commodities in Pakistan was done by ul Haque (2012). Variations in the margin over time might be attributable to marginal marketing costs under perfect competition, and additional factors such as seasonality, technological changes, and sales volume may also explain variations in the margin.

Swat and Cholistan are host to many vulnerable and important (economically and medicinally) plant species. But only a few of these species are marketed and reach the final consumer. Therefore, the top five marketed species from each area were selected to estimate their marketing margins (Table 12). These species were selected on the basis of frequency (those which were reported more by collectors and assemblers of that specific area).

Table 12: Top 5 Marketed Medicinal Plants from the Study Area

S. No.	Swat Valley	Cholistan Desert
1	*Viola serpens* (Banafsha)	Farsetia hamiltonii (Fareed booti)
2	*Morchella esculenta* (Guchi)	Fagonia indica (Dhamasa)
3	*Paeonia emodi* (Mameekh)	Neurada procumbens (Chapri booti)
4	*Trillium govanianum* (Matar jarri)	Citrullus colocynthis (Kaurr tuma)
5	*Bergenia ciliata* (Mushkebala)	Withania somnifera (Asgand)

Common names of the medicinal plants are given in brackets.

Marketing margins of collectors

Collectors were selling medicinal plants to other stakeholders at the average prices given in Tables 13 and 14 for Swat and Cholistan respectively. Gross margins are the difference between avg. sale price and collection cost. Net margins are calculated by subtracting total costs from gross margins. Total costs for collectors include marketing cost and transportation costs.

Table 13: Marketing Margins for Collectors for the Top 5 Plants in Swat Valley (Rs. / kg)

Name of Plant	Avg. Sale Price	Collection Cost	Gross Margin	Total Costs	Net Margin	Net Profit as % Sale Price
Viola serpens (Banafsha)	400	135	265	90 (33.97%)	175 (66.03%)	43.75
Morchella esculenta (Guchi)	18,500	13,100	5400	1,300 (24.08%)	4,100 (75.92%)	26.15
Paeonia emodi (Mameekh)	170	88	82	50 (60.98%)	32 (39.02%)	18.82
Trillium govanianum (Matar jarri)	2,250	1,195	1,055	327 (31%)	728 (69%)	32.35
Bergenia ciliata (Mushkebala)	283	109	174	89 (51.15%)	85 (48.85%)	30.03

Table 14: Marketing Margins for Collectors for the Top 5 Plants in Cholistan Desert (Rs. / kg)

Medicinal Plant	Avg. sale Price	Collection Cost	Gross Margin	Total Costs	Net Margin	Net Profit as % Sale Price
Farsetia hamiltonii (Fareed booti)	100	45	55	33 (60%)	22 (40%)	22
Fagonia indica (Dhamasa)	80	38	42	29 (69.05%)	13 (30.95%)	16.25
Neurada procumbens (Chapri booti)	110	53	57	37 (64.92%)	20 (35.08%)	18.18
Citrullus colocynthis (Kaurr tuma)	60	31	29	20 (68.97%)	9 (31.03%)	15
Withania somnifera (Asgand)	220	104	116	53 (45.69%)	63 (54.31%)	28.63

Marketing margins of assemblers

Assemblers (shopkeepers) were purchasing plants and selling to other stakeholders at the costs given in Tables 15 and 16 in Swat and Cholistan respectively. Gross margins are the difference between avg. sale price and avg. purchase price. Net margins are calculated by subtracting total costs from gross margins. Total costs for assemblers include transportation cost, labor cost, and market fee, utility bill charges, and rent paid.

Table 15: Marketing Margins for Assemblers in Swat Valley (Rs. / kg)

Name of Plant	Avg. sale Price	Avg. Purchase price	Gross Margin	Total Cost	Net Margin	Net Profit as % Sale Price
Viola serpens (Banafsha)	650	400	250	57 (22.8%)	193 (77.2%)	29.69
Morchella esculenta (Guchi)	24,800	18,500	6,300	230 (3.66%)	6,070 (96.34%)	24.47
Paeonia emodi (Mameekh)	400	170	230	54 (23.48%)	176 (76.52%)	44
Trillium govanianum (Matar jarri)	2,870	2,250	620	112 (18.07%)	508 (81.93%)	17.7
Bergenia ciliata (Mushkebala)	545	283	262	59 (22.52%)	203 (77.48%)	37.24

Table 16: Marketing Margins for Assemblers in Cholistan Desert (Rs. / kg)

Name of Plant	Avg. Sale Price	Avg. Purchase Price	Gross Margin	Total Cost	Net Margin	Net Profit as % Sale Price
Farsetia hamiltonii (Fareed booti)	180	100	80	34 (42.5%)	46 (57.5%)	25.56
Fagonia indica (Dhamasa)	200	80	120	49 (40.84%)	71 (59.16%)	35.5
Neurada procumbens (Chapri booti)	240	110	130	45 (34.62%)	85 (65.38%)	35.41
Citrullus colocynthis (Kaurr tuma)	150	60	90	36 (40%)	54 (60%)	36
Withania somnifera (Asgand)	500	220	280	53 (18.93%)	227 (81.07%)	45.4

The findings of gross margins and net margins generally show that collectors are less aware about economic importance of medicinal plants in both study areas. Their low margins also imply that they do not have the access to large markets. They were selling medicinal plants at fairly low prices as compared to assemblers. The margins of collectors in Cholistan are lesser than those of collectors in Swat perhaps showing that markets in Cholistan are comparatively less developed than those in Swat.

MARKETING PROBLEMS

Understandably, there were other problems in the marketing chains we discovered besides the weak linkages already discussed. In the previous analysis we identified specific relationships where the marketing chain linkage was the weakest: assembler to wholesaler in Swat and collector to assembler in Cholistan. Below, we break down the reasons behind these weak linkages, by the different stakeholders in the marketing chain, as found in our survey results for Swat and Cholistan.

Potential problems we found associated with plant collectors in Swat are low prices received for plants, taxes charged by forest the department, absence of government support, and difficult access to larger markets (Table 17).

Table 17: Major Problems of Collectors in Swat Valley

Problems	Yes	No
Low Price Received for Plants	32 (80%)	8 (20%)
Tax from Forest Dpt.	25 (62.50%)	15 (37.50%)
No Government Support	35 (87.50%)	5 (12.50%)
Difficult Access to Larger Markets	31 (77.50%)	9 (22.50%)
Sample size: 40 collectors		

Potential marketing problems faced by assemblers in Swat were interference from the forest department, the absence of a pricing policy for plants, absence of storage facilities, transport and trade permit problem, and complex and lengthy system for duty payment (Table 18). Sher *et al.* (2014) also provides analysis of the marketing problems for Swat valley showing that many of the medicinal plants currently being collected in Swat district are now being produced and exported to other countries, including India and China.

Table 18: Major Problems of Assemblers in Swat Valley

Problems	Yes	No
Interference of Forest Dpt.	12 (60%)	8 (40%)
Absence of Pricing Policy	14 (70%)	6 (30%)
Absence of Storage Facilities	15 (75%)	5 (25%)
Transport & Trade Permit Problem	10 (50%)	10 (50%)
Complex & Lengthy System of Duty Payment	12 (60%)	8 (40%)
Sample size: 20 assemblers		

Wholesalers buy the medicinal plants from different collectors at very low rates and sell in bulk at higher rates to bigger markets like Lahore. A few are now trying to export the items directly. For this, a concrete export policy needs to be implemented in this region.

Potential problems identified by plant collectors in Cholistan are the absence of proper markets and selling points for plants, high transportation and travel costs, lower prices of plants (variation in prices), absence of governmental and institutional support, and difficult access to larger plant markets in other parts of country (Table 19).

Table 19: Major Problems of Collectors in Cholistan Desert

Problems	Yes	No
Absence of Proper Market/Selling Point	38 (95%)	2 (5%)
High Transportation Charges/ High Travel Costs	28 (70%)	12 (30%)
Low Prices Received	34 (85%)	6 (15%)
No Government Support	30 (75%)	10 (25%)
Difficult Access to Markets in Other Cities	32 (80%)	8 (32%)
Sample size: 40 collectors		

Potential marketing problems faced by assemblers in Cholistan were the absence of a proper separate market for medicinal plants, high transportation costs, the monopoly of big dealers, and absence of a pricing policy for plant species (Table 20). The results of the study are also supported by Nasir *et al.* (2014) who found that several factors like over harvesting, deforestation, over grazing, loss during collection and storage, unmonitored trade, and little knowledge of market requirement are resulting in depletion of plant population. Berhans (1991) reported that increasing demand for wild products, costs borne to implement conservation and pricing policies, and importance of collection for rural economies (as this was a source of livelihood for them) were causing over exploitation of wild plants.

Table 20: Major Problems of Assemblers in Cholistan Desert

Problems	Yes	No
Absence of Separate Market for Plants	16 (80%)	4 (20%)
High Transportation and Other Costs	13 (65%)	7 (35%)
Monopoly of Big Dealers	11 (55%)	9 (45%)
Absence of Pricing Policy for Plants	17 (85%)	3 (15%)
Sample size: 20 assemblers		

In Cholistan in particular, the economy is predominantly pastoral and people have been practicing a nomadic lifestyle for centuries. The nomads own smaller to larger herds of camels, cattle, sheep, and goats. The interior desert area is not connected by a modern communication system, and sandy desert tracks are used for travel by camel or jeep. Local people use camels as a mode of transportation. Habitations are small and extremely scattered. The economy of these nomads entirely depends on fragile and meager natural resources associated with inconsistent rainfall patterns. Job opportunities are confined to labor in agricultural fields or other minor activities due to lack of education or skilled training. Most of the nomads live below the poverty line and in the absence of basic human needs like clean drinking water, sufficient food, health, and education for their children. Livestock breeding, improvement of performance, or range management is not practiced scientifically. It was observed that the there was no awareness about the value of medicinal plants in desert area. After collection, they hand over the plants to the wholesale dealers and hakims (traditional healers) free of cost, or at a very marginal price. No market was found in the vicinity for selling/purchase of medicinal plants. Government actions in addressing these issues, and framing relevant policies, will help improve the condition of these people.

CONCLUSION

This project was focused on three major topics: the conservation of natural plant resources using modern molecular techniques, creating awareness of the need for determining the active ingredients of these plants through biochemical profiling, and investigating the marketing channels and issues for these plants via surveys. Biochemical profiling of twelve selected plants, followed by their comparison with marketed products, revealed that the plants contained highly valuable compounds that could have commercial applications. DNA barcoding was optimized, and DNA sequences of many of the plant species were deposited in GenBank and BOLD; 200 sequences of 88 species can be accessed on www.boldsystems.org. Four barcoding regions (*matK*, *rbcL*, ITS and *trnH-psbA*) were evaluated for species discrimination success. For the phylogenetic and evolutionary study of biodiversity, a large dataset of sequences is required, which is possible only by expanding the barcode sequence library.

The socio-economic objectives of the study were to identify the marketing channels of medicinal plants, costs and margins of stakeholders involved, and factors responsible for poor trade and decreasing populations of these plants in the two study areas (Swat valley and Cholistan desert). Lack of awareness regarding the importance of medicinal plants, the fact that no pricing policy exists, a monopoly of a few big dealers, and high transportation costs were the main marketing problems faced by stakeholders. Medicinal plants markets were well developed in Swat as compared to Cholistan, which lack adequate market structure. Recommendations made on the basis of the project outcomes for the establishment of well-structured markets for medicinal plants along with conservation measures to make full use of medicinal plant products are given below.

RECOMMENDATIONS

- Molecular documentation, by DNA barcoding, should be done for important medicinal plant species of the country.
- Biochemical profiling should be conducted, at least for the most valuable plant species, which will increase their economic value.
- Locals should be informed of the economic importance of medicinal plants and trained regarding proper collection techniques to ensure sustainable populations of medicinal plants.
- The government should facilitate the private sector to establish a cohesive medicinal plants industry. Well-structured and smooth functioning markets should be established in the area to ensure a sustainable flow of plant products.
- The government should create a policy framework regarding the pricing and conservation of medicinal plants.
- Export policies for medicinal plants need to be framed and implemented under the control of a regulatory authority.
- Infrastructure development for the people living in deep desert areas should be undertaken to make access to markets in urban areas easier.
- Institutions and plant based industries should cooperate with each other to make full use of the industrial potential of medicinal plants.
- Photographic representation of important plant species should be prepared showing plants at different stages of their lives to help understand their nature. Teaching and research institutions should be encouraged to develop libraries of medicinal plants for knowledge dissemination.

- Areas supporting large numbers of medicinal plant species should be declared as protected areas from animals and nomadic people.
- Seed banks should be established for providing good quality seeds of medicinal plants. Cultivation of important medicinal plants should be adopted/encouraged at the farm level.
- *Pansaries* should be registered in the national council for Herbal Medicine, and they should be made to hold a diploma. Steps should be taken on a scientific basis for promoting Herbal Medicine.

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APPENDIX 1: BARCODED MEDICINAL PLANT SPECIES

Table A.1: Description of Barcoded Medicinal Plant Species Investigated Under the Study

Field ID/Collection Site	Plant Name	Herbarium Voucher No.	BOLD Process ID	matK Seq. Length	rbcL Seq. Length	ITS Seq. Length	Barcode Compliant
cholistan93	Abutilon muticum	822-29-2014c	DBMPP093-14	800[0n]	552[0n]	352[1n]	Yes
cholistan90	Acacia jacquemontii	822-30-2014c	DBMPP090-14	815[0n]	553[0n]	326[0n]	Yes
cholistan89	Acacia modesta	822-31-2014a	DBMPP089-14	809[0n]	553[0n]	308[0n]	Yes
cholistan99	Acacia nilotica	822-32-2014c	DBMPP099-14	839[0n]	552[0n]	0	Yes
cholistan101	Acacia nilotica	822-32-2014a	DBMPP101-14	0	552[0n]	224[0n]	No
cholistan10	Achyranthes aspera	822-4-2014c	DBMPP010-14	803[0n]	552[0n]	319[0n]	Yes
cholistan23	Aerva javanica	822-5-2014c	DBMPP023-14	803[0n]	544[8n]	325[0n]	Yes
cholistan15	Aizoon canariense	822-58-2014a	DBMPP015-14	817[0n]	552[0n]	313[2n]	Yes
cholistan30	Asphodelus tenuifolius	822-8-2014b	DBMPP030-14	827[0n]	553[0n]	358[0n]	Yes
cholistan126	Calligonum polygonoides	822-41-2014c	DBMPP126-14	806[0n]	552[0n]	344[0n]	Yes
cholistan21	Calotropis procera	822-6-2014a	DBMPP021-14	823[0n]	540[12n]	365[0n]	Yes
cholistan61	Capparis aphylla	822-17-2014b	DBMPP061-14	788[0n]	532[20n]	342[0n]	Yes
cholistan108	Cenchrus ciliaris	822-36-2014a	DBMPP108-14	771[0n]	552[0n]	344[0n]	Yes
cholistan72	Chenopodium album	822-19-2014a	DBMPP072-14	785[0n]	552[0n]	355[0n]	Yes
cholistan27	Cirsium arvense	822-9-2014b	DBMPP027-14	815[0n]	519[0n]	349[0n]	Yes
cholistan87	Citrullus colocynthis	822-25-2014c	DBMPP087-14	758[0n]	552[0n]	0	Yes
cholistan25	Conyza bonariensis	822-10-2014a	DBMPP025-14	809[0n]	553[0n]	333[0n]	Yes
cholistan136	Corchorus depressus	822-49-2014b	DBMPP136-14	759[0n]	552[0n]	360[0n]	Yes
cholistan109	Crotalaria burhia	822-35-2014c	DBMPP109-14	0	552[0n]	345[0n]	Yes
cholistan104	Cymbopogon jwarancusa	822-37-2014b	DBMPP104-14	773[0n]	552[0n]	297[3n]	Yes
cholistan85	Cyperus conglomeratus	822-62-2014b	DBMPP085-14	0	551[1n]	0	No
cholistan58	Dipterygium glaucum	822-18-2014b	DBMPP058-14	786[0n]	552[0n]	342[0n]	Yes
cholistan81	Euphorbia prostrate	822-27-2014c	DBMPP081-14	753[0n]	530[22n]	347[0n]	Yes
cholistan150	Fagonia indica	822-50-2014a	DBMPP150-14	767[0n]	552[0n]	331[2n]	Yes
cholistan64	Farsetia hamiltonii	822-16-2014b	DBMPP064-14	0	0	313[0n]	No
cholistan6	Gisekia pharnacioides	822-1-2014c	DBMPP006-14	809[0n]	533[19n]	349[0n]	Yes
cholistan68	Haloxylon solicornicum	822-20-2014b	DBMPP068-14	797[0n]	551[1n]	331[0n]	Yes
cholistan66	Haloxylon stocksii	822-21-2014a	DBMPP066-14	747[0n]	553[0n]	328[2n]	Yes
cholistan50	Heliotropium strigosum	822-15-2014c	DBMPP050-14	779[0n]	547[5n]	340[3n]	Yes
cholistan53	Heliotropium europaeum	822-14-2014c	DBMPP053-14	809[0n]	519[0n]	366[0n]	Yes
cholistan116	Indigofera hochstetteri	822-38-2014c	DBMPP116-14	803[0n]	552[0n]	336[0n]	Yes
cholistan113	Lasiurus scindicus	822-40-2014c	DBMPP113-14	760[0n]	552[0n]	343[1n]	Yes
cholistan36	Launaea nudicaulis	822-59-2014c	DBMPP036-14	0	0	348[0n]	No
cholistan32	Leptadenia pyrotechnica	822-7-2014c	DBMPP032-14	0	0	366[0n]	No
cholistan4	Limeum indicum	822-2-2014b	DBMPP004-14	803[0n]	552[0n]	337[0n]	Yes
cholistan154	Neurada procumbens	822-54-2014	DBMPP154-14	0	552[0n]	0	No
cholistan41	Oligochaeta ramose	822-60-2014b	DBMPP041-14	0	552[0n]	343[0n]	Yes
cholistan146	Peganum harmala	822-51-2014b	DBMPP146-14	0	552[0n]	0	No
cholistan98	Prosopis cineraria	822-33-2014a	DBMPP098-14	0	552[0n]	0	No
cholistan45	Pulicaria crispa	822-13-2014a	DBMPP045-14	0	0	347[0n]	No
cholistan78	Salsola imbricate	822-22-2014b	DBMPP078-14	0	0	345[0n]	No
cholistan47	Salvadora sp.	822-61-2014b	DBMPP047-14	775[0n]	522[0n]	357[0n]	Yes
cholistan121	Solanum nigrum	822-44-2014a	DBMPP121-14	792[0n]	552[0n]	331[0n]	Yes
cholistan134	Solanum virginianum	822-45-2014a	DBMPP134-14	802[0n]	552[0n]	334[0n]	Yes
cholistan34	Sonchus arvensis	822-11-2014b	DBMPP034-14	0	552[0n]	326[0n]	No
cholistan74	Suaeda fruticosa	822-23-2014c	DBMPP074-14	0	0[0n]	352[0n]	No
cholistan142	Tamarix aphylla	822-47-2014b	DBMPP142-14	800[0n]	552[0n]	369[0n]	Yes
cholistan139	Tamarix dioica	822-48-2014b	DBMPP139-14	800[0n]	552[0n]	369[0n]	Yes
cholistan153	Tribulus longipetalous	822-57-2014a	DBMPP153-14	803[0n]	552[0n]	334[0n]	Yes
cholistan158	Tribulus pentandrus	822-52-2014c	DBMPP158-14	801[0n]	551[1n]	334[0n]	Yes
cholistan155	Tribulus terrestris	822-53-2014c	DBMPP155-14	809[0n]	552[0n]	334[0n]	Yes
cholistan131	Withania somnifera	822-46-2014a	DBMPP131-14	803[0n]	552[0n]	314[0n]	Yes
cholistan2	Zaleya pentandra	822-3-2014a	DBMPP002-14	780[0n]	553[0n]	331[2n]	Yes
cholistan123	Zizyphus nummularia	822-43-2014b	DBMPP123-14	808[0n]	552[0n]	339[0n]	Yes
sw-2	Achyranthes aspera	821-2-2014	DBMPP165-14	0	330[0n]	340[0n]	No
sw-31b	Adiantum incisum	821-31-2014b	DBMPP197-14	0	0	0	No
sw-32b	Adiantum venustum	821-32-2014b	DBMPP195-14	0	552[0n]	355[0n]	Yes
sw-1	Amaranthus hybridus	821-1-2014	DBMPP166-14	809[0n]	552[0n]	343[0n]	Yes
sw-4	Anethum graveolens	821-4-2014	DBMPP163-14	806[0n]	552[0n]	347[0n]	Yes
sw-5	Asparagus adscendens	821-5-2014	DBMPP162-14	827[0n]	552[0n]	348[2n]	No
sw-9	Berberis lyceum	821-9-2014	DBMPP175-14	0	0	0	No
sw-36	Bergenia ciliate	821-36-2014	DBMPP191-14	0	0	0	No
sw-11b	Cannabis sativa	821-11-2014b	DBMPP172-14	818[0n]	552[0n]	253[0n]	Yes

sw-27	Cedrus deodara	821-27-2014	DBMPP187-14	0	0	0	No
sw-14	Convolvulus arvensis	821-14-2014	DBMPP170-14	0	0	346[0n]	No
sw-15	Dioscorea bulbifera	821-15-2014	DBMPP169-14	0	512[0n]	0	No
sw-16c	Dryopteris ramosum	821-16-2014c	DBMPP199-14	0	552[0n]	0	No
sw-18a	Euphorbia helioscopia	821-18-2014a	DBMPP182-14	777[0n]	486[0n]	341[0n]	Yes
sw-24	Ficus palmate	821-24-2014	DBMPP190-14	769[0n]	523[0n]	360[0n]	Yes
sw-3	Foeniculum vulgare	821-3-2014	DBMPP164-14	801[0n]	552[0n]	348[0n]	Yes
sw-20	Indigofera heteantha	821-20-2014	DBMPP180-14	815[0n]	552[0n]	339[0n]	Yes
sw-8	Inula grandiflora	821-8-2014	DBMPP160-14	0	0	330[0n]	No
sw-21b	Juglans regia	821-21-2014b	DBMPP178-14	0	552[0n]	0	No
sw-21a	Juglans regia	821-21-2014a	DBMPP179-14	0	0	344[0n]	No
sw-40b	Lantana camara	821-40-2014b	DBMPP201-14	815[0n]	552[0n]	352[0n]	Yes
sw-22	Mentha royleana	821-22-2014	DBMPP177-14	756[1n]	552[0n]	359[0n]	Yes
sw-25	Morus alba	821-25-2014	DBMPP189-14	812[0n]	553[0n]	357[0n]	Yes
sw-31a	Oxalis corniculata	821-31-2014a	DBMPP198-14	772[1n]	552[0n]	350[0n]	Yes
sw-28	Pinus wallichiana	821-28-2014	DBMPP186-14	0	0	0	No
sw-26	Plantago lanceolata	821-26-2014	DBMPP188-14	0	552[0n]	0	No
sw-29	Plantago lanceolata	821-29-2014	DBMPP185-14	0	0	351[0n]	No
sw-30	Rumex nepalensis	821-30-2014	DBMPP184-14	0	552[0n]	0	No
sw-34	Salix tetrasperma	821-34-2014	DBMPP193-14	782[0n]	552[0n]	294[0n]	Yes
sw-23	Salvia moorcroftiana	821-23-2014	DBMPP176-14	769[0n]	552[0n]	307[0n]	Yes
sw-10	Sarcococca saligna	821-10-2014	DBMPP174-14	0	0	0	No
sw-12	Silene apetala	821-12-2014	DBMPP171-14	0	0	0	No
sw-38a	Urtica dioica	821-38-2014a	DBMPP206-14	0	552[0n]	325[0n]	Yes
sw-7	Xanthium strumarium	821-7-2014	DBMPP161-14	811[0n]	552[0n]	371[0n]	Yes

APPENDIX II: TAXON-ID TREES

Figure A.1: ITS TaxonID Tree (Part I)

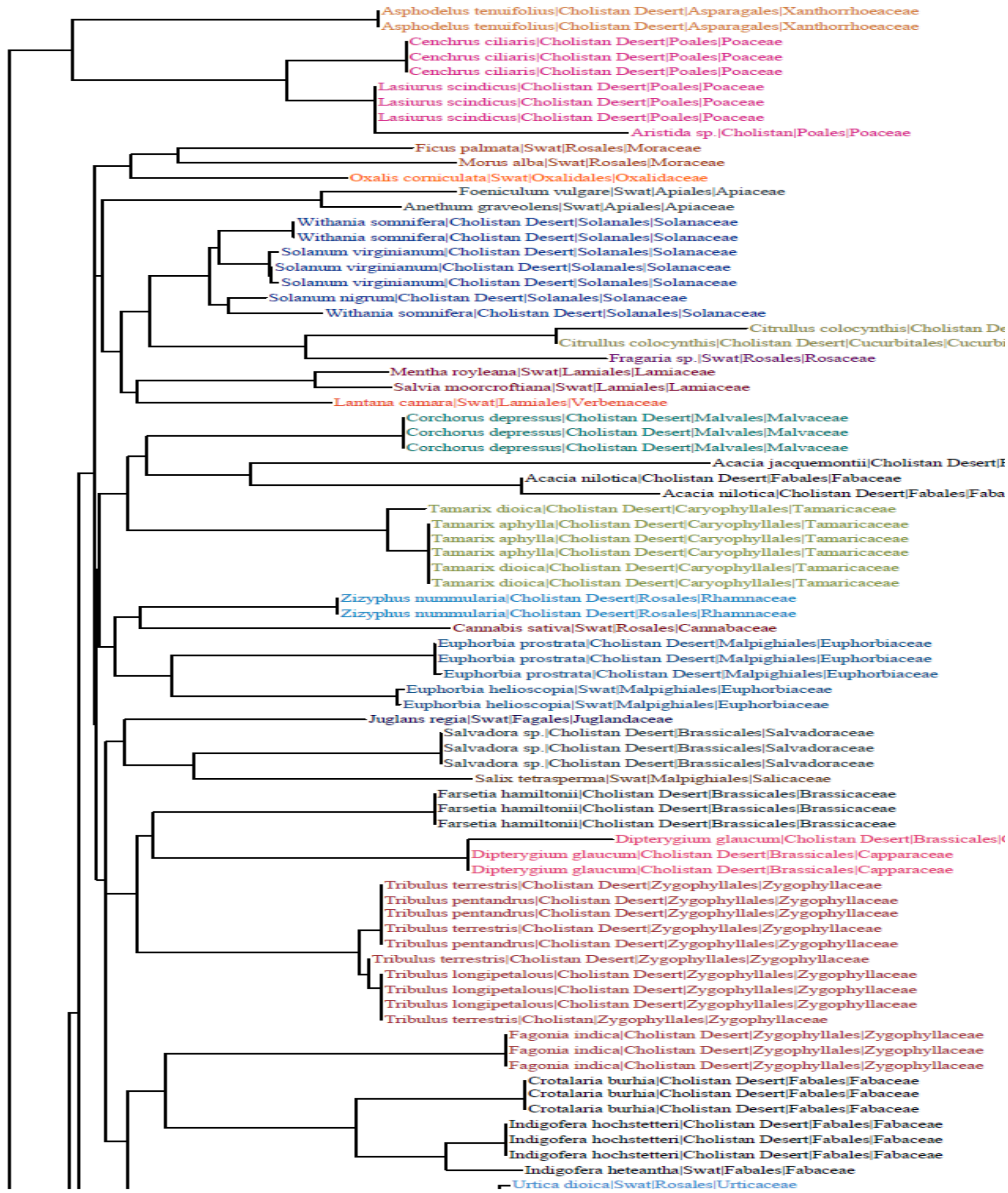


Figure A.1: (Part II)

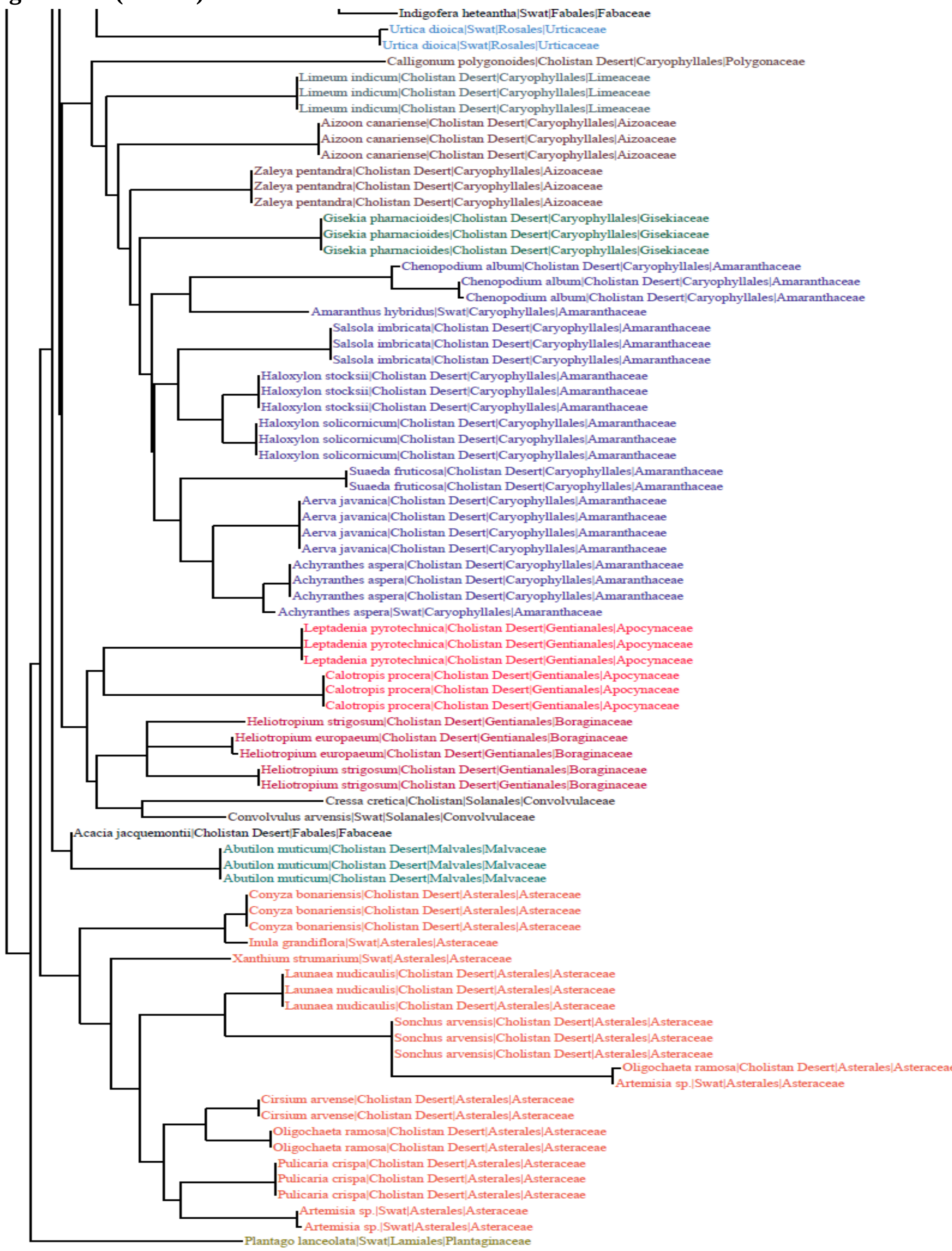


Figure A.2: rbcL TaxonID Tree (Part I)

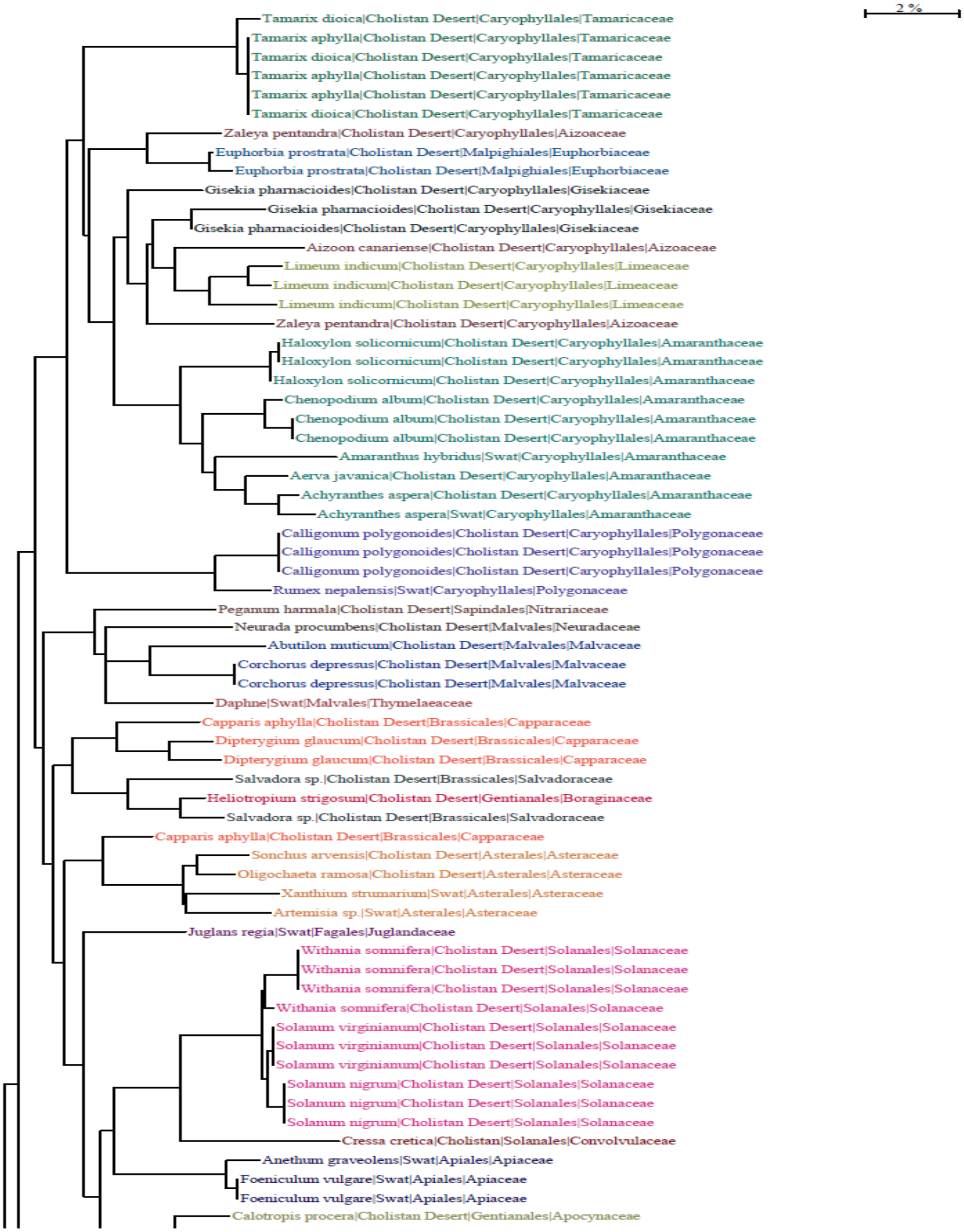


Figure A.2: (Part II)

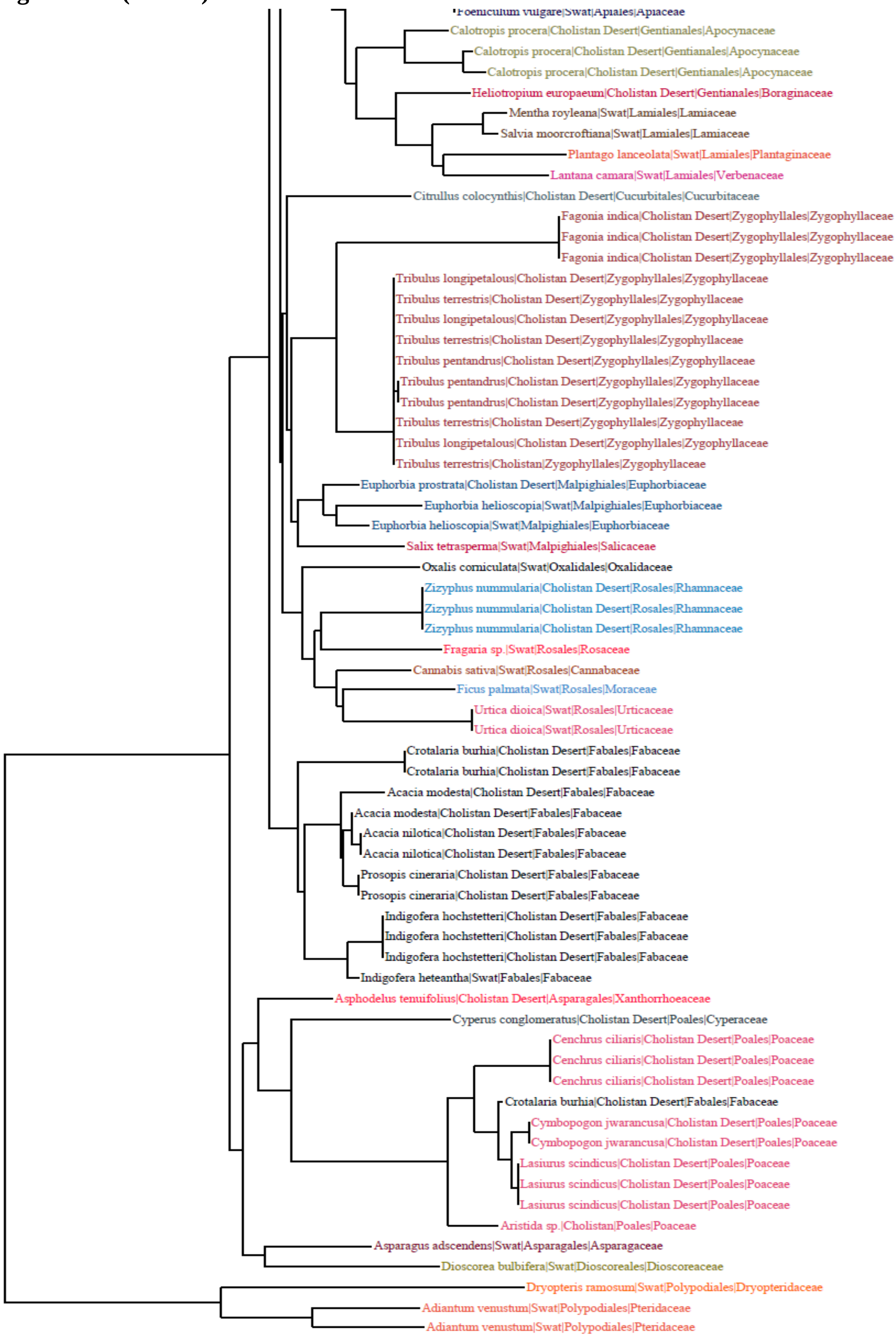
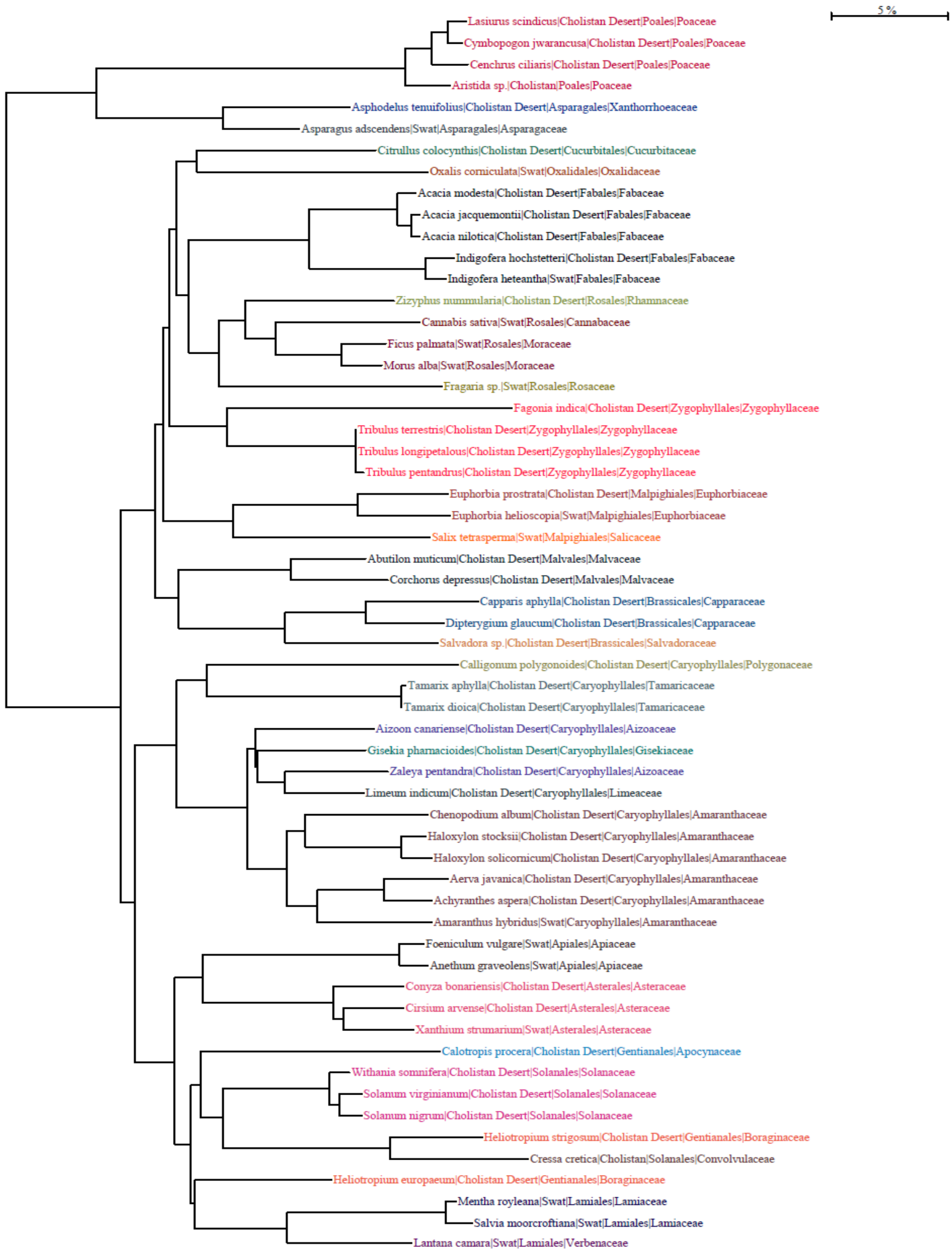


Figure A.3: matK TaxonID Tree



APPENDIX III: LIBRARY SEARCH

Table A.2: List of Compounds (by Library Matching) Present in Each Medicinal Plant with their Medicinal Use

Compounds with their Reported Activities	Medicinal Plant Species											
	Dryopteris ramose	Bergenia ciliata	Qurcus baloot	Isodon rugosus	Fragria bucharia	Valeriana jatamansi	Trillium govanianum	Solanum surattense	Calligonum polygonoides	Fagonia indica	Suaeda fruticosa	Heliotropium strigosum
Phenylacetic acid Anticancer Activity (Neish, 1971)	+	+	+	+	+	+	+	+	+	+	+	+
Malon Saeure Phenyl	+	+	+	+	+	+	+	+	+	+	+	+
Daidzin Antidrinking Activity (Keung et al., 1995)	+											
Daidzein Anticancer Activity (Guo et al., 2004)	+	+										
Indole-3-carboxylic acid Antiinflammatory activity (Manish et al., 2011)	+		+	+	+	+	+		+	+	+	+
Mimosine Anticancer Activity (Zalatnai et al., 2003)	+	+										
Genistein Against genetic diseases (Wegrzyn et al., 2010)	+	+	+				+				+	+
Genistin Anticancer Activity (Hamdy et al., 2011)	+										+	
Trimethoxy- 2-indolcarbon	+	+	+					+	+		+	
L-Tryptophan Antidepressent (Thomson et al., 1982)	+	+	+	+		+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+
Indole-3-acetic acid Treatment of Acne (Huh et al., 2012)	+			+	+	+	+	+	+	+	+	+
Enhydrin Antibacterial Activity (Choi et al., 2010)	+	+	+								+	
Maytansin Anticancer Activity (Bell-McGuinn et al., 2011)	+	+	+	+	+	+	+	+	+	+	+	+
Dihydro-Parthenolide Antiinflammatory activity (Feltenstein et al., 2004)	+		+	+	+	+	+	+	+	+	+	+
Maltophilin	+		+			+		+				
4-Hydroxy-3-nitro-benzoic acid Vasodilative Compounds (Seki et al., 2010)			+	+				+		+		
Quercetin Against Interstitial Cystitis (Katske et al., 2001)			+	+	+				+	+		+
2-Methoxybenzoic Acids			+									
Cinnamic acid Antidiabetic Activity (Adisakwattana et al., 2009)			+									
L-Phenylalanin Antidepressent (Birkmayer et al., 1984)			+									
Piericidin Anticancer Activity (Hwang et al., 2008)				+								
Psilostachyn A				+								
Shikimic acid RT 1.38 Antiviral Activity (Krämer et al., 2003)				+								

Shikimic acid RT 1.11	+						
Antiviral Activity (Krämer et al., 2003)							
Damsin	+						+
L-Tyrosin		+		+	+		
Against Parkinson's Disease (Morais et al., 2003)							
Indole-3-propionic acid		+					
2,3-Dihydroxybenzoic acid			+				
For Sepsis-Induced lung Injury (Sharpe et al., 1984)							
Melampodinin				+		+	
Melampodin A				+			
Protocatechuic acid					+		
Cancer Chemo-preventive Activity (Tanaka et al.,							
Psilostachyn A							+

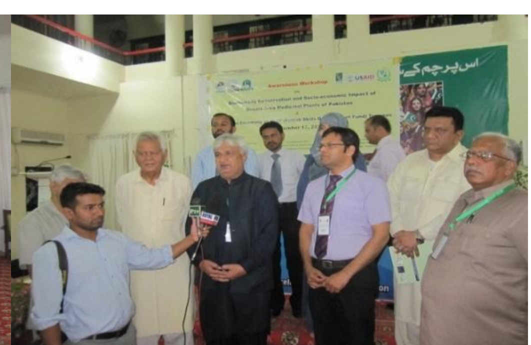
APPENDIX IV: AWARNESS WORKSHOPS

Two awareness workshops were conducted. The workshop in the Swat valley was conducted on May 22, 2013in collaboration with the Institute of Plant Sciences and Biodiversity Conservation, University of Swat. A good number of participants attended the workshop. The event was used to collect data from stakeholders of medicinal plants.

The workshop in Cholistan was conducted in collaboration with the Cholistan Institute for Desert Studies (CIDS) in Islamia University of Bahawalpur on September 17, 2013. This workshop was also attended by stakeholders of medicinal plants who interacted very keenly in the discussion and gave valuable information regarding medicinal plants and their marketting chain in the area. An MoU was also signed between University of Agriculture Faisalabad and CIDS for future research in this important area.

Pictures of Workshop in Swat Valley

Pictures of Workshop in Cholistan Desert



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