DNABarcodingand MolecularPhylogeneticstudyof *Ophiocordycepssinensis*(Berk.) (Yarshagumba)ofNepal.

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Submittedto
CENTRALDEPARTMENTOFBIOTECHNOLOGY
TribhuvanUniversity
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Forthepartialfulfillmentoftherequirementfordegreein
MasterofScienceinBiotechnology

MiraTamang
ExamRoll No.:BT068-068
T.U.Regd. No.:5-2-37-612-2007

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Mira Tamang

ABSTRACT

Ophiocordycepssinensis (Berk.) Sung (Syn. *Cordycepssinensis*(Berk) Sacc.) is one of the highly prized medicinal fungus distributed in Tibetan plateau of China, high altitude and grassland of Nepal, Bhutan and India ranging from 3000-5000m asl.This study has attemptedDNA sequencing of Cytochrome 'C' Oxidase (COI), Internal Transcribed Spacer (ITS) and D1/D2 Domain of nuclear ribosomal DNA 28S Large Sub-Unit (LSU) of different *Ophiocordycepssinensis*populations to generate barcode sequences for their identification and understanding their phylogenetic relationship. Total 34 samples were collected from four different districts *viz*.Dolpa, Manang, Bajhang and Darchula and genomic DNA was extracted both from fungal and insect parts. Polymerase Chain Reaction (PCR) amplification of nuclear ribosomal DNA (nrDNA)ITS, D1/D2 domain of LSU and COI region were carried out using genomic DNA. Sequencing of three loci were able to identify samples at the species level by comparing nucleotide sequences with Genbank database by using bioinformatics tools. TheBLASTn search of ITS loci of 26sample of present study showed 98-100% similarity with *Ophiocordycepssinensis.* Similarly BLASTn search of 28s LSUof 20 samples of present study showed 98-99% similarity with *Ophiocordycepssinensis*.AgainBLASTn search of COI locusof 11samples of present study showed 97-99% similarity with *Hepialus* larvae. Overall, all three barcode markerswere able to identify *Ophiocordycepssinensis*and *Hepialus* spp. at species level either individually or in combination. Molecular phylogenetic analysis of *O. sinensis* based on three barcode sequences showed it to be monophyletic and *O. nepalensis* and *O. nutans* to be sister species.

Keywords: COI,D1/D2 Domain,ITS,*Ophiocordycepssinensis*, maximum parsimony, model-based phylogeny.

LIST OFFIGURESANDTABLES

FIGURES

TABLES

CONTENTS

Abbreviation and Acronyms

µg	micro gram
µl	micro liter
mg	milli gram
MgCl	Magnesium Chloride
mL	milli liter
mm	milli meter
mM	milli Molar
ADP	Adenosine diphosphate
ASL	Above Sea Level
AFLP	Amplified Fragment Length Polymorphism
AICc	Akaike's Information Criteria corrected
Bp	base pair
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
CBOL	Consortium for barcode of life
cDNA	complementary DNA
CI	consistancy index
DHR	Dhorpatan Hunting Reserve
DNA	deoxyribo nucleic acid
dNTPs	Deoxynucleotide phosphate
DOF	Department of forest
EDTA	Ethylene Diaminetetraacetic acid
EtBr	Ethidium Bromide
GLB	gel loading buffer

GTR	general time reversible
HKY	Hasegawa, Kishino and Yano
MAPs	Medicinal and Aromatic Plants
MEGA	Molecular Evolutionary Genetic Analysis
MPTs	Maximum Parsimony Tree
NAST	Nepal Academy of Science and Technology
NCBI	National Center for Biotechnology
NJ	Neighbour Joining
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SSR	Simple Sequence Repeat
STS	Sequence Tagged Sites
QTL	Quantitative Trait Loci
TPM1uf	Kimura 3-parameter with unequal frequency
TPM2uf+G	Kimura 3-parameter with unequal frequency +Gamma Distribution
TVM+I	Transversion Model with invariable sites
UPGMA	Unweighted Pair Group Method with ArithmeticMean

1.1 Background

Nepal, the country with world's highest mountain peaks, is situated in the central Himalaya and it has diverse physiographic zones, climatic contrasts and altitudinal variations (Bhuju*et al.,* 2007). Physically, Nepal is a land-locked country, stretched about 885km from east to west and a width of 145 to 248km from north to south (Jha*et al.,* 2013). Nepal is located between the latitudes 26°22' and 30°27'North and longitude 80°40' and 88°12' East(NTNC, 2001).Nepal is blessed with rich floral and faunal diversity. The study of flora and fauna of Nepal started for the first time in the early 1820s(Katuwal *et al.*, 2013). Nepal is actually a storehouse of Medicinal and Aromatic plants (MAPs) diversity. Over 1950 species of medicinal and aromatic plants (MAPs) have been reported to be found in Nepal(Ghimire, 2008). Among them, *Ophiocordycepssinensis*(*Cordycepssinensis*) is one of the most important medicinal species that comprises of a fungus colonizing the moths of Hepilopteran family.It is found in sub-alpine to alpine regionsat altitudesranging from 1300-4000 aslof Nepal(Wojcikowski *et al.*, 2004).

The name *Cordyceps*derive from the Latin words 'cord' and 'ceps' meaning 'club' and 'head', respectively. 'Dong Chong Xia Cao' (DCXC), is the chinese name. It is generally called 'DCXC' in Chinese and 'Tochukaso' in Japanese. It is also called "winter worm summer grass''. It may have beennamed due to fungi infect to larvae on summer or autumn and it is covered by mycelia and in winter is converted into worm so it is called winter worm summer grass(Xuanwei Zhou, 2009).

Ophiocordycepssinensis is surrounded by and interspersed with towering mountain ranges, its complex geomorphology and climate generate substantial interregional variation which results in remarkable faunal and floral diversity and high levels of endemism (Li & Fang, 1999).This distribution pattern suggests that the evolution of *O. sinensis*was significantly influenced by the tectonic movements during the strong uplift of the Tibetan Plateau. The spread of *O. sinensis*, depending on the shooting ofascospores, is limited to defined areas in a mountain andis unlikely through discontinuous mountains. Therefore,gene flow between different populations on discontinuous mountains is expected to be low, and divergence mayhave occurred (Chen *et*

al., 1999). Due to geographical variation, 27 Himalayan districts of Nepal has the potentiality of producing the host caterpillar of *Ophiocordycepssinensis*(Baral *et al.*, 2015).

Ophiocordycepssinensis is one of the most important and highly traded species of Nepal, commonly known as the Chinese caterpillar fungus. It has been widely used in traditional Chinese medicine for the treatment of bronchial asthma, lung inflammation, and other diseases (Zhu *et al.*, 1998). The medicinal activity is from the complexofthemothcaterpillarparasitizedvby the fungal stromalbiomass, whichbelongtothe familyHepialidae,live undergroundinsoil burrowsandfeedonplantroots.Whenaninfectionis established,thefungusgrowswithinbodycavityof the insectlarvaeand eventuallykills andmummifiesthemintheundergroundburrows.Fungalfruitingbodyemergesfromthefrontendoft hecaterpillarandpokesoutof the groundin springandearly summer (Buenz *et al.*, 2005).

This species is being traded globally as high value Medicinal species of Nepal.Its trade in Nepal is also closely linkedwith livelihood of rural people. Due to land fragmentation, degradation, over exploitation and climate change, high value MAPs including *O. sinensis*has been threatened, which is causing another big challenge for Nepal Himalayas. Many counterfeit species (or products) are being released in the market which look like *O. sinensis*but have low medicinal and economical importance (Paudel, 2007).

In this context, DNA barcoding technique has emerged as a powerful molecular tool for accurate species identification and for counterfeitspecies identification (Xiang *et al.*, 2013). DNA barcoding technology can be a highly potential technology for regulating illegal trade of biological materials of animal, plants or fungal origin (Taylor & Harris, 2012). It can be considered as a gold standard technique for the identification of counterfeit species and new speciesand where taxonomist are unable to identify species (Hebert *et al.*, 2003).This technique is one of the most reliable and quick molecular identification techniques, which can be applied even if the sample is in trace amount. Besides being a useful tool for taxonomy (Hebert & Gregory, 2005),it also assists in identification of species at communitylevel. It has grown

exponentially in terms of the number of sequences generated as barcodes as well as its applications. Sequence generated for DNA barcoding can also be used for the phylogenetic and evolutionary studies utilizing bioinformatics tools (Kress & Erickson, 2012).

In this research genomic DNA was extracted and PCR amplification of theDNA was carried out by respective primers of ITS and D1/D2 for fungi and COI for larva. After that the selected amplicons were sent to Macrogenkorea for sequencing, after that sequence were retrived and analysed by using MEGA 5 and jModeltest version 2.2.4 for evolutionary and phylogenetic relationship analysis. This type of research plays a vital role to unravel its biological secrets in order to achieve full benefit from it. It is highly anticipated that in near future by doing this type of scientific research, scientists will definitely prove the way toward conservation and sustainable utilization of this highly prized medicinal species (Zhang *et al.*, 2012).

1.2 DNA barcoding

Identification of a species through DNA barcoding is a critical tool for varied areas such as plant,fungi and animal. These species are generally identified using morphological characteristics, which requires special training, on-hand identification tools (like books, flora information, scientific papers etc.) and most importantly identified with the help of reproductive structures. In the absence of reproductive structures, it is almost impossible for many species to be correctly identified. First of all the term "DNA barcode" was coined by Paul Hebert in 2003 for the identification of global species(Hebert *et al.*, 2003).The Barcode of Life Data (BOLD) system provides an integrated bioinformatics platform that supports all phases of the analytical pathway from specimen collection to tightly validated barcode library. A repository for the specimen and sequence records forms the basic data unit of all barcode studies. It is also a workbench that aids the management, quality assurance and analysis of barcode data. It also provides a vehicle for collaboration across geographically dispersed research communities by coupling flexible security and data entry features with web-based delivery (Ratnasingham & Hebert, 2007).

The DNA barcode is a very short, standardized DNA sequences from coding and non-coding parts of the genome (Kress & Erickson, 2008). It provides a way to identify the species to which a plant, animal or fungus belongs. The Consortium for the Barcode of Life (CBOL) is promoting international partnerships that will enable people in all countries to better understand and protect their biodiversity using DNA Barcoding technology (Ratnasingham & Hebert, 2007).

1.3. Research Hypothesis

1) H_0: There is no population level genetic differentiation in all tested loci of *O. sinensis* of Nepal.

H_1: There is population level genetic differentiation in all tested loci of *O. sinensis* of Nepal.

2) H_0: It is not possible to identify different population of *O. sinensis*of Nepal using DNA barcoding technique.

H_1: It is possible to identify different population of *O. sinensis*of Nepal using DNA barcoding technique.

Where, H_0: Null Hypothesis H_1: Alternate Hypothesis

1.4 Objectives

The general objectives of this study is to generate standard DNA barcode sequences (ITS, D1/D2 and COI) using DNA, extracted from fungal and insect parts of *Ophiocordycepssinensis*, collected from different geographical locations of Nepal for the development of molecular diagnostics of *O. sinensis* and their phylogenetic study based on different barcode loci.

The Specific objectives of this study are:

- To optimize DNA extraction protocol for fungal and insect part of *Ophiocordycepssinensis*.
- To optimize PCR reaction parameters and thermal cyclic conditions for the amplification of barcode loci of both fungal and insect portion of *Ophiocordycepssinensis*.
- To generate ITS, D1/D2, COI sequences for DNA Barcoding.
- To perform phylogenetic analysis.

1.5 Rationale and scope of study

Molecular diagnostics based on DNA barcodes can be powerful identification tools in the absence of distinctive morphological characters for distinguishing between closely related species(Davis *et al.*, 2011). It requires a highly trained and judgmental specialist in order to distinguish subtle anatomical differences between closely connected species. This is tedious and time consuming. Nowadays DNA barcoding is gaining popularity because it does not require taxonomists and objectively identifies species even from small, damaged, or industrially processed material. Barcoding allows comparing newly found species with the older ones in order to identify their evolutionary and phylogenetic relationship (Kress & Erickson, 2012).

*Ophiocordyceps*is usedas a traditional medicine for lung protection, reproductive invigoration and to maintain the fundamental energy of life. It is a parasitic organism that grows on a caterpillar until a caterpillar dies and the mushroom sprouts from the caterpillars head(Li Xiang, 2013). Due to lack of molecular studies in Nepal, there is no established DNAbarcode database of *O. sinensis*and its related specie. Due to its over exploitation, pollution and illegal trading there has been great depletion of the species which might cause extinction in the future. As a biodiversity discovery tool, DNA barcoding is one of the most important tool for the fast and accurate identification method to distinguish *O. sinensis* from counterfeit and closely related species to ensure its quality control (Li Xiang, 2013).

2.1 History of discovery of *O. sinensis*

About 1500 years ago in Tibet, a Herdsmen observed that their livestock became energetic and healthy after eating certain mushroom (Sharma, 2004), which was none other than *Ophiocordycepssinensis*(Berk) Sung *et al*.(Syn. *Cordycepssinensis* (Berk.)) (Sung *et al.*, 2007). It has a 1500 years history of use as a medicine by Tibetan monk (Devkota, 2006). At that time they just collected stromal part of the organism and used as energy giving food and to exchange greetings. Historically,*Ophiocordycepssinensis* is called as 'medicinal mushroom' but it is not exactly the mushroom (Holliday & Cleaver, 2004). *Ophiocordycepssinensis* was historically classified in the family *Clavicipitaceae*(Zhang *et al.,* 2012). Local people of Dolpa called to *Ophiocordycepssinensis*as *Yarsagumba*, *Jara* (Root), *Kira* (Insect), *Jeevanbuti* (Life tonic), *Chyau* (Mushrooms) and *ChyauKira*(mushroom insect). In 1843 A.D. it was described as *Sphaeriasinensis*Berk by Berkley who was a great British mycologist. Later in 1878, Saccardo renamed it as *Ophiocordycepssinensis*. Finally, it was scientifically named as*Ophiocordycepssinensis*(Berk) (based on the fungal part of *Ophiocordycepssinensis)*(Li *et al.*, 2006).

2.2 Family Clavicipitaceae

Clavicipitaceae is a family of the order Hypocreales in the class Pyrenomycetes of the phylum Ascomycota (Spatafora & Blackwell, 1993). This family is phylogenetically divided into three clades :Clavicipitaceae clade 'A', Clavicipitaceaeclade 'B' and Clavicipitaceae clade 'C'. *Cordyceps* species belongs to clade 'C' and *Ophiocordyceps* species belong to clade 'B' (Sung *et al.*, 2007). This family contain 43 genera and includes more than 400 species (Mains, 1958). The family Clavicipitaceae contain a variety of mutualistic or pathogenic symbiont of plants or fungi or of invertebrate animal(Kuldau *et al.*, 1997). The family Clavicipitaceae contains fungal pathogens exploiting hosts across three kingdoms of life in a pattern that features multiple inter-kingdom host shifts among plants, animals, and fungi. Fungal pathogens that cause ergot are linked morphologically with Clavicipitaceae(Kepler *et al.*, 2012). Clavicipitaceous fungi and related

anamorphs contain diverse econutritional groups.Among clavicipitaceous fungi *Ophiocordyceps*and related anamorphs are the potential sources for bioactive molecules and medicines such as ergot alkaloids,have a great potential as biocontrol agents (Sung *et al.*, 2001).One of the most important genusof family Clavicipitaceae is *Ophiocordyceps* which is endoparasite, ascomycetous with a diversity of insect hosts(Internet visit 2, 2015).The most important species of clavicipitaceae family are *O. sinensis*, *C. cicada*, *C. militaris* and *E. ophioglossoides*, the best known is *O. sinensis*(Zhang *et al.,* 2012).

2.3 Genus *Ophiocordyceps*

Ophiocordyceps is a genus of fungi within the family *Clavicipitaceae*, based on its cylindrical asci, thickened ascus apices, and filiformascospores that often disarticulate into part-spores (Mains, 1958). *Ophiocordyceps*is characterized and distinguished from other genera of the family by its production of superficial to completely immersed perithecia on stipitate and often clavate to capitatestromata and its ecology as a pathogen of arthropods and the fungal genus *Elaphomyces* (Kobayasi, 1941). It contains about 440 species that grow on insects(Hollingsworth *et al.*, 2009).

2.4 *Ophiocordyceps* species

Species of the genus *Ophiocordyceps* are the fungi found growing on insect larvae.Globally, more than 400 species of *Ophiocordyceps* have been reported of which only three species have been reported from Nepal(Devkota, 2006), namely, *O. sinensis* (Balfour –Browne, 1955)*, O. nutans*(Shrestha, 1985)and *O. nepalensis*(Zang and Kinjo, 1998), but according to Jha*et al.,*2013, nine species of *Ophiocordyceps* are found in Nepal: *O. formicarum, O. gracilis, O. kangdigensis, O.multiaxialis, O. nepalensis, O.nutans, O.sinensis, O. sphecocephala, O. tricentri*(Thapa,1998). Among them, *Ophiocordycepssinensis* has the high value inmedicinal, economic, social and ecological parts(Zhang *et al.*, 2012).

2.5*Ophiocordycepssinensis*(Berk)(Sung *et al.*, 2007)

Ophiocordycepssinensis (Berk) Sung. is the fruiting body of the fungus arising out of the dead body of a caterpillar (Hsu *et al.*, 2002). Like all other fungi*Ophiocordycepssinensis* is also a non-chlorophyllous, annual fungus. After the infection of insect larvae by fungi of Clavicipitace family the larva starts to die due to fungal infection then the fruiting structure of this species comes from the anterior head of larva of caterpillar (Ali, 2012). It is an entomogenous fungus found in high altitude.It has two components: the lower part is dead caterpillar and the upper part is the fungus. The fungus has a small spike with dark brown fructification and yellowish white stalk. The size of the fungus is about 4 to 12 cm in length and 0.14 to 0.4 cm in girth. The meaning of Yarshagumba is 'summer-grass winter-worm' in Tibetan language. Yarshagumba with both the caterpillar and fungal part in an intact single piece is an item of commerce(Bhushan Shrestha 2010). Some of the article says that *Ophiocordycepssinensis* and *cordycepssinensis*are just synonyms of a single species (Sung *et al.,* 2007) but some author says that they are different species (Shrestha, 2012) (Jha*et al.,*2013) . *Ophiocordycepssinensis* have pleasant, sweet taste with nutty-mushroom flavor. No known harmful or negative side effects even for pregnant women have been reported until today. It is the most valuable combination, but the genes responsible for biosynthesis of bioactive components, insect pathogenicity and the control of sexuality and fruiting have not been determined (Peng Zheng, 2011).

Fig.2.1whole*O. sinensis* of this study.

A single *Ophiocordycepssinensis* weight ranges from 0.3 to 0.5 gm. It can grow in the soil having pH 7-7.5 and habitats ranging from 2800-4500 meters it germinates during April – June/July and collection time is April/May – June/July (Baral *et al.*, 2015).Scientist has gone so far as to think that there is no type specimen of *O. sinensis* or even wonder what *O. sinensis*actually is(Bhushan Shrestha 2010). Due to nice combination of fungi and larvae has led to unique assemblage of metabolites including proteins, nitrogenous compounds, polysaccharides, sterols, nucleosides, fatty acids and their derivatives, vitamins and inorganic compounds (Shrestha *et al.*, 2013).

2.6 Morphology of *Ophiocordycepssinensis*

The surface of the head is granular due to projecting openings or ostioles of perithecia, its body surface consist of fatty acids. Temperature, soil pressure, environmental condition and light intensity are the main parameter for their growth (Guo *et al.*, 2015). *Ophiocordycepssinensis*is the name given to the stroma (fungal part), whereas the caterpillar belongs to different species of *Thitarodes* and other similar genera of the family Hepialidae, commonly known as the ghost moth, bat moth or swift moth. Mature *O. sinensis* consists of two parts, the basalinsect part sclerotium and the upperfungalstroma. Caterpillar is yellowish, while the stroma is dark brown or black in color. The length of sclerotium ranges from 3.5–4 cm, but the stroma is longer, normally 4–10 cm. Caterpillar of *Ophiocordycepssinensis* is clearly divided into head, neck, body segment and legs. The stromais further distinguished into two parts: the basal stem and upper head. The stem is 2.5–8.5 cm long and 2–3 mm wide, while head is 1–2.5 cm long and 3–5 mm wide. The stem is also slender, glabrous, but longitudinally furrowed or ridged; the headis slightly swollen, sub-lanceolate or fusiform, and distinct from the stem. Laterally compress and furrowed; 2–4 mm long and 2–4 mm wide (Shrestha *et al.*, 2010).

2.7 Distribution of *Ophiocordycepssinensis*

The latitude range of *Ophiocordycepssinensis* is N 25°45′-38°49′, and the longitude range is E 99° 34′-102°90′(Xiao & Zhong, 2007). About 203 localities are found to be the distribution sites of *O. sinensis,* of which 106 are considered as confirmed distribution sites, 65 as possible distribution sites, 29 as excluded distribution sites and 3 as suspicious distribution sites (Li *et al.*, 2011). These localities are Tibetan Plateau and its surrounding regions, along with Tibet, Gansu, Qinghai, Sichuan, and Yunnan provinces in China and certain areas Southern Himalayas, in the countries of Nepal, Bhutan and India (Li *et al.*, 2011). Globally, among the 91 species of larvae found in 13 genera of Hepialidae,57 species are considered as compatible host insects of *Ophiocordycepssinensis*, whilst eight as indeterminate hosts and 26 as non-hosts. Host insects are also found in the high altitude of around 2800 to 5100 m. (Wang & Yao, 2011). Grasslands of high altitude are the main habitats for larvae of *Ophiocordycepssinensis,*hence it is endemic Larvae prone to infection by *O. sinensis* normally live 15 cm (6 inches) underground. The *Ophiocordyceps* fungus parasitizes larvae of moths, larva dies by the fungal infection and larva converted into sclerotia from which the fungal stroma grows to the grasslands and shrub lands at an altitude of 3,000 to 5,000 m of different countries of Asia (ALI, 2012).

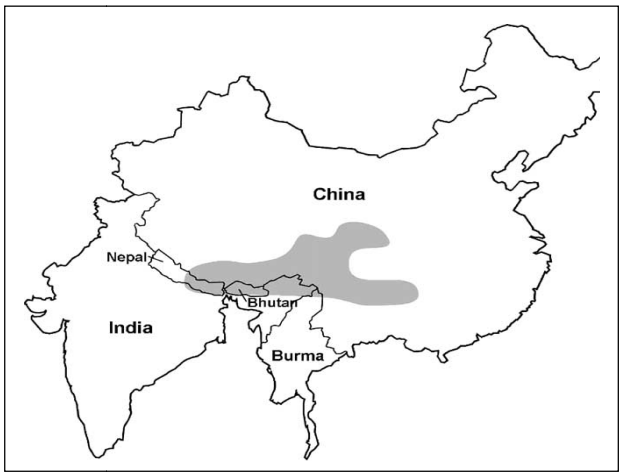


Fig 2.3. Worldwide distribution of *Ophiocordycepssinensis*(Buenz *et al.*, 2005).

In Nepal, its distribution is reported mainly in various localities of Dolpa, Rukum, Manang, Myagdi, Gorkha, Bajhang, Darchula and Jumla district (Shrestha & Bawa, 2014).

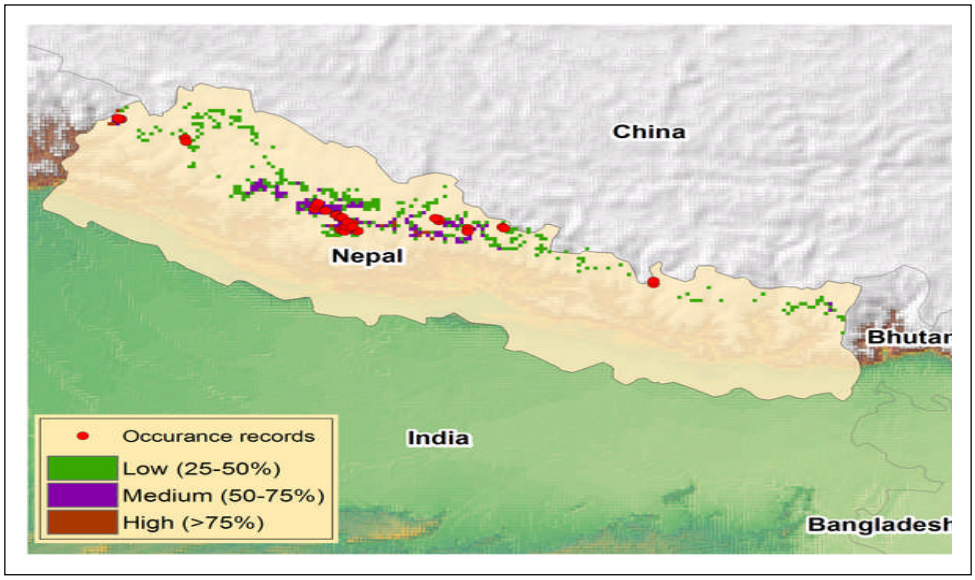


Fig 2.4. Distribution of *Ophiocordycepssinensis* in Nepal (Shrestha & Bawa, 2014).

2.8 Taxonomy of *Ophiocordycepssinensis*

The taxonomy of *O. sinensis*has generally been conceived as problematic and controversial due to lack of molecular study and confusion in anamorph and teleomoph stage of *O. sinensis*. *Ophiocordycepssinensis* name came through mis-understanding as *Clavariaentomorhiza*. Dickson (1785) reported *Clavariaentomorhiza* from England as *Sphaeriaentomorhiza* Dicks. After that *Clavariaentomorhiza* was identified as new species of fungi and new name *Sphaeriasinensis*was given by Berkeley (1843)(Bhushan Shrestha 2010). Saccardo (1883) gave the full description of *S. sinensis* Berkeley (1843) in Latin along with the group *C. sinensis* as an imperfectly identified and listed the scientific name from *Sphaeriasinensis* to *Ophiocordycepssinensis* (Berk) Sacc. (Sung *et al.*, 2007). Some latest literature revised and classified Genus *Ophiocordyceps* into four genera *Cordyceps, Elaphocordyceps, Metacordyceps* and *Ophiocordyceps* based on molecular phylogeny and morphology (Shrestha, 2012).Today, eventually scientists have learned to classify the organism using genetic sequences.It is classified as:

Kingdom: Fungi
Divison: Ascomycota
Order: Sordariomycetes
Class: Hypocreales
Family: Clavicipitaceae
Genus: *Ophiocordyceps*(Internet visit 7, 2015)
Local Name: Yarsagumba, Jivanbuti, Buti
Chinese Name: Dong Chong Xia Cao
English Name: Caterpillar Mushroom, Cordyseps

Entomopathogenic fungi are generally common natural enemies of arthopods worldwide, attracting attention as potential biological control agent(Hajek & St. Leger, 1994). *Ophiocordycepssinensis* belongs to entomopathogenicfungi these type of fungi usually attach to the external body surface of insects in the form of microscopic spores (Sung *et al.*, 2007).

Table2.1: Current classification of entomopathogenic fungi (Roy *et al.*, 2006).

Division	Class	Order	Family	Genus
Zygomycota	Zygomycetes	Entomophthorales	Entomophoraceae	Entomophaga Entomophthora Erynia Eryniopsis Furia Massospora Strongwellsea Pandora Tarichium Zoophtthora
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Beauveria Cordyceps Cordycepioideus Lecanicillium

2.8.1 Anamorph and teleomorph of *Ophiocordycepssinensis*

Ophiocordycepssinensis has two steps in its life cycle respectively sexual (teleomorph) and asexual (anamorph).*Ophiocordycepssinensis* is present in the plant roots during the anamorph life cycle, to deal with harsh conditions in alpine habitats and have an in-creased opportunity to infect the larvae. This finding will help to increase the annual yield of *Ophiocordycepssinensis* by giving information to biology and ecology of *Ophiocordycepssinensis*(Zhong *et al*., 2014).

Due to the lack of clear link between sexual and asexual phase of *Ophiocordycepssinensis,* it is very problematic to distinguish anamorph and teleomorph of *Ophiocordycepssinensis*(Chioza & Ohga, 2014). Hence, molecular study of different stage of *Ophiocordycepssinensis* is very important task. The result obtained after molecular study shows that *Hirsutellasinensis* and *Ophiocordycepssinensis* are the different phase of the life cycle of the same organism, therefore *Hirsutellasinensis* is anamorph of *Ophiocordycepssinensis*(Chen *et al.*, 2001). Both morphological and molecular methodology also prove that *Hirsutellasinensis* isanamorph(Chioza & Ohga, 2014),fully developed fruiting body is teleomorph and mycelium form of *Ophiocordycepssinensis* before larval infection is called anamorph(Chen *et al.*, 2004). But the anamorph of *Cordycepsgunni* is *Pacilomyceshawkesii*, anamorph of *C. brongniartii*is*Beauveriabrongniartii*, anamorph of *C. brittlebankisoides*is*Metarhiziumanisopliae*, anamorph of *C. soboliferai*s*Beauveriasobolifera*, anamorph of *C. pruinosa* is *Mariannaeapruinosa* and anamorph of *C. militaries* is*Paecilomycesmilitaris*(Liu *et al.*, 2002, Xiang *et al.*, 2013, Zhong *et al.*, 2014). From1st January 2013 the International Code of Nomenclature started giving just a single name for single fungus, that is why anamorph*Hirsutellasinensis*has changed to*Ophiocordycepssinensis*(Lo *et al.*, 2013).

2.9 Artificial cultivation

Its biology is still under discovery and its commercial cultivation is still a dream (Zhang *et al.*, 2012). Even though many practices are been done for the artificial cultivation of this treasure herb,some of them produces simple stromata of *Ophiocordyceps*spp by injecting a suspension of its hyphal bodies into larvae (Sato & Shimazu, 2002). *Ophiocordyceps* fungi infects compatible host larvae. Infection to all larvae is not possible because every organisms over the

earth has their own defense system. Difficulty in cultivation is due to the low percentage of fungal infection on artificially inoculated host insect. Cloning of cuticle-degrading serine protease gene (csp1 and csp2) from *O. sinensis* has been done in order to better understand the infection mechanism(Yongjie Zhang 2008). In-vitro stroma production of this fungus was examined by injecting a suspension of its hyphal bodies into three species of lepidopteran pupae (*Mamestrabrassicae, Spodopteralitura, Bombyxmori*) and a species of coleopteran pupae (Tenebriomolitor) (Sato & Shimazu, 2002). *Ophiocordycepssinensis* infects insect larvae in soil in mid-August because at that time shedding of old cuticles and forming new cuticles occur. Larvae at less advanced stages are seldom infected probably because of their limited movement and food intake, and those at more advanced stages are seldom infected probably because of increased resistance. After the fungal infection of larvae they become less active in 6-10 days and die in 15-25 days; infected larvae, however, often move to positions 2-5 cm beneath the soil surface before dying, which facilitates the growth of the fruiting body and its emergence from the soil in the next year. A small stroma bud often grows from the head of infected larvae before the soil freezes in winter. Local people have observed larvae with small stroma buds that were alive and able to move(Zhang *et al.*, 2012). Development of special capillary curve, identify the quantity of *O.sinensis* in *Thitarods* larvae, was detected rapidly in four tissues of its host larvae, the maximum content of *O. sinensis*parasitized in the fat-body and next came body-wall both of them were much larger than that observed in the haemolymph and intestinal-wall (Lei *et al.*, 2013). Compatible and nice combination of larva and fungi in yarsagumba is primarily based on its larva fungi depend upon even in small things such as feeding (Wei Lei, 10 January, 2011).

2.10 Life cycle of *Ophiocordycepssinensis*

Life cycle of *Ophiocordycepssinensis* follows by the process of Alternation of generation (Guo *et al.*, 2015). Selection of host moth is according to the locality and altitude. Its life cycle completes within 2-6 year, varying according to the species and altitude (Baral *et al.*, 2015).

Fig2.5 life cycle of*Ophiocordycepssinensis*(Zhang *et al.,* 2012*).*

Its life cycle include two stages asexual phase and sexual phase (Zhong *et al.*, 2014). Naturally *Hepialus* moths require 3-4 years to complete their life cycle. In general the caterpillar need 6 years, but they may have 7-8 years in special environment. If Hepialus moths are raised in lab they can finish life cycle in 1-2.2 year(Xiao & Zhong, 2007). *Ophiocordyceps sinensis* has an unusual life cycle in nature. In late autumn, the fungus infects underground larvae of ghost moths of the family Hepialidae. The mode of fungal infection is still unknown, it may be either by inhaling fungal spore by larva, perhaps a fungal hypha penetrates a spiracle of the insect, or perhaps an ascospore or a conidium adhering to the insect surface germinates and directly penetrates the insect's cuticle. After entering the caterpillar's body, the fungus grows vegetatively and fills the caterpillar with thread like hyphae. Even infected by the fungus, the larvae can still move from positions 5-25cm below to positions 2-5 cm beneath the soil surface before dying with the head upward. The fungus then grows out from the dead host and forms a small stroma bud before soil freezing in winter. In the following spring, the stroma bud grows

upward, emerging above the soil surface and forming a stalked fruiting body. Thread-like ascospores are released from the perithecia and can presumably infect new caterpillars. In addition to the ascospores, *O. sinensis* can also produce asexual conidia. The anamorph of the fungus is widely recognized as *Hirsutella sinensis*. The conidia are produced by conidiogenous cells formed on vegetative hyphae or germinated ascospores (Zhang *et al.*, 2012).

2.11 Importance of *Ophiocordycepssinensis*

It has high economic, biological and pharmaceutical importance.Natural product of *Ophiocordycepssinensis* origin play very important role in biological and pharmaceutical industries. Till today no harmful activity about *Ophiocordycepssinensis* have been reported (Yue *et al.*, 2013).

2.11.1 Medicinal importance of *Ophiocordycepssinensis*

Ophiocordycepssinensis is reported to have diverse medicinal properties such as antitumor properties, it is also helpful in kidney, liver and heart disease, acute and chronic hepatitis to kidney, heart, liver. It is important medicine for respiratory disease, kidney , headache, insomnia, it also help to stimulatory immune system(Xiao & Zhong, 2007). As a valued Chinese herb and tonic, *Ophiocordycepssinensis* has a long history of use and a high reputation of value in Nepal, China and abroad. In Traditional Chinese Medicine (TCM), the fungus is believed to nourish the lungs and kidneys (Wu 1757). It has also been shown in recent studies to have multiple pharmacological effects, including immune modulating(Wu *et al.*, 2006), hypocholesterolemic(Koh *et al.*, 2003), hypoglycemic (Zhang *et al.*, 2006), anti-tumor(Yalin *et al.*, 2005), anti-oxidation (Dong and Yao 2008) and anti-aging (Ji et al. 2009) activities(Ji *et al.*, 2009). *Ophiocordycepssinensis*parasitize larvae of Thitarodes(ZHI-WEN ZOU1, 2011) which is categorized as a warm medicinal substance and is commonly administered in tonic form to replenish immune function and to help against respiratory disease, fatigue, night sweating, arrhythmias heart diseases, hypo-sexuality, hyperglycemia, hyperlipidemia, myocardial mitochondrial ATP generation, and renal failure. Its polysaccharide fractions modulate immune

activity and inhibit tumor growth(STEWART, 2011). This treasure herb is used for tonifying lungs,replenishing kidneys,stopping bleeding and resolving phlegm for treating chronic cough,debilitating asthma, consumptive cough with hematemesis, impotence, spermaturia, lumber and knee pains (Pharmacopoeia commission, 2005).More than 30 types of bioactivities such as immuno-modulatory, antitumor, anti-inflammatory and antioxidant activities have been reported from wild *O. sinensis*. These bioactivities are derived from over 20 bioactive ingredients mainly extracellular polysaccharide, intracellular polysaccharide, cordycepin, adenosine, mannitol and sterol. *Hirsutellasinensis* has the ability to accelerate leucocytes recovery (Hui-chen lo, 2013). It has been described as an exotic medicinal mushroom in traditional Chinese and Tibetan medicine (internet visit4, 2015).Amchi of high Himalayan district even in the absence of sophisticated equipment they diagnose disease and prescribe *Ophiocordycepssinensis*(Lama *et al.*, 2000).

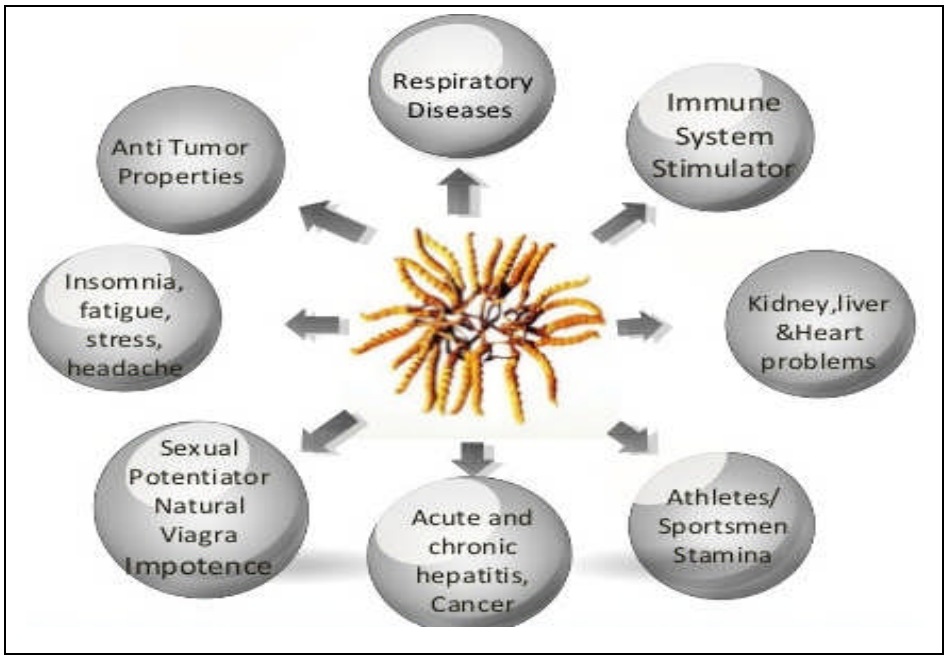


Fig. 2.6 Medicinal importance of *O. sinensis* (Zhu, Halpern et al. 1998).

Over dose consumption of *Ophiocordycepssinensis* does not have any side effect, even though appropriate dosage is recommended for an healthy individual to get health benefit like energy and vitality, thereby increasing physical performance, reducing blood pressure and lowering

cholesterol level, relieving indigestion and improving appetite, strengthening the immune system and providing relief from cough and cold, anti-aging ,reduce chronic fatigue, refreshing the mind, sharpening the memory.*Ophiocordycepssinensis* per day 0.3-0.7 gram (1-2 pieces) whole mushroom in the form of powder or with boiling water is prescribed, according to health problem dosage can be increased (Zhu *et al.*, 1998, Shrestha *et al.*, 2013).Extract from *Ophiocordycepssinensis*improved the activity of superoxide dismutase glutathione peroxidase and catalase and lowered the level of lipid per-oxidation and mono amine oxidase activity in the aged mice as a result acting as anticancer and antitumor agent (Ji *et al.*, 2009).*Ophiocordycepssinensis* has a history of hundreds of years in medicine, ithas great role for the treatment or prevention of diverse chronic diseases such as cancer, blood pressure, hyper tension, renal and hepatic diseases, inflammation and tumor (Xuanwei Zhou, 2009).

2.11.2 Economic value of *Ophiocordycepssinensis*

Year by year fluctuation in the quantity and quality of *Ophiocordycepssinensis*occured. This decrease in number and quality of *Ophiocordycepssinensis* may be due to many reason, it may be due to overharvesting, pollution done by collector and visitor, decrease in moth and larval populations, ecological imbalance, cattle over grazing etc. The annual collection of *O. sinensis* in Nepal during 2004 was 50,000 Kg, 3.1 kg was marketed in 2002. Harvesting of *O. sinensis* decreased from 260 to only 7 pieces per person in 2006 then increased to 125 pieces in 2010, reaching 872.4 kg in 2009, then plummeting to 473.8 kg in 2011. The national trade volume of *Ophioordyceps* throughout Nepal was 2 442.4 kg in 2009 which declined to 1170.8 kg in 2011. No data are available from 2012 to date (Baral *et al.*, 2015).

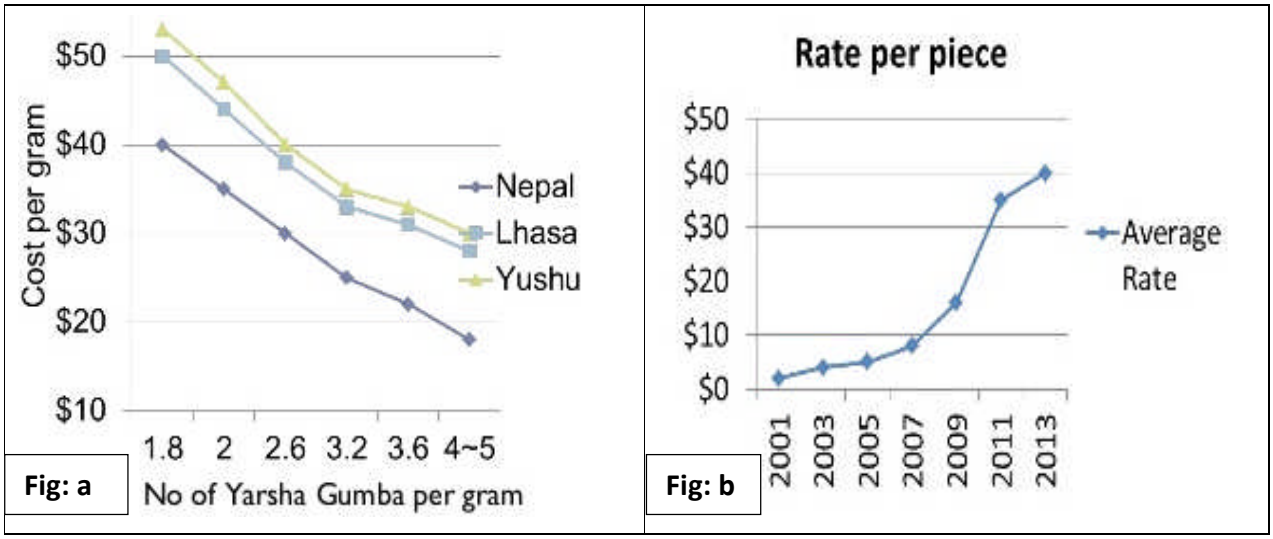


Fig 2.7: Financial analysis of *O. sinensis* from 2001 to 2013, fig: a) shows that the drastic increase in the rate of *O.sinensis* per gram, 17$ to 40$ in Nepal, 27-50 in Lhasa and 30$ - 55$ in Yushu. Fig b) Rate of *O.sinensis*per piece in 2001 was 0$ but in 2013 it increases to 40$ per piece (internet visit 5, 2015).

From this data, we can say that price of *Ophiocordycepssinensis* increased dramatically from 2001 to 2011 market price climbed by 2300% in Nepal and in Tibet it is increased by 900%. In 2001 A.D., local people of Dolpa used to sale in NRs. 20-25 per piece for Yarshagumba, which is very cheap in comparison to this year (2015 AD). After that year, consequently rate of each yarshagumba is increasing. They received NRs 200-600 per piece in 2011, representing an increase of 900-2300%. Similarly, local traders sold one kg to wholesalers/exporters for NRs 80,000- 130,000 in 2001, but received NRs 1,100,000-1,600,000/kg in 2011 an increase of 275-1131%. This drastic change in rate of yarshagumba may be due to literacy, awareness about health market demand, timing and location of trading (Shrestha & 2012).*Ophiocordycepssinensis* changes life standard, gives job to local collector, increase income opportunities, improve livelihood, enhance rural income and raise the national revenue (Devkota, 2006). *Ophiocordycepssinensis* has been used as food and herbal medicine in Asian country, they generally take *Ophiocordycepssinensis* in the form of power, syrupand whole organism (Kuo *et al.*, 2005). It is widely used as a tonic and medicinal food in traditional Chinese

medicine (TCM), as it possess wonderful health benefits(Shashidhar *et al.*, 2013). To support its functional attributes, various investigations have been carried out to find out its adaptogenic, aphrodisiac, anti-oxidant, anti-aging, neuroprotective, immune modulatory, anti-cancer and hepatoprotective role(Shashidhar *et al.*, 2013).

2.11.3 Chemical composition of *Ophiocordycepssinensis*

Ophiocordycepssinensis contains crude fats, proteins, fiber, carbohydrate, cordycepin, cordycepic acid, polysaccharide and a series of vitamins, etc. The therapeutic applications of *Ophiocordyceps* centered primarily on the key effects of increased oxygen utilization and ATP production and as well the stabilization of blood sugar metabolism. Cordycepin, cordycepic acid and the polysaccharides, vitamins and trace elements are found (Shrestha *et al.*, 2014). The molecular weight of cordycepin with molecular formula is 251($C_{10}H_{13}N_5O_3$). Its melting point is 230°C-231°C and maximum absorption is at 259 nm. Cordycepic acid is an isomer of quinic acid.

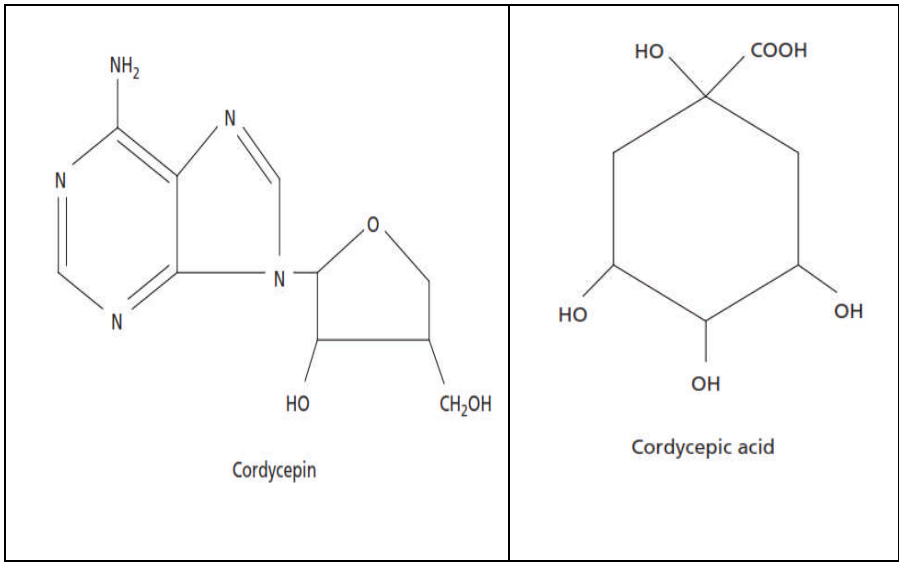


Fig.2.8 Chemical structure of cordycepin and cordycepic acid.

Fruiting body of *O.sinensis* provides 3-8% polysaccharide of total weight of *O. sinensis*, polysaccharide play an important role in the maintenance and healing of health

problem.*Ophiocordycepssinensis* also produces important bioactive component such as lipid, protein, polysaccharides, nucleosides etc.(Ali, 2012).

Nucleotides are the main class of active component in *O. sincensis*. There are some health food products of *O.sinensis* available in the international market. Adenosine, uridine and guanosine are the main nucleotide component of *cordyceps* mushroom (Wah, 2003). Ergosterol is a sterol unique to fungi and is an important precursor of vitamin D, which has important medicinal value. The crude protein in *O.sinensis,*ranges from 29.1–33%, which contain 18 amino acids. Saturated or unsaturated fatty acids comprise of carbon, hydrogen and oxygen and are the major components of lipids, phospholipids and glyco-lipids. Unsaturated fatty acid plays an important role for the maintenance of physiology, decreases blood lipids and protecting against cardiovascular disease (Zhou *et al.*, 2009). Chemical components of *O. sinensis* help to stimulate immune reactivity and to inhibit the growth of tumor, act as antibacterial agent, strengthen heartbeat and lower heart disease. It also helps to promote or suppress immuno-modulating factors such as T-cells and *β*-cells. Bioactive component extracted from these *O. sinensis* also help to protect kidney, liver and heart(Zhou *et al.*, 2009, Yue *et al.*, 2013).Tumor is the one of the major death causing problem in the world and researcher have found that polysaccharide, sterol, lipids found in the *O. sinensis* has the potentiality to inhibit tumor (E.J. Buenz, 2005).

2.12 Identification of *Ophiocordycepssinensis*

Identification of any species is the initial and fundamental part of research. Correct identification of any species is very important part of research work which can be done by employing morphological, anatomical, physiological, behavioral and molecular methods. *Ophiocordycepssinensis*is identified by morphological and molecular method.

2.12.1 Morphological identification

Morphological identification is done by observing the discription and distribution ,growth and culture morphological characteristic of *Ophiocordycepssinensis*(Chih-Sheng Chen, 2011).

2.12.1.1 Macroscopically

The *Ophiocordyceps* species their counterfeit can be distinguished by macroscopic identificationby observing the length, shape, color, pair of leg of host insect, diameter of stroma and sclerotium. The stroma of *C. gunnii* is stout and rough with sterile bulgy or branched apex, the stroma of *C. liangshanensi* is thread-like, *C. gracilis* does not contain stroma. The larvae of *C. barnesii* have a pair of teeth whereas *C. militaris* does not have larvae. The stroma of *C. barnesii* is without perithecia,*C. gunnii*, *C. liangshanensis*and *C. gracilis*are without bristles on the larva body. These differences allow *C. sinensis* and its counterfeits to be easily distinguished (Kinjo & Zang, 2001, Liu *et al.*, 2011).

2.12.1.2 Microscopically

By observing transverse section of larval body, andstroma and surface section of stroma,*C. sinensis* and its counterfeits can be distinguished easily(Liu *et al.*, 2011)(Mondini *et al.*, 2009). Anatomical identification is also done by another way of species identification (Li *et al.*, 2015).

2.12.2 Molecular identification

Molecular identificationis based on either restriction fragment analysis of DNA or PCR Amplification or both, for ecological, evolutionary, taxonomical, phylogenic and genetic studies of any species.DNA markers are useful in both basic and applied research.Protein polymorphisms were the first markers(Marsjan & Oldenbroek, 2006, Marsjan & Oldenbroek, 2007)**.**

2.12.2.1 Molecular marker and their application

Molecular marker should be polymorphic and evenly distributed throughout the genome,provide adequate resolution of genetic differences, generate multiple independent and reliable markers (Sung *et al.*, 2007). Even in the trace amount of DNA it should be reliable, should have linkage with phylogenetic relation, should not concern with the prior information about the genome of an organism (Mondini *et al.*, 2009).The benefits of molecular technique

are as follow 1) At any stage of life of an organism can be used as template DNA which contains introns, exons and regulation region, mitochondria and any of these regions can be used as template DNA, 2) It does not contain epistatic effects, distinguish polymorphisms which doesnotnot produce phenotypic variation and Some of them being co-dominant (Pandey *et al.*, 2010).

2.12.2.2 Non-PCR based Marker

2.12.2.2.1 Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphisms sites are identified using restriction enzymes that cleave the DNA only at precise "restriction sites" (Botstein *et al.*, 1980). At present, the most frequent use of RFLPs is downstream of PCR (PCR–RFLP), to detect alleles that differ in sequence at a given restriction site. A gene fragment is first amplified using PCR, and then exposed to a specific restriction enzyme that cleaves only one of the allelic forms. The digested amplicons are generally resolved by electrophoresis(Botstein *et al.*, 1980). RFLP is done for the RAPD product by applying some precaution mention in Sambrook book (Bowen *et al.*, 1996).

2.12.2.2 PCR-based Markers

2.12.2.3.1 Amplified fragment length polymorphisms

AFLP is one of the most reliable test for the identification of unknown species and it is also useful for the identification of evolutionary and phylogenetic relationship (El-Fadly *et al.*, 2008). In this process, DNA restriction enzyme is used and desired fragment are identified by PCR amplification technique based on DNA fingerprinting technique. Variations at many loci can be assayed simultaneously to detect a single nucleotide variations of unknown genomic regions (Zeid, 2003). This technique has lots of benefit over normal PCR. Such as the generation of a large number of markers in a single PCR reaction,a high level of polymorphism and a high reproducibility and reliability due tostringent PCR conditions has the capability to detect various polymorphisms in different genomic regions simultaneously (internet visit 6,2015).AFLP has

higher reproducibility, resolution and sensitivity as it has the capacity to amplify between 50 and 100 fragments at one time (Vos *et al.*, 1995).

2.12.2.3.2 Microarray technology

The DNA microarray is a tool used to determine whether the DNA from a particular individual contains a mutation in genes**.** Microarray technique is useful to measure gene transcription at a large scale, identification of genes involved in a number of traits, including adaptive traits, and polymorphisms causing functional genetic variation. Molecular techniques is useful in the investigation of the origin and domestication of livestock species, and their subsequent migrations, as well as providing information on evolutionary relationships, and identifying geographical areas of admixture. However, the number of polymorphic loci that can be assayed, and the level of polymorphisms observed at the loci are often low, which greatly limits their application in genetic diversity studies. With the development of new technologies, DNA polymorphisms have become the markers of choice for molecular-based surveys of genetic variation (internet visit 10, 2015). Microarray technology is useful for the discovery of new gene. Besides, this technique also identifies the functioning and expression of that gene. This technique is useful for the detection of disease like heart diseases, mental illness, infectious disease and especially the study of cancer, by recognizing disease. It helps the pharmaceutical group to formulate suitable medicine and to discover new drug. Microarray technology is also applicable for the toxicological researchestablishes correlation between responses to toxicants and the changes in the genetic profiles (internet visit 7, 2015).

2.12.2.3.3 Microsatellites

Microsatellites are the most popular markers in livestock genetic characterization studies. Their high mutation rate and co-dominant nature permit the estimation of within and between breed genetic diversity, and genetic admixture among breeds even if they are closely related. Microsatellites are of relatively small size and can therefore be easily amplified using PCR from DNA extracted from a variety of sources including blood, hair, skin or even faeces(Glover & Hames, 1995). Polymorphisms can be visualized on a sequencing gel and the availability of

automatic DNA sequencers allows high-throughput analysis of a large number of samples. They often show tens of alleles at a locus that differ from each other in the numbers of the repeats Microsatellites are hyper variable, they are still the markers of choice for diversity studies as well as for parentage analysis and Quantitative Trait Loci (QTL) mapping. Minisatellites has the same characteristics as microsatellites, but the repeats are ten to a few hundreds basepair long (internet visit 8, 2015).

2.12.2.3.4 Sequences-tagged sites (SCARs, CAPS, ISSRs)

The concept of STS was introduced by Olson *et al* in 1989. STSs are DNA sequences that occur only once in a genome, in a known position. They need not be polymorphic and they are used to build physical maps. STS is a relatively short, easily PCR-amplified sequence (200 to 500 bp) and detected in the presence of all other genomic sequences and whose location in the genome is mapped easily.The advantage of STSs over other mapping landmarks is that, the means of testing for the presence of a particular STS can be completely described as information in a database and anyone who wishes to make copies of the marker would simply look up the STS in the database, synthesize the specified primers, and run the PCR under specified conditions to amplify the STS from genomic DNA (Green & Green, 1991)Unlike PCR with arbitrary primers, sequence-tagged sites (STS) are primers that are based on some degree of sequence knowledge. These unique sequence-specific primers detect variation in allelic, genomic DNA. STS have a particular advantage over RAPDs in that they are co-dominant, that is, they can distinguish between homozygotes and heterozygotes. They also tend to be more reproducible, because they use longer primer sequences.The DNA sequence of an STS may contain repetitive elements, sequences that appear elsewhere in the genome, but as long as the sequences at both ends of the site are unique and conserved, researches can uniquely identify this portion of genome using tools usually present in any laboratory (Perry & Bousquet, 1998).

2.12.2.3.5 Single Nucleotide Polymorphism

SNPs are variations at single nucleotides which do not change the overall length of the DNA sequence in the region. They are highly abundant and are present at one SNP in every 1000 bp in the human genome. Most SNPs are located in non-coding regions, and have no direct impact on the phenotype of an individual. However, some introduce mutations in expressed sequences or regions influencing gene expression (promoters, enhancers) and may induce changes in protein structure or regulation (Bowen *et al.*, 1996).

2.12.2.4 DNA Sequence based markers

DNA barcoding system employs short standardized segment to identify species in all kingdom of life(Seifert *et al.*, 2007, Hollingsworth *et al.*, 2009). Identification of species using the other method mentioned above is sometimes problematic because of a number of reason including necessity of well-trained taxonomist requirement of a good habits, such technique are almost insufficient in regulating illegal trade of biological material as they are traded in distinguished form. Species identification is also done by sequence based PCR amplification of different DNA region such as COI,ITS, D1/D2 one of them is by PCR amplification of ITS region by primer ITS1-ITS4 (Li *et al.*, 2008, Kress *et al.*, 2009). Identification of species by ITS region is greater than 97%.DNA based species identification is done by amplification of the nuclear ribosomal 28s large subunit region (Abliz *et al.*, 2004).

2.12.2.4.1 Cytochrome 'C' oxidase (COI)

As like ITS region is the most reliable loci for fungi and plant barcoding , cytochrome C oxidase region (COI) gene has been used as barcode region (Wang *et al.*, 1998). For almost all animal groups including larvae, birds, fish, butterfly *etc*, it can identify sample at species level because in COI amplification involve the amplification of mitochondrial DNA rather than nuclear DNA,It is short enough to sequence and to observe variation (Robideau, De *et al*. 2011).

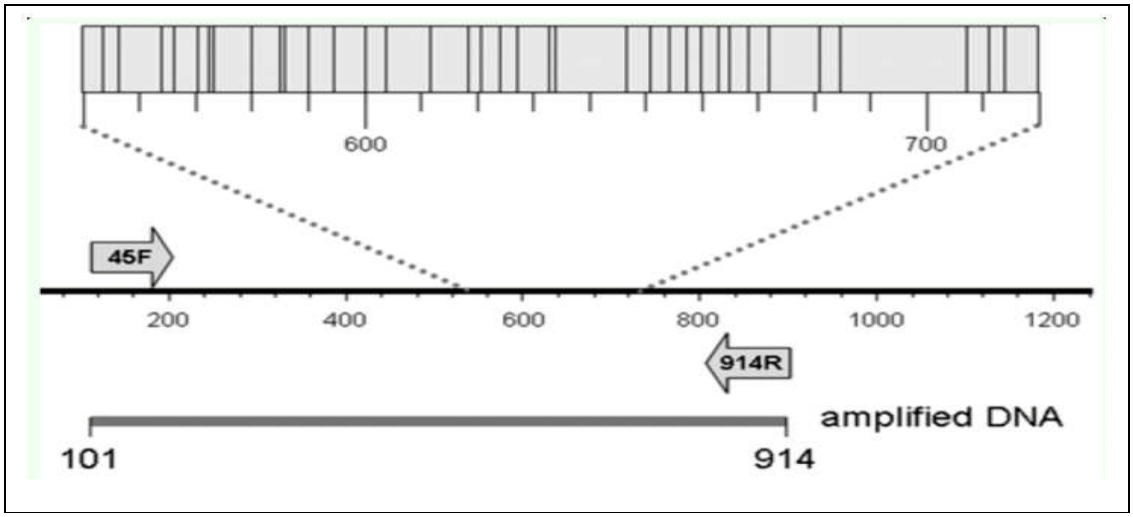
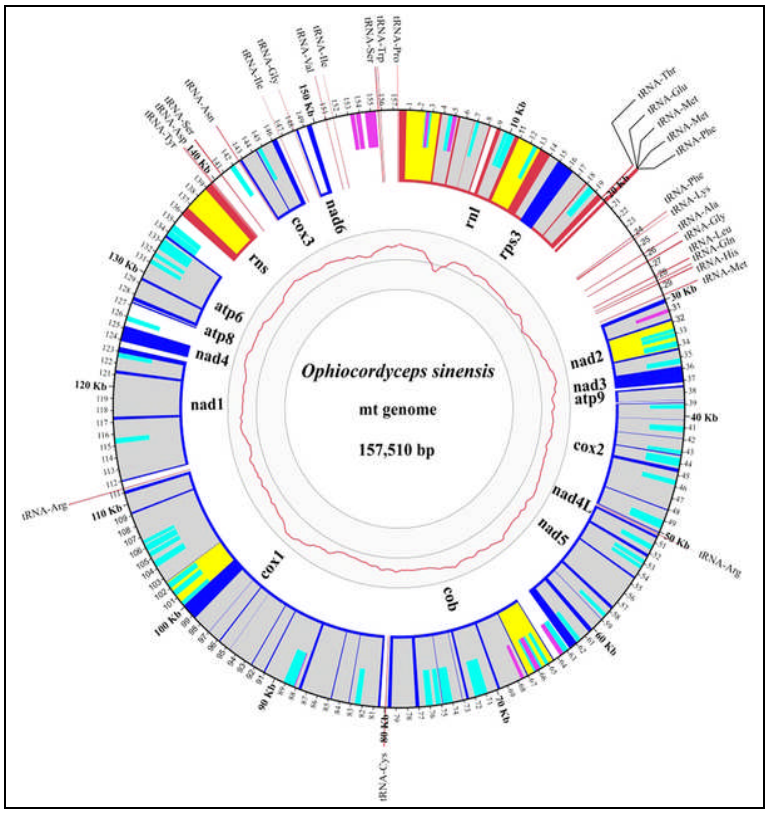


Fig2.9 Diagram of the COI region used for barcoding. Arrows indicate primers used for PCR amplification(Internet visit 9, 2015).



GGTCAACAAATCATAAAGATATTGG-LCOI
TTAGTATTTCTATAACCAACTTTATATTTTATTTTTGGTATTTGATCAGGAATAGTAGGAACATCTTTAA
GAATAATAATTCGAACAGAATTAGGAAATCCCGGATCTTTAATTGGAGATGATCAAATTTATAATGTAAT

TGTTACAGCTCACGCTTTTATTATAATTTTTTTTATGGTAATACCAATTATGATTGGTGGTTTTGGAAAC
TGATTAATTCCTTTAATGTTAGGGGCCCCCGATATAGCATTCCCACGACTTAATAATATAAGATTTTGAT
TATTACCCCCATCATTAATATTATTAATTTCTAGGAGAATTGTAGAAAACGGGGCAGGAACAGGTTGAAC
TGTATATCCCCCATTATCATCAAATATTGCACACTCAGGGGCATCAGTAGATTTAGCTATTTTTTCTTTA
CATCTTGCAGGAATTTCATCAATTTTAGGAGCAATTAATTTTATCACTACTGTAATTAATATACGATCAA
AAAGAATATCTTTTGACCGCATACCATTATTTGTGTGAAGAGTTGTAATTACTGCATTATTACTTTTACT
ATCATTACCTGTATTGGCAGGAGCTATTACTATATTATTAACAGATCGAAACTTAAATACTTCCTTTTTC
GACCCTGCGGGAGGGGGTGACCCTATCTTATTTGAAGTCCCACTGGTTTTTTGG
TAAACTTCAGGGTGACCAAAAAATCA-HCOI

Fig.2.10 Mitochondrial DNA of *Ophiocordyceps sinensis*.The COI sequence extracted from GenBank is shown above along with the indication of primer binding sites.

2.12.2.4.2 Internal Transcribed Region

ITS region consist of ITS1 intergenic spacer,5.8s rDNA and ITS2 intergenic spacer, with the size ranging from 600-800bp(Hinrikson *et al.*, 2005),formally proposed for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life, with the possibility that supplementary barcodes may be developed for particular narrowly circumscribed taxonomic groups. ITS region of the fungal ribosomal DNA(rDNA) are high variable sequence of great importance in distinguishing species (Martin & Rygiewicz, 2005). ITS region have high variation strong enough for the evolutionary and phylogenetic analysis (Tiwari *et al.*, 2011), among the regions of the ribosomal cistron, the internal transcribed space(Sato & Shimazu, 2002) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation (Schocha *et al.*, 2012). ITS region is strong enough molecular tool for the phylogenetic analysis of fungal species(Gardes & Bruns, 1993) .

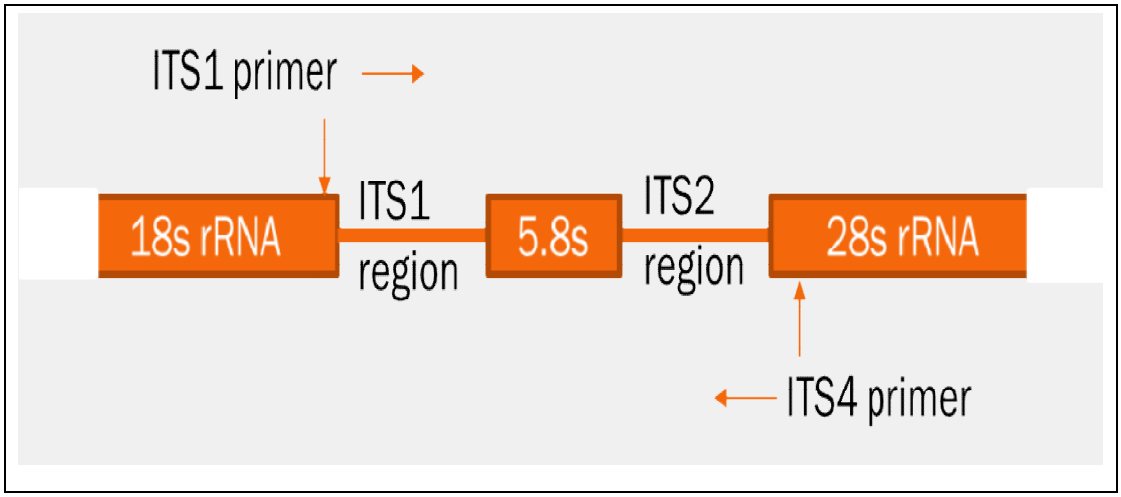


Fig.2.11 Schematic diagram of nuclear ribosomal DNA repeat unit including 18S ITS and 28S ITS region.

2.12.2.4.3 D1/D2 Domain of Large Sub-unit

D1/D2 region is a portion of ribosomal DNA consisting of 800-1300 nucleotides. This have been proven as the most important region for the molecular identification of *Ophiocordycepssinensis* by amplifying tandem region of 28S LSU rDNA(Sonnenberg *et al.*, 2007),the divergence region, symbolic representation as D, is numbered in5'-3' direction in the mature LSU region of rDNA gene complex.The highly variable part of the nuclear rDNA, the large subunit D1-D2 fragment has been used widely in DNA barcoding (Hall *et al.*, 2004) and has been one of the most important molecular tool for the identification of species and for the evolutionary and phylogenetic analysis(Abliz *et al.*, 2004).

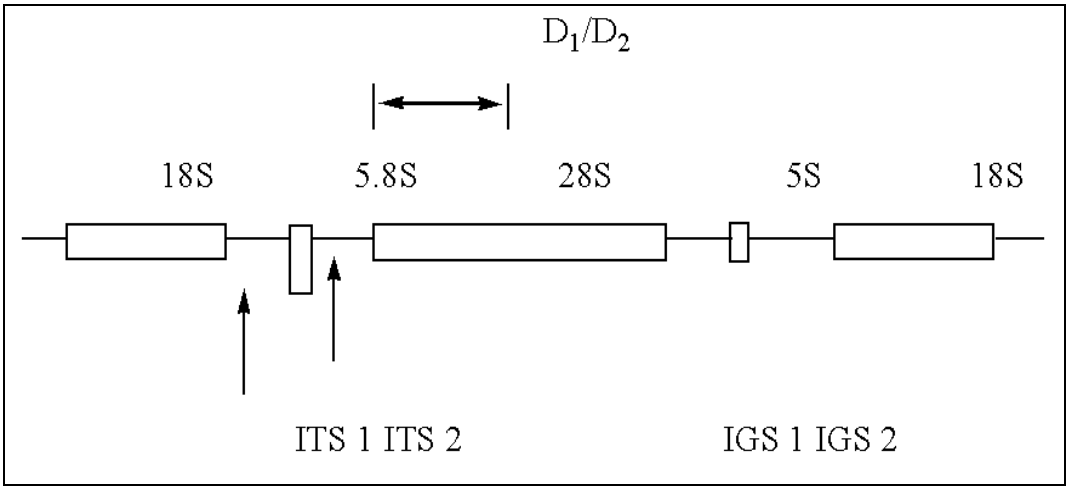


Fig 2.12 Diagrammatic representation of D1/D2 Domain of large subunit

28 S rDNA large subunit is suitable molecular marker for the differentiation of species by PCR amplification(Dagar *et al.*, 2011). The 5'D1-3'D2 region about 600 nucleotide is one of the most important tool for phylogenetic relationship identification(Kurtzman & Robnett, 1995) to provide a more rapid and exact identification of fungi(Hinrikson *et al.*, 2005).Even though, it has been highly reported that D1/D2 domain of fungus LSU-rDNA can be well amplified with primers NL1 and NL4 (Abliz *et al.*, 2004, Dagar *et al.*, 2011).

2.13 Phylogenetic Analysis

Phylogenetic analysis is important technique for comparative analysis of homologous gene sequences either from multigene families or from different species with a special emphasis on inferring evolutionary relationships and patterns of DNA and protein evolution. There are many software such as PAUP for constructing parsimony tree, PHYLIP and MEGA also for phylogenetic analysis, due to easy accessibility and free of cost MEGA is one of the most common software (Kumar *et al.*, 2008, Tamura *et al.*, 2011). Comparative analysis of molecular sequence data is essential for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species. By the releaseof Molecular Evolutionary Genetics Analysis version 6 (MEGA6),evolutionary and phylogenetic analysis has been easy. MEGA6 is specially designed to reduce the time needed for mundane task in data analysis and to provide statistical method of molecular evolutionary genetic analysis (Tamura *et al.*, 2011). To assess the reliability of a phylogenetic tree,MEGA provides the bootstrap test. This test uses the bootstrap resampling strategy, according to the number of replicates entered(Hillis & Bull, 1993).For the phylogenetic analysis these step are the necessary step. Selection of sequence of interest is main part of phylogenetic relationship analysis, followed by identifying homologs,aligning the sequences of interest and the homologous region for generating a sequence data matrix (Tamura, Peterson et al. 2011). The polymorphism of nrDNA ITS sequences suggested that *O. sinensis* spread from a center of origin to southern regions of south Asia and subsequently to northern areas. These results suggest that southern

populations are important reservoirs of genetic diversity and should be taken into account in conservation programs(Zhang *et al.*, 2009).

2.13.1 Sequence Alignment

Way of arranging sequence of DNA,RNA or protein to identify similar region is called sequence alignment, that may be the functional, structural or evolutionary relationship between the sequences (Mount & Mount, 2001), pairwise sequence alignment and multiple sequence alignment is most important step for the phylogenetic analysis(Sievers *et al.*, 2011). Sequence alignment of DNA,RNA or protein is done by adding gaps in between sequences of identical or similar characters(Mount & Mount, 2001). The multiple sequence alignment that are mostly targeting protein or short DNA sequence and are based on progressive alignment include CLUSTALW(Thompson *et al.*, 1994), MULTALIGN(Barton & Sternberg, 1987), T-COFFEE(Notredame *et al.*, 2000), MAFFT/PartTree(Katoh *et al.*, 2002), MUSCLE(Edgar, 2004), PROBCONS(Do *et al.*, 2005) and Clustal omega(Sievers *et al.*, 2011). Among these all software the best known, trustworthy, easy to use, easily available and reliable system is CLASTALW(Batzoglou, 2005).CLUSTALW uses command line or command console interface for the sequence alignment(Thompson *et al.*, 2002).

2.13.2 Phylogenetic tree construction

A phylogenetic tree is a diagrammatic representation of the evolutionary relationships between species, gene, genome that share a common ancestor which are more close and make close relationship in diagram. It should be possible to infer evolutionary relationship from the pattern of similarity among organism(evolution 2013).A table of evolutionary distance between each pair of taxa is generated (Tamura *et al.*, 2011). There are various methods for phylogenetic tree construction(Nei & Kumar, 2000).They can be classified into distance method, parsimony method and likelihood method (Tamura *et al.*, 2011).

2.13.2.1 Distance based method

In the simple case, the distance represents the evolutionary distance between each pair of taxa. The distance based method can be broadly categorized as minimum evolution(ME),neighbor joining(NJ) and unweight pair group method with arithmetic mean (UPGMA) (Tamura *et al.*, 2011).

2.13.2.2 Parsimony method

Maximum parsimony (MP) is one of the most widely used and accepted phylogeny reconstruction methods. MP builds a phylogenetic tree that minimizes the total number of changes (or branches) to illustrate evolutionary relationship (Wiley online,2015). Maximum parsimony is based on the principle of Occam's (or Ockham's) Razor according to this principle if all else is equal, the simplest tree is chosen. Given set of data (e.g., a multiple sequence alignment), phylogenetic trees that represent alternative possible relationships between the operational taxonomic units (OTUs) in the datasetis given a score. A score is the result of the measure of the number of evolutionary changes (e.g., G changing to C) that would be required to generate the data given that particular tree. Of the possible number of trees, the one considered most likely to represent the true history of the OTUs is the one with the lowest score (i.e., the one requiring the fewest evolutionary changes), which is also called as the most parsimonious tree or maximum parsimony tree (evolution,2015).For a given topology, sum of the minimum possible substitutions over all sites is known as the tree length. In this term, the topology with the minimum tree length is known as the maximum parsimony tree.MP uses subtree pruning and regrafting (SPR)for the construction of tree (evolution,2015).

2.13.2.3 Likelihood method

Likelihood method have been designed to provide a statistical framework for phylogenetic reconstruction. It is designed to overcome the drawbacks of MP and distant based method, because these processes create a single phylogenetic tree over another based on some criterion(evolution,2015). To search for tree with the highest probability.Maximum likelihood

(ML) trees are scored based on a character dataset,and the tree with the best score is selected(Tamura *et al.*, 2011).

2.13.3 Testing Best-fit model

Appropriate model selection has a long history in the statistical literature. As model-based approaches begin dominating systematic biology, increased attention has focused on how models should be selected for distance-based, likelihood based and parsimony based methods. Statistical analysis, evolutionary processes, and review methods that have been applied to model selection in phylogenetics: likelihood-ratio tests, AIC, BIC, and performance based approaches (Sullivan & Joyce, 2005). Measures of goodness of fit typically summarize the discrepancy between observed values and the values expected under the model and test over certain criteria how well it fits a set of observations (Tamura *et al.*, 2011). Despite the relevant role of models of nucleotide substitution in phylogenetic analysis choosing among different models remains a problem (Posada & Crandall, 2001). jModelTest(Darriba & Posada, 2014) and MEGA 5.0(Tamura *et al.*, 2011) are tools to carry out statistical selection of best fit model. These computer based software work under some strategy such as hierarchical and dynamic likelihood ratio test (hLRT and dLRT), Akike and Bayesian information criteria (AIC and BIC) and a decision theory method (Sullivan & Joyce, 2005).

3.1. Sample collection

Samples of *O. sinensis*were collected from Dolpa, Manang, Bhajhang and Darchula,sample were collected safely and kept in tight cap vial containing alcohol as preservative agent.

Fig3.2 Map of Nepal showing sample collected area in color.

A total of 34*Ophiocordycepssinensis* samples were collected from different localities of NEPAL ranging from 3000 to 5000 m elevations. From different four areas respectively from Dolpacontain 10 samples, Manang contain 10 samples, Darchula contain 8samples and Bhajhang contain 6 samples.

3.2 Samples Detail

The further classification of the samples collected from four different district of Nepal has been listed as shown in the table 3.2

Table 3.1 Samples detail of this study

Bhajhang					
S.N.	Species	Location	Collection number	Accession code	
				Fungal part	Insect part

1	*O. sinensis*	Far Western Nepal 726 m. to 7036 m	Yar B01	BF 01	--------
2	*O. sinensis*	,,	Yar B02	BF02	BI02
3	*O. sinensis*	,,	Yar B03	BF03	BI 03
4	*O. sinensis*	,,	Yar B04	BF04	BI 04
5	*O. sinensis*	,,	Yar B05	BF05	BI 05
6	*O. sinensis*	,,	Yar B06	BF05	BI 06
Darchula					
S.N.	Species	Location	Collection number	Accession code	
				Fungal part	Insect part
1	*O. sinensis*	Far Western Nepal 518 meters to 7134 meters	Yar-56	Daf 56	DaI 56
2	*O. sinensis*	,,	Yar-57	Daf 57	DaI 57
3	*O. sinensis*	,,	Yar-96	Daf 96	DaI 96
4	*O. sinensis*	,,	Yar-99	Daf 99	DaI 99
5	*O. sinensis*	,,	Yar-116	Daf 116	DaI 116
6	*O. sinensis*	,,	Yar-117	Daf 117	DaI 117
7	*O. sinensis*	,,	Yar-118	Daf 118	DaI 118
8	*O. sinensis*	,,	Yar-137	Daf 137	------
Manang					
S.N.	Species	Location	Collection number	Accession code	
				Fungial part	Insect part
1	*O. sinensis*	Western Nepal 3,519 metres	MF	MF	MI
2	*O. sinensis*	,,	M4	MF 4	MI4
3	*O. sinensis*	,,	M6	MF 6	MI 6

4	*O. sinensis*	,,	M8	MF 8	------
5	*O. sinensis*	,,	M 10	MF 10	------
6	*O. sinensis*	,,	M 11	MF 11	MI 11
7	*O. sinensis*	,,	M013	MF 013	------
8	*O. sinensis*	,,	M020	MF 20	MI 20
9	*O. sinensis*	,,	M 255	MF 255	------
10	*O. sinensis*	,,	M 258	MF 258	------
Dolpa					
S.N.	Species	Location	Collection number	Accession code	
				Fungal part	Insect part
1	*O. sinensis*	Mid-Western Nepal 1,525 to 7,625 m	Plot 6	DF6	------
2	*O. sinensis*	,,	Plot 8	DF8	------
3	*O. sinensis*	,,	Plot 10	DF10	------
4	*O. sinensis*	,,	Plot 12	DF12	------
5	*O. sinensis*	,,	Plot 22	DF22	-------
6	*O. sinensis*	,,	Plot 30	DF30	------
7	*O. sinensis*	,,	Plot 34	DF34	-------
8	*O. sinensis*	,,	Plot 35	DF35	-------
9	*O. sinensis*	,,	Plot 54	DF54	-------
10	*O. sinensis*	,,	Plot 58	DF58	-------

3.3 Surface sterilization of sample

All samples were preserved in an absolute alcohol. Then the preserved samples were washed with distilled water to remove unnecessary raw materials. After that,each sample was divided into two parts, thestroma and sclerotia. Both the partswere placed in a separate petriplate and washed by distilled water. After that both parts were dipped in 0.7% sodium hypochloride(NaOCl) for 10 min and washed with distilled water followed by washing with 70%

ethanol to remove excess chlorine residue. Finally, it was washed by distilled water for three times.

3.4 Molecular method of *Ophiocordycepssinensis* identification

3.4.1 DNA extraction and DNA Quantification

DNA extraction protocol for both host insect and fungal portion of *Ophiocordycepssinensis*was described by(Aljanabi & Martinez, 1997), was followed. There after homogenization of tissue was carried out in mortar and pestle containing 400µl of sterilized salt homogenizing buffer (0.4M NaCl 10mMTris-HCl of PH 8.0 and 2Mm EDTA of PH 8.0), followed by addition of 40µl of 20% sodium dodecyl sulphate (SDS) (2% final concentration) and 8µl of 20mg/ml proteinase K (400mg/ml final concentration) were added and mixed well. Then samples were incubated at 55-65°C for at least an hour or overnight. After that 300µl of 6M NaClwas added to each sample. Then the samples were vortexed for 30s at high speed followed by spinning for 30 minutes at 10000g.Then the supernatants were transferred to fresh Eppendroff tubes and equal volume of isopropanol was added to each sample. All these were incubated at -20°C for 1 hr or overnight for proper precipitation. Finally,the samples were centrifuged at 4°C for 20min at 10000g. After removing the supernatant,the pelletwas washed with 70% alcohol and dried for some time in laminar airflow.The dried pellet was re-suspended pellets in 300-500µlTE with RNase and finally stored at -20°C for subsequent use.Quantification of DNA was carried out by spectrophotometric method using Biophotometer (Eppendorf-AG22331, Germany). Both the concentration and purity were observed**(Appendix I)**.

3.4.2Optimization of PCR component

The PCR reaction conditions were optimized by varying the concentration of different PCR components, namely template DNA, $MgCl_2$, primers, dNTPs, and *Taq* polymerase**(Table 3.2).**The different sets ofprimers used in this study are given in Table. 3.4.

Table 3.2: Concentrations of various components used for optimization of PCR

S.N.	Components	Concentrations
1.	DNA	10, 15, 20, 25,35,45ng
2.	$MgCl_2$	0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5mM
3.	Primers	0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6,1.8,2 µM
4.	dNTPs	0.1, 0.2, 0.3, 0.4, 0.5 mM
5.	Taq polymerase	0.5, 1.0, 1.5, 2.0 U/µL

3.4.3Optimization of PCR thermal cyclereaction condition

Four different cyclic conditions as described by (Chen *et al.*, 1999), (Liu *et al.*, 2002), (Sonnenberg *et al.*, 2007) and(Chih-Sheng Chen, 2011)were tried in this study for the amplification of different Barcode loci. The PCR conditions highlighted in bold was found to be the best PCR thermocyclic condition (Table 3.3). The PCR conditions for the COI region of *O. sinensis* samples fromManangis as follow : with initial denaturation for 5 min at 94°C, followed by 35 cycle of 1 min at 94°C,1 min at 50.5°C,1.3 min at 72°C,finalextension at 72°C for 7 min. For Bhajhang and Darchula insect part sample : the initial denaturation at 95°C for 3 min,followed by 35 cycle of 30 sec at 95°C, 1 min at 30°C, 1 min at 72°C, final extension at 72°C for 7 min. For ITS and D1/D2 Region amplification of four district of Nepal best PCR conditions are as follow : theinitial denaturation for 2 min at 96°C,followed by 35 cycle of 45 sec at 96°C, 45 sec at 52°C, 2 min at 72°Cfollowed by final extension at 72°C for 10 min.

Table 3.3 The optimization ofPCR thermal cycler condition

(Liu *et al.*, 2002)			(Sonnenberg *et al.*, 2007)			(Chen *et al.*, 1999)		
Temp.	Time	Cycle	Temp.	Time	Cycle	Temp.	Time	cycle
97°C	1’		94°C	4’		95°C	3.5’	
97°C	1’	35 cycle	94°C	0.3’	45 cycle	94°C	1’	40 cycle
48°C	1’		52.5°C	0.3’		36°C	1’	

72°C	3′		72°C	1.5′		72°C	2′	
72°C	7′		72°C	8′		72°C	5′	
Thermocyclic condition for insect part of Manang			**Thermocyclic condition for insect part of Bhajhang and Darchula**			**Thermocyclic condition for fungi (Chih-Sheng Chen, 2011)**		
94°C	**5′**		**95°**	**3′**		**96°**	**2′**	
94°C	**1′**	**35 cycle**	**95°**	**0.5′**	**35 cycle**	**96°**	**0.75′**	**35 cycle**
50.5°C	**1′**		**30°**	**1′**		**52°**	**0.75′**	
72°C	**1.5′**		**72°**	**1′**		**72°**	**2′**	
72°C	**7′**		**72°**	**7′**		**72°**	**10′**	

3.4.4 PCR amplification

PCR amplification was performed in Thermal cycler (BIOER, China). All PCR reactions were carried out in 25µl reaction volume containing 1µl of 25mM $MgCl_2$, 12.5µl of 2X master mix(dNTPs, Taq polymerase and PCR buffer), 1.5µl of 10mM ITS-4 primer, 1.5µl of 10Mm ITS-5 primer, 1µl of 45ng template DNA, 7.5 µl nuclease free water for the amplification of Internal Transcribes Region.Following component were used for the amplification of Cytochrome 'C' Oxidase loci : 1.5µl of 25mM $MgCl_2$, 12.5µl of 2X master mix (dNTPs, Taq polymerase and PCR buffer), 2µl of 10mM HCOI primer, 2µl of 10mM LCOI primer, 1µl of 45ng T-DNA and 7µl nuclease free water were used, finally for the amplification of COI region of Bajhang and Darchula 1.5µl of 25mM MgCl2, 12.5µl of 2X master mix (dNTPs, Taq polymerase and PCR buffer), 1.5µl of 10mM each primer, 1µl of 45ng T-DNA and 7µl nuclease free water were used.

Table3.4 List of Primers used in the amplification of ITS,D1/D2 Domain and COI region of *O. sinensis.*

DNA Region	Primers	Sequences	Remarks
ITS	ITS5 F ITS4 R	(5′-GGAAGTAAAAGTCGTAACAAGG-3′) (5′-TCCTCCGCTTATTGATATGC-3′)	(Sonnenberg *et al.*, 2007)

D1/D2 Domain	NL1 F(5'-GCATATCAATAAGCGGAGGAAAAG-3') (Guo *et al.*, 2015) NL4 R (5'-GGTCCGTGTTTCAAGACGG-3')
COI	LCOI (5'-GGTCAACAAATCATAAAGATATTGG-3') (Paterson, 2008) HCOI (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') CIJ-1632 (5'-TGATCAAATTTATAAT-3')(Simon *et al*., 1 994) CIN-2191 (5'-GGTAAAATTAAAATATAAACTTC-3')

3.4.5Agarose Gel Electrophoresis

PCR Amplified product of ITS, D1/D2 Domain and COI loci were analyzed on 1.5% agarose gel in tris acetate (TAE) buffer (1X) at 80V for one and half hour respectively using EMBI TEC(Santigo,CA)gel tank ,EtBr 5µl of (10mg/ml Promega) was added for 100 ml of total volume agarose gel(Sambrook and Russell,2011). Total volume loaded in well was 5µl PCR Product plus 1µl Gel loading buffer(6X) for both extracted DNA analysis and PCR product analysis. After running, agarose gels were visualized on a gel documentation system(IN GENIUS,Syngene Bio-imaging,UK) and gel pictures documented.

3.4.6 DNA Sequencing

Out of 35 samples used in this study, only ten samples from Dolpa, ten samples from Manang, five samples from Bajhang and five samples from Darchula were selected for DNA sequencing.Similarly, five PCR amplicones of COI region of host from Bajhang, five from Manang and five from Darchulawere selected for DNA sequenced. DNA sequencing was done at Macrogen Inc. South Korea.

These samples representating three developmental region were selected for sequencing samples DF 6, DF 8, DF 10, DF 12, DF 22, DF 34, DF 35, DF 54, DF 58 were selected from Dolpa population, MF, MF1, MF4, MF6, MF10, MF11, MF013, MF20, MF255, MF258 were selected from Manang population, BF01, BF02, BF03, BF04, BF05, BF06 were selected from Bajhang population and DaF56, DaF96, DaF99, DaF116, DaF117, DaF118, DaF137 were selected from Darchula population and for COI region BI01, BI02, BI05, BI06 from Bajhang. MI01, MI, MI4, MI11, MI20 from Manang, DaI116, DaI117, DaI56, DaI96, DaI99 from Darchula.

A total of 21µl(60ng) each of amplified DNA samples were sent for bidirectional sequencing to Macrogen (Macrogen Inc.), South Korea.

3.4.6.1 Data Analysis

The DNA Sequence obtained respective forward and reverse primers were retrieved and analyzed using codon code Aligner Ver 4.0.4. After end trimming and assembling of the reverse and forward sequences of respective accession of *Ophiocordycepssinensis* consensus sequences were generated by using CodonCode Aligner software. Thus obtained ITS, D1/D2 and COI sequence were separately analyzed usingBLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) (NCBI,2015, (Zhang *et al.*, 2000, Romanelli *et al.*, 2010)The most similar sequences were aligned with the assembled sequences using CodonCode Aligner version 4.0.4 and adjusted manually. Multiple sequence alignment was performed using ClustalW(Thompson *et al.*, 1994)of Molecular Evolution Genetics Analysis (MEGA) version 5.2 (Tamura *et al.*, 2011).The phylogenetic analysis was performed by maximum parsimony (MP) analysis with MEGA v.5.2.2 software and intraspecific and interspecific P-distance was calculated using CLUSTALW (Thompson *et al.*, 1994)in which gaps were treated as missing data (MEGA,2015,(Thompson *et al.*, 2002). Likewise best fit model was tested and the consensus tree were built accordingly using jModelTestver 2.1.4 software (Sullivan & Joyce, 2005)jModeltest 2015).

3.4.6.2Phylogenetic Analysis

Maximum Parsimony (MP) trees based on ITS, D1/D2 and COI szuequenceswere constructed in MEGA v. 5.2 with 1000 bootstrap replications to determine clade support and tree resolution. Branch swapping algorithm used was Subtree-Pruning-Regrafting (SPR) algorithm. Branch swapping algorithm was used to optimize a cladogram, because manipulation of addition sequences alone generally yields a local optimum. To construct Maximum Parsimony Tree, only those nucleotide sites at which there are at least two different kinds of nucleotides, each represented at least twice are used. These sites are called parsimony-informative sites (Yue *et al.*, 2013).In this method, the nucleotide of ancestral sequences are inferred at each nucleotide site for a given tree topology is called a number of steps or tree length. Tree length is computed

for all possible topology and the topology, which shows the smallest tree length, is chosen as the final tree (Shashidhar *et al.*, 2013).

3.4.6.3 Model Based Phylogeny

For the model based phylogenetic trees,the best fit model of nucleotide substitution were selected for respective ITS,D1/D2 and COI sequences using jModelTestVer 2.1.4(Darriba & Posada, 2014)under Bayesian Information criterion(BIC). Based on selected nucleotide substitution models,the strict consensus phylogenetic tree was constructed under 95% confidence interval.

4.1 DNA Extraction and quantification

The genomic DNA was successfully extracted from both stroma and sclerotia of 34 samples of *Ophiocordycepssinensis*. The concentration and purity of extracted DNA was assessed byusing Biophotometer (Eppendorff,Germany). DNA concentration of stromal regionof *O. sinensis* samples ranged from 19.5 µg/ml - 1475.6µg/ml and DNA Concentration of extracted sclerotiaregionof *O. sinensis* samples ranged from 22µg/ml - 1927.7µg/ml. The A260/280 ranged from 1.02-2.02 in the case of sclerotium and 1.4-2.01 in the case of stroma(Appendix II).

4.2 PCR optimizationand amplificationof different Barcode loci

Of the various PCR parameters assessed for PCR optimization of ITS region, the amplification using genomic DNA of 45ng extracted from stromal samples, $MgCl_2$concentration of 1mM, primer concentration of 1.5pM, dNTPs concentration of 0.5mM and taq polymerase concentration of 0.3U were found to be the best(Table4.1). PCR was carried out in Bioer PCR machine using universal forward primer ITS-5 and reverse primer ITS-4 which gave rise to DNA fragment of 650bp (fig. 4.1). The ITS region of this barcode comprises ITS1 (rapidly evolving), 5.8S (very conserved) and ITS2 (moderately rapid) domain as a DNA Barcode to identify fungal species.

Table 4.1PCR parameters tested, tested range and optimum concentration obtained for ITS PCR optimization experiment.

S.N.	PCR parameters	Tested range	Optimum condition selected	Remarks
1.	DNA concentration(ng)	10, 15, 20, 25,35,45,50 ng	45ng	45 ng DNA concentration produces desirenible bands so is selected as the optimum concentration. Most of the concentrations of template produced faint bands.
2.	$MgCl_2$ concentration(Mm)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5mM	1mM	Most of the concentration of $MgCl_2$ produced reproducible bands. 1mM concentration produced desireable bands so was

				taken as optimum concentration.
3.	Primer concentration (Pm)	0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6,1.8,2 Pm	1.5Pmol	1.5 Pmol concentration of primer produces discernible bands and was selected as optimum concentration.
4.	dNTP Concentration(Mm)	0.1, 0.2, 0.3, 0.4, 0.5 mM	0.5mM	At 0.5mM concentration, bright and single bands were observed so selected as optimum concentration.
5.	*Taq* polymerase concentration	0.3, 0.5, 1.0, 1.5, 2.0 U/µL	0.3 U	At the concentration of 0.3U bright and single bands were observed so selected as optimum.

Among the several cyclic conditionstried(Chan *et al.*, 2011),(Chih-Sheng Chen, 2011),(Xiang *et al.*, 2013) for ITS-PCR amplification cycling conditions described by Chih-Sheng Chen was found to be the best (Table 4.2). It consisted of PCR program withinitial denaturation for 2 min at 96°C,followed by 35 cycle of 45 sec at 96°C, 45 sec at 52°C,2 min at 72°C,followed by final extension at 72°C for 10 minutes.

Table 4.2: Different PCR program used for amplification of ITS locus and best program selected (bolded one) for subsequent PCR amplification.

Thermal cycler condition for ITS locus								
(Xiang *et al.*, 2013)			(Chan *et al.*, 2011)			(Chih-Sheng Chen, 2011)		
Temp.	Time(min)	Cycle	Temp.	Time(min)	Cycle	**Temp.**	**Time**	**cycle**
97°C	1		95°	5		**96°**	**2 min**	
97°C	1	30 cycle	95°	1	35 cycle	**96°**	**45 sec**	**35 cycle**
48°C	1		53°	1		**52°**	**45 sec**	
72°C	3		72°	1		**72°**	**2 min**	
72°C	7		72°	5		**72°**	**10 min**	

Of the various PCR parameters assessed for PCR optimization of D1/D2 domain amplification using genomic DNA extracted from stromal samples DNA concentration of 45ng,

$MgCl_2$concentration of 1mM, primer concentration of 1.5PM, dNTPs concentration of 0.5mM and taq polymerase concentration of 0.4U were found to be the best (Table4.3).

On the basis ofnuclear ribosomal locus D1/D2 domain the species were molecularly identified. PCR was carried out in Bioer PCR machine using universal forward primer NL4 and reverse primer NL1.

Table 4.3PCR parameters tested, tested range and optimum concentration obtained for D1/D2 PCR optimization.

S.N.	PCR parameters	Tested range	Optimum condition selected	Remarks
1.	DNA concentration(ng)	10, 15, 20, 25,35,45,50 ng	45ng	45 ng DNA concentrationproduces desirenible bands so is selected as the optimum concentration. Most of the concentrations of template produced faint bands.
2.	$MgCl_2$ concentration(Mm)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5Mm	1mM	Most of the concentration of $MgCl_2$ produced reproducible bands. 1mM concentration produced desireable bands so was taken as optimum concentration.
3.	Primer concentration (Pm)	0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6,1.8,2 Pm	1.5Pmol	1.5 Pmolconcentration of primer produces discernible bands and were selected as optimum concentration.
4.	dNTP Concentration(Mm)	0.1, 0.2, 0.3, 0.4, 0.5 Mm	0.5mM	At 0.5mM concentration bright and single bands were observed so selected as optimum concentration.
5.	*Taq* polymerase concentration	0.5, 1.0, 1.5, 2.0 U/µL	0.4 U	At the concentration of 0.4U bright and single bands were observed so selected as optimum.

Among the several cyclic conditions(Xiang *et al.*, 2013), (Hebert *et al.*, 2003), (White *et al.*, 1990) tried for D1/D2 Barcode locus and sample, following shown in bold (text)are the best PCR thermal cycler condition and best PCR reagent mixture for D1/D2 locus. The PCR program is

initial denaturation for 2 min at 96°C,followed by 35 cycle of 45 sec at 96°C, 45 sec at 52°C,2 min at 72°C,followed by final extension at 72°C for 10 min. The amplified fragment is found to be of 650bp band size (fig. 4.1)

Table 4.4 Different PCR program used for amplification of D1/D2 locus and the best program selected (bolded one) for subsequent PCR amplification.

Thermal cycler condition for D1/D2 locus								
(Xiang *et al.*, 2013)			(Chan *et al.*, 2011)			(Chih-Sheng Chen, 2011)		
Temp.	Time	Cycle	Temp.	Time	Cycle	**Temp.**	**Time**	**cycle**
97°C	1 min		95°	5 min		**96°**	**2min**	
97°C	1 min		95°	1 min		**96°**	**45sec**	
48°C	1 min	30 cycle	53°	1 min	35 cycle	**52°**	**45sec**	**35 cycle**
72°C	3 min		72°	1 min		**72°**	**2min**	
72°C	7 min		72°	5 min		**72°**	**10min**	

Using optimized PCR reaction and cycling conditions ITS and D1/D2 loci were amplified for all samples and amplification success rate was 100% for both ITS and D1/D2 under study. The amplified fragments were approximately 650bp long for ITS and D1/D2 domain.

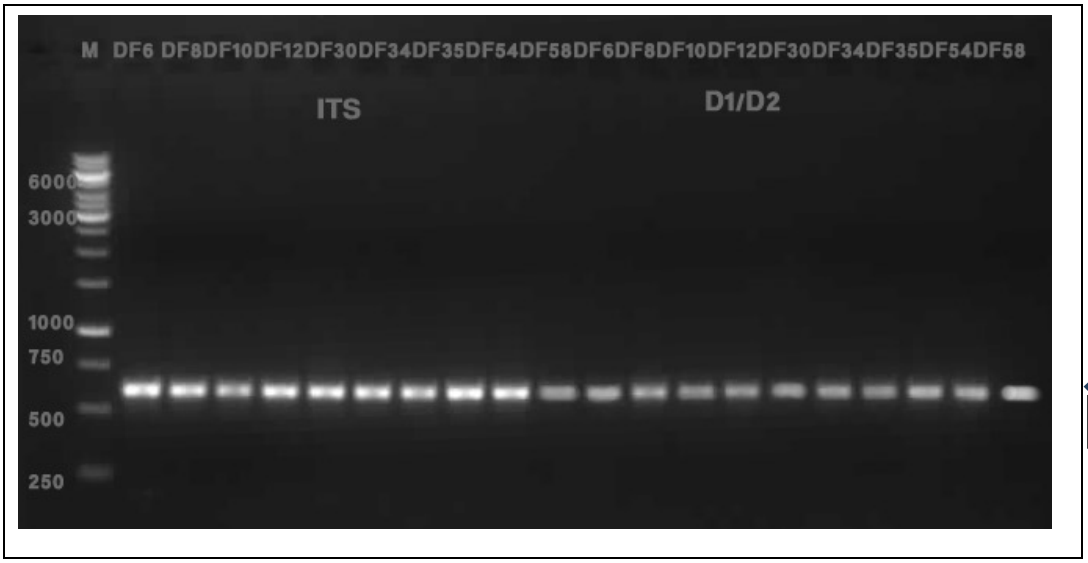


Fig.4.1 PCR amplified fragments of fungal ITS and D1/D2 domains of *O. sinensis*samples from Dolpa. Lane marked M is 1kb DNA Ladder,Lanes DF6-DF58 are *O. sinensis* accessions amplified by ITS primer ladder and Lane DF6-DF58 are *O. sinensis* accession amplified by D1/D2 primer (for sample abbreviation refer to appendix).

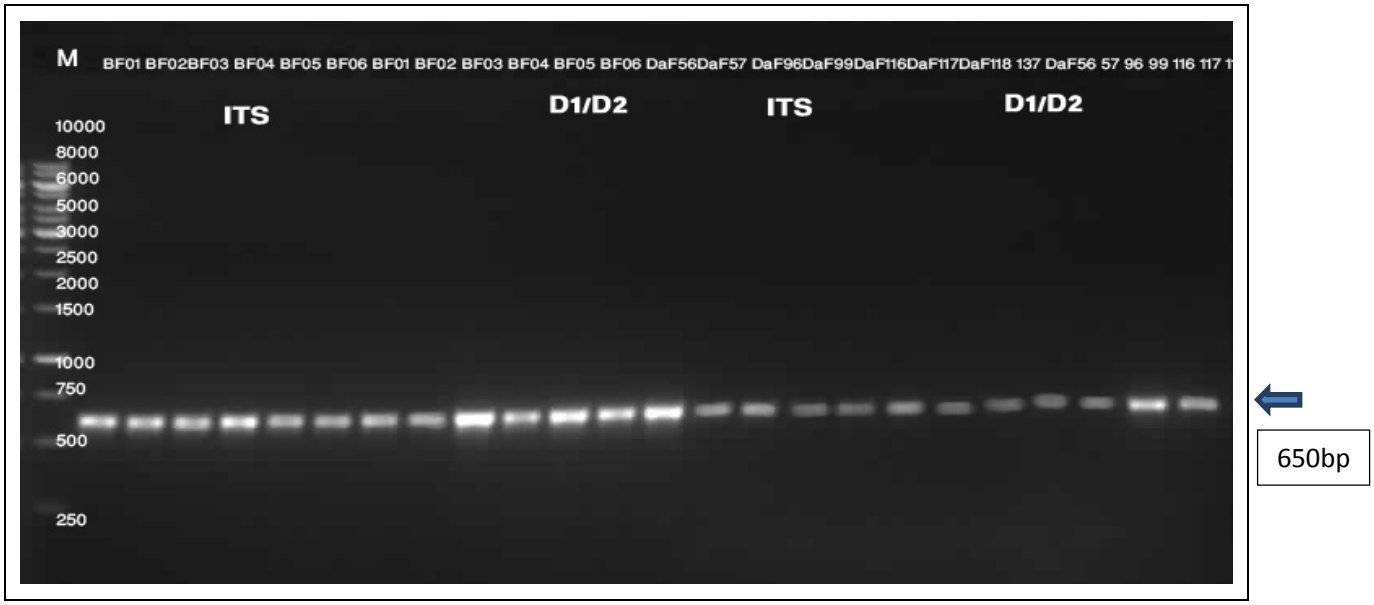


Fig 4.2:PCR amplified fragment of fungal ITS and D1/D2 domains of *O. sinensis*samples from Bhajhang and Darchula. Lane marked M is 1kb DNA Ladder, Lanes BF01-BF06 are *O. sinensis* accessions amplified by ITS primer ladder and Lane BF01-BF06 are *O. sinensis* accession amplified by D1/D2 primer, Lane DaF56-DaF118 are *O. sinensis* accession amplified by ITS primer, Lane DaF56-DaF118 are *O. sinensis* accession amplified by D1/D2 primer (for sample abbreviation refer to appendix).

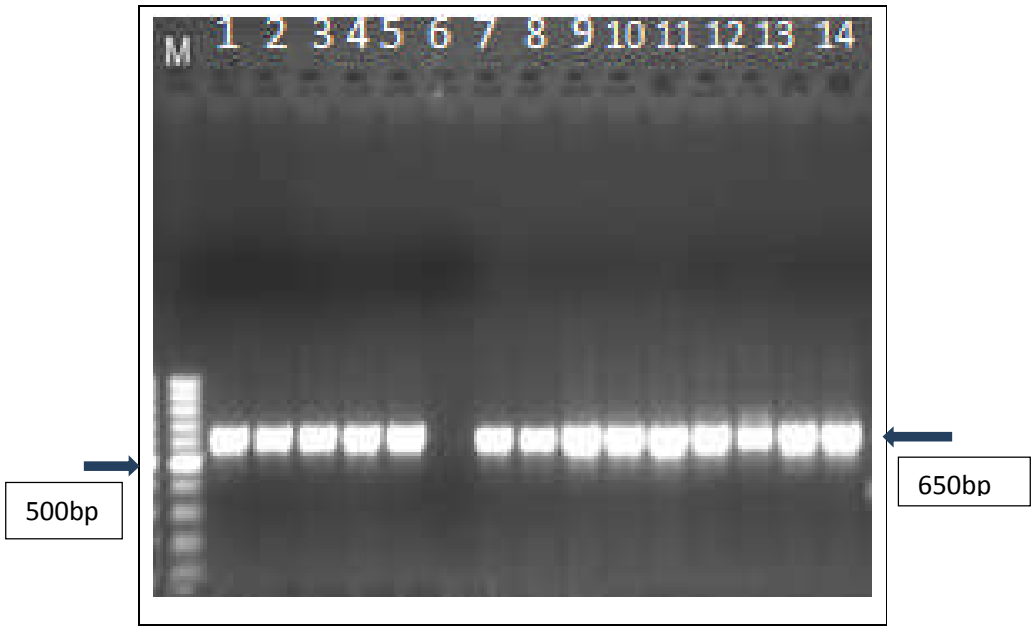


Fig.4.3PCR amplified fragments of fungal D1/D2 domain of *O. sinensis*samples from Dolpaand Manang. Lane marked M is 100bp DNA Ladder, Lane 1-7 are D1/D2 amplified fragment*O. sinensis* accessions from Dolpa, Lane 8-14 are D1/D2 amplified fragment *O. sinensis* accession from Manang(for sample abbreviation refer to appendix).

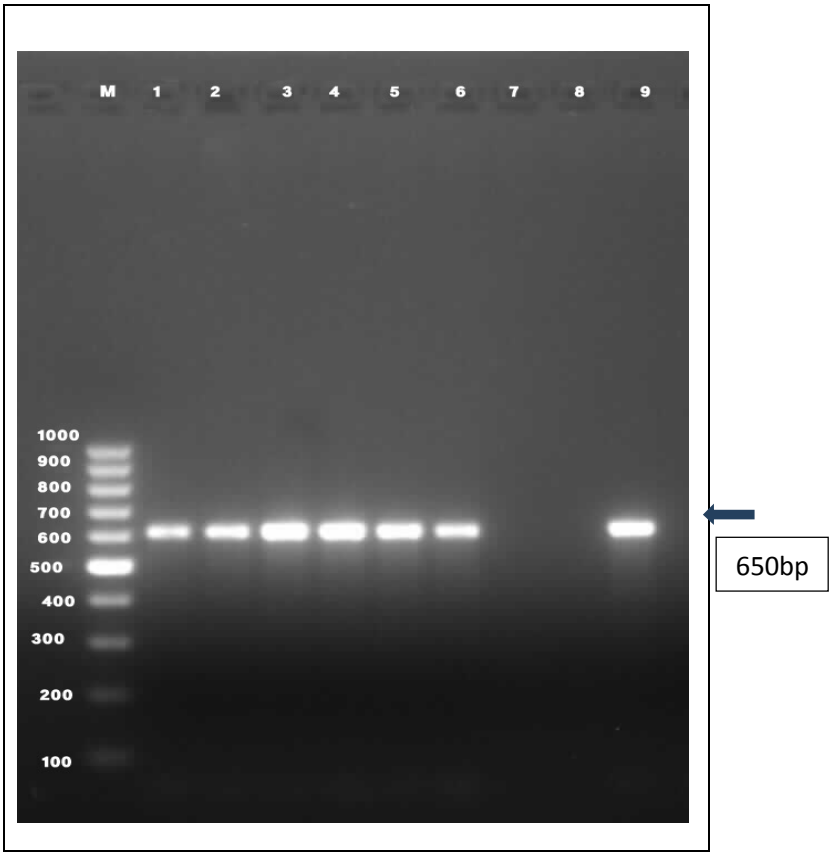


Fig.4.4PCR amplified fragments of fungal D1/D2 domain of *O. sinensis*samples from Bhajhang. Lane marked M is 100bp DNA Ladder, Lane 1-8 are D1/D2 amplified fragment *O. sinensis* accessions from Bhajhang(for sample abbreviation refer to appendix).

Of the various PCR parameters assessed for PCR optimization of COI region amplification using genomic DNA extracted from sclerotium samples DNA concentration of 45ng, $MgCl_2$concentration of 1.5mM, primer concentration of 2Pm, dNTPs concentration of 0.5mM and taq polymerase concentration of 0.5U were found to be the best (Table4.5).

Table 4.5 PCR parameters tested, tested range and optimum concentration obtained for COI PCR optimization.

S.N.	PCR parameters	Tested range	Optimum condition selected	Remarks
1.	DNA concentration(ng)	10, 15, 20, 25,35,45,50 ng	45ng	45 ng DNA concentration produces desirenible bands so is selected as the optimum

				concentration. Most of the concentrations of template produced faint bands.
2.	$MgCl_2$ concentration(Mm)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5mM	1.5mM	Most of the concentration of $MgCl_2$ produced reproducible bands. 1.5mM concentration produced desireable bands so was taken as optimum concentration.
3.	Primer concentration (Pm)	0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6,1.8,2 Pm	2Pmol	2 Pmol concentration of primer produces discernible bands and were selected as optimum concentration.
4.	dNTP Concentration(mM)	0.1, 0.2, 0.3, 0.4, 0.5 mM	0.5mM	At 0.5mM concentration bright and single bands were observed so selected as optimum concentration.
5.	*Taq* polymerase concentration	0.5, 1.0, 1.5, 2.0 U/µL	0.5 U	At the concentration of 0.5 U bright and single bands were observed so selected as optimum.

Among the several cyclic conditions(Hebert *et al.*, 2003),(Laiho & Ståhls, 2013) tried for COI Barcode locus. Following shown in boldare the best PCR thermal cycler condition and best PCR reagent mixture for COI locus. PCR program is initial denaturation for 5 min at 94°C,followed by 35 cycle of 1 min at 94°C, 1 min at 50.5°C,1.5 min at 72°C,followed by final extension at 72°C for 7 min.On the basis ofCytochrome 'C' Oxidase (COI) sequences the species were molecularly identified. PCR was carried out in Bioer PCR machine using universal forward primer LCOI and reverse primer HCOI.

Table 4.6 Different PCR program used for amplification of COI locus and best program selected (bold) for subsequent PCR amplification.

Thermal cycler condition for COIlocus								
(Laiho & Ståhls, 2013)			(Hebert *et al.*, 2003)			Optimized condition		
Temp.	Time(min)	Cycle	Temp.	Time(min)	Cycle	**Temp.**	**Time (min)**	**cycle**

95°C	2		94°	1		**94°**	**5**	
94°C	0.5	30 cycle	94°	1	5 cycle	**94°**	**1**	**35 cycle**
49°C	0.5		45°	1.5		**50.5°**	**1**	
72°C	2		95°	1	35 cycle	**72°**	**1.5**	
72°C	7		50°	1.5		**72°**	**7**	
			72°C	1				
			72°C	5				

Using optimized PCR reaction and cycling conditions COI locus was amplified for all samples and amplification success rate was 75%. The amplified fragments were approximately 700bp long for COI sequence.

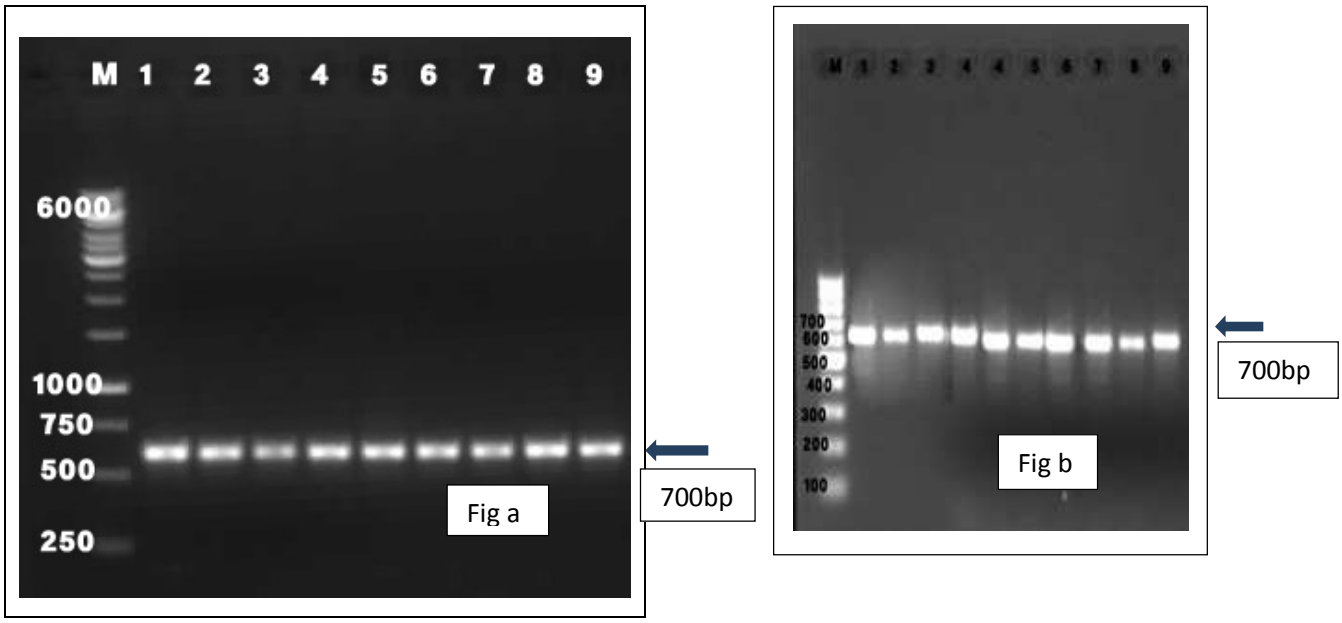


Fig 4.5PCR amplified fragment of larval COI sequence of *O. sinensis*samples. Lane marked M is 1kb DNA Ladder**Fig a)** Lane 1-9 are COI amplified fragment accession from Bhajhang and Darchula**Figb)** Lane 1-9 are COI amplified fragment accession from Manang(for sample abbreviation refer to appendix).

Table 4.7 PCR parameters tested, tested range and optimum concentration obtained for COI sequence PCR optimization of Bhajhang and Darchula district.

S.N.	PCR parameters	Tested range	Optimum condition selected	Remarks
1.	DNA concentration(ng)	10, 15, 20, 25,35,45,50 ng	45ng	45 ng DNA concentrationproduces desirenible bands so is selected as the optimum concentration. Most of the concentrations of template produced faint bands.
2.	$MgCl_2$ concentration(mM)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5mM	1.5mM	Most of the concentration of $MgCl_2$ produced reproducible bands. 1.5mM concentration produced desireable bands so was taken as optimum concentration.
3.	Primer concentration (Pm)	0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6,1.8,2 Pm	2Pmol	2 Pmol concentration of primer produces discernible bands and were selected as optimum concentration.
4.	dNTP Concentration(mM)	0.1, 0.2, 0.3, 0.4, 0.5 mM	0.5mM	At 0.5mM concentration bright and single bands were observed so selected as optimum concentration.
5.	*Taq* polymerase concentration	0.5, 1.0, 1.5, 2.0 U/µL	0.6 U	At the concentration of 0.6 U bright and single bands were observed so selected as optimum.

Among the several cyclic conditions(Xiang *et al.*, 2013), (Hebert *et al.*, 2003), (White *et al.*, 1990) tried for different Barcode loci and sample. Following shown in boldare the best PCR thermal cycler condition and best PCR reagent mixture for COI locus. PCR program is initial denaturation for 3 min at 95°C,followed by 35 cycle of 30 sec at 95°C, 1minut at 30°C,1 min at 72°C,followed by final extension at 72°C for 7 minutes.

Table4.8 Different PCR program used for amplification of COI sequence and best program selected (bolded one) for subsequent PCR amplification.

Thermal cycler condition for COIlocus								
(Laiho & Ståhls, 2013)			(Hebert *et al.*, 2003)			Optimized condition		
Temp.	Time	Cycle	Temp.	Time	Cycle	**Temp.**	**Time**	**cycle**
95°C	2 min		94°	1 min		**95°**	**3 min**	

94°C	30 sec	30 cycle	94°	1 min	5 cycle	**95°**	**30 sec**	**35 cycle**
49°C	30 sec		45°	1.5 min		**30°**	**1 min**	
72°C	2 min		95°	1 min	35 cycle	**72°**	**1 min**	
72°C	7 min		50°	1.5 min		**72°**	**7 min**	
			72°C	1 min				
			72°C	5 min				

Using optimized PCR reaction and cycling conditions COI locus was amplified for all samples and amplification success rate was 75%. The amplified fragments were approximately 700bp longusing universal forward primer Ci-J and reverse primer Ci-N.

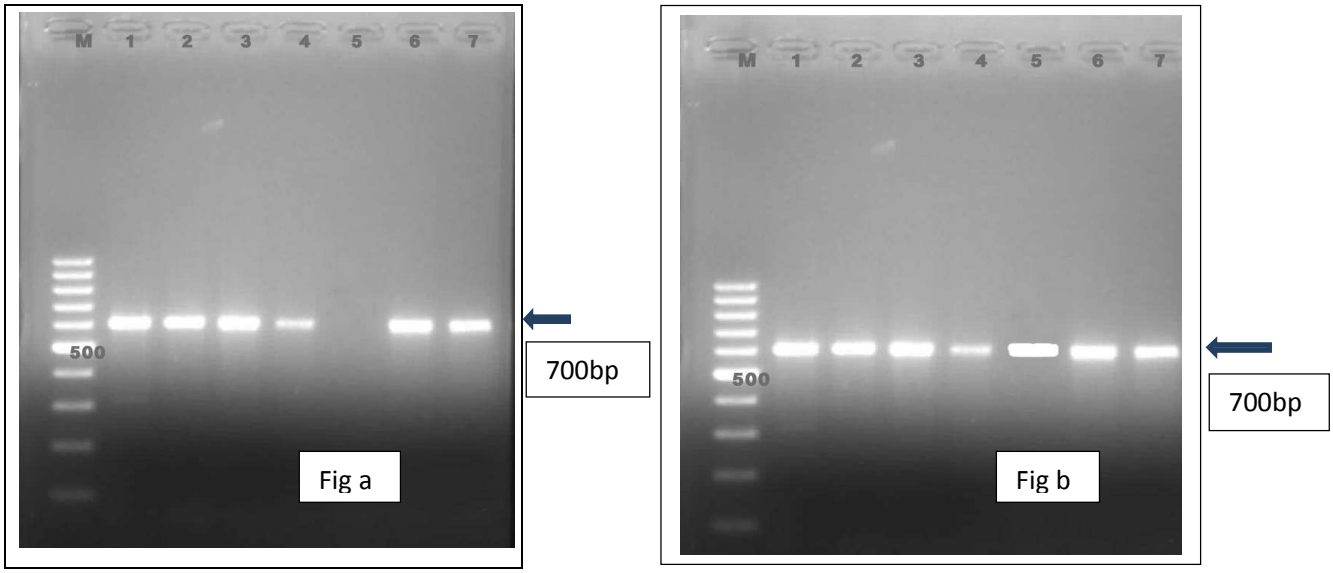


Fig.4.6PCR amplified fragments of larval COI sequence of *O. sinensis*samples. Lane marked M is 100bp DNA Ladder **Fig a)** Lanes 1-9 are COI amplified fragment accessions from Bhajhang**Figb)** Lane 1-9 are COI amplified fragment accession from Darchula (for sample abbreviation refer to appendix).

4.3 DNA Sequencing and Sequence analysis of different Barcode loci

The success rate of sequence obtained was 57%for ITS sequence, 50%for D1/D2 domain 30% for COI region sequence. Then the retrieved sequences wereedited assembled and consensus sequences were prepared using CodonCode aligner v.4.2.2 software. After end trimming and assembling of the forward and reverse sequence of respective accession of *O. sinensis*, for this the reference sequences were selected from the NCBI Genbank. ITS sequence of *O. sinensis*[(GI: gbEU570946)] was used as reference sequence to edit ITS sequences, while for *D1/D2*, domain of *O. sinensis* [GI: JX968032] and in case of *COI*sequence, *Hepialus spp. sequence* [gb|HM595868|]was used as reference sequence to edit COI sequences. After that BLASTn done in NCBI than evolutionary relationship and phylogenetic tree construction was done in MEGA 6 and jModelTest software, best fit model test was done using jModelTest software.

During consensus generation ITS sequences of BF02, BF03, BF05, BF06 did not assembled and were not used in the study and COI sequences of BI01, BI02, BI03, BI04, BI05, BI06, DaI56, DaI57, DaI96, DaI99, DaI116, DaI117, DaI 118 did not assembled but as per the suggestion of supervisors its forward sequences were of high quality so were used in the study.

4.4Phylogenetic analysis of *Ophiocordycepssinensis* and model based phylogeny

Best fit model for the phylogenetic analysis was selected by using jModelTest(Santorum *et al.*, 2014), for the different accessions used in the study using corrected Bayesian Information Criterion (BIC_c). In jModelTest likelihood calculation model based phylogeny method was used with number of substitution scheme 11 (num model 88) including model with equal of unequal base frequencies(+F),including model with /without a proportion of invariable sites(+I) and including models with/without rate variation among sites(+G).After completing the computation of likelihood score ,Bayesian Information Criteria(BIC) calculation found TVMef+I, TIM1ef, HKY+I model as a best fit model at 95 % confidence interval.

Table 4.9 Best fit model of nucleotide substitution as determined using jModelTest.

Best fit model	**ITS**	**D1/D2**	**COI**
JModelTest	TVMef+I	TVMef+I	HKY+I
BICvalue	3321.8285	4694.1730	3088.2822

Where, TVMef+I = Transversion Model equal base frequencies, +I = invariable sites

HKY = Hasegawa-Kishino-Yano)

Lower BIC values signify the best model among all others. jModelTest based calculation showed the lowest BIC score to be 3321.8285for TVMef+I Model for *ITS* sequence. Similarly, TVMef+I model was found to be the best nucleotide substitution model for *D1/D2* sequence with lowest BIC score of 4694.1730. For *COI* sequence, HKY+I model showed lowest score of 3088.2822 and hence was found to be the best model.

4.4.1 Phylogenetic analysis based on ITS sequence

Phylogenetic trees were constructed to depict the evolutionary relationship between different accessions of *Ophiocordycepssinensis.*Using jModelTest (v. 2.1.4), a model based phylogenetic tree was constructed and using MEGA (v. 6.0) a maximum parsimony (MP) tree was constructed for ITS barcode locus. Using best fit nucleotide substitution model from jModelTest (v. 2.1.4), strict consensus trees were generated with confidence interval of 95%.

Phylogenetic analyses of ITS was carried out using reference sequences retrieved from NCBI nucleotide database. ITS sequences of different accessions were compared with ITS sequences of *O. sinensis*taken from NCBI genbank : (gbEU570946), *O. sinensis*(gb|KP185268.1|), *O. sinensis*(gb|KP185265|), *O. sinensis*(gb|KP185252 |), *O. sinensis*(gb|KP185255|), *O. nutans*(gb|AB544491|) and *O. nepalansis* (gb|AJ309358|), *O.sinensis*(gb|EU570926|), *O.sinensis* (gb|JQ325150|).

4.4.2 Multiple and pair-wise alignment of ITS sequence

Multiple and pair-wise alignment of 29nucleotide sequences of 533 bpof nrDNA ITS were carried out using reference sequence from NCBI nucleotide database. The multiple and pairwise alignments of these sample were performed in MEGA6 using CLUSTALW. Results are shown in following Table 4.3.

Table 4.10 Evaluation of ITS sequences and sequence alignment analysis

Parameters	**Analysis**
Universal ability of ITS 5/ITS 4 primers for amplification of ITS region	Yes
Percentage sequencing success	57
Aligned sequence length	533
Number of conserved sites	312
Number of variable sites	194
Distribution of variable sites	Dispersive
Number of sample	29

With the help of jModelTest best model,model average phylogeny was computed with TVMef+Imodel. In model-average phylogeny criteria for tree weights was BIC and strict consensus type was chosen with confidence interval 95%. The unrooted strict consensus tree was constructed according to the TVMef+Imodel.

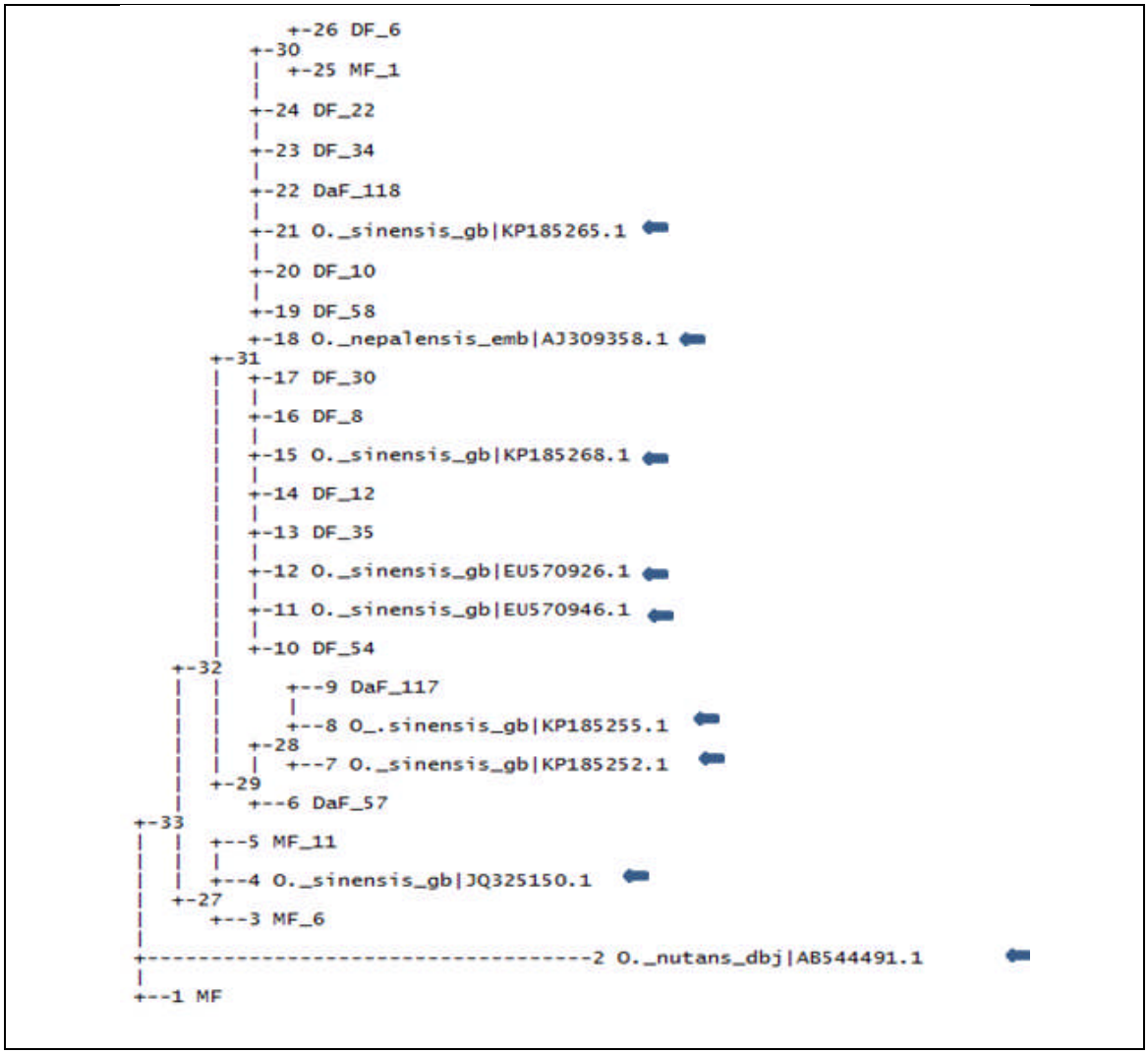


Fig 4.7 Strict consensus tree of fungi by ITS primer generated with confidense interval of 0.95 and selection criteria by BIC using TIM1ef model in jModelTest. Labeled number in tree indicate the branch length(**Note:** shown in arrow are sample taken fron NCBI genbank).

Branch length are the expected number of substitution per site. From the above figure five major clade were seen in strict consensus tree(Fig.4.7)eventhough that five clades are formed but the tree shows that there is no genetic variation because MF ITS make separate clade on the bottom however all the manag fungal are not making single clade they are mixed with Dolpa and Darchula,from this tree we can conclude that the tree is paraphyletic.Out of the 26 accession under this study, 24 accessions formed a major monophyletic clade. In this major clade three subclade were separated, first monophyletic clade was formed by *O. sinensis* accession from Dolpa, Darchula, one accession of Manang and *O. nepalensis* accession. Second

subclade was formed by *O. sinenesis* accession of Darchula and *O. sinensis* accession taken from Genbank . Similarly third subcladewas formed by *O. sinensis* accession of Manang and *O. sinensis* accession taken from Genbank. Genbank accession of *O. nutans*was showndiversed from all other accession in this study.

Maximum parsimony tree was constructed using the subtree pruning regrafting (SPR)algorithm with 1000 bootstrap replication. The most parsimonius tree length of 193 is shown in figure.

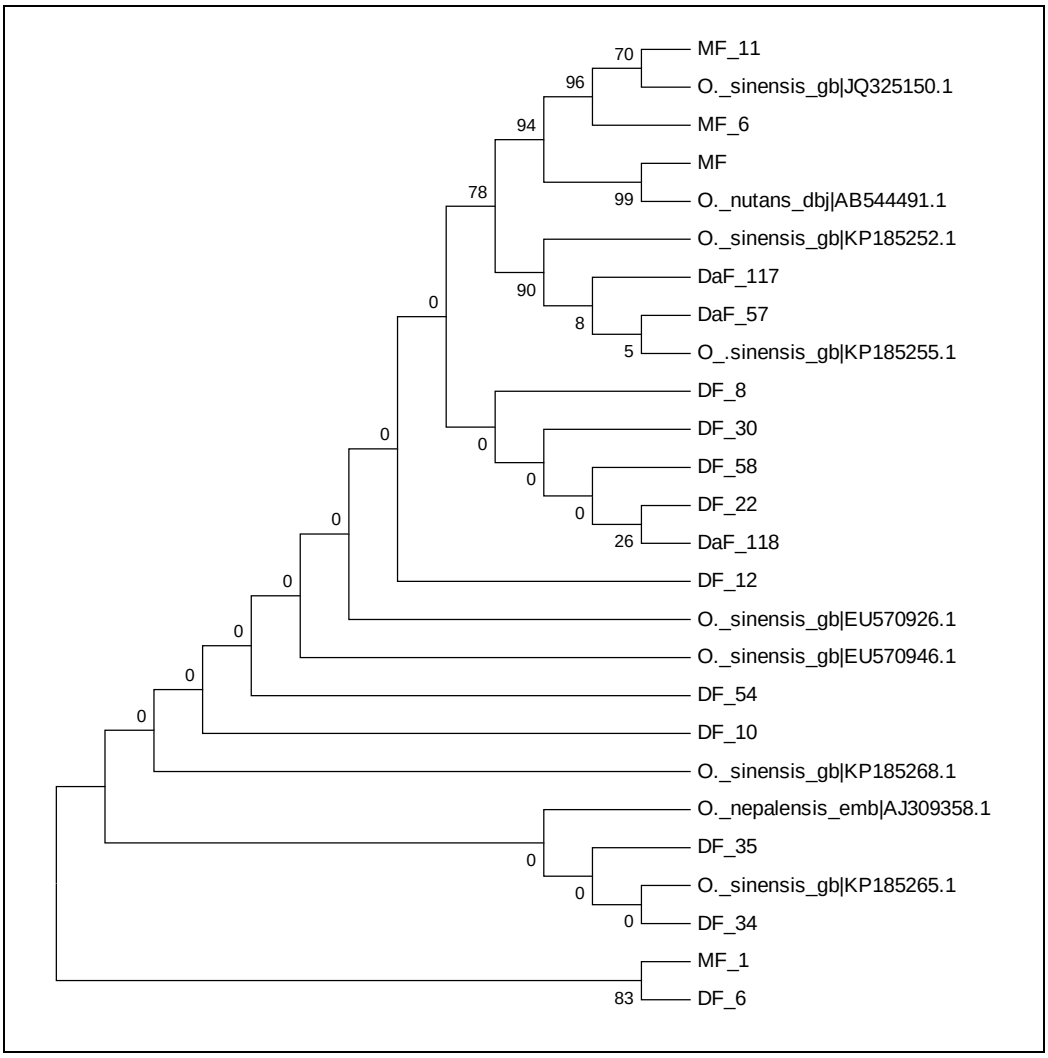


Fig.4.8 Maximum Parsimony tree generated from ITS with reference sequences from NCBI Genbank obtained using the subtree-pruning regrafting (SPR) algorithm in MEGA 6.(**Note:** shown in frame are sample taken fron NCBI genbank)

The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 10 most parsimonious trees (length = 213) is shown. Theconsistency index is (0.967742), the retention index is (0.974359), and the composite index is 0.969785 (0.942928) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 533 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. In this tree major three monophyletic clade were separated, first monophyletic clade was formed by *O. sinensis* accession from Dolpa, Darchula, Manang and *O. nutans* accession. Second clade was formed by*O. sinensis* accession of Dolpa with *O. nepalensis*. Similarly third clade was formed by *O. sinenesis* accession of Manang and Dolpa. Rest of the sample from Genbank accession of *O. nepalensis, O. sinensis*and*O. sinensis* accession of Dolpa formed polytomy.

Comparison of branching patterns of different sequences between phylogenetic trees generated by jModelTest and maximum parsimony tree generated by MEGA revealed similar branching pattern thereby confirming the validity of phylogenetic analysis. ITS sequences were able to distinguish the accession sequences from outgroup sequences and placed all the accessions in single clade.

4.2.4.3 Estimates of evolutionary divergence between sequences with reference sequence from genbank.

Data are shown in AppendixII, data shows that the probability of accepting or rejecting null hypothesis by estimating the P-values which are shown below the diagonal. P-value smaller than or equal to 0.05 (marked with bold) are considered significant. The estimates of the disparity index per sites are shown for each sequence pair above the diagonal. Higher the disparity index per site, lower is the P-value and test is more significant. The result showed that the null hypothesis can be rejected in 31% of the gene at 5% significance level Analyses were

conducted using the Maximum parsimony model. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 551 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Estimates of Evolutionary Divergence between Sequences with reference sequence shows that *Ophiocordycepsnepalensis* is more closer than *Ophiocordycepsnutans* with our samples.

Table 4.11 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for ITS sequence with reference sequence from genbank.

	A	**T**	**C**	**G**
A	-	*2.78*	*3.31*	**36.19**
T	*1.89*	-	**12.77**	*4.41*
C	*1.89*	**10.72**	-	*4.41*
G	**15.53**	*2.78*	*3.31*	-

Table 4.11 shows the probability of substitution (r) from one base to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. The transition rate is higher for G to A while the transversional substitutions are minimal. The nucleotide frequencies are 15.27% (A), 22.42% (T/U), 35.59% (C), and 26.71% (G). The transition/transversion rate ratios are k_1 = 8.207 (purines) and k_2 = 3.858 (pyrimidines). The overall transition/transversion bias is R = 2.709, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The analysis involved 26 ITS sequences representing various *O. sinensis* samples under this study.

4.5 Phylogenetic analysis based on D1/D2 Domain of LSU

Phylogenetic and evolutionary relationship were analyzed by using jModelTestver 2.1.4 software and MEGA6. By using MEGA 6 software for each nucleotide sequences maximum parsimony method was analyzed. D1/D2 Domain of different accessions were compared with D1/D2 sequences of *O. sinensis* (NCBI JX968032, JX968033, HQ025954, JX968031, JX968030, and HM595876.

4.5.1Multiple and pair-wise alignment of D1/D2 domain

Multiple and pair-wise alignment of 26nucleotide sequences of 551bpofD1/D2 domain was carried out using reference sequence from NCBI nucleotide database. The multiple and pairwise alignments of these sample were performed in MEGA6 using CLUSTALW. Results are shown in following Table 4.12.

Table 4.12 Evaluation of D1/D2 Sequences and sequences alignment analysis

Parameters	**Analysis**
Universal ability of D1/D2 primers for amplification of D1/D2 region	Yes
Percentage sequencing success	50
Aligned sequence length	586
Number of conserved sites	379
Number of variable sites	204
Distribution of variable sites	Dispersive
Number of sample	20

With the help of jModelTest best model,model average phylogeny was computed with TVMef+Imodel. In model-average phylogeny criteria for tree weights was BIC and strict consensus type was chosen with confidence interval 95%.the unrooted strict consensus tree was constructed according to the TVMef+Imodel.

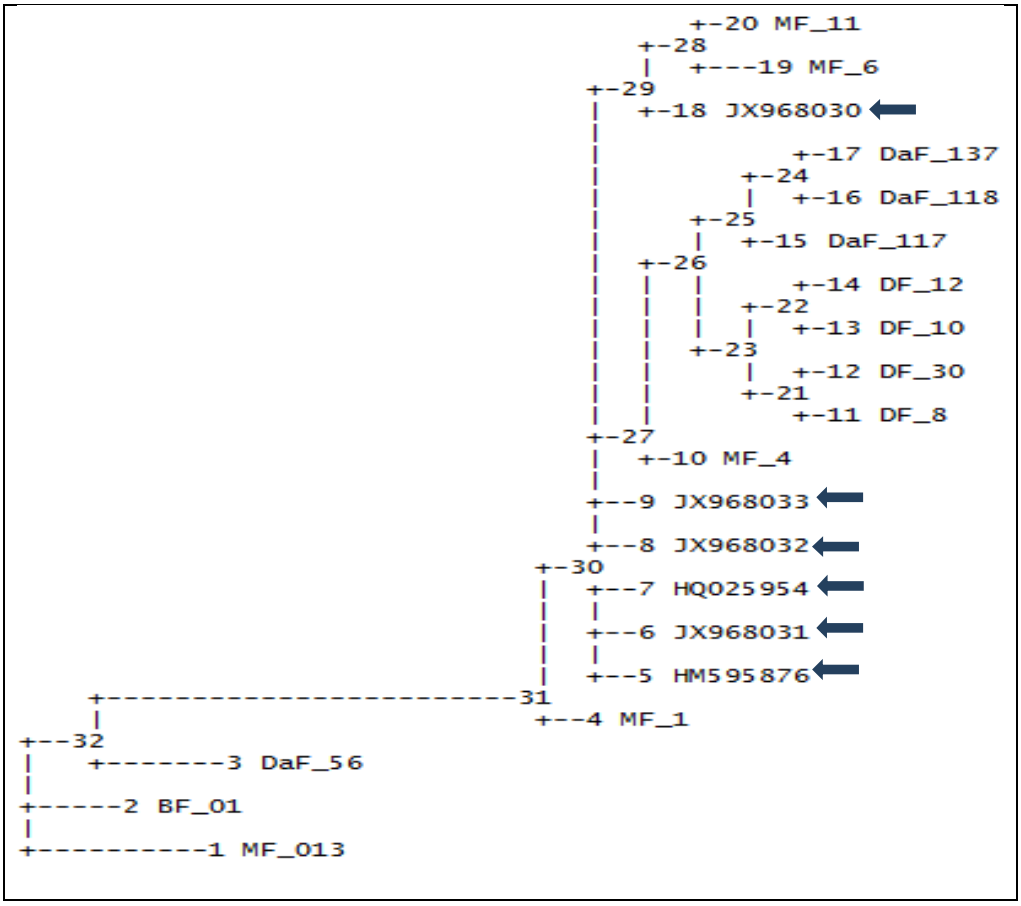
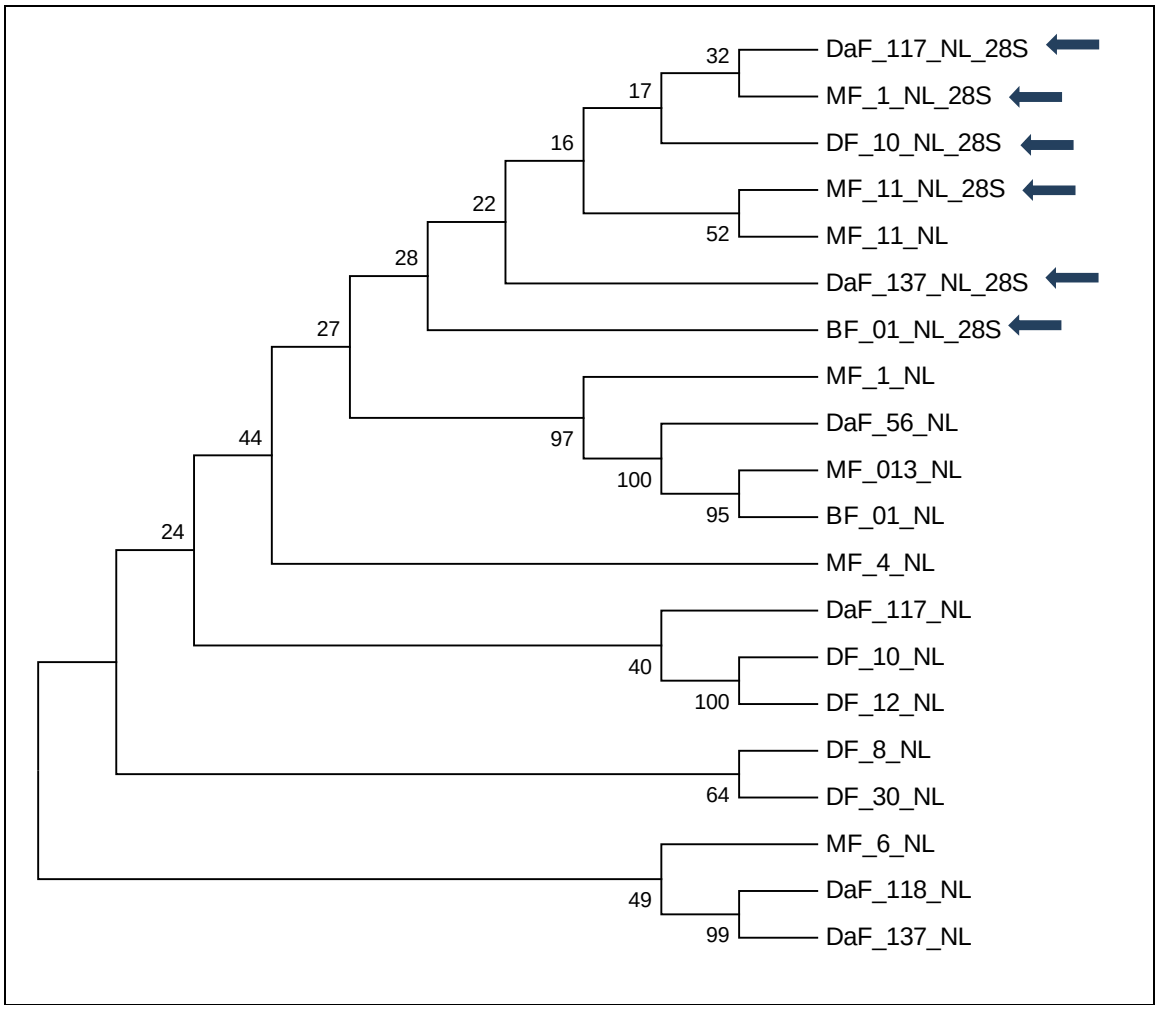


Fig.4.9 Strict consensus tree generated from D1/D2 sequences using jModelTest. Labeled number in tree indicate the branch length (**Note:** samplesshown in arrow are sample taken fron NCBI genbank).

Branch length are the expected number of substitution per site. From the above figure two major clade were seen in strict consensus tree (Fig. 4.7)eventhough that twelve subclade are formed but the tree shows that there is no genetic variation because MF013 make separate clade on the bottom however all the Manang fungal are not making single clade they are mixed with Dolpa, Bajhanag and Darchula,from this tree we can conclude that this tree is polytomy.Out of the 20 accession under this study 17 accession formed a monophyletic clade. In this tree major two monophyletic clade were separated, first monophyletic clade was formed by *O. sinensis* accession from Dolpa, Darchula and Manang. Second clade was formed by *O. sinenesis* accession of Manang, Darchula, Bajhang and Dolpa. Some accession of Bhajhang and Manangwas showndiversed from all other accession in this study.

Maximum parsimony tree was constructed using the subtree pruning regrafting (SPR)algorithm with 1000 bootstrap replication the most parsimonius length of 223 is shown in figure(Fig. 4.10).



(**Note:** shown infram are sample taken fron NCBI genbank)

Fig.4.10 Maximum Parsimony tree generated from D1/D2 with reference sequences from NCBI Genbank obtained using the subtree-pruning regrafting (SPR) algorithm in MEGA 6.

The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 8 most parsimonious trees (length = 272) is shown. The consistency index is (0.759358), the retention index is (0.812500), and the composite index is 0.678079 (0.616979) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the

branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 586 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The parsimony informative site is 128 over 586, singleton site 74 over 586 variable sites 204 over 586, conserved site 379 over 586. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 20 nucleotide sequences. There were a total of 538 positions in the final dataset. In the cladogram two nodes majorly represent the group of sub-clades with 22% and 49% bootstrap value.

Out of the 20 accession under this study, 19 accessions formed a major monophyletic clade. In this major clade two subclade were separated, first monophyletic clade was formed by *O. sinensis* accession from Bhajhang, Darchula and Manang with genbank accession. Second subcladewas formed by *O. sinenesis* accession of Darchula, Dolpa and Manang. Single accession of Manang (MF4)was showndiversed from all other accession in this study.

Comparison of branching patterns of different sequences between phylogenetic trees generated by jModelTest and maximum parsimony tree generated by MEGA revealed similar branching pattern thereby confirming the validity of phylogenetic analysis. D1/D2 sequences were able to distinguish the accession sequences from out-group sequences and placed all the accessions in single clade.

Pairwise distance value between nucleotide sequences of D1/D2 region

Data are shown in AppendixII, data shows that the probability of accepting or rejecting null hypothesis by estimating the P-values which are shown below the diagonal. P-value smaller than or equal to 0.05 (marked with bold) are considered significant. The estimates of the disparity index per sites are shown for each sequence pair above the diagonal. Higher the disparity index per site, lower is the P-value and test is more significant. The result showed that the null hypothesis can be rejected in 31% of the gene at 5% significance level Analyses were

conducted using the Maximum parsimony model. The number of base substitutions per site from between sequences is shown for each sequences. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 586 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The result showed that the null hypothesis can be rejected in 31% of the gene at the 5% significance level. From the above table we can observe that divergence ranges from 0.000-0.3. (Table shown in appendix)

Table4.13 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution of D1/D2 Domain with reference sequence from genbank.

	A	**T**	**C**	**G**
A	-	*7.56*	*10.48*	**5.85**
T	*6.13*	-	**13.77**	*9.09*
C	*6.13*	**9.93**	-	*9.09*
G	**3.94**	*7.56*	*10.48*	-

In above table each entry shows the probability of substitution (r) from one base to another base (column). For simplicity, the sum of 'r' values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. The nucleotide frequencies are 18.42% (A), 22.73% (T/U), 27.32% (C), and 31.52% (G). The transition/transversion rate ratios are k_1 = 0.643 (purines) and k_2 = 1.314 (pyrimidines). The overall transition/transversion bias is R = 0.51, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The analysis involved 20 nucleotide sequences.

CLUSTAL O (1.2.1) multiple sequence alignment based on D1/D2 Domain

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MF       ATTGTCATTATCGAGTTACACAACTCCCAAACCCCCTGTGAACCTTACCCCACGCGTTGC 82
BF06     AAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 78
BF3      ----------------------------------------------CCACAGCAGTTGC 13
DaF137   ----------------------------------CTGCGAA---CACCACAGCAGTTGC 22
DaF56    GAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 60
MF1      GAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 75
DF12     GGGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 75
DF8      GAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 78
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DF22     GGGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 75
DF34     GAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 79
DaF96    ---------ATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 46
DaF99    --------TATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 47
DF58    TTTGACATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 175
DF6      AGGGACATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 75
DF35     GGGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 77
DF54     GGGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 76
MF11     GAGATCATTATCGAGTCA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 75
DF10     AGGGACATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 70
MF6      GAGATCATTATCGAGTCA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 77
DaF116   --------------GTCA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 41
DaF117   ---------------------------------------AA---CACCACAGCAGTTGC 17
DF30     GAGATCATTATCGAGTTA--CCACTCCCAAACCCCCTGCGAA---CACCACAGCAGTTGC 77
DaF118   ---------------------------------------AA---CACCACAGCAGTTGC 17
                             ** **   *****

MF       CTCGGCGGGACCGCCCCGGCGCCCCAGCGGCCCGGACCAAGGCGCCCGCCGGAGGACCCC 142
BF06     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 137
BF03      CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 72
DaF137   CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 81
DaF56    CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 119
MF1      CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 134
DF12     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 134
DF8      CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 137
DF22     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 134
DF34     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 138
DaF96    CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 105
DaF99    CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 106
DF58     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 234
DF6      CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 134
DF35     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 136
DF54     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 135
MF11     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 134
DF10     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 129
MF6      CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 136
DaF116   CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 100
 DaF117 CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 76
DF30     CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 136
 DaF118 CTCGGCGGGACCGCCCCGGCGC-CCCAGGGCCCGGACCAGGGCGCCCGCCGGAGGACCCC 76
      ********************** **   *********** ********************

MF       CAGACTCTCCTGTCGCAGCGGCATTTCTCTCCGAGTCAAAAAAAACAAGCAAATGAATCA 202
BF06     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 191
BF03     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 126
DaF137   CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 135
DaF56    CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 173
MF1      CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 188
DF12     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 188
DF8      CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 191
DF22     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 188
DF34     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 192
DaF96    CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 159
DaF99    CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 160
DF58     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 288
DF6      CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 188
DF35     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 190
DF54     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 189
MF11     CAGACCCCCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 188
DF10     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 183
MF6      CAGACCCCCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 190
DaF116   CAGACCCCCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 154
DaF117   CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 130
DF30     CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 190
DaF118   CAGACCCTCCTGTCGCAGTGGCATCTCTCA------GTCAAGAAGCAAGCAAATGAATCA 130
      ***** * ********** ***** ****          ** ** ***************

MF       AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 262
BF06     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 251
BF03     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 186
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DaF137  AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 195
DaF56   AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 233
MF1     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 248
DF12    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 248
DF8     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 251
DF22    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 248
DF34    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 252
DaF96   AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 219
DaF99   AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 220
DF58    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 348
DF6     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 248
DF35    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 250
DF54    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 249
MF11    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 248
DF10    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 243
MF6     AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 250
DaF116  AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 214
DaF117  AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 190
DF30    AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 250
DaF118  AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT 190
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MF      AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG 322
BF06    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 311
BF03    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 246
DaF137  AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 255
DaF56   AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 293
MF1     AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 308
DF12    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 308
DF8     AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 311
DF22    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 308
DF34    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 312
DaF96   AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 279
DaF99   AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 280
DF58    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 408
DF6     AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 308
DF35    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 310
DF54    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 309
MF11    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 308
DF10    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 303
MF6     AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 310
DaF116  AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 274
DaF117  AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 250
DF30    AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 310
DaF118  AAGTAATGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGAACGCACATTGCGCCCG 250
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MF      CCAGTACTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAGCCCCCCTCCGC 382
BF06    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 371
BF03    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 306
DaF137  CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 315
DaF56   CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 353
MF1     CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 368
DF12    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 368
DF8     CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 371
DF22    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 368
DF34    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 372
DaF96   CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 339
DaF99   CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 340
DFI58   CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 468
DF6     CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 368
DF35    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 370
DF54    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 369
MF11    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 368
DF10    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 363
MF6     CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 370
DaF116  CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 334
DaF117  CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 310
DF30    CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 370
DaF118  CCAGCACTCTGGCGGGCATGCCTGTCCGAGCGTCATCTCAACCCTCGAGCCCCCCGCCTC 310
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MF      CCCCGGCGGCGGCGGGGCTCGGCGTTGGGGGTCCCGGCCCACGCCCCCCCGAGGGCGGTC 442
BF06    TTGGTGGGGGGGGCTGGTTTTG-----GGTGATTTTTTTTTTTTGGAGTGTGGTAGGTGT 426
BF03    GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCCCGCCCCCCCCCCGCCCCTAGGGGGTG 361
DaF137  GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCACTCCGCCCCCAGGGGGTG 370
DaF56   GCGGCGGCGGGGCCCGGCTTTG-----GGGGTCCGGCCCCCCTGCGCCCCTAGGGGTGGG 408
MF1     GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCGCCCGCCCCCTGGGCGGGG 423
DF12    GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCGCCCGCCCCCTAGGGTGTG 423
DF8     GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCGCGCCGCCCCCTAGGGCAG 426
DF22    GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCCCTCCGCCCCCTGGGGGCT 423
DF34    GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCCCGCCGCCCCCTGGGGCTG 427
DaF96   GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCACGCCGCCCCCTAGGGCAG 394
DaF99   GCGGCGGCGGGGCCCGGCCTTG-----GGGGTCACGGCCCCACCCCGCCCCCTAGGGCAG 395
DF58    GCGGCGGGGGCCTTGGGGGGGG-----AGGTTATGGGGCCTTTTGAGCCCCGCGCCGCCC 523
DF6     GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 413
DF35    GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 415
DF54    GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 414
MF11    GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 413
DF10    GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 408
MF6     GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 415
DaF116  GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 379
DaF117  GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 355
DF30    GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 415
DaF118  GCGGCGGCGGGGCCCGGCCTTG--------------GGGGTCACGGCCCCGCGCCGCCC 355
*  *      **    *
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Fig. 4.11 CLUSTAL O (1.2.1) multiple sequence alignment based on D1/D2 Domain

4.6Phylogenetic analysis basedon COI barcode locus

Phylogenetic and evolutionary relationship was analyzed by using jModelTestver 2.1.4 software and MEGA6.By using MEGA6 software for each nucleotide sequences maximum parsimony method was analyzed. COI sequence samples of this study and COI sequence of *Hepialus* NCBIgb|KF696592|-MI11 COI gene, MICOI similar- gb|HM744695|, MICOI gene-gb|JQ325879|, MI4 COI gene-gb| KC994972|, MI01 COI similar- gb|HM595868|, MI01COIgene-gb|JQ325941| were analyzed.

4.6.1 Multiple and pair-wise alignment of COI sequences of Manang

Multiple and pair-wise alignment of 10 nucleotide sequences of 604 bp of COI region were carried out using reference sequence from NCBI nucleotide database. The multiple and pairwise alignments of these sample were performed in MEGA6 using CLUSTALW. Results are shown in following table.

Table 4.14 Evaluation of COI sequence and sequence alignment analysis

Parameters	**Analysis**
Universal ability of COI primers for amplification of COI region	Yes

Percentage sequencing success	30
Aligned sequence length	604
Number of conserved sites	514
Number of variable sites	86
Distribution of variable sites	Dispersive
Number of sample	10

4.6.2 Amino acid Composition analysis

The nucleotide sequence analysis based on the mtDNA COI sequence, considering all the codon position, the average amino acid frequencies in COI nucleotide of some of the samples were 23.324% Leucine, 13.353% tyrosine, 8.2799% glutamin, 8.863% Glutamic and so on.

Table 4.15 Amino acid concentration found in COI sequences of *O. sinensis*

	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu
MI11 COI	1.1696	0	4.6784	7.6023	8.1871	4.6784	7.0175	8.1871	1.7544	21.637
MI11 COIgene	1.1561	1.1561	4.6243	8.0925	7.5145	4.0462	6.9364	8.6705	1.7341	21.965
MI4 COI	0.5848	0	4.0936	7.6023	7.6023	4.6784	8.1871	8.1871	2.3392	21.637
MI COI	0.5848	0	5.2632	9.9415	7.6023	2.3392	7.0175	11.111	1.7544	22.222
MI COIsimilar	0.5814	0	5.814	9.3023	7.5581	2.907	6.9767	9.8837	1.7442	24.419
MI COIgene	0.5814	0	5.2326	9.8837	8.1395	2.3256	5.814	11.047	1.7442	26.163
MI1 COI	0	0	4.7059	10.588	7.6471	1.7647	7.0588	10.588	2.3529	22.353
MI4 COIgene	1.1696	0.5848	4.6784	7.0175	7.6023	5.2632	7.6023	8.7719	1.7544	22.222
MI01 COIsimilar	0.5814	0	4.6512	8.7209	8.1395	3.4884	6.9767	9.8837	1.7442	24.419
MI01 COIgene	0.5814	0	5.2326	9.8837	8.1395	2.3256	5.814	11.047	1.7442	26.163
Avg.	0.6997	0.1749	4.898	8.863	7.8134	3.3819	6.9388	9.7376	1.8659	23.324

	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
MI11 COI	1.1696	0.5848	4.6784	7.6023	0.5848	1.1696	3.5088	1.7544	0.5848	13.45
MI11 COIgene	1.1561	0.578	4.6243	8.0925	0	1.1561	3.4682	1.7341	1.1561	12.139
MI4 COI	1.1696	0	4.0936	8.1871	0.5848	1.7544	4.0936	1.7544	0.5848	12.865
MI COI	2.3392	0.5848	4.0936	8.7719	0	1.1696	0	1.7544	0	13.45
MI COIsimilar	2.3256	0.5814	2.907	8.1395	0	0.5814	1.1628	1.1628	0	13.953
MI COIgene	1.7442	0.5814	1.7442	8.7209	0	0	0.5814	1.7442	0	13.953
MI1 COI	2.3529	0.5882	4.1176	8.8235	0.5882	1.7647	0	1.7647	0	12.941
MI4 COIgene	1.1696	0.5848	4.0936	7.6023	0.5848	1.1696	3.5088	1.7544	0	12.865
MI01 COIsimilar	2.3256	0.5814	2.907	8.1395	0	0	1.1628	2.3256	0	13.953
MI01 COIgene	1.7442	0.5814	1.7442	8.7209	0	0	0.5814	1.7442	0	13.953
Avg.	1.7493	0.5248	3.4985	8.2799	0.2332	0.8746	1.8076	1.7493	0.2332	13.353

With the help of jModelTest best model,model average phylogeny was computed with HKY+G model. In model-average phylogeny criteria for tree weights was BIC and strict consensus type was chosen with confidence interval 95%.the unrooted strict consensus tree was constructed according to the HKY+Gmodel.

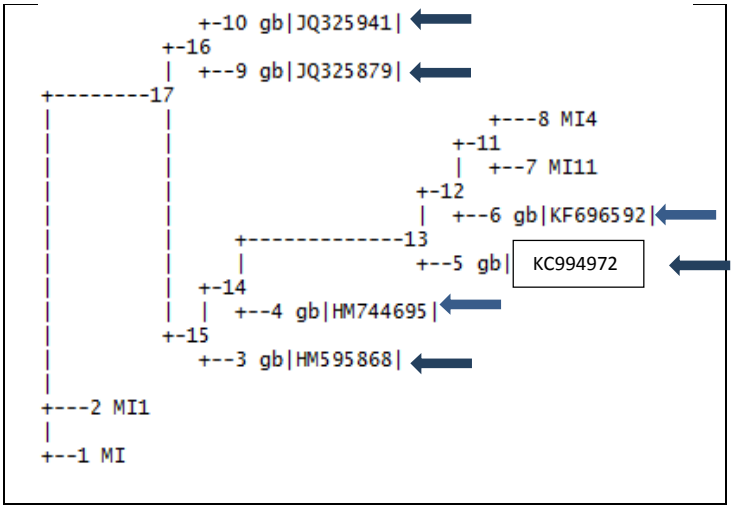

Fig 4.12 Strict consensus tree generated from COI sequences using jModelTest. Labeled number in tree indicate the branch length.

Branch length are the expected number of substitution per site.From the above figure seven major clade were seen in strict consensus tree (Fig.4.11)eventhough that eight clade are formed but the tree shows that there is no genetic variation because manang fungi make separate clade on the bottom however all the manag fungal are not making single clade they are mixed with dolpa and darchula ,from this tree we can conclude that through the help of air spore of fungi are distributed everywhere in Nepal so they have same genetic makeup.Out of the 10 accession under this study, 8 accessions formed a major monophyletic clade. In this major clade two subclade were separated, both monophyletic clade were formed by *Hirsutellasinensis* accession of Manang. Some *Hirsutellasinensis* accession of them shown diversed from all other accession in this study.

Maximum parsimony tree was constructed using the subtree pruning regrafting (SPR)algorithm with 1000 bootstrap replication. The most parsimonius length of 118 is shown in **Fig (4.13)**

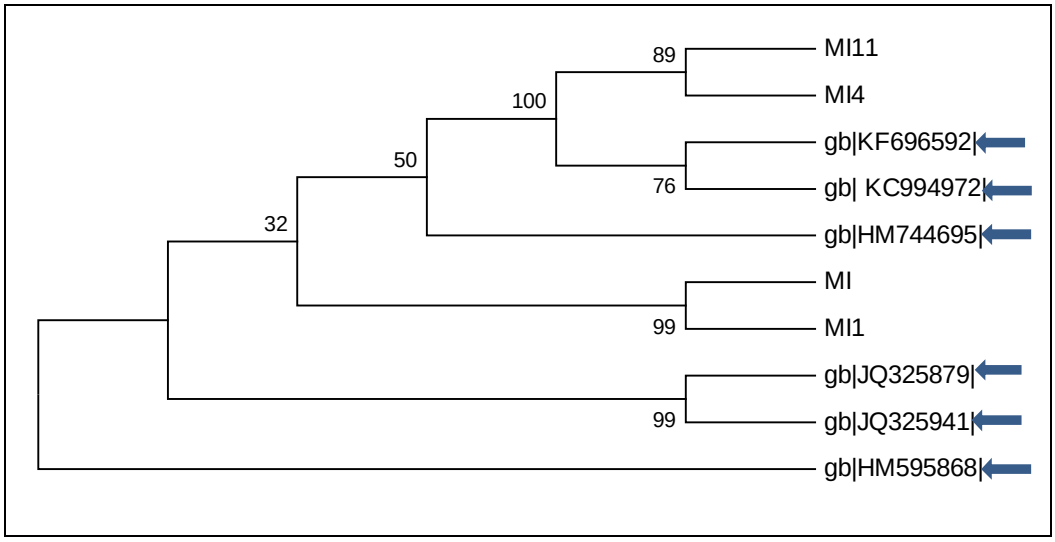

Fig 4.13: Maximum Parsimony tree for COI region with reference sequence from genbank obtained using the subtree-pruning regrafting (SPR) algorithm in MEGA 6.

The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 3 most parsimonious trees (length = 127) is shown. The consistency index is (0.773196), the retention index is (0.848276), and the composite index is 0.701330 (0.655883) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 604 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Out of the 10 accession under this study, 9 accessions formed a major monophyletic clade. Single accession taken from Genbankwas showndiversed from all other accession in this study.All positions containing gaps and missing data were eliminated. There were a total of 591 positions in the final dataset. In the cladogram two nodes majorly represent the group of sub clades with 34% and 98% bootstrap value.

Comparison of branching patterns of different sequences between phylogenetic trees generated by jModelTest and maximum parsimony tree generated by MEGA revealed similar branching pattern thereby confirming the validity of phylogenetic analysis. COI sequences were able to distinguish the accession sequences from outgroup sequences and placed all the accessions in single clade.

4.6.3 Evolutionary distance between nucleotide sequences of COI region

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum parsimony model. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 591 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. From the above table we can observe that the divergence between research sample *Ophiocordyceps* larvae and reference sequence taken from NCBI genbank. Their evolutionary and genetic distance is very low so they are not genetically diverse.

Table4.16 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution of COI locus in *O. sinensis* of Mannag sample

	A	**T**	**C**	**G**
A	-	*6.38*	*2.53*	**5.74**
T	*5.53*	-	**13.58**	*2.53*
C	*5.53*	**34.19**	-	*2.53*
G	**12.55**	*6.38*	*2.53*	-

The probability of substitution (r) from one base (Bowen *et al.*) to another base (column) is shown in Table 4.14. For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. The transition rate is higher for G to A while the transversional substitutions

are minimal. The nucleotide frequencies are 32.61% (A), 37.56% (T/U), 14.91% (C), and 14.92% (G). The transition/transversion rate ratios are k_1 = 2.267 (purines) and k_2 = 5.363 (pyrimidines). The overall transition/transversion bias is R = 1.648, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The analysis involved 10 nucleotide sequences.

```
CLUSTAL O (1.2.1) multiple sequence alignment

MI4        ------------------------------TCAGAAAAATGAAGTATTTAAGTTTCGATCT 31
MI11       ------------------------------------------------------------ 0
MI1        -------------------------------CAAAAAATGAAGTATTTAAATTTCGATCT 29
MI         ATAAGGCGCGTCCAGCACATAGCATAGAGATCAAAAAATGAAGTATTTAAATTTCGATCT 60

MI4        GTTAATAATATAGTAATAGCTCCTGCCAATACAGGTAATGATAGTAAAAGTAATAATGCA 91
MI11       ----------TAGTAATAGCTCCTGCCAATACAGGTAATGATAGTAAAAGTAATAATGCA 50
MI1        GTTAATAATATAGTAATAGCTCCTGCTAATACTGGTAATGATAATAAAAGTAATAAGGCT 89
MI         GTTAATAATATAGTAATAGCTCCTGCTAATACTGGTAATGATAATAAAAGTAATAAGGCT 120
                     *************** ***** ********** ************ **

MI4        GTAATTACAACTCTTCATACAAATAATGGTATGCGGTCAAAAGATATTCTTTTTGATCGT 151
MI11       GTAATTACAACTCTTCATACAAATAATGGTATGCGGTCAAAAGATATTCTTTTTGATCGT 110
MI1        GTAATTGCAACACTTCATACAAATAATGGTATTCGATCAAAAGATATTCTTTTTGATCGT 149
MI         GTAATTGCAACACTTCATACAAATAATGGTATTCGATCAAAAGATATTCTTTTTGATCGT 180
           ****** **** ******************** ** ************************

MI4        ATATTAATTACAGTAGTAATAAAATTAATTGCTCCTAAAATTGATGAAATTCCTGCAAGA 211
MI11       ATATTAATTACAGTAGTAATAAAATTAATTGCTCCTAAAATTGATGAAATTCCTGCAAGA 170
MI1        ATATTAATTACGGTAGTAATAAAATTAATTGCCCCTAAAATTGATGAAATTCCTGCTAAA 209
MIC        ATATTAATTACGGTAGTAATAAAATTAATTGCCCCTAAAATTGATGAAATTCCTGCTAAA 240
           *********** ******************** ********************** * *

MI4       TGTAAAGAAAAAATAGCTAAATCTACTGATGCCCCCGAGTGTGCAATATTTGATGATAAT 271
MI11      TGTAAAGAAAAAATAGCTAAATCTACTGATGCCCCCGAGTGTGCAATATTTGATGATAAT 230
MI1       TGTAAAGAGAAAATAGCTAAATCTACAGATGCTCCAGAATGTGCAATATTTGATGATAAT 269
MI        TGTAAAGAGAAAATAGCTAAATCTACAGATGCTCCAGAATGTGCAATATTTGATGATAAT 300
          ******** ***************** ***** ** ** *********************



MI4       GGGGGATATACAGTTCAACCTGTTCCTGCCCCGTTTTCTACAATTCTCCTAGAAATTAAT 331
MI11      GGGGGATATACAGTTCAACCTGTTCCTGCCCCGTTTTCTACAATTCTCCTAGAAATTAAT 290
MI1       GGTGGATATACAGTTCAACCTGTTCCTGCCCCATTTTCTAAAATTCTTCTAGAAATTAAT 329
MI        GGTGGATATACAGTTCAACCTGTTCCTGCCCCATTTTCTAAAATTCTTCTAGAAATTAAT 360
          ** ***************************** ******* ****** ************

MI4       AATATTAATGATGGGGGTAATAATCAAAATCTTATATTATTAAGTCGTGGGAATGCTATA 391
MI11      AATATTAATGATGGGGGTAATAATCAAAATCTTATATTATTAAGTCGTGGGAATGCTATA 350
MI1       AATATTAATGAAGGAGGTAATAATCAGAATCTTATATTATTAAGTCGTGGAAATGCTATA 389
MI        AATATTAATGAAGGAGGTAATAATCAGAATCTTATATTATTAAGTCGTGGAAATGCTATA 420
          *********** ** ********** ********************** *********

MI4       TCGGGGGCCCCTATTATTAAAGGAATTAATCAATTCCCAAAACCACCAATCATTTTGGGT 451
MI11      TCGGGGGCCCCTAATATTAAAGGAATTAATCAGTTCCCAAAACCACCAATCATAATTGGT 410
MI1       TCAGGGGCTCCTAATATTAAAGGAACTAATCAATTTCCAAATCCACCAATTATAATTGGT 449
MI        TCAGGGGCTCCTAATATTAAAGGAACTAATCAATTTCCAAATCCACCAATTATAATTGGT 480
          ** ***** **** *********** ****** ** ***** ******** **  * ***
```

```
MI4       TA--CTACAAATAAAATTATTAAAAAGTCGTGAGCGGGGACTATTACTTTTTAAATTTTT 509
MI11      ATTACTATAAAAAAAATTATAATAAAAGCGTGAGCTGTAACAATTACATTATAAATTTGA 470
MI1       ATTACTATAAAGAAAATTATAATAAAGGCATGGGCTGTAACAATTACATTATAAATTTGA 509
MI        ATTACTATAAAGAAAATTATAATAAAGGCATGGGCTGTAACAATTACATTATAAATTTGA 540
             *** *** ******** * ***  * ** ** *  ** ***** ** *******

MI4       TCGTCGCCGGGGAAAGATCCTTTATTTTTTTTTCTCAGTGTGAGAAATTAATATTCCCCA 569
MI11      TCATCTCCAATTAAAGATCCGGGATTTCCTAACTCTG-CTCGAATTATTATTCTTAAAGA 529
MI        TCATCTCCAATTAAAGATCCAGGATTTCCTAATTCTG-TTCGAATTATTATTCTTAAAGA 568
MI        TCATCTCCAATTAAAGATCCAGGATTACCTAATTCTG-TTCGAATTATTATTCTTAAAGA 599
          ** ** **    ********   ***   *           **   **** * **    *
```

Fig.4.14 CLUSTAL O (1.2.1) multiple sequence alignment

4.7 Phylogenetic analysisBased on COI locus of *O. sinensis* samples from Bhajhang and Darchula

Phylogenetic and evolutionary relationship was analyzed by using jModelTestver 2.1.4 software and MEGA6 .By using MEGA6 software for each nucleotide sequences maximum parsimony method was analyzed. Due to the improper sequencing of some sample of larval portion of *O. sinensis*contig formation was problematic but fortunately its forward sequence was of very nice quality so in this project we are using just forward sequence only for the phylogenetic and evolutionary study for larval analysis of Bhajhang and Darchula, on the other hand its fungal sequence was of high grade so phylogenetic and evolution study was done through contig.Cytochrome 'C' oxidase sequences were compared with that of sequences of *HepialidaeCOI* sequences were compared with that of NCBI reported sequences (gb| KF696584 |), (gb| KC994967|), (gb|KC994924|), gb(|KC994967|), gb(|JQ325928|) and (gb| KC994926|).

4.7.1 Multiple and pair-wise alignment of COI sequences

Multiple and pair-wise alignment of 10 nucleotide sequences of 604 bp of COI region were carried out using reference sequence from NCBI nucleotide database. The multiple and pairwise alignments of these sample were performed in MEGA5 using CLUSTALW. Results are shown in following table 4.15.

Table4.17 Evaluation of COI sequence and sequence alignment analysis.

Parameters	Analysis
Universal ability of COI primers for amplification of COI region	Yes
Percentage sequencing success	30

Aligned sequence length	609
Number of conserved sites	512
Number of variable sites	82
Distribution of variable sites	Dispersive
Number of sample	14

With the help of jModelTest best model,model average phylogeny was computed with HKY+G model. In model-average phylogeny criteria for tree weights was BIC and strict consensus type was chosen with confidence interval 95%.the unrooted strict consensus tree was constructed according to the HKY+G model.

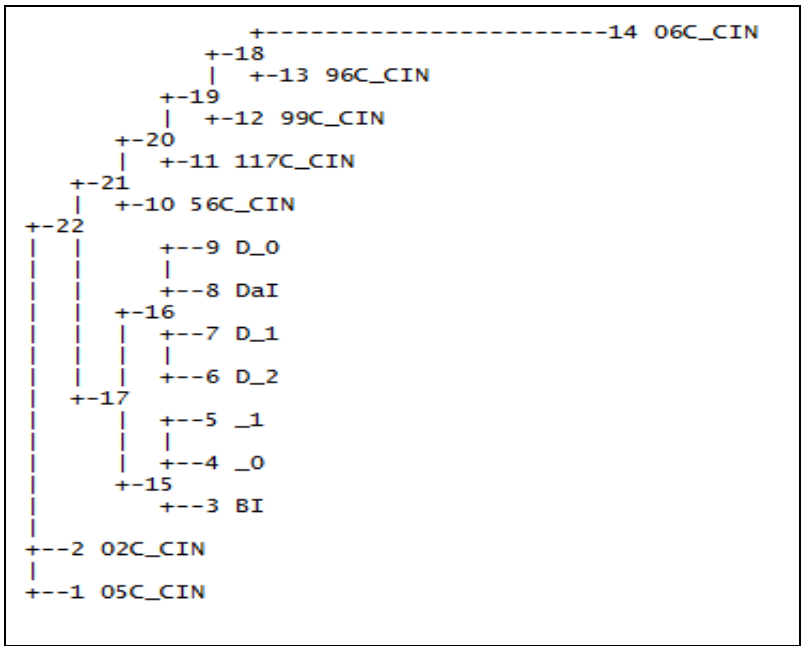

Fig. 4.15 Strict consensus tree of larva by CI-J CIN primer, generated with confidense interval of 0.95 and selection criteria by BIC using HKY+Imodel in jModelTest. Labeled number in tree indicate the branch length.

Branch length are the expected number of substitution per site. Two major clades were seen in strict consensus tree (Fig.4.13). Two samples of bhajhang formed separate clades at the bottom of phylogenetic tree however other sample are mixed together.Out of the 14 accession under this study, 12 accessions formed a major monophyletic clade. In this tree major two

subcladeswere separated, both clade were formed by *Hepialus* accession from Bhajhang and Darchula. Some accession of Bhajhang were shown diversed from all other accession in this study.

Maximum parsimony tree was constructed using the subtree pruning regrafting (SPR)algorithm with 1000 bootstrap replication. The most parsimonius tree had the treelength of 90 (fig. 4.15)

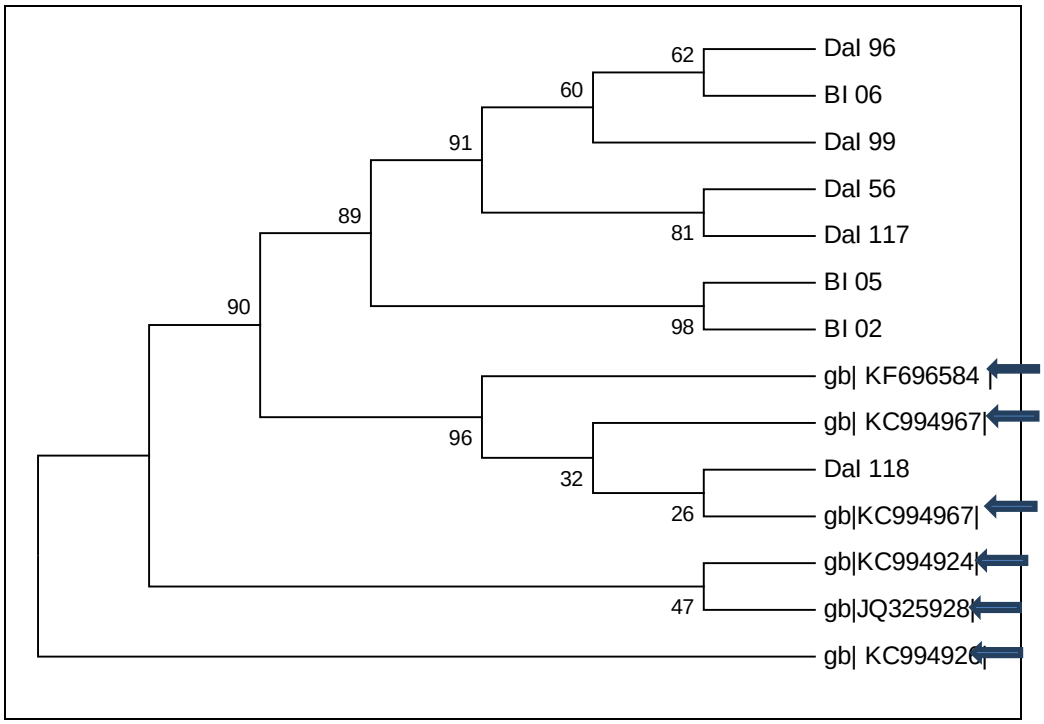

Fig. 4.16 Maximum Parsimony tree for COI region of Bajhang and Darchula with reference sequence from genbank obtained using the subtree-pruning regrafting (SPR) algorithm in MEGA6

The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 8 most parsimonious trees (length = 98) is shown. The consistency index is 0.794872, the retention index is 0.896104, and the composite index is 0.822953 (0.712288) for all sites and parsimony-informative sites (in parentheses). The parsimony informative site is 7 over 604, variable sites 82 over 604, conserved site 512 over 604. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown

next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 14 nucleotide sequences. There were a total of 609 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. In the cladogram there are two major clade and other subclade with 32% and 98% bootstrap value.Out of the 14 accession under this study, all accessions formed a major monophyletic clade. In this tree major two subclade were separated, first monophyletic clade was formed by *Hepialus* accession only from Bhajhang and Darchula, second subclade was formed by *Hepialus* accession of Darchula and Bhajhang along with sample taken from Genbank.

Comparison of branching patterns of different sequences between phylogenetic trees generated by jModelTest and maximum parsimony tree generated by MEGA revealed similar branching pattern thereby confirming the validity of phylogenetic analysis. *CO*I sequences were able to distinguish the accession sequences from outgroup sequences and placed all the accessions in single clade.

4.7.2Evolutionary distance between nucleotide sequences based on COI region

Analyses were conducted using the Maximum parsimony model. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 591 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. From the above table we can observe that the divergence between research sample *Ophiocordyceps* larvae and reference sequence *Hepialus* species taken from NCBI genbank. Their evolutionary and genetic distance is very low so they are close to each other (data are shown in appendix).

Table4.18 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution of COI sequences of Bhajhang and Darchula.

	A	**T**	**C**	**G**
A	-	*2.41*	*1.2*	**7.96**
T	*2.92*	-	**19.48**	*1.32*
C	*2.92*	**39.27**	-	*1.32*
G	**17.59**	*2.41*	*1.2*	-

Above table show each entry shows the probability of substitution (r) from one base to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. The transition rate is higher for T to C while the transversional substitutions are minimal. The nucleotide frequencies are 37.19% (A), 30.74% (T/U), 16.83% (C), and 15.25% (G). The transition/transversion rate ratios are k_1 = 6.022 (purines) and k_2 = 16.265 (pyrimidines). The overall transition/transversion bias is R = 4.586, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The analysis involved 14 nucleotide sequences. There were a total of 573 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

5.1 Discussion

Ophiocordycepssinensis is a highly valued medicinal species of Nepal and is reported to be found in Dolpa, Rukum, Manang, Myagdi, Gorkha,Bhajhang, Darchula, and Jumla district of high altitude of Nepal (Shrestha & Bawa, 2014). Every year rural people of different district visit these remote location for the collection of *O. sinensis* as this valuable commodities is linked with the livelihood of these rural people.Scientific invention pertinent to its conservation, characterization and sustainable utilization has now became crucial.Taking these into consideration, government has also listed this species as highly prioritized species. This study was conducted for the evolutionary and phylogenetic analysis of *O.sinensis*samplesof different localities of Nepal, using molecular technique. Sequence based barcode markers were generated for accessions coming from different populations and their phylogenetic analysis was performed. This project was initiated with objectives of generating DNA barcode of *O. sinensis* samples collected from various geographical location creating database of its DNA sequences for documentation and identification as well as to elucidate evolutionary and phylogenetic relationship of different population. For the current study, the samples were collected from four district*viz*. Manang, Dolpa, Bhajhang and Darchulaof Nepal representing three development regions.

5.1 DNA Extraction and Quantification

Genomic DNA extraction was done by using modified universal high salt DNA extraction protocolasdescribed by (Aljanabi & Martinez, 1997). Thus extracted DNA was quantified by using UV Biophotometer, however the purity of DNA of some samples was not satisfactory as A 260/280 was in the range of 1.4-2.0. DNA purity depend upon the kind of material and quality of sample(Pan *et al.*, 2006). Although theDNA extraction protocol is reliable but in this DNA extraction protocol no RNAse were used so purity of DNA is not satisfactory as it contains high amount of RNA so was cured by treating with RNAse.

5.2 PCR Optimization and PCR amplificationofdifferent barcode loci

PCR amplification of stroma and sclerotium is carried out by different PCR condition due to improper PCR amplification(Obradovic *et al.*, 2013).In order to generate DNA barcode sequences, PCR reaction and cycling parameter were optimized prior to sequencing. For the successful PCR amplification appropriate combination of PCR reaction mixture is important. Of the various PCR reagents $MgCl_2$ concentration,DNA concentration, polymerase and buffer types,dNTPsconcentration are important to increase directly chelate a proportional number of Mg (++) ions, an increase in the concentration of dNTPs decreases the concentration of free Mg (++) available to influence polymerase function(Innis & Gelfand, 1999). Various optimization strategies had tried in this study including touchdown PCR and hot-start PCR (Romano & Palumbi, 1997).

The template DNA concentration is one of the main parameter to be optimized in PCR. But the concentration of DNA depend upon the kind of material and purity of extracted DNA(Pan *et al.*, 2006). In this study DNA concentration ranges from 10-55 ngin 25µL reaction volume but the best DNA concentration was found to be 45ng. Generally DNA concentration ranges from 50-100ng in 25-50 µL reaction volume (Padmalatha & Prasad, 2006).

$MgCl_2$concentration optimization is done by two ways either by determining the optimal primer/template combinationor varying $MgCl_2$ concentration for fixed primer/template combination (Shrestha *et al.*, 2011). The range of $MgCl_2$ tested was 0.5 mM to 4mM, in which bright and single band was observed at 2.5mM and was selected as optimum for this study. Magnesium acts as a cofactor for thermostable DNA polymerase and in the absence of adequate free magnesium,*Taq*polymerase is inactive whereas excess of free magnesium reduces enzyme fidelity (Eckert & Kunkel, 1990).

Primer concentration is another most important parameter for PCR reaction.Primer range tested in this project is 0.3- 4 µl primer of 10Pmol concentration and the concentration of 1µl for ITS and 1.5µl for COI was found to be the best primer concentration. High concentration of primer promotes mispriming, accumulation of non-specific products and formation of primer dimer wheras low concentration leads to failure in amplification (Innis *et al.*, 2012). dNTPs were

tested in the range of 0.1-0.5mM concentration among which the concentration at 0.3mM gave single and bright band. dNTPs are known to chelate magnesium and high concentration of dNTPs increasing the error rate of *taq* polymerase(Padmalatha & Prasad, 2006).Concentration of 0.1 -0.2 mM of dNTPs have been quoted as being optimal for the most reactions (Innis & Gelfand, 1999).

Taq polymerase range tested is 0.3- 2.5 U. PCR bands were observed in all above concentration but single sharp and bright bands were observed at 1U, hence this concentration was selected as optimum concentration for PCR amplification. *Taq* polymerase is the most common enzyme used for PCR amplification because of its thermostability and processivity. It has extension rate of 35 to 100 nucleotides per second at 72^{o}C which is the most common extension temperature (Innis *et al.*, 1988). A recommended range of *Taq* concentration is between 1 and 2.5 units per 100 µL reaction volume (Lawyer *et al.*, 1989, Innis & Gelfand, 1990). If the enzyme concentration is too high, nonspecific background products may accumulate and if too low, an insufficient amount of desired product is yielded (Grunenwald, 2003).

In the polymerase chain reaction (PCR) technique mainly three steps DNA denaturation, primer-template annealing and DNA synthesis by a thermo stable DNA polymerase play an important role. For the purity and yield of the reaction mixture annealing temperature play an important role.Optimization of annealing temperature,optimum melting temperature is the melting temperature of less stable primer (Rychlik *et al.*, 1990) to be successful in PCR reaction require efficient and specific amplification of the product (Bio-rad, 2015). Failure to amplify under certain PCR condition is due to by generation of multiple undefined and unwanted product, presence of nonspecific background band due to mispriming, formation of primer-dimer, mutation or heterogeneity due to misincorporation that compete for amplification with the desired product (Babec,2015).

Optimization of annealing temperature is one of the most important steps during PCR. Annealing temperature calculation is started with calculating melting temperature of the primer template pair (Roux, 2009). The simplest rule of thumb is that Tm in degree centigrade of perfect primer is sum of four times the (G+C)'s and two times (A+T)'s (Roux, 2009), although

after calculating the Tm of a primer it may be needed to determine the annealing temperature empirically. It includes trying PCR reaction at above and below the calculated Tm temperature of the primer. The optimum annealing temperature is the one that result in highest yield with no nonspecific amplification (Bio-rad,2015). After trying Tm value for COI, ITS and D1/D2 primer pairs, optimized annealing temperature was found to be 50.5°C for COI primer,52.5°C for ITS and D1/D2 primer pairs and 39°C for CIJ-CIN primer.

PCR reaction was performed after optimization of PCR condition and reaction. The PCR amplification success rate for D1/D2, ITS and COI was 99%, 80% and 72% respectively. Even though the PCR reaction and condition are optimized, the PCR amplification rate was not 100%. It may be due to requirement of different PCR conditions for different samples from different locations(Kwiatkowski *et al.*, 2012). Poor PCR amplification may be due to one of many causes such as temperature for denaturation, annealing and extension may not be propertemplate, may not be in appropriate amount or reagent may be at sub-optimal concentration(Babec,2015). The PCR works readily with a DNA template of up to two to three thousand base pair in length and above this size product yields often decreases, as with increasing length premature termination by the polymerase begin to affect the efficiency of the PCR. In addition, helices with a high G-C content possess a higher melting temperature, often impairing PCR (Strachan T and Read, 1999).ITS region of nrDNA were amplified using forward primer ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and a reverse primer of ITS4R(5'-TCCTCCGCTTATTGATATGC-3') PCR mixture contained 1µl $Mgcl_2$(25Mm),1.5µl taq buffer(10X),dNTPs0.3µl(10Mm),0.1µl taq polymerase,0.6µl primer (10pm) 1µl T-DNA(45ng). The D1/D2 domain was amplified with forward primer NL4 (5'-GCATATCAATAAGCGGAGGAAAAG-3')and the reverse primer NL1 (5'-GGTCCGTGTTTCAAGACGG-3'). The COI region was amplified by forward primer LCOI (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCOI (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). Again COI region of Bhajhang and Darchula was amplified by next primer sets CIJ-1632 (5'-TGATCAAATTTATAAT-3') and CIN-2191 (5'-GGTAAAATTAAAATATAAACTTC-3'). The samples were amplified by using BIOER PCR.

5.3DNA sequencing and sequence analysis

Total 68 samples included in this study PCR amplification were successful for all loci. PCR amplification is a molecular based technique mainly ITS and D1/D2 loci was used for fungal identification and COI was used for animal identification (Robideau, De et al. 2011).All DNA regions *viz*, ITS, D1/D2 and COI showed 99%, 100% and 72% success of PCR amplification in the chosen accessions.The pairwise and multiple alignments were performed using CLUSTAL W in MEGA6 for respective sequences of a mentinedthree DNA Regions. In the three tested DNA regions, D1/D2 has the highest variable sites of 204 and lowest variable sites of 82 of COI loci. Using BLASTn(Altschul *et al.*, 1990) found in the NCBI web interface a sequence similarity search was performed. The pairwise sequence matches were ranked by statistical significance. The significance scores help to distinguish evolutionary related sequences from unrelated ones. Due to outgroup species taken in this study variable sites was high for all three loci. As such all three barcode markers used in this study i.e. ITS, D1/D2 and COI region can be assigned as a true barcode marker for*O. sinensis*.

5.4 Phylogenetic inference

Phylogenetics is the study of evolutionary history of living organisms using tree-like diagrams to represent pedigrees of the organisms. It is clear that the closely related species invariably get clustered in same clade(Xiong, 2006). Phylogenetic tree is a branching diagram that represents the evolutionary history of a group of organisms. Such a tree can provide a huge amount of information. For any particular group of animals this tree could identify the ancestors and closest relatives of the group (Hebert PDN *et al.,* 2003).

This research suggest that the ITS, D1/D2 and COI region are a valuable DNA barcode to authenticate *O. sinensis* from its counterfeit and closely related species and for the molecular phylogenetic study of *O. sinensis*.Out of 34 samples thus observed,the genomic DNA was extracted and ITS, D1/D2 and COI region were amplified using universal primer respectively. The identification process was made more accurate by using bioinformatics tools. The sequence data showed that D1/D2 domain varied from 585 to 712base pair, ITS length varied from 545 to 700 base pair and COI ranges from 600 to 714 base pair this result showed approximation to

reported data(Vrijenhoek, 1994, Abliz *et al.*, 2004, Hall *et al.*, 2004). The genetic variation between and within the species was analyzed by MEGA 6(Kimura, 1981) in ITS, D1/D2 and COI region. In this study the result of phylogenetic analysis revealed that all the experimental samples were identified as species of *O. sinensis* regardless of the geograpic origin. However, the BLASTn analysis of ITS and D1/D2 showed 99% similarity with that of *Hirsutella sinensis* which comes under same order indicating that *Hirsutella sinensis* is the anamorph of *O. sinensis* (Chen *etal*., 2004).

Using MEGA v.6 Maximum Parsimonious (MP) trees for each locus was constructed and Consistency Index (CI) of all parsimonious trees was computed. The value of CI for ITS, D1/D2 and COI was found to be 0.909091, 0.786667 and 0.768421 respectively. CI value indicates the realness of clades present in generated cladograms(Shrestha, 2001). CI is widely used method to measure homoplasy, which corresponds to the minimum amount of possible evolutionary change divided by actual tree length (i.e. the number of actual genetic changes in the tree). Lower values of consistency indices indicate that many characters are contradictory to the evolutionary tree. When a maximum parsimony tree is generated, the given set of data may yield a set of very similar trees with same tree length and generating a consensus tree may be useful to represent common features of this set of trees (Rohlf, 1982). In this studyML and MP trees revealed identical phylogenetic relationship among the species. The phylogenetic relationship among the species was clearly established, and similar species were clustered under same nodes while dissimilar species were clustered under separate nodes with both high and low bootstrap value support. Low bootstrap values in analysis indicates their position within respective families to be uncertain (Creˆte-Lafrenie`re A *etal.,* 2012).

Comparing model averaged phylogeny and maximum parsimony trees ITS and D1/D2 sequences of all *O. sinensis* accessions formed monophyletic clade. ITS phylogenetic analysis revealed that evolutionary relationship between the species of *Ophiocordyceps*. Some *O. sinensis* of Manang (MF11) sample showed close relationship with the accession of genbank (gb|JQ325150|), However, the single sample of Dolpa (DF6) and Manang (MF1) showed close relationship forming a single clade. Interestingly single sample of Manang (MF) is showing very close relationship with *O. nutans* gb|AB544491|. D1/D2 phylogenetic tree suggest that all

accession of *O. sinensis*made single monophyletic clade, among the monophyletic clade many subclade are formed. First subclade was formed by *O. sinensis* sample taken from genbankexcept single sample of Manang (MF11) showed close relation with *O. sinensis* of gb|JX968030|. Second subclade was formed by the sample from Manang (MF1, MF 013), Bhajhang (BF01) and Darchula (DaF56). Next subclade was formed by O. siensis sample taken from Dolpa (DF 8, DF 10,DF 12, DF 30). Finally, another subclade was formed by O. sinensis sample taken from Manang (MF6 and DaF 117, DaF 118, DaF 137). At last single and separate clade is formed by *O. sinensis*sample of Manang (MF4).

In case of *COI* sequences, all the accessions of *Hepialus* appeared as a single clade, Hepialus species of manang (MI 11 and MI 4) made single clade. Likewise single clade was made by MI and MI1. Hepialus accession taken from genbank made single subclase (gb|KF696592| and gb|KC994972| (Fig 4.12 and Fig 4.13). COI sequence accession of Bhajhang and Darchula made separate subclade with accession from genbank except single accession of Darchula (DaI 118) showed close relation with genbank accession gb|KC994967|(Fig. 4.15 and 4.16).

Based on ITSandD1/D2 sequences, although all accessions under study formed a single monophyletic clade, all accessions formed a subclade inside the large clade indicating all population being diverged from two other populations. As this locus is variable, it should be further explored for its potential use as a diagnostic marker for *O.sinensis*. In case of ITS sequence, it was observed that all the accessions and the reference sequence of *O. sinensis* from nucleotide database a single monophyletic clade while *O. nutans* was shown as sister species of *O.sinensis.* Thus, ITS holds a great promise as a potential barcode for identification of *O.sinensis.* In case of *D1/D2* the accessions from different locations were clustered as single monophyletic clade. Thus, *D1/D2* also holds a promise for identification of *O. sinensis*. This work DNA Barcoding and Molecular Phylogenetic study of *Ophiocordycepssinensis*(Berk)of Nepal is the first work in Nepal and thus this research has opened a new avenue for further research of *O. sinensis*in Nepal.

Summary

The purpose of this study was to determine the phylogenetic and evolutionary relationship of *O. sinensis*of Nepal. In this study, total 34 *O. sinensis* samples were taken from Dolpa, Manang, Bhajhang and Darchula representing three developmental regions.Isolated genomic DNA was amplified using universal primer pairs and sequenced bi-directionally for nuclear ribosomal ITS region, D1/D2 domain of LSU rDNA for fungi and COI region for insect identification. These samples were analyzed and characterized by PCR amplification and sequencing of ITS region, D1/D2 domain and COI region for fungal and insect identificationas well as to study their phylogeny.Sequences were generated having sequence length of 606 bp in average, ranging from 549-652 bp.Some of the sample gave no good sequence, so was discarded. The consensus sequences were assembled and edited using codon code aligner ver 4.2.2 and were compared with the genbank database. The sequence showed >99% identicalto *O. sinensis* and >98% identical to *Hepialus* species. Thus, the sequence information helped to identify the samples upto species level. The sequence of ITS, D1/D2 domain and COI were then aligned and analysed by using MEGA 6.The inter and intra species nucleotide differences were found to be range from 0.00- 0.05. This result suggested the sufficient variability of ITS and D1/D2 domain for the identification and characterization fungal species. Besides, the homogeneity of substitution patterns was tested between sequences to check the probability of accepting or rejecting the null hypothesis by estimating the p-value <0.05.

The clustal W alignment using ITS, D1/D2 sequences were done with 26 and 20 samples with the total sequence length of 533bp and 586bp. The number of conserved and variable sites were found to be 312 and194 for ITS and 379 and204 for D1/D2 respectively. The number of singleton sites was 166 and parsimonious informative sites were 20. The number of singleton sites was 74 and parsimonious informative sites were 129. The maximum parsimony informative (MP) tree was constructed with 1000 bootstrap test. By the phylogenetic analysis of D1/D2 domain of LSU rDNA*O. sinensis*and *O. nepalensis* showed close relationship forming sister groups with the common lineage. Interestingly single sample of Manang (MF) showed close relation with *O. nutans* accession taken from genbank. They also showed geography specific except some samples.

The clustal W alignment using COI sequences were done with 10 and 14 samples with the total sequence length of 604bp and 609bp. The number of conserved and variable sites were found to be 514 and 86 for COI sequences of *O. sinensis* of Manang and 512 and 82 for COI sequences of *O. sinensis* of Bajhang and Darchula. The number of singleton sites was 61and parsimonious informative sites were 24 for COI sequences of *O. sinensis* of Manang. The number of singleton sites was 56and parsimonious informative sites were 24 COI sequences of *O. sinensis* of Bajhang and Darchula.The maximum parsimony informative (MP) tree was constructed with 1000 bootstrap test. By the phylogenetic analysis of ITS, D1/D2 and COI sequence shows that

*Hepialus*spp is the anamorph of*O. sinensis, O. sinensis*and *O. nepalensis*have low genetic diversity than *O. nutans*and also confirm that ITS, D1/D2 and COI loci are reliable barcode loci for molecular phylogenetic analysis of *O. sinensis*.

Overall result suggest that ITS and D1/D2 barcode loci were able to identify *O. sinensis* at molecular level and also able for molecular phylogenetic analysis of this species likewise COI barcode locus was able to identify Hepilopteran family at species level and also able for molecular phylogenetic analysis of this species. In summary,this result shows that DNA barcoding technique can be very effective technique in the context of *O. sinensis* identification.

Conclusion

Molecular characterization based on DNA sequencing and bioinformatics generate overwhelming information on phylogeny, phylogeograpgy, conservation genetics etc.which in-turncan be utilized in various human endeavours pertinent to biodiversity conservation and utilization. Yarsahagumba consist of different chemical constituent which are very important for human health and well-being. Due to its medicinal importance it has been considered as highly valued medicine,as a result it is exportedin very high price to many countries throughout the globe. Due to its high demand in national and international market, different counterfeit products are also being traded which looks like *O. sinensis* (yarsahagumba). DNA barcoding can play critical role in fighting against wildlife crime including illegal trade of high value medicinal species such as *O.sinensis*.

DNA barcoding technique has evolved as one of the most important molecular technique for biodiversityassessment, bio monitoringand documentation in the changing climate scenario. *Ophiocordyceps* samples from different localities of Nepal have been analyzed and identified at species level by PCR amplification and DNA sequencing of standard genetic loci designated for the authentication of fungi and insect species. Cytochrome 'C' Oxidase, Internal Transcribed Spacer and D1/D2 Domain of large subunit loci were PCR amplified with their respective universal primer pairs. BLAST result of these samples shows 97-100% similarity with *Ophiocordycepssinensis*, evolutionary relationship and phylogenetic tree were also constructed with the help of MEGA 6 and jModelTest 2.1.4.Phylogenetic study of ITS barcode locus of *O. sinensis* showed that *O. sinensis* samples are more closer to *O. nepalensis* rather than *O. nutans*. Interestingly single sample of Mannag (MF) showed close relationship to *O. nutans*. D1/D2 barcode locus showed that single monophyletic clade are formed proving no genetic diversity at species level.

Overall result suggest that ITS and D1/D2 barcode loci were able to identify *O. sinensis* at molecular level and also able for molecular phylogenetic analysis of this species likewise COI barcode locus was able to identify Hepilopteran family at species level and also able for molecular phylogenetic analysis of this species.

This work DNA Barcoding and Molecular Phylogenetic study of *Ophiocordycepssinensis*(Berk)of Nepal is the first work in Nepal and thus this research has opened a new avenue for further research of *O. sinensis*in Nepal.

LIMITATIONS AND RECOMMENDATION

LIMITATIONS

1. Due to the limitation of funding in this project sequencing of COI, ITS, D1/D2 region of some samples couldnot be carried out and β-tubulin sequencing of all samples could not also be carried out.
2. Due to resource and time limitations, samples from all over Nepal could not be considered in this study.

RECOMMENDATIONS

1. Due to its increasing demands in national and international markets, research on artificial cultivation of yarsagumba needs to be prioritized.
2. Over exploitation and illegal trade of yarshagumba from its natural habitats needs to be strongly discouraged and molecular diagnosis of *O. sinensis* should be used check its illegal trade.
3. Due emphasis should be given in sustainable harvesting of *O. sinensis*.
4. Further research aiming at molecular genetics and phylogeny the DNA-barcoding has to be carried out on *O. sineneis* samples collected from all over the country in order to DNA Barcode purpose can authenticate reference history of all species of Ophiocordyceps.
5. Climate change can be a great threat to this valuable species so, timely actions needs to be taken for its long-term survival in its natural habitats.
6. Based on chemical characterization of Yarshagumba synthetic biological and chemical techniques need to be employed for the mass production of active chemical constituents of *O. sinensis*for future human consumption as therapeutic agent.

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