African Journal of Biotechnology Vol. 10 (2), pp. 159-167, 10 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.865
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Molecular identification and food source inference of constructive plants, native to the *Ophiocordyceps sinensis* habitat

Wei Lei, Hai Chen, Gu-ren Zhang, Shao-song Li, Qing-yun Peng, Xin Zhong and Xin Liu*

Food and Health Engineering Research Center of State Education Ministry, School of Life Sciences, Sun Yat-sen University, Guangzhou, China.

Accepted 5 November, 2010

Ophiocordyceps sinensis **is a precious and effectual Traditional Chinese Medicine, native to Tibetan Plateau. Its development originated from parasitization of *Hirsutella sinensis* to *Hepialus* larvae, which was deemed to be of great importance including feeding food habit. Hereby in the present, study on food resource plants and their molecular identification technique were performed by some methods such as vegetation investigation, morphological taxonomy, sequence alignment, phylogenetic inference, primer design and correlation analysis and so on. In consequence, molecular identification system of constructive plants native to the *O. sinensis* habitat was established, and then feeding food habit of *Hepialus pui* larvae and its correlation with genesis mechanism of *O. sinensis* were preliminarily analyzed and discussed. It is very significant for phylogenetic systematicbotany research of plateau plants on a relative large scale, and it is the first time to study feeding food habit of *Hepialus* larvae and its related infected process on the molecular level.**

Key words: Phylogenetic inference, molecular identification, constructive plant, *Ophiocordyceps sinensis*, *Hepialus pui.*

INTRODUCTION

Ophiocordyceps sinensis (Berkeley) Saccardo, subordinated to ascomycete phylum, is distributed on some alpine regions in the Tibetan Plateau. Modern pharmacological studies have proved that *O. sinensis* takes possess of various active constituents such as mannitol, nucleosides, ergosterol, aminophenol, trace elements etc., which have a broad therapeutic function (Zhou et al., 2009; Ko et al., 2010); for example, cordycepin and adenosine were considered to play an important role in immunity regulation, kidney meridian and organ transplants and so on (Russell and Paterson, 2008). Therefore, date back to 15th century, *O. sinensis* is employed as a well-known and high-valuable medicinal mushroom in Oriental countries qua Traditional Chinese Medicine and Traditional Tibetan Medicine (Halpern and Miller, 2002; Winkler 2008).

*Corresponding author. E-mail: lsslx@mail.sysu.edu.cn. Tel: +86-20-84112299. Fax: +86-20-84112299.

O. sinensis is an entomogenous fungi, obligately parasitizing some larval insects of *Hepialus* genus on partial regions of Tibetan Plateau. When the anamorph of *O. sinensis*, called as *Hirsutella sinensis* Liu et al., attacks a host, the conidia or mycelia invades and eventually replaces the host tissue, resulting in exhaustive contribution of host insect to mushroom growth. After germination, elongation and fattening of hyphae, the fruiting body (stroma) develops gradually and matures consequently (Li and Yang, 2009). The entire fungus-larva combination is collected for medical use at last. To say at least, the larvae of *Hepialus* species parasitized by *H. sinensis* are the material foundation and energy resource of *O. sinensis* whose scientific development and sustainable utilization were demonstrated to depend on the study on *Hepialus* insects. Recently, more and more attention was paid on cultivation and breeding of host biotype *Hepialus* insects, and thus it is significant to investigate feeding habit of the larvae especially in original ecological environment. Traditionally, the main

Figure 1. Topography and geomorphology of sample areas. Site A marked "Tibetan Plateau characteristic resources science workstation of Sun Yat-sen University", sample area No.1, No.2 and No.3 were pointed out, respectively. Green star marked the study area.

methods were field investigation, artificial feeding and chemical tracer, prone to deviation caused by human disturbance (Xu, 2004; Zhu and He, 2007; Chen et al., 2009). However, as a direct and natural approach, plant detection of food residue in wild type larvae foregut seems still nonfeasible to adopt when lacking of species identification technique of constructive plants.

Hepialus pui, newly designated by our laboratory (Zhang et al., 2007), live mainly on Nyingchi in Mt. Segyi La of the Tibetan Plateau (Zhang et al., 2007). As excellent hosts of *H. sinensis*, *H. pui* are being studied deeply to protect and produce this rare medical epiphyte. However, due to little information about predominant plants native to the *O. sinensis* habitat, it is not available to identify the vegetative food resources, and then it is necessary to investigate vegetation condition and establish identification system of constructive plants, which helps to study on feeding habit by design and application of species specific primers. In the present, molecular identification technique based on phylogenetic inference was constructed, and further applied on feeding habit of *H. pui* and genesis mechanism of *O. sinensis*.

MATERIAL AND METHODS

Vegetation investigation and plants morphological identification

Nyingchi in Mt. Segyi La was considered as the excellent *O. sinensis* habitat and the *H. pui* cradleland, where the "Tibetan Plateau characteristic resources science workstation of Sun Yat-sen University" (4156 m Alt., 29°36′ N, 94°35′ E) have been founded for *O. sinensis* original ecological and biological studies. Three sample areas for this experiment were located near the workstation as follows: Number 1(4181 m Alt., 29°36′ N, 94°36′ E), Number 2(4205 m Alt., 29°36′ N, 94°36′ E) and Number 3(4144 m Alt., 29°35′ N, 94°35′ E) (Figure 1). The vegetation composition was investigated by chessboard sampling pattern, and the plants species were identified through the typical morphological method. Then the coverage of every plant species in the entire experimental areas was eyeballing evaluated and statistics analyzed (Table 1).

Molecular systematic identification and species specific primer design

The tender roots tissue and the vegetable food residues were collected from each plants species and foregut of

Table 1. Vegetation Composition and Molecular Identification System

Plant Name	Family	GenBank Accession No.	Average coverage±SD	Species specific primer	Tm(℃)
Deschampsia caespitosa	Poaceae	GU444008	21.697±14.777	5′-TCCTCGACAACCTCCTCTTC-3′ 5′-AACACCGGCAGCCACATC-3′	57
Phleum alpinum	Poaceae	GU444005	0.957±0.842	5′-AGTCCTCGATAACCTCGTCT-3′ 5′-TTATAGGGTCCTTCAAGGC-3′	54
Polygonum viviparum	Polygonaceae	GU444007	25.413±15.452	5′-CACTCGGTCACCCGGTGT-3′ 5′-CGAGGGTCCTCTTGACTCC-3′	54
Polygonum filicaule	Polygonaceae	GU444019	1.035±0.716	5′-CGCTTTCCCTCAAATCAAC-3′ 5′-GAGGACCACGGAAGACGC-3′	56
Ranunculus brotherusii	Ranunculaceae	GU444012	0.639±0.852	5′-CCGATCCAGTCCGCTTGT-3′ 5′-CGTCATTTTGTCTTTGGAGG-3′	56
Caltha scaposa	Ranunculaceae	GU444013	0.449±0.556	5′-CGTGAAACAAACTATGGTC-3′ 5′-GTCTATTTCTGTGAGGATGAG-3′	57
Pachypleurum xizangense	Umbelliferae	GU444009	11.112±4.703	5′-GAATCCCTGGTAGGTGGC-3′ 5′-GTCGAAGCGCACAGAGTG-3′	54
Pleurospermum hookeri	Umbelliferae	GU444010	1.534±1.353	5′-AAAACACTGGGCAAGCGAC-3′ 5′-GACGAAGGTGACGGGCAT-3′	57
Pedicularis sherriffii	Scrophulariaceae	GU444016	0.705±0.730	5′-CACGTTAAACCATATTGGGAC-3′ 5′-CCGACGGACCACTAAAAAC-3′	57
Veronica ciliata	Scrophulariaceae	GU444014	0.676±0.450	5′-AGGAAAACTTAAAAGAAGCG-3′ 5′-ACAGCCGATGTAATGACG-3′	53
Potentilla fruticosa	Rosaceae	GU444027	8.000	5′-AAGGAACTTGAATGAAAGAGC-3′ 5′-GGGTCAGGAGATCCAGCA-3′	56
Spiraea myrtilloides	Rosaceae	GU444028	52.500	5′-CGGGCGTACAAACGAAAAC-3′ 5′-CGACTTGCGACAGAGGTCTC-3′	57
Cyananthus macrocalyx	Campanulaceae	GU444026	0.801±0.807	5′-AGGGCGGACTGTTCTTAG-3′ 5′-AAAGTATAAGGCGCATCAGG-3′	54
Cyananthus lobatus	Campanulaceae	GU444030	5.676±8.897	5′-AAGAACACCGGGAAAGTG-3′ 5′-GACTCCGTTTTGAGCCAG-3′	57
Taraxacum tibetanum	Asteraceae	GU444020	0.587±0.547	5′-ATCCTCAACACCTCCCAG-3′ 5′-CCTATTTTGACCAACCACAC-3′	54
Anaphalis nepalensis	Asteraceae	GU444021	0.678±0.357	5′-GTTTGATCCTTAACTGCC-3′ 5′-CGAAGGTTTTATCAATCAC-3′	57
Ligularia pubifolia	Asteraceae	GU444022	37.000±4.472	5′-CTTGGTATCGGGCACTTGT-3′ 5′-CAACCGCACCATAGGAAC-3′	54
Aster tongolensis	Asteraceae	GU444006	13.945±10.029	5′-ATGGGTTGAGCGTTAGTT-3′ 5′-TGGGTCTATTAAGTTGCAC-3′	54
Epilobium sikkimense	Onagraceae	GU444011	1.822±1.407	5′-AATCCTGCATAGCAGAACA-3′ 5′-GGTTGGGTGACGAATAGA-3′	53
Cerastium fontanum	Caryophyllaceae	GU444015	0.661±0.856	5′-GAGTTGTAGGTAGCCTTGTG-3′ 5′-GCTCTACATTCAAAGGGTT-3′	57
Juncus leucanthus	Juncaceae	GU444017	0.479±0.492	5′-ACTCTTTAAGGGCTGCGTC-3′ 5′-GGTCTTTGTCCGAGGTTG-3′	57
Primula alpicola	Primulaceae	GU444018	2.600±5.814	5′-TTCATCCTCGCTGGGTAT-3′ 5′-AAATGGTGCTAAGGGTCA-3′	55
Salix gilashanica	Salicaceae	GU444023	10.000	5′-CGTGGAGGGACGCATCTG-3′ 5′-ACCGTGTCCGAGGGTCTCT-3′	56
Parnassia mysorensis	Saxifragaceae	GU444024	1.689±2.533	5′-GATAAATCACGGTTGAAGAG-3′ 5′-ACAGCACGCTACAAAGTC-3′	53
Kingdon-Wardia racemosa	Gentianaceae	GU444025	1.261±1.321	5′-GAAAACAAGAAAGGGATGG-3′ 5′-GGGTCAAGGAGTCTCCTAAC-3′	55

Table 1. Cont..

Rhododendron vellereum	Ericaceae	GU444029	2.000	5′-GCTTTCCCCTGGCGAGTAG-3′ 5′-CCGACCGAGCACGAATGT-3′	56

SD, Standard deviation; No., number.

some *H. pui* larvae, respectively. These samples were cleaned for total genomic DNA extraction, and subsequently the quality of DNA was determined by agarose gel electrophoresis according to the manufacturer's instructions (Axygen, USA). Nuclear ribosomal DNA internal transcribed spacer (ITS) partial sequences were amplified with eucaryotic universal primers ITS1 (5′-TAGGTG AACCTGCGGA AGGATCA-3′) and ITS2 (5′-TTCCCTGTTCACTCGCCGTTACT -3′) (Zhao et al., 1999). Polymerase chain reaction (PCR) amplification was implemented in a 20.0 µl mixture containing 13.4 µl ddH_2O, 2.0 µl 10 × PCR Buffer (Mg^{2+} Plus), 5.0 nmol dNTP Mixture, 12.5 pmol of each primer, 4.0 ng DNA template and 0.5 units of r*Taq* DNA polymerase (TaKaRa, Dalian, China). The PCR program was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturing for 1 min at 94°C, annealing for 1 min at 58°C and extending for 1 min at 72°C, and with final extension for 7 min at 72°C. After gel purification with DNA gel extraction kit (Axygen, USA), the PCR product was ligated into pMD19-T vector (TaKaRa, Dalian, China) and introduced into competent *Escherichia coli* strain DH5α cells. Finally, Recombinant plasmids recovered from the positive colonies were sequenced for BLASTn verification.

On the basis of the corroborant plant ITS sequences, a series of species specific primers were designed by Primer Premier 5 software, and then used for PCR amplification with DNA template from the different plants in order to verify the corresponding one.

Sequence alignment and phylogenetic analysis

A matrix of ITS sequences were selected from BLASTn search of specimen in GenBank, and these congeneric or coordinal members were aligned using the AlignX module of the Vector NIT software package with manual adjustment as necessary.

After *de novo* aligning among each relative species cluster, a phylogenetic tree was constructed by Neighbor-Joining method with 1000 replicates and reliability of each node was established based on bootstrap calculation through MEGA4.1 software. The bootstrap values were showed on branches.

Feeding habit and infected process of *H. pui* larvae

A total of 100 *H.pui* larvae aged three (3) and above instars were collected from experimental areas. The larvae surface was sterilized with 75% ethanol and then insect bodies were anatomized. The food residue in larval foregut were microscopic observation, and subsequently collected, cleaned and precipitated, and prepared for extraction of the genomic DNA of food residue using Multisource Genomic DNA Miniprep Kit (Axygen, USA).

On the template of total DNA, PCR amplification was carried out with the plant specific primers to identify the botanical species, and estimated the feeding habit according as the population coverage. Another nest PCR reaction was amplified to detect *H. sinensis* with the help of its specific primers (ITS3: 5′-TCCTCCGCTTATT GATATGC-3′ and ITS4: 5′-GGAAGTAAAAGTCGTAACAAGG-3′; ITS5: 5′-TGT CGCAGTGGCATCTCTCAGT-3′ and ITS6: 5′-TGGTTTCACGGCGTGACCGCCT -3′) (White et al., 1990; Chen et al., 2004), elucidating candidate infected process involved in the feeding habit of *H. pui* Larvae.

RESULTS AND DISCUSSION

Vegetation investigation and species identification

There were 26 species of plants to be recorded in the three sample area, including 22 species herbaceous plants and 4 species shrubby ones. By the method of typical morphological taxonomy, these plants were identified and classified as follows: 25 genera in 17 families (Table S1). The coverage of each species was also monitored, calculated and compared. The results manifested that the proportion of herbage and shrub was 3:1 and 2:1 in respective sample areas Number 1 and 2, which both were categorized as mixing region of alpine shrubbery and meadow, and no shrub distributed in Number 3 area defined as the alpine meadows region.

Molecular identification system and phylogenetic relationship inference

ITS sequences from each plant species were submitted for BLAST to verify homology and credibility, and thereby species ITS was confirmed for specificity responding to morphological taxonomy. As a result, molecular identification system was established based on species specific ITS sequences, which are reported for the first time except some partial sequences from *R. brotherusii*, *C. scaposa*, *A. nepalensis*, *P. fruticosa* and *C. lobatus*. These newly-acquired ITS partial sequences have been submitted into GenBank (Accession Numbers listed in Table 1).

Molecular polygram analysis of constructive plants

As shown in Figure 2, nine phylogenetic trees were constructively combined with sequence identity and morphological character, including Scrophulariaceae (Group 1), Polygonaceae (Group 2), Umbelliferae (Group 3), Rosaceae (Group 4), Poaceae (Group 5), Campanulaceae (Group 6), Ranunculaceae (Group 7), Asteraceae (Group 8), and other individual family (Group 9). After aligning, it is believed that they were highly identical within each clade designating one genus. The alignment result illustrated there was so high homology between specimen and congener plants on the molecular level, covering with the analysis result of morphological taxonomy. In addition, with inference to phylogenetic relationship, close Sibship existed in one genus plants

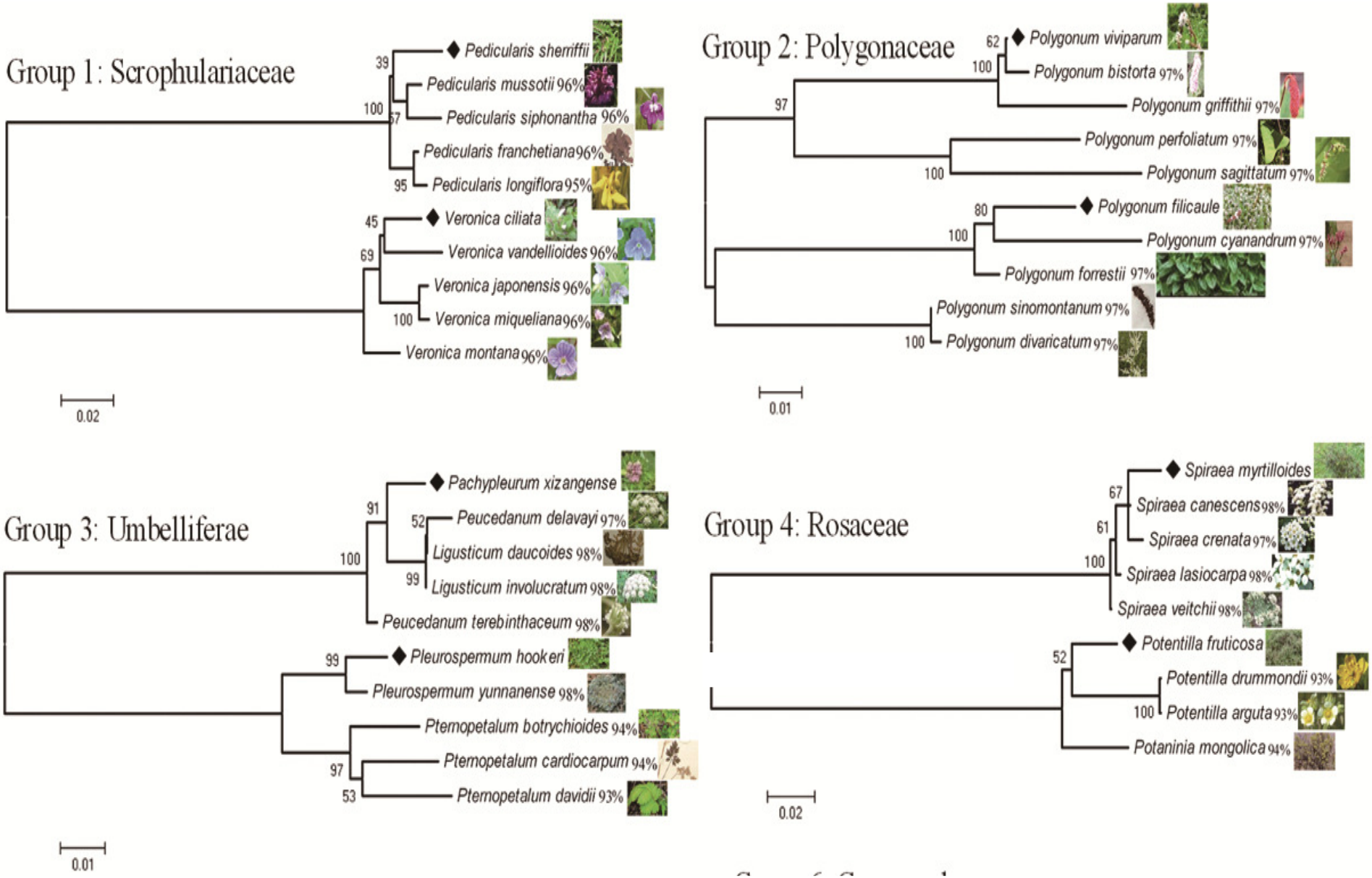

Figure 2. Molecular polygram analysis of specimen plants and their relative species. Phylogenetic trees were constructed by Neighbor-Joining method (based on 1000 replicates) using MEGA4.1 software, and the bootstrap values were shown on branches. specimen plant species were marked as ♦, and the relative species were not marked.

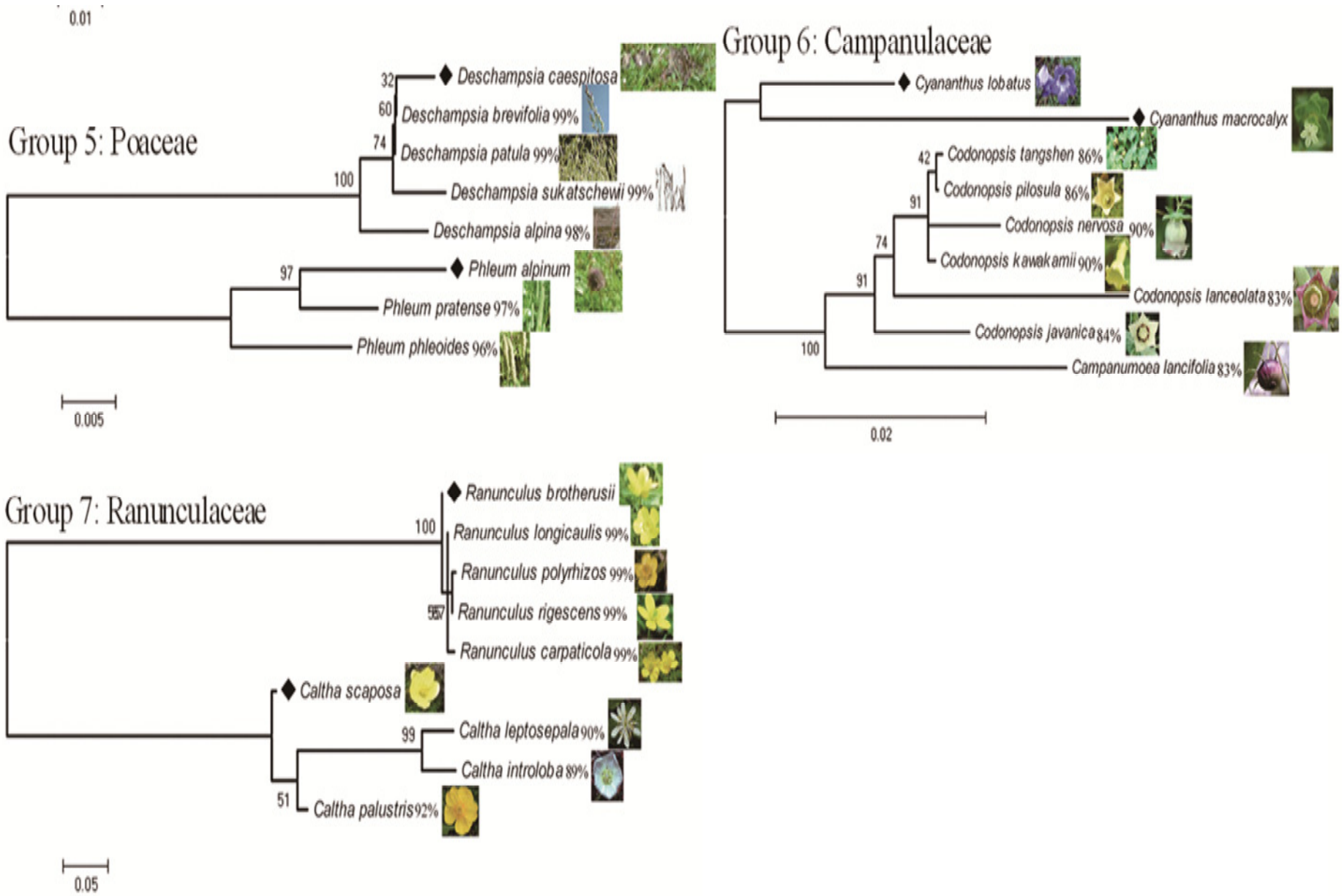

Figure 2. Continued.

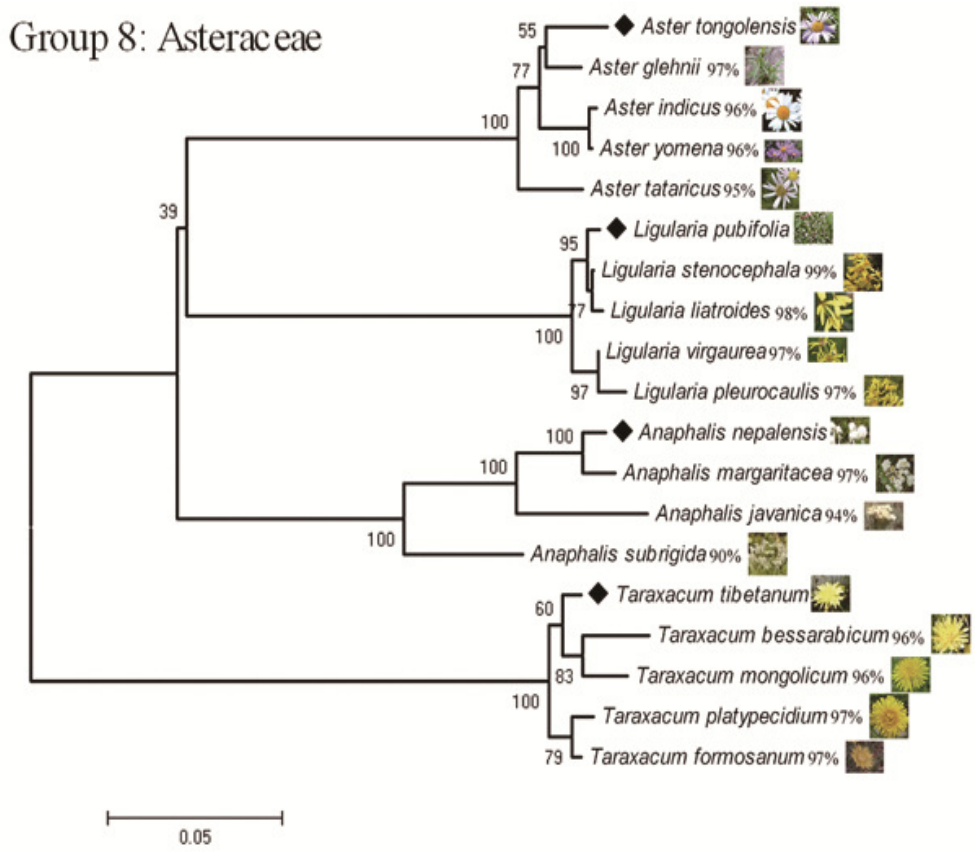

Figure 2. Continued.

but not discovered between different genera. And thus identification and classification of the target plants were further corroborated on the view of genetics and evolution.

In result, the system analysis consisting of morphological taxonomy, homological alignment and phylogenetic inference, provided the evidence that ITS sequences from plants specimen possess of high specificity to distinguish between interspecific plant, so they can be used to identify plant or plant tissue. Furthermore, oligonucleotide primers pairs were designed on the basis of ITS sequences, respectively, which were verified by PCR amplification with the corresponding plant DNA template, and finally these primer pairs were certain to identify plant species as the effective tools. So far, the molecular identification system was established for the feeding habit of *H. pui* Larvae.

Feeding habit and infected process of *H. pui* larvae

Food residue samples from *H.pui* larvae foregut were microscopically observed and imaged. Plant cells were observed clearly and widely, and some root hairs were even observed to exist on the edge of the debris region (Figure 3). Hence, it appears that plants tender roots were the main food of *H. pui* larvae, answering to the previous report (Sheng et al., 1983; Zhu et al., 2004). ITS sequences of the various plants were amplified with their own specific primers and the total DNA from the food residue, followed by sequenced, aligned and analyzed, and then plant species of the food residue were detected and recognized. The feeding habit of *H. pui* larva in original ecological environment was studied combining with vegetation composition and feeding experiments in the insectary. The results showed that *H. pui* larva was a polyphagous phytophagous insect mainly feeding on tender roots of almost all alpine plants belonging to 16 families and 24 genera. The larva's favourite foods were regarded as the roots of *R. brotherusii*, *C. macrocalyx*, *J. leucanthus* and *V. ciliate*.

Besides, after surveying *H. sinensis* in the food residue of *H. pui* foregut, four cases were positive in larvae test from Number 1 and seven cases from Number 2 area, but no one from Number 3. The highest fungal detection rate presented to some larvae taking more food of *R. brotherusii*, *C. macrocalyx*, *J. leucanthus* and *V. ciliate*, which was consistent with food preference. Hence, these plants may lead to higher invading rate, and feeding food habit of *H. pui* played important role on infection process of *H. sinensis*. It is obviously possible for *H. pui* larvae to ingest *H. sinensis* thalli in the process of taking food, and

Figure 2. Continued.

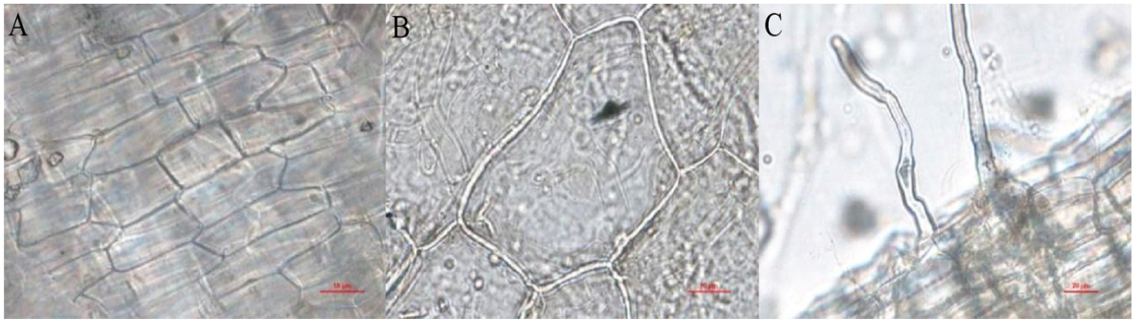

Figure 3. The micrograph of *H. pui* larvae's intestinal debris. A: Cytoarchitecture of debris (Scale bar = 20 µm); B: Cytoarchitecture of debris (Scale bar = 10 µm); C: root hair in debris (Scale bar = 20 µm).

this intestinal flora may infect insect body, leading to growth and development of *O. sinensis*.

In conclusion, three sample areas of different vegetation types were chosen in the *O. sinensis* habitat at the altitude of about 4156 m in Mt. Segyi La of the Tibetan Plateau. The vegetation compositions of these sample areas were investigated and the ITS were cloned and sequenced from 26 species of constructive plants. By the bioinformatic analysis and phylogenetic inference as well as species specific primer pairs design, a molecular identification system was established based on plant distinguishability and classification. At last, this system was applied on research of feeding habit of *H. pui* larvae, which positively correlated with the infection rate of *H. sinensis*, expecting further demonstration about the possible pathway of this parasitic process and genesis mechanism of *O. sinensis*. It is evident that the ITS-derived molecular technique presented herein was a very useful and important tool to species diagnosis based on traditional morphological criteria. Therefore, this study provided the potent strategy to plant diversity protection of plateau vegetation and sustainable utilization of *O. sinensis* resource.

ACKNOWLEDGMENT

We are grateful to the reviewers for valuable suggestions and comments on the manuscript. This research was supported by the National Key Technology R&D Program in the 11th Five-year Plan of China (2007BAI32B05 & 2007BAI32B06), the Research Project of the State Council Leading Group Office of Poverty Alleviation and Development (2009-3A) and the National Key Technology R&D Program in the 12th Five-year Plan of China (2011BAI13B06).

Group 5: Poaceae
Group 1: Ranunculaceae
Group 1: Asteraceae

REFERENCES

Chen D, Yuan J, Xu S, Zhou X, Zhang Y, Xu X, Zou Z, Zhang G, Wang J (2009). Stable carbon isotope evidence for tracing the diet of the host *Hepialus* larva of *Ophiocordyceps sinensis* in the Tibetan Plateau. Sci. in China Series D-Earth Sci. 52(5): 655-659.

Chen YQ, Wang N, Qu LH, Li TH (2004). Characteristic Nucleotide Acids Sequence and Method of *Ophiocordyceps Sinensis* Identification. Patent Number: ZL00114160.0

Halpern MG, Miller AH (2002). Medicinal Mushrooms. M. Evans and Company Inc., New York, pp. 64-65.

Ko WS, Hsu SL, Chyau CC, Chen KC, Peng RY (2010) Compound *Cordyceps* TCM-700C exhibits potent hepatoprotective capability in animal model. Fitoterapia, 81: 1-7.

Li TX, Yang JS (2009) A review and prospect on the studies of *Cordyceps Sinensis* (Berk.) Sacc. Chinese J. Med. Guide, 11(8): 1310-1316.

Russell R, Paterson M (2008). *Cordyceps*- A traditional Chinese Medicine and another fungal therapeutic biofactory? Phytochemistry, 69: 1469-1495.

Sheng NY, Zeng L, Zhang XC, Zhou ZR, San Z, Wei SL (1983). Study on feeding food habit of *Hepialus armoricanus* Oberthur. Special Wild Econ. Anim. Plant Res. 3: 19-20.

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA genes for Phylogenetics. PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315-322.

Winkler D (2008) *Yartsa Gunbu* (*Ophiocordyceps sinensis*) and the Fungal Commodification of Tibet's Rural Economy. Econ. Bot. 63(2): 291-305.

Xu HF (2004) Study on biological characteristic of *Hepialus lagii* in Qinghai. J. Sichuan Grassland, 9: 4-5.

Zhang GR, Gu DX, Liu X (2007) A New Species of *Hepialus* (Lepidoptera, Hepialidae) from China. Acta Zootaxonomica Sinica 32(2): 473-476.

Zhao J, Wang N, Chen YQ, Li TH, Qu LH (1999) Molecular identification for the asexual stage of *Ophiocordyceps sinensis*. Acta Scientiarum Naturalium Universitatis Sunyatseni, 38(1): 121-123.

Zhu DX, He RH (2007). The relationship and countermeasure of continuable utilize aweto resources in Tibet. Edible Fungi of China, 26(6): 18-20.

Zhu HF, Wang LY, Han HX (2004). Insecta Lepidoptera Hepialidate In: Fauna Sinica. Science Press, Beijing, 38: 3-194.

Zhou XW, Gong ZH, Su Y, Lin J, Tang KX (2009). Cordyceps fungi: natural products, pharmacological functions and developmental products. J. Pharm. Pharmacol. 61: 279-291.