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Mycorrhizal and dark septate endophytic fungi of *Pedicularis* species from northwest of Yunnan Province, China

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Abstract Colonization of mycorrhizal and root endophytic fungi in 14 *Pedicularis* species from northwest of Yunnan Province, southwest China, was examined. These species included: *Pedicularis gracilis* Wall., *Pedicularis longipes* Maxim., *Pedicularis axillaris* Franch., *Pedicularis cephalantha* Franch., *Pedicularis tenuisecta* Franch., *Pedicularis tapaoensis* Tsoong, *Pedicularis likiangensis* Franch., *Pedicularis dichotoma* Bonati, *Pedicularis yui* Li, *Pedicularis rhinanthoides* Schrenk, *Pedicularis rex* C.B. Clarke, *Pedicularis longiflora* Rudolph., *Pedicularis siphonantha* Don, and *Pedicularis oxycarpa* Franch., among which nine are endemic to China (one to Yunnan). Three types of potentially beneficial fungi associated with roots of these species were observed, namely, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and dark septate endophytic fungi (DSEF), with DSEF as the most common colonizers. An unexpected high colonization level was detected in this hemiparasitic genus. Of the 19 sampling sites examined, 10 gave colonization frequency of above 50% and 6 showed a colonization index of above 50. Heavy colonization suggested a significant ecological role of these fungi and their potential to be applied to successful cultivation of these intractable plants.

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Introduction

With about 600 species worldwide, *Pedicularis* L. (Orobanchaceae) was known as one of the largest genera of angiosperms in the northern hemisphere, mainly distributed in frigid, high-latitude alpine belts in the North Temperate Zone (Yang et al. 1998). Plants belonging to this genus often bear attractive and long-standing showy flowers (with an average florescence of 2 to 3 months). Meanwhile, these species show dramatic diversity in interspecific morphological variation, particularly in their galea (the upper part of a corolla), which can be variously elongated, curved, toothed, beaked, and crested (Ree 2005), giving them a great potential in developing into popular ornamental plants. Also, many members of this genus have special pharmacologic effects (Wang and Jia 1995; Zhu 1997; Wu et al. 2002) and thus make *Pedicularis* L. a precious treasure of Chinese traditional herbal drugs. Unfortunately, widespread in natural habitats as they are, these species have a reputation for being uncultivable, which hinders their exploitation to a large extent. The majority of botanists tend to ascribe the difficulties encountered in cultivation to their hemiparasitism and assume that cultivation of *Pedicularis* species may be possible only when their proper host plants are discovered (Terry 2000; Petrů 2005). Experiments on *Pedicularis lanceolata* showed, however, that a poor survival rate (35%) was achieved even in the presence of their host plants (Lackney 1981). We believe that there may be other more important limiting factors, though these still remain mysterious.

Mycorrhizal fungi and other root endophytes such as some dark septate endophytic fungi (DSEF), which often give plant growth promoting effects, are playing an ever growing important part in producing healthy seedlings in nurseries and becoming increasingly significant in fostering forests in adverse environments and introducing wild trees (Zhang 1998; Barrow 2003; Fuchs and Haselwandter 2004). As mentioned above, *Pedicularis* L. is often found in mountain areas with poor soil, unfavorable weather, and a short growing season. To survive in such potentially stressful conditions, plants must have developed some adaptation mechanisms, among which forming mutualistic symbioses may be an important one (Mullen et al. 1998). Thus, mycorrhizal fungi or some other beneficial microorganisms are probably essential for *Pedicularis* L. to survive.

As far as we know, little work has been done concerning the relationships between *Pedicularis* and soil microorganisms, not to mention more specific investigations with individual soil microbes. Wang et al. (1996) reported root nodules in *Pedicularis dolichorrhiza* for the first time. Once considered as a nonmycorrhizal species (Olsson et al. 2004), *Pedicularis* L. was pointed out to be colonized by both arbuscular mycorrhizal fungi (AMF) (Chen and Guan 2001) and ectomycorrhizal fungi (EMF) (Kohn and Stasovski 1990). Findings of Treu et al. (1996) showed that some species developed mutualistic associations with DSEF. Almost all the previous reports are rather casual businesses, usually as a tiny part of a mycorrhizal survey in a particular ecosystem. To the best of our knowledge, no systematic studies were conducted regarding mycorrhizal or root endophytic fungal associations with *Pedicularis* and the colonization status of the genus is largely unknown.

When it comes to the Chinese species, mycorrhizal investigations of *Pedicularis* are even fewer. Only four species were studied so far (Chen and Guan 2001). With 105 species endemic to China and 52 species to Yunnan (Li et al. 2002), *Pedicularis* L. is in great abundance in Yunnan province, particularly in the northwest area known as an important part of the Hengduan Mountain range, a place that is believed to be one of the evolution centers of this genus (Ree 2005; Cai et al. 2004). Accordingly, the present study evaluates the mycorrhizal status of 14 *Pedicularis* species (belonging to 19 populations) from the northwest of Yunnan. We aimed to answer the following questions: (1) Are there any potentially beneficial fungi associated with these species? (2) What types of fungi are detected? (3) Are these fungi in abundance or only detected occasionally? By answering the above questions, we may predict the application potential of beneficial root-associated fungi to the cultivation of the genus *Pedicularis* and thus contribute to the introduction of these intractable plants.

Materials and methods

Root sampling and site description

Fourteen common species representing all the four major evolutionary corolla types described in Li's system (Li 1951) were collected from the northwest of Yunnan province, mainly from Dali (99°58′–100°27′ E and 25°25′–25°58′ N), Lijiang (99°23′–101°31′ E and 25°59′–27°56′ N), and Zhongdian (also known as Shangrila, 90°35′–100°19′ E and 26°52′–29°16′ N) in July 2005. Annual average temperatures for the three areas are 15.1, 12.6–19.8, and 5.4°C, and annual average rainfalls are about 1,078.9, 1,000, and 634 mm, respectively. For the widely distributed species, samples were collected from more than one population with different habitats. In each population, five plots were chosen and at least three plants were collected in each plot with a random sampling method. Samples were put into formalin–acetic acid–alcohol fixation immediately after they were removed from soil. Detailed information about the sampling sites is described in Table 1.

Identification of plant species and classification of mycorrhizas

Identification of species of *Pedicularis* was based on Flora Reipublicae Popularis Sinicae (Tsoong 1963). The voucher specimens are deposited in Kunming Botanic Garden of Kunming Institute of Botany, Chinese Academy of Sciences. The voucher numbers are also given in Table 1. Mycorrhiza types were designated according to Harley and Smith (1983) and DSEF tissues were named according to Jumpponen and Trappe (1998).

Preparation of root material

Before examination under a dissecting microscope, the root samples were soaked in water for 4 h then adhering soil particles were carefully removed with the aid of a dissecting needle. Only rootlets less than 2 mm in diameter were picked out for examination of target fungi. The samples were processed according to the method of Phillips and Hayman (1970) with slight modification. Rootlets were cut into 1-cm segments and cleared in 10% KOH solution at 90°C in a water bath for 1 h. After the rootlets were rinsed in running water for 30 s, they were transferred into alkaline H_2O_2 solution (tender fine rootlets were skipped over this step) and softened for 5–10 min, depending on the lignification degree of individual samples. Acidification was conducted in 5% lactic acid solution and lasted for 3–4 min. Then, the rootlets were put directly into trypan blue (0.5 g/l) in 1:1:1 lactic acid/glycerol/deionized water

Table 1 Environmental characteristics and plant community in sampling sites of *Pedicularis* L.

Plant samples						Site description					
Species	Voucher number	Endemism in China	Corolla type	Developmental stage	Location	Long.	Lat.	Altitude (m)	Dominating life forms	Soil moisture	pH of soil
P. rex C.B. Clarke (1)	P050701	–	Erostrate, toothed	Adult	DL	E:100°07'	N:25°39'	2,830	Forbs, grass	Moist	8.0
P. rex C.B. Clarke (2)	P050707	–	Erostrate, toothed	Juvenile	LJ	E:100°16'	N:27°12'	3,010	Forbs	Moist	7.3
P. rex C.B. Clarke (3)	P050709	–	Erostrate, toothed	Juvenile	LJ	E:100°15'	N:27°10'	3,220	Forbs	Dryish	7.0
P. gracilis Wall.	P050702	?	Rostrate, short tube	Juvenile	DL	E:100°07'	N:25°39'	2,845	Shrubs, forbs	Moist	7.2
P. longipes Maxim.	P050703	+	Rostrate, long tube	Adult	DL	E:100°07'	N:25°38'	3,175	Shrubs	Dry	6.4
P. axillaris Franch.	P050704	+	Rostrate, long tube	Adult	DL	E:100°06'	N:25°37'	3,250	Forbs	Moist	6.2
P. cephalantha Franch.	P050705	+	Rostrate, long tube	Juvenile	LJ	E:100°13'	N:27°01'	2,750	Forbs	wet	5.7
P. tenuisecta Franch.	P050710	+	Rostrate, long tube	Juvenile	LJ	E:100°15'	N:27°04'	3,190	Shrubs	Moist	8.1
P. tapaoensis Tsoong	P050712	+	Rostrate, long tube	Adult	ZD	E:99°58'	N:27°43'	3,670	Forbs, grass	Dryish	7.1
P. rhinanthoides Schrenk	P050713	–	Rostrate, short tube	Adult	ZD	E:100°15'	N:27°10'	3,665	Grass	Dryish	7.8
P. likiangensis Franch.	P050715	+	Erostrate, toothless	Adult	ZD	E:99°00'	N:28°23'	4,300	Shrubs	Dry	6.3
P. yui Li	P050716	+	Rostrate, short tube	Adult	ZD	E:99°03'	N:28°25'	4,315	Shrubs	Dry	6.6
P. longiflora Rudolph.	P050717	–	Rostrate, long tube	Juvenile	ZD	E:99°02'	N:28°25'	4,330	Grass	Saturated	5.5
P. siphonantha Don (1)	P050720	–	Rostrate, long tube	Adult	ZD	E:99°46'	N:27°45'	3,370	Grass	Moist	6.8
P. siphonantha Don (2)	P050721	–	Rostrate, long tube	Juvenile	ZD	E:99°34'	N:27°43'	3,410	Forbs	Dryish	6.0
P. siphonantha Don (3)	P050714	–	Rostrate, long tube	Adult	ZD	E:99°14'	N:27°46'	3,430	Forbs	Dry	7.3
P. oxycarpa Franch. (1)	P050722	+	Rostrate, short tube	Adult	ZD	E:99°43'	N:27°37'	3,290	Grass, forbs	Dryish	7.2
P. oxycarpa Franch. (1)	P050719	+	Rostrate, short tube	Adult	ZD	E:99°39'	N:27°53'	3,350	Grass, forbs	Moist	7.9
P. dichotoma Bonati	P050718	+	Rostrate, short tube	Juvenile	ZD	E:99°38'	N:27°54'	3,328	Grass	Dry	8.0

Different numbers attached to the same species in the first column indicates samples from different sites. Location of sampling sites: DL stands for Dali, LJ for Lijiang, and ZD for Zhongdian (Shangrila); "?" indicates the amphibolous endemism status of a certain species.

Table 2 Mycorrhizal and root endophytic fungal colonization status in examined species of *Pedicularis* L.

Species	F%	CI	Types of fungi and visible fungal tissue
P. gracilis Wall.	95	71	AMF (H, A, V); DSEF (H, MS)
P. siphonantha Don (2)	85	72	AMF (H, A, V); DSEF (H, MS)
P. likiangensis Franch.	85	58	DSEF (H, MS)
P. longipes Maxim.	78	53	AMF (H, A, V); DSEF (H, MS)
P. rex C.B. Clarke (2)	71	35	DSEF (H, MS)
P. tenuisecta Franch.	68	53	AMF (H, A, V); DSEF (H, MS)
P. rhinanthoides Schrenk	64	39	DSEF (H, S)
P. rex C.B. Clarke (3)	61	54	AMF (H, S, A, V); DSEF (H, MS)
P. oxycarpa Franch. (2)	55	26	DSEF (H, MS); EMF
P. yui Li	53	38	DSEF (H, MS)
P. siphonantha Don (1)	48	39	AMF (H, A, V); DSEF (H)
P. rex C.B. Clarke (1)	47	37	AMF (H, A, V); DSEF (H, MS)
P. oxycarpa Franch. (1)	46	35	DSEF (H, MS)
P. cephalantha Franch.	45	39	DSEF (H, MS)
P. longiflora Rudolph.	44	38	DSEF (H, MS)
P. axillaris Franch.	43	21	DSEF (H, MS); EMF
P. tapaoensis Tsoong	39	17	AMF (H, A, V); DSEF (H, MS); EMF
P. dichotoma Bonati	28	17	DSEF (MS)
P. siphonantha Don (3)	25	19	AMF (H, S, A, V); DSEF (H)
Average	57	40	

F% Frequency of colonization, *CI* colonization index, *AMF* arbuscular mycorrhizal fungi, *EMF* ectomycorrhizal fungi, *DSEF* dark septate endophytic fungi *H* hypha, *S* spore, *V* vesicle, *A* arbuscular, *MS* microsclerotium

(by volume) and stained for 30 min at 90°C in a water bath. After that, samples were destained in 1:1 water/glycerol (by volume) for several times. For better slide mounting quality, 10–15 like-sized fragments were mounted under the same cover slip. All microscopical determinations were made at ×200 magnification using an Olympus optic microscope. Micrographs were recorded at ×80, 100, or 1,000 magnification on an Olympus or Nikon digital microscope camera.

Determination of colonization level

Colonization level of target fungi were obtained with colonization frequency (F%) and colonization index (CI). Considering the potentially beneficial sides of all three fungal types and the difficulty to distinguish some fungal tissues of one type from another, e.g., the hyphae of AMF and those of DSEF, we give in this study only the total values though there were frequently two or even three types of fungi that coexisted. Because EMF were detected only occasionally with extremely low colonization level, colonization values in this article actually reflect mainly the colonization status of AMF and DSEF. Individual fragment was scored based on the whole percentage of colonized part in the entire fragment using category values from 0 to 4. Numbers indicate the proportion of root cortex colonized by the fungi, i.e., 0, without colonization; 1, less than 25%; 2, above 25% but less than 50%; 3, above 50% but less than 75%; and 4, more than 75%. F% and CI were calculated by the following equations:

$$F\% = \frac{\text{number of fragments colonized by target fungi}}{\text{total number of fragments examined}} \times 100\%$$

$$CI = \frac{\sum (\text{category value} \times \text{corresponding number of fragments})}{\text{the highest category value} \times \text{total number of fragments examined}} \times 100$$

Results

Three types of fungi were detected in the roots, namely, AMF, EMF, and DSEF. Table 2 shows the occurrence of mycorrhizal and endophytic fungi in the examined species, with an average F% of 57% and mean CI of 40.

Among 14 species tested, six were found to be colonized by both AMF and DSEF, namely, *Pedicularis rex* C.B. Clarke, *Pedicularis gracilis* Wall., *Pedicularis longipes* Maxim., *Pedicularis tenuisecta* Franch., *Pedicularis tapaoensis* Tsoong, and *P. siphonantha* Don (Figs. 1 and 2). Coexistence of DSEF and EMF was detected in *Pedicularis*

Fig. 1 Colonization by AMF in the genus *Pedicularis*. **a** Vesicles in root cortex of *P. siphonantha* Don. *Bar* 40 μm. **b** Intraradical and extraradical hyphae in roots of *P. siphonantha* Don. *Bar* 40 μm. **c** Intraradical hyphae and vesicle in root cortex of *P. tenuisecta* Franch. *Bar* 40 μm. **d** Extraradical hyphae and vesicles detected in roots of *P. gracilis* Wall. *Bar* 80 μm. *EH* Extraradical hypha, *IH* intraradical hypha, *V* vesicle
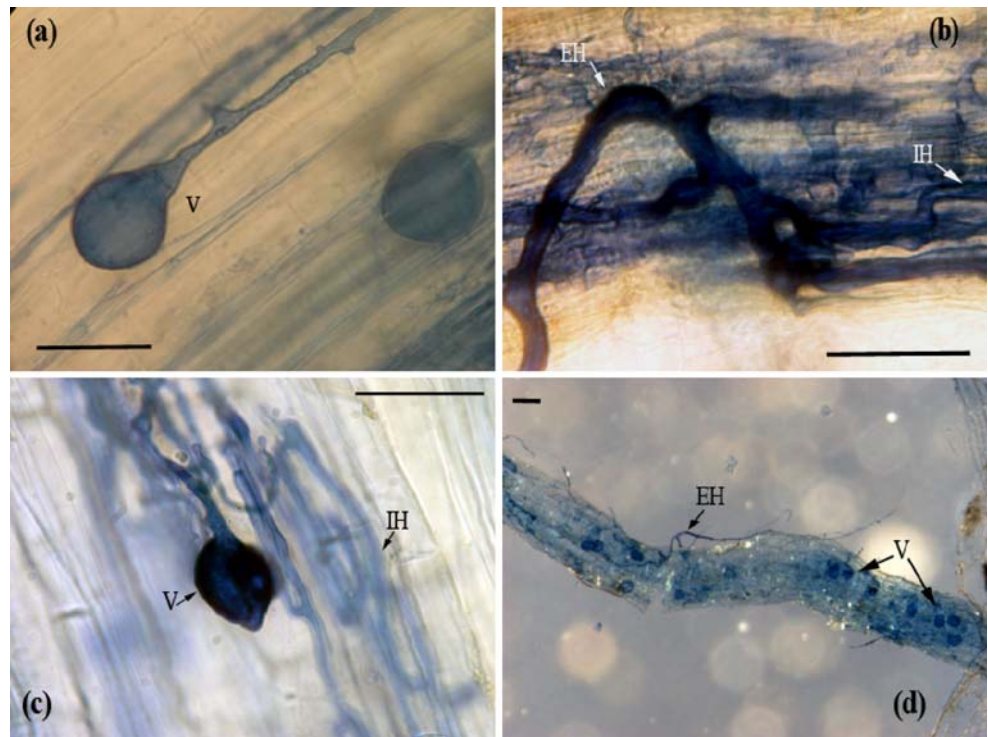
axillaris Franch., *P. tapaoensis* Tsoong, and *Pedicularis oxycarpa* Franch. (Fig. 2). The rest of the species hosted only DSEF (Fig. 3). Of all the species studied, *P. tapaoensis* Tsoong was the only one with coexistence of all three fungi types despite a relatively low colonization level. No detection of cocolonization by AMF and EMF without the presence of DSEF was observed.

Table 2 shows that DSEF were the most frequently encountered type in natural habitats of *Pedicularis* and were found in all the sampling sites involved. With an occurrence frequency of 47.4% in tested sites, AMF ranked the second in this survey. When it came to the species level, 6 out of 14 were able to develop AM associations. On the other hand, EMF showed a rather low colonization level in this genus, detected sporadically only in three sampling sites.

Sampling in different plots from the same population does not allow detailed statistical analysis. Nevertheless, results in Table 2 suggest interspecific variation in colonization level. Meanwhile, based on the dramatic intraspecific variances in terms of both F% and fungal community among different populations, the environmental factors seem to impose a stronger influence on the colonization status.

Discussion

Species of *Pedicularis* L. are common herbaceous plants in alpine and subalpine areas. Slow decomposition and mineralization of organic matter is typical of nutrient cycling in such ecosystems (Schmidt et al. 1999). That is,

Fig. 2 Coexistence of target fungi in the same species of *Pedicularis*. **a** Coexistence of DSEF and EMF in roots of *P. oxycarpa* Franch. *Bar* 1,000 μm. **b** Microsclerotium of DSEF and vesicle of AMF in root cortex of *P. rex* C.B. Clarke. *Bar* 40 μm. *AMF* Arbuscular mycorrhizal fungi, *DSEF* dark septate endophytic fungi, *EMF* ectomycorrhizal fungi, *MS* microsclerotium, *V* vesicle

Fig. 3 Colonization by DSEF in *Pedicularis* species.
a Melanized cortical cells adjacent to vascular cylinder in roots of *P. likiangensis* Franch. *Bar* 40 μm. **b** Cortical cells filled with microsclerotia in roots of *P. longiflora* Rudolph. *Bar* 40 μm.
c Septate intraradical and extraradical hyphae in roots of *P. siphonantha* Don. *Bar* 40 μm.
d Microsclerotium and hyaline hyphae detected in roots of *P. gracilis* Wall. *Bar* 40 μm. *HH* Hyaline hypha, *IH* intraradical hypha, *MC* melanized cell, *MS* microsclerotium, *PS* penetration site, *RH* runner hypha, *VC* vascular cylinder
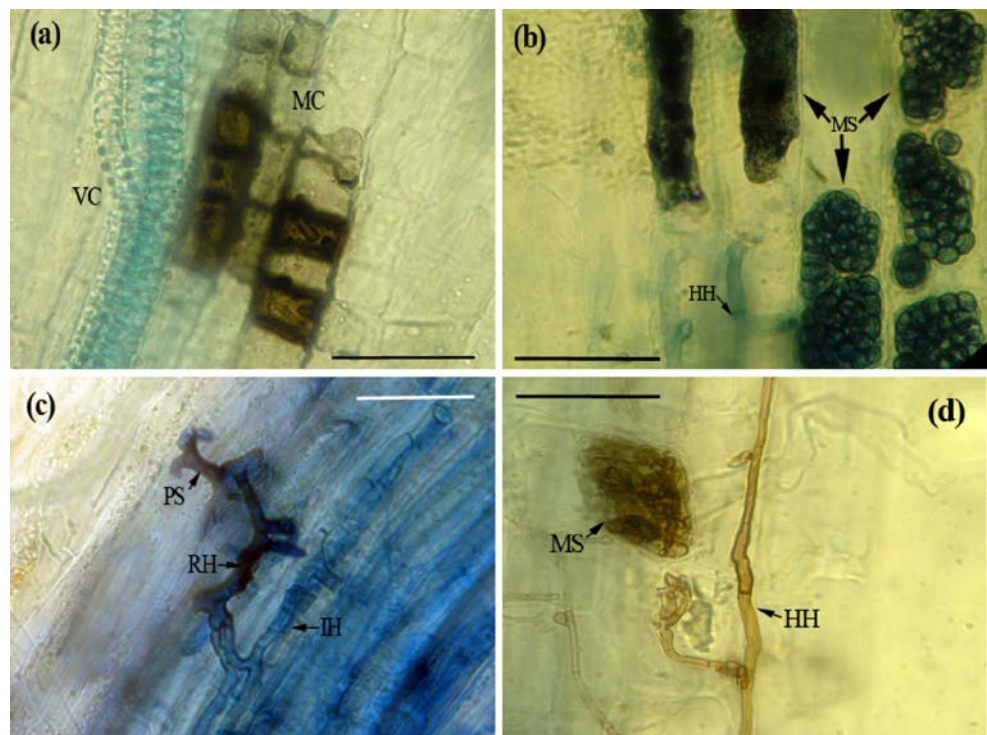
nutrient availability is usually low in natural habitats of *Pedicularis* L. Plants in such areas must have developed various kinds of mechanisms to activate the otherwise unavailable organic matters and increase their nutrient utilization efficiency to survive. With plenty of extraradical mycelia and intraradical mycelia to form a rather huge hyphal network that functions as a bridge in the process of nutrient transport and redistribution between root and soil and among plant individuals, mycorrhizal fungi have this capacity to help (Koide and Mosse 2004). Based on this viewpoint, mycorrhization might serve as an important adaptation mechanism for some alpine and subalpine plants, especially for those with underdeveloped roots such as the genus *Pedicularis*.

As the results showed, there were at least three types of fungi associated with roots of *Pedicularis*, with DSEF as the most dominant colonizers that form melanized hyphae and microsclerotia in the root cortex and the epidermis or on the root surface. Because we used trypan blue for staining, a conventional fungus stain binding specifically to chitin (a component of most fungal walls), hyaline hyphae of DSEF with low chitin concentration were not evident (Barrow and Aaltonen 2001). In other words, we probably underestimated the colonization level by DSEF.

Although different opinions persist regarding whether DSEF should be treated as mycorrhizal fungi or not, there seems to be an agreement that at least in some cases some members do play an important role in soil ecosystems, functioning much like mycorrhizal fungi (Jumpponen

and Trappe 1998; Jumpponen 2001). It was reported that DSEF possess a wide range of enzymes needed for relatively efficient utilization of organic matters (Caldwell et al. 2000), which may partially explain why DSEF can exist in soils with low organic matter. Previous reports also show that abundant DSEF exist in various ecosystems, particularly in highly stressed environments such as arctic and alpine habitats (Treu et al. 1996), and they seem to be alternative symbiotic partners for plants growing in alpine habitats where AMF can no longer persist due to ecological stress (Haselwandter and Read 1980). Their great abundance in natural environments and better adaptation to various habitats than other symbiotic fungi, particularly in potentially stressful conditions, suggest an even more important ecological role of DSEF. By this token, plants of *Pedicularis* will doubtlessly benefit greatly from the associations once they establish mutualistic symbioses with DSEF.

As we all know, being parasitic and mycotrophic are both evolutionary adaptation mechanisms for surviving in stressful conditions. While obligate parasitic plants are seldom reported to be mycorrhizal, some root hemiparasites may have maintained the two mechanisms at the same time, which can be doubtlessly deduced from the transitional status of the genus *Pedicularis*. Moreover, according to previous findings, interactions exist among mycorrhizal fungi, host plants, and parasitic plants involved in a microniche (Davies and Graves 1998). Therefore, the high colonization level of mycorrhizal and endophytic fungi in roots

of *Pedicularis* L. suggests a potentially significant eco-logical function. Future research should be directed toward isolation and identification of the fungal species associated with this genus and their possible contribution to the sur-vival of *Pedicularis*. If these plants show strong depen-dence on their symbiotic fungi, a breakthrough in their cultivation may be achieved by the application of the beneficial microorganisms.

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