A novel technique for isolating orchid mycorrhizal fungi

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Zhu, G.S., Yu, Z.N., Gui, Y. and Liu, Z.Y. (2008). A novel technique for isolating orchid mycorrhizal fungi. Fungal Diversity 33: 123-137.

We describe a technique for isolating mycorrhizal fungi from roots of orchids. This technique involves selection and treatment of roots, preparation of pelotons, treatment of pelotons, culture of pelotons so that fungal hyphae grow out and strain purification. The technique is considered better because 1) problems of fungal and bacterial contamination are resolved, 2) endophytic bacteria are suppressed and also used to promote hyphal growth from the pelotons, 3) live and dead pelotons, and those from which fungi are culturable or unculturable can easily be identified, providing increased isolation efficiency, 4) a single taxon can be isolated from a single peloton containing several mycorrhizal taxa, 5) slow-growing mycorrhizal taxa can easily be isolated. The implications and potential use of this technique in future studies is discussed.

Key words: *Ceratorhiza*, *Epulorhiza*, isolate, *Moniliopsis*, mycorrhiza, orchid, peloton

Article Information
Received 8 August 2008
Accepted 15 October 2008
Published online 30 November 2008
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Introduction

The *Orchidaceae* is the world's largest plant family with estimates of more than 25,000 species (Jones, 2006). The seeds of orchids are minute and contain sparse food reserves, while the protocorms (early small, sphaerical, food-storing underground stems, which are formed after the germination and growth of epiphytic orchid seeds), young seedlings and some adult plants cannot produce carbon as they lack chlorophyll. It is therefore difficult for orchids to propagate in the wild. Much research has been carried out on how to resolve this problem. Wahrlich (1886) and Janse (1897) first noted the occurrence of mycorrhizal fungi in the roots of temperate and tropical orchids. Orchid mycorrhizal fungi have also been found in protocorms (Zelmer *et al*., 1996; Hayakawa *et al*., 1999; Kristiansen *et al*., 2001a; Takahashi *et al*., 2005; Zettler *et al*. 2005) and occasionally from rhizomes (horizontal, usually underground stems that often send out roots and shoots from nodes and form after the germination and growth of edaphic orchid seeds) (Warcup, 1985; Yagame *et al*., 2008), tubers and corms (Kusano, 1911; Fuchs and Ziegenspeck, 1925). Mycorrhizae can provide or increase uptake of inorganic and organic nutrients by orchids (Rasmussen, 1995; Smith and Read, 1997; Dearnaley, 2007). This includes carbon (Hadley, 1984; Alexander and Hadley, 1985; Trudell *et al*., 2003; Abadie, 2006; Cameron *et al*., 2006), phosphorus (Alexander *et al*., 1984; Cameron *et al*., 2007), nitrogen (Wolff , 1932, Burgeff, 1936; Trudell *et al*., 2003; Cameron *et al*., 2006), water (Yoder *et al*., 2000) and vitamins (Harvais and Pekkala, 1975). Mycorrhizal fungi in orchids are thought to 1) promote germination of seeds (Warcup, 1973; Masuhara and Katsuya, 1989; Zettler and Hofer, 1998; McKendrick *et al*., 2002; Sharma *et al*., 2003a; Otero *et al*., 2004; Chou and Chang, 2004; Leake *et al*., 2004; Shimura and Koda, 2005; Dearnaley, 2007; Takahashi *et al*., 2007; Batty *et al*., 2007;

Stewart and Kane, 2006, 2007), and 2) stimulate the development and growth of protocorms, seedlings and juveniles, some adult plants, and tubers (Hadley and Williamson, 1971; Masuhara and Katsuya, 1989; Richardson *et al*., 1992; Zettler and Hofer, 1998; Bayman, 2002; Leake *et al*., 2004; Shimura and Koda, 2005; Kazuhiko *et al*., 2005; Kazuhiko *et al*., 2006; Dearnaley, 2007; Takahashi *et al*., 2007; Batty *et al*., 2007).

Techniques used for the isolation of orchid mycorrhizal fungi include plating fragments of surface-sterilized roots (PFSSR) on nutrient agar (Bernard, 1904; Currah *et al*., 1987, 1988, 1990; Zettler, 1997; Zettler *et al*., 2005; Sharma, 2003b; Stewart, 2002; Stewart and Kane, 2006, 2007) and plating individual carefully separated fungal pelotons (PICSFP) on nutrient agar (Bernard, 1909; Constantin and Dufour, 1920; Warcup *et al*., 1967; Rasmussen *et al*., 1990; Taylor, 1997; Rasmussen, 1995; Zelmer, 1995; Otero, 2002; Bayman, 2002; Shan, 2002; Dearnaley, 2005). There are however, many problems associated with these isolation methods. These include 1) problems of fungal and bacterial contamination that cannot be avoided without destroying the pelotons, 2) the unknown role of endophytic bacteria in isolation, 3) a low isolation efficiency as dead pelotons or those from which fungi cannot be isolated could not be identified, 4) a single peloton may contain several mycorrhizal taxa and all of these cannot usually be isolated and analysed, and 5) it is difficult to isolate slow growing mycorrhizal fungi. These problems have resulted in difficulties in understanding the taxonomy and role of mycorrhizal fungi of orchids and thus make it hard to successfully apply these fungi to promote seed germination, development and growth of protocorms, seedlings, juveniles, tubers and adult plants without chlorophyll.

Improved methods are needed to prevent contamination and improve isolation efficiency. The objective of this paper is to introduce methodology that 1) resolves problems of fungal and bacterial contamination, 2) uses endophytic bacteria to promote isolation, 3) increases isolation efficiency, 4) allows isolation of a single taxon from a single peloton containing several mycorrhizal taxa, and 5) allows isolation of slow-growing mycorrhizal taxa. When utilised, this methodology will improve the understanding of orchid mycorrhizal fungi biology and improve application.

Materials and Methods

Orchids used in the study

Cremastra appendiculata (D. Don) Makino (Fig. 1-1) was obtained from Shibing County, Guizhou Province; *Pleione bulbocodioides* (Franch.) Rolfe (Fig. 1-2) from Liupanshui area, Guizhou Province and *Pleione yunnanensis* (Rolfe) Rolfe (Fig. 1-3) from Leigong Mountain, Leishan county, Guizhou Province, China.

Fig. 1. Orchids used in this study**. 1-1.** *Cremastra appendiculata*. **1-2.** *Pleione bulbocodioides*. **1-3.** *Pleione yunnanensis*.

Isolation of orchid mycorrhizal fungi: method of Currah et al. (1987)

Root segments are surface sterilized in a 20% solution of household bleach for 1 minute, rinsed twice in sterile distilled water, and decorticated with a sterile scalpel. Clumps of cells are removed from the inner cortex, macerated in a drop of sterile water, and plated in molten modified Melin-Norkran's agar cooled to 55°C. Plates are allowed to solidify and are incubated in the dark at 18°C until hyphae grow from the cortical cells into the media. Hyphal tips are transferred to potato dextrose agar (PDA, Difco) and serially transferred until pure cultures are obtained.

Isolation of orchid mycorrhizal fungi: method of Warcup and Talbot (1967)

Orchid roots with external *Rhizoctonia*-like mycelium are selected and washed thoroughly in tap water and cut into segments. Segments are teased apart in sterile water using two needles, releasing the pelotons into isolation plates. These are mixed with cooled, molten agar with 50 ug/ml streptomycin and 20 ug /ml tetracycline and poured in Petri dishes to obtain fungi growing from the pelotons.

Novel technique for isolating mycorrhizal fungi from orchid

1. Selection of roots

Whole wild healthy orchid plants are dug out with soil, packaged in plastic bags and taken to the laboratory and soil is removed gently (Fig. 2-1). Roots are carefully washed in running tap water to remove soil and surface debris (Fig. 2-2). Slight yellowish (Fig. 2-3) or opaque roots and those with *Rhizoctonia*-forming fungal mycelia on the surface (Fig. 2-4) are cut at root insertion. No more than 50% of roots of the orchids are removed so that the plants can be replanted.

Fig. 2. Selection of roots. **2-1.** The whole plant dug out from the soil. **2-2.** Soil and surface debris removed. **2-3.** The faintly yellowish roots, arrowed. **2-4.** Root with *Rhizoctonia*-forming fungal mycelia on surface, arrowed.

2. Treatment of roots

Root hairs, epidermis, velamen and other attachments are peeled or scraped off with a scalpel, needles and forceps (Figs 3-1, 3-2). Roots with pelotons are selected by microscopic examination and rinsed with sterile distilled water five times. After that the roots are immersed in 10 ml sterile distilled water with 150 ug/ml streptomycin sulphate and 150 ug/ml potassium Penicillin G for 10 minutes. They are then washed again with sterile distilled water.

Fig. 3. Treatment of roots. **3-1.** Root with hairs, epidermis, velamen and other attachments. **3-2.** Roots where hairs, epidermis, velamen and other attachments are removed.

3. Preparation of pelotons

Roots with pelotons (Fig. 4-1) are cut into segments (Fig. 4-2). Those with several separate mycorrhizal sites are cut into segments with only one segment per mycorrhizal site. Roots with continuous mycorrhizal sites are cut into segments at 2 cm intervals. Segments are teased apart using a needle and forceps. This releases individual pelotons from the cortex cells which are placed in a 60 cm sterile Petri dish containing 10 ml sterile distilled water (Figs 4-3, 4-4). Pelotons from all roots can be teased 5 times in 5 different plates from the exodermis to the endodermis.

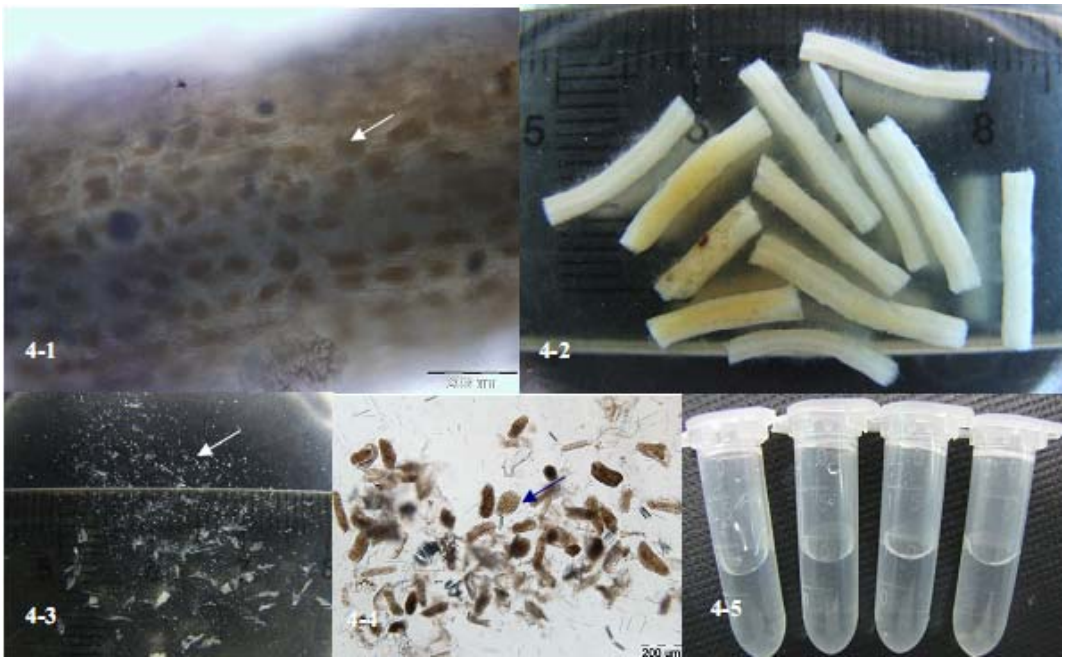

Fig. 4. Preparation and treatment of pelotons. **4-1.** Root with pelotons (arrowhead). **4-2.** Roots cut into segments. **4-3.** Pelotons teased into sterile distilled water (arrowed). **4-4.** Teased pelotons at higher magnification (arrowhead). **4-5.** Pelotons transferred into Eppendorf tubes and incubated at 18°C.

4. Treatment of pelotons

There are few living pelotons in old roots and it is difficult to select out the living pelotons from the numerous teased pelotons in the Petri dishes. Therefore pelotons from old roots are transferred into 2 ml Eppendorf tubes and incubated for 4~24 hours at 18°C to induce growth of fungi (Figs 4-5). Streptomycin sulphate (100 ug/ml) and potassium Penicillin G (100 ug/ml) is added to cultures incubated for more than 10 hours. This provides an easy method for selecting living pelotons because

the emerging hyphae can be observed. Growth of hyphae from pelotons from young roots occurs at a high rate, so young pelotons do not require incubation.

5. Selection and culture of pelotons on agar disks

Incubated pelotons from old roots in Eppendorf tubes are transferred to sterile 6 cm diam. Petri dishes and 10 ml of sterilized distilled water is added. These are placed under a dissecting microscope at low power to observe the pelotons suspended in water. A 50 µl solution containing 1 peloton with emerging hyphae (Fig. 5-1) from old roots is transferred to 1 cm^2 PDA disks (Fig. 5-2) with 100 ug/ml streptomycin sulphate and 100 ug/ml potassium Penicillin G. using a 1 ml Eppendorf micropipette. A 50 µl solution containing 3-5 pelotons from young roots, is also transferred to 1 cm^2 PDA disks. They are cultured at 18°C until hyphae emerge.

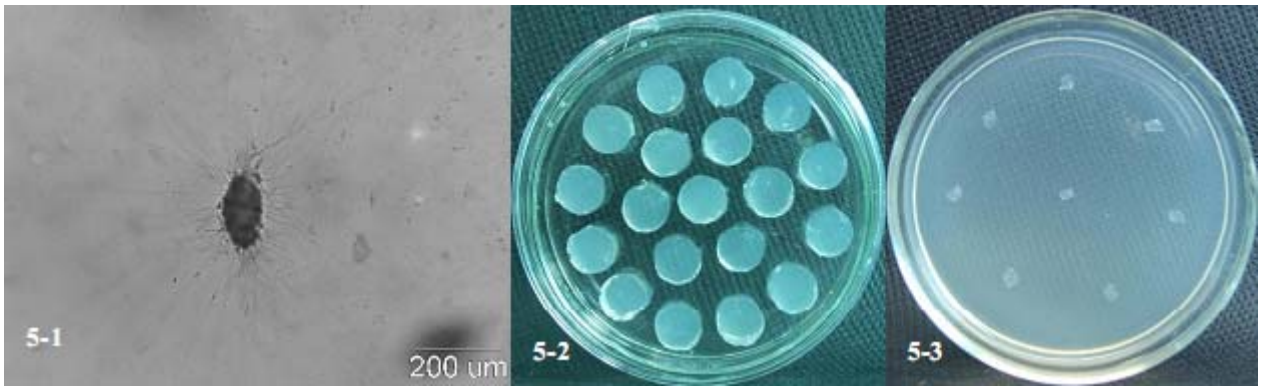

Fig. 5. Selective culture and purification of strains. **5-1.** Peloton in sterile distilled water with emerging hyphae. **5-2.** Selective culture of individual pelotons on agar disks. **5-3.** Purification of pelotons from which hyphae have emerged.

6. Purification of strains

The incubated pelotons on agar disks are observed under a dissecting microscope and pelotons with emerging hyphae are individually cut out from the agar disks (Fig. 5-2). These are transferred to PDA media and incubated at 24°C until the hyphae grow out to more than 0.5 cm long. Tips of the hypha growing from the pelotons are cut and transferred to PDA in test tubes for purification. The strains contaminated by bacteria can be purified using one of the following methods.

Method 1. The agar disk containing the growing fungal hyphae (Fig. 6-1) is carefully inverted and placed into the cover of the Petri dish (Fig. 6-2). The mycelia in the media is cut out (Fig. 6-3) and cultured on PDA medium containing antibiotics at 18°C until the hyphae grow more than 1 cm long (Fig. 6-4). The purified fungi can then be obtained by one or more transfers based on this method as bacterial contamination can be removed as the fungi growing into the agar are free from bacteria.

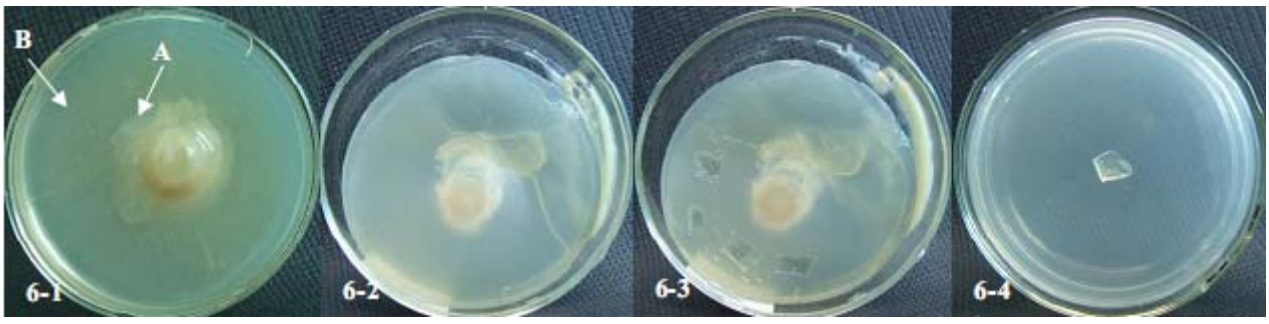

Fig. 6. Purification of fungi contaminated by bacteria. **6-1.** Fungal strains contaminated by bacteria. **A.** Bacteria contamination. **B.** Fungi growing into the media. **6-2.** The PDA medium is turned over carefully and placed into the cover of the petri dish. **6-3.** The medium with fungal hyphae cut out. **6-4.** The medium is cut out and transferred onto PDA medium with antibiotics.

Method 2. An agar disk with growing hyphae is transferred to a sterile Petri dish (Fig. 7A) and several small PDA disks (Fig. 7B) containing antibiotics (PDA + 100 ug streptomycin sulphate and 100 ug Potassium penicillin G per milliliter water) are placed near the main agar disk. The Petri dish is then inverted and 5 ml of sterile water is added to the cover to maintain humidity. Hyphae grow from the central agar disks onto the small PDA trapping disks. These are then transplanted to PDA media with antibiotics and incubated at 24°C. The purification of fungi can be achieved by one or more attempts based on this method because 1) bacteria reproduce and spread out more slowly than the growing fungal hyphae and thus the hyphae lacking bacterial contamination colonize the trapping disks, and/or 2) different fungi grow at different rates and in different directions so different fungi can be isolated from different trapping disks.

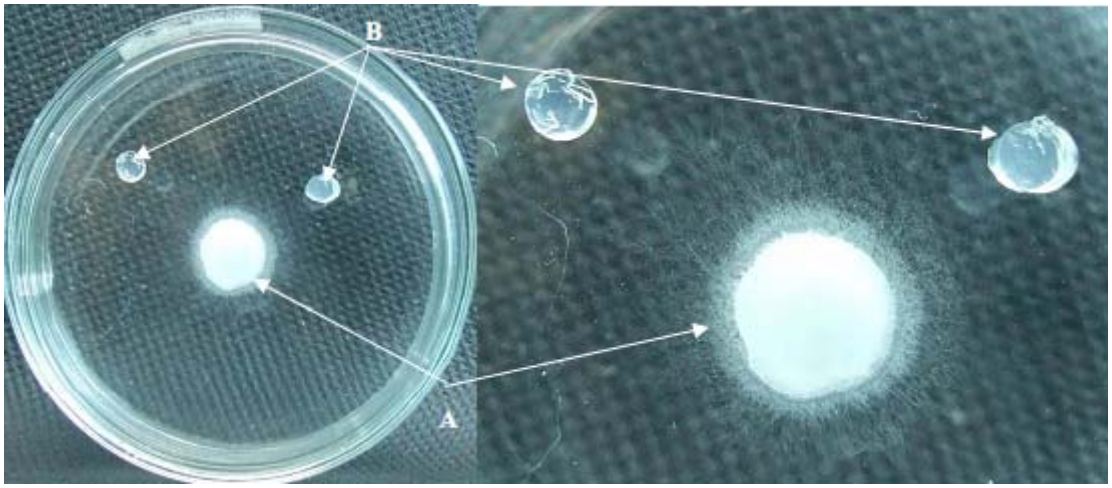

Fig. 7. Purification of fungal strains. A medium disk with orchid mycorrhizal fungi cultured in sterile water. **A.** An agar disk with several mycorrhizal fungal species or contaminated by microorganisms. **B.** Medium disks used to trap the advancing mycelium.

Result and analysis

The ratio of pelotons emerging fungal hyphae and mycorrhizal fungal species incubated at different conditions

In this study it was found that the ratio of pelotons with emerging hyphae was lower in sterile distilled water with streptomycin sulphate and potassium Penicillin G, than in sterile distilled water without antibiotics (Table 1). It is well known that streptomycin sulphate inhibits the growth of G^- bacteria and potassium Penicillin G inhibits the growth of G^+ bacteria. Thus the growth of bacteria is inhibited in the sterile distilled water with antibiotics as compared to that without antibiotics. All other conditions are identical. Therefore, it is likely that bacteria presence may be the only factor that promotes hyphae to grow out from the pelotons. Some bacteria grow in or near the pelotons in water agar without antibiotics (Fig. 8), but no bacteria were found in water agar with antibiotics. We also found that hyphae emerged from seven pelotons of *P. bulbo-codioides* and their growth was promoted by bacteria whereas in 12 pelotons hyphal growth was inhibited by bacteria. Thus, certain endophytic bacteria can promote mycorrhizal hyphae to grow out from orchid root pelotons whereas some other species could not. *Ceratorhiza* sp. GZAAS 0003 and *Ceratorhiza* sp. GZAAS 0006) were isolated from pelotons incubated in sterile distilled water with antibiotics while many more species (*Epulorhiza* sp. GZAAS 0001, *Epulorhiza* sp. GZAAS 0002, *Epulorhiza* sp. GZAAS 0005, *Ceratorhiza* sp. GZAAS 0003, *Ceratorhiza* sp. GZAAS 0004, *Ceratorhiza* sp. GZAAS 0006, *Ceratorhiza* sp. GZAAS 0007, *Ceratorhiza* sp. GZAAS 0008) were isolated from pelotons incubated in sterile distilled water without antibiotics (Table 2, Fig. 9). All mycorrhizal fungal species isolated from pelotons incubated in sterile distilled water with antibiotics could also be isolated in sterile distilled water without antibiotics, but some species of *Ceratorhiza*

Table 1. The ratio of pelotons with emerging hyphae incubated under different conditions.

Orchid	Pelotons incubated in sterile distilled water without antibiotics			Pelotons incubated in sterile distilled water with antibiotics		
	Total pelotons	Pelotons with emerging hyphae	Ratio of pelotons with emerging hyphae (%)	Total pelotons	Pelotons with emerging hyphae	The ratio of pelotons with emerging hyphae (%)
P. bulbocodioides	52	43	82.7	61	11	18.0
P. yunnanensis	95	62	65.3	90	10	11.1
C. appendiculata	610	413	67.7	-	-	-

"-"= Experiment not carried out.

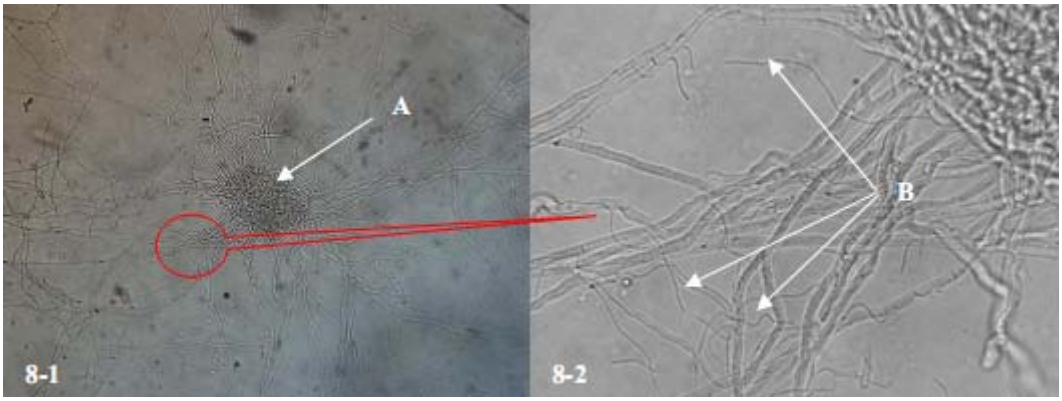

Fig. 8. Bacteria growing with growth peloton**. 8-1.** A growing peloton, **A.** peloton; **8-2.** The part marked in 8-1, **B.** Bacteria

and all species of *Epulorhiza* were not isolated in sterile distilled water with antibiotics. Thus, Epulorhizal taxa and some Ceratorhizal taxa within pelotons may need endophytic bacteria to promote their growth. It was found that pelotons of *Epulorhiza* could easily be contaminated by bacteria, but they could still grow into the agar. Purification was easily achieved by Method 1. It is interesting that species of *Epulorhiza* could grow slowly without bacteria when they were in pure culture. In the roots, the bacteria may regulate the growth of endophytic *Epulorhiza*.

Table 2. Species isolated from pelotons incubated under different conditions.

Orchid	Fungi isolated from pelotons incubated in sterile distilled water		Fungi isolated from pelotons incubated in sterile distilled water with antibiotics	
	Genus	Strains	Genus	Strains
P.bulbocodioides	*Epulorhiza*	*Epulorhiza* sp. GZAAS 0001	*Ceratorhiza*	*Ceratorhiza* sp. GZAAS 0003
		Epulorhiza sp. GZAAS 0002		
	Ceratorhiza	*Ceratorhiza* sp. GZAAS 0003		
		Ceratorhiza sp. GZAAS 0004		
P.yunnanensis	*Epulorhiza*	*Epulorhiza* sp. GZAAS 0005	*Ceratorhiza*	*Ceratorhiza* sp. GZAAS 0006
	Ceratorhiza	*Ceratorhiza* sp. GZAAS 0006		
		Ceratorhiza sp. GZAAS 0007		
		Ceratorhiza sp. GZAAS 0008		
C. appendiculata	*Epulorhiza*	*Epulorhiza* sp. GZAAS 0009	-	-
	Ceratorhiza	*Ceratorhiza* sp. GZAAS 0010		
		Ceratorhiza sp. GZAAS 0011		
		Ceratorhiza sp. GZAAS 0012		
	Monilioposis	*Monilioposis* sp. GZAAS 0013		
		Monilioposis sp. GZAAS 0014		
		Monilioposis sp. GZAAS 0015		

"-" = Experiment not carried out.

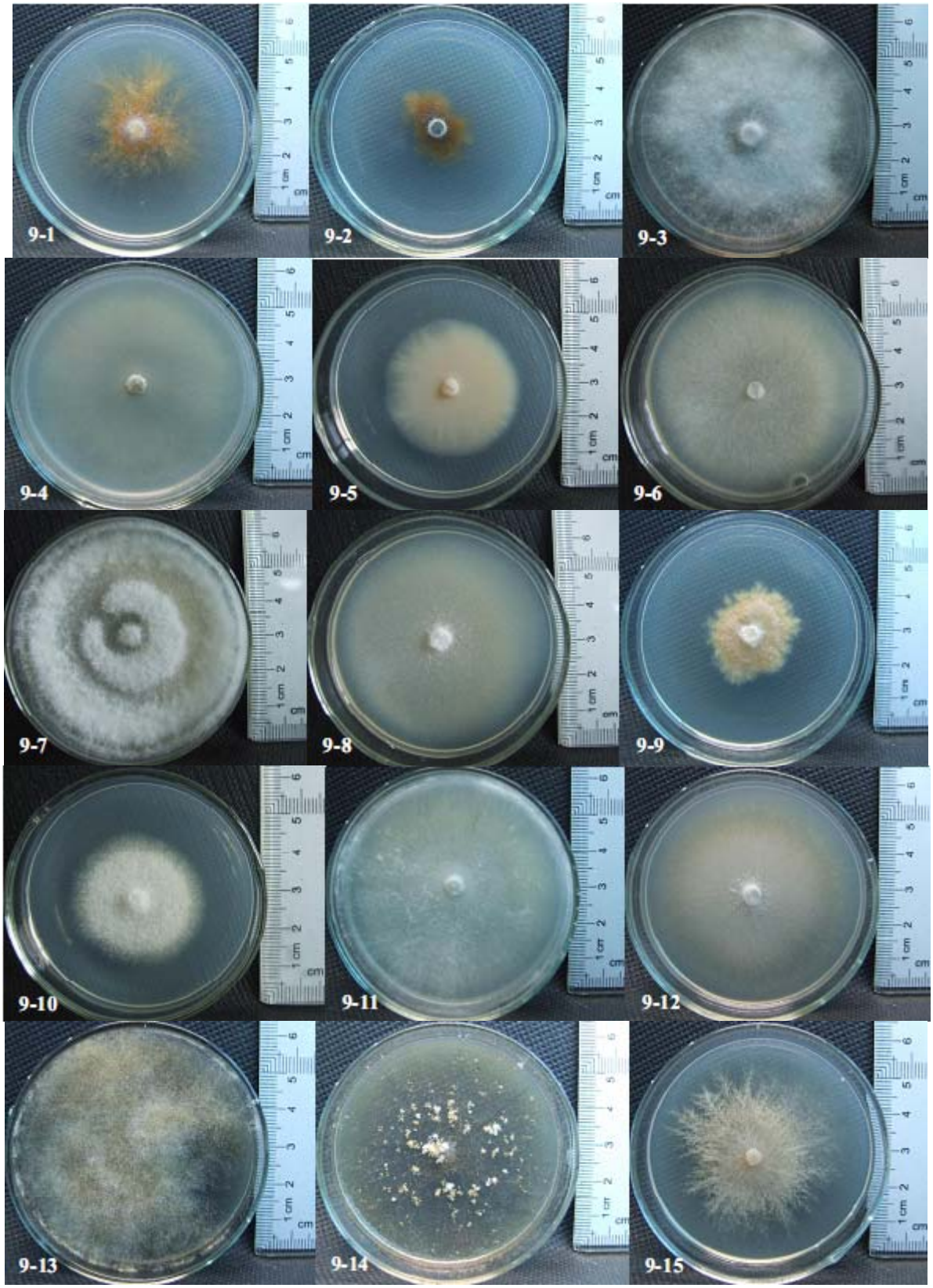

Fig. 9. Species isolated from pelotons from three orchid roots incubated in sterile distilled water. All cultured on BD DIFCO PDA medium. **9-(1-4).** Being isolated from *P. bulbocodioides*. **9-1.** *Epulorhiza* sp. GZAAS 0001; **9-2.** *Epulorhiza* sp. GZAAS 0002. **9-3.** *Ceratorhiza* sp. GZAAS 0003. **9-4.** *Ceratorhiza* sp. GZAAS 0004. **9-(5-8).** Being isolated from *P. yunnanensis*. **9-5.** *Epulorhiza* sp. GZAAS 0005. **9-6.** *Ceratorhiza* sp. GZAAS 0006. **9-7.** *Ceratorhiza* sp. GZAAS 0007. **9-8.***Ceratorhiza* sp. GZAAS 0008. **9-(9-15).** Being isolated from *C. appendiculata*. **9-9.** *Epulorhiza* sp. GZAAS 0009. **9-10.** *Ceratorhiza* sp. GZAAS 0010. **9-11.** *Ceratorhiza* sp. GZAAS 0011. **9-12.** *Ceratorhiza* sp. GZAAS 0012. **9-13.** *Monilioposis* sp. GZAAS 0013. **9-14.** *Monilioposis* sp. GZAAS 0014. **9-15.** *Monilioposis* sp. GZAAS 0015.

The treatment of the pelotons is a key step for isolating mycorrhizal fungi from orchids since bacteria play a key role in promoting hyphae to grow out from the pelotons. If bacteria reproduce too quickly, the growth of fungi from the pelotons is inhibited. Therefore, some method is needed to slow down the reproduction of the bacteria. One way is to shorten the incubation period. Hyphae from pelotons from young roots grow quickly from the PDA disks and do not require incubation in sterile distilled water. Pelotons from old roots needed be incubated in sterile distilled water without antibiotics for up to 10 hours to induce growth of bacteria. The bacteria may then produce some substances which promote mycorrhizal hyphae to grow out from the pelotons. If the incubation period is longer than 10 hours, antibiotics should be added to control the bacteria, otherwise the hyphae growing out from the pelotons would be inhibited. A second way is to lower the incubation temperature. Both fungi and bacteria grow slowly at 18°C, but the fungi grow relatively faster. A third method is to control the amount of bacteria. The incubated pelotons are placed in sterile distilled water containing antibiotics and the bacteria are diluted and inhibited so that the bacteria can be controlled and used effectively.

Factors influencing the ratio of pelotons with emerging hyphae and the correct method to select roots

Huynh *et al*. (2004) found that pelotons in the cortex cells had undergone both formation and digestion? The young pelotons were less compact and living, but the digested old pelotons were compact and dead. The young less compact pelotons could be easily cultured (Fig. 10). The state of pelotons can change with root age and their distribution and position in the roots. The ratio of pelotons with emerging hyphae is higher from young roots than those

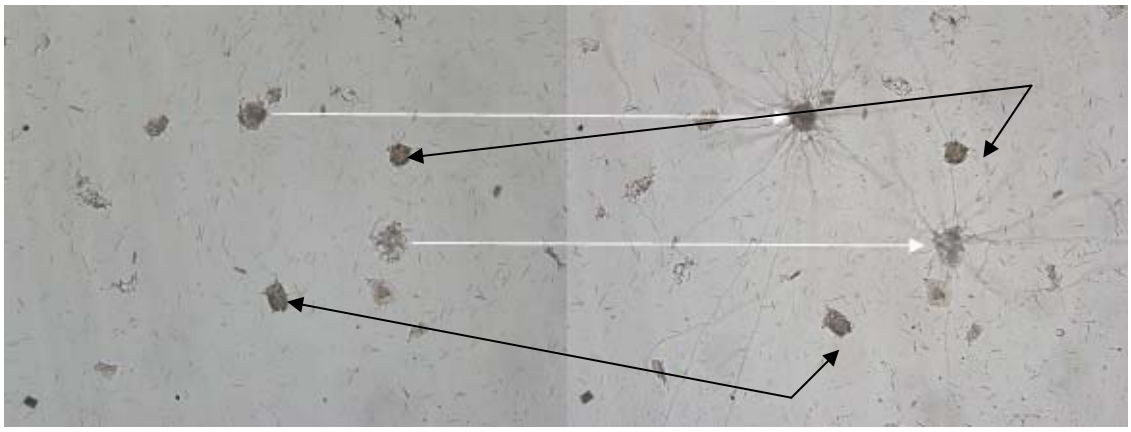

Fig. 10. The growth of loose pelotons. White **arrow**: the hyphae grow out of less compact pelotons. Black arrow: the compact pelotons without hyphae growing out.

from old roots. In general, young roots are suitable for isolating mycorrhizal fungi, but isolates obtained from young roots of *C. appendiculata* were different from those of old roots. *Epulorhiza* sp. GZAAS 0009, *Ceratorhiza* sp. GZAAS 0011, *Ceratorhiza* sp. GZAAS 0012 and *Monilioposis* sp. GZAAS 0015 were only isolated from old roots, but *Ceratorhiza* sp. GZAAS 0010, *Monilioposis* sp. GZAAS 0013 and *Monilioposis* sp. GZAAS 0014 were only isolated from young roots. Therefore if we want to isolate the complete range of mycorrhizal fungi from an orchid, we should isolate from roots of different ages.The ratio of pelotons with emerging hyphae is higher from exodermis cells (outer and secondly layers) than those from endodermis cells (fourth and fifth inner cell layers). Old roots of *C. appendiculata* were teased five times to obtain pelotons from the exodermis to endodermis (Table. 3). Our results are in good agreement with those of some other researchers (Kristiansen *et al*., 2001a) in that, pelotons from exodermis cells were suitable for isolating mycorrhizal fungi. However, a greater number of species were isolated from inner cortex cells (third layer) than from other layers. *Ceratorhiza* sp. GZAAS 0012 and *Monilioposis* sp. GZAAS 0015 were isolated from all five layers, but strains of *Epulorhiza*

sp. GZAAS 0009 were only isolated from cells in the central cortex cells and only two strains were isolated. The reason why *Epulorhiza* sp. GZAAS 0009 was only isolated from the third layer and why so few strains of *Epulorhiza* sp. GZAAS 0009 were isolated may be because species of *Epulorhiza* are distributed only in the middle cortex cells and most of them are digestion pelotons. *Ceratorhiza* sp. GZAAS 0011 was not isolated from the roots. This indicates that mycorrhizae in different roots of the same age are not always identical. This was also found in *P. bulbocodioides*. *Epulorhiza* sp. GZAAS 0001 and *Epulorhiza* sp. GZAAS 0002 were only isolated from one root. To isolate all mycorrhizal fungi, we should select several roots with the same age from several plants. The pelotons in the middle cortex cells of each root should be teased out so that more mycorrhizal fungi can be isolated.

Table 3. Ratio of pelotons with hyphae growing out from different layers of old roots of *C. appendiculata*.

Layers*	Total pelotons	Pelotons with hyphae growing out	Ratio of pelotons with hyphae growing out	Species
Firstly layer	53	12	22.6%	*Ceratorhiza* sp. GZAAS 0012 *Monilioposis* sp. GZAAS 0015
Secondly layer	111	31	27.9%	*Ceratorhiza* sp. GZAAS 0012 *Monilioposis* sp. GZAAS 0015
Thirdly layer	106	7	6.6%	*Epulorhiza* sp. GZAAS 0009 *Ceratorhiza* sp. GZAAS 0012 *Monilioposis* sp. GZAAS 0015
Fourthly layer	87	9	10.3%	*Ceratorhiza* sp. GZAAS 0012 *Monilioposis* sp. GZAAS 0015
Fifthly layer	76	9	11.8%	*Ceratorhiza* sp. GZAAS 0012 *Monilioposis* sp. GZAAS 0015

"*" = Sequence arranged from exodermis to endodermis.

The best method for isolating as many mycorrhizae as possible is to select roots and pelotons from roots of different ages. Different plants should also be selected out and the pelotons in middle cortex cells should be teased out.

Discussion

The methods presented in this paper help to solve the problem of fungal and bacterial contamination. Fungal contamination is reduced because 1) endogenous fungi such as pathogens and root epiphytic fungi are eliminated by selecting healthy orchids and removing root hairs, epidermis, velamen and other attachments, 2) exogenous air fungal contaminations are eliminated by selecting a peloton and observing the culturing process under a microscope. Bacterial contamination is reduced because 1) streptomycin sulphate and potassium Penicillin G were used to inhibit the growth of G+ and G- bacteria, 2) fungi are separated from bacteria by culturing them at 18°C because fungi grow much more quickly than bacteria at this temperature, 3) fungi are separated from bacteria by using the characteristic that fungi grow into the media while bacteria do not. Pure cultures of fungi can be easily obtained by transplanting the under-side medium with mycelia in a Petri dish.

This method presented here is considered to be better than the methods of Currah (1987) and Warcup and Talbot (1967) because contamination is reduced effectively without loss of hyphae from pelotons (Table 4), more effective isolation of slow growing fungi, a higher diversity of taxa isolated, higher efficiency and the fact that several orchid mycorrhizal fungi in a single peloton could be separated and obtained in pure culture.

Better isolation of slow growing fungi

The slow growing fungi in orchid roots are easily isolated by this technique and is considered better than methods of Currah *et*

Table 4. Comparison of the contamination reduction of our techinique with that of other methods.

		Our technique	Currah (1987) PFSSR	Warcup (1967) PICSFP
Isolation materials		Single peloton	Clumps of cortex cells with pelotons	Single peloton or several pelotons
Methods used to Reduce fungal contamination	Pathogenic fungi	Removed by selecting healthy orchid roots		
	Epiphytic fungi	Removed by selecting only one peloton	Not removed because epiphytic fungal hyphae would exist among cells and pelotons	Removed by selecting only one peloton
	Exogenous air fungi	Solved by being selected under microscope	No methods used	No methods used
Methods used to Reduce bacterial contamination		Cultured under 18℃ Antibiotics used Seprate fungi from bacteria	Cultured under 18℃ Disinfectors used Antibiotics used	Antibiotics used
Bacteria used to promote fungal hyphae grow out from pelotons		Used	Not used	Not used
Peloton damage		No	Yes by disinfector	Yes by molten medium

al., (1987) and Warcup and Talbot (1967) (Table 5). This is because 1) a single peloton could be selected out easily and hyphae pure cultured, 2) endophytic bacteria are used to promote slow growing hyphae from pelotons, 3) some unknown substances from roots released into the peloton solution can promote hyphae growing out of pelotons. With this technique, some slow growing fungi such as *Epulorhiza* sp. GZAAS 0009 and *Epulorhiza* sp. GZAAS 0005 were isolated from *C. appendiculata* and *P. yunnanensis*. The ITS region of these taxa were sequenced and aligned in GenBank accessions using a Blast search. It was found that most "uncultured" species in GenBank have a close relation with them.

Table 5. Isolation of slow growing fungi using our technique as compared with other methods.

	Our technique	Currah(1987) PFSSR	Warcup(1967) PICSFP
Outgrown by other fast growing mycorrhizal fungi	No	Yes	Yes
Growth promoted by endophytic bacteria	Yes	No	No
Hyphal growth promoted by unknown root extracts	Yes	No	No

More mycorrhizal fungal taxa isolated

A greater number of mycorrhizal fungal taxa were isolated using our technique than other methods (e.g. Currah, 1987; Warcup and Talbot, 1967) (Table 6) This is due to, 1) some slow growing or rare fungi can easily be isolated by this method. For example *Epulorhiza* sp. GZAAS 0009, a strain isolated by our technique was not isolated using the methods of Currah (1987) and Warcup and Talbot (1967), 2) those fungi needing bacteria to promote growth can be isolated using our technique. For example *Monilioposis* sp. GZAAS 0015, 3) those fungi needing extracts from orchid roots to promote growth can be isolated, and 4) those fungi within pelotons alongside other fast growing fungi could be isolated, such as *Epulorhiza* sp. GZAAS 0005 which grows together with *Ceratorhiza* sp. GZAAS 0006 in one peloton.

Better isolation efficiency

There are thousands of pelotons per root. But the vitality of pelotons are different in

Table 6. Comparison of species isolated from three orchids with different methods.

	Our technique	Currah(1987) PFSSR	Warcup(1967) PICSFP
P.bulbocodioides	*Epulorhiza* sp. GZAAS 0001	*Ceratorhiza* sp. GZAAS 0003	*Epulorhiza* sp. GZAAS 0001
	Epulorhiza sp. GZAAS 0002	*Ceratorhiza* sp. GZAAS 0004	*Epulorhiza* sp. GZAAS 0002
	Ceratorhiza sp. GZAAS 0003		*Ceratorhiza* sp.GZAAS 0003
	Ceratorhiza sp. GZAAS 0004		*Ceratorhiza* sp.GZAAS 0004
P.yunnanensis	*Epulorhiza* sp. GZAAS 0005	*Ceratorhiza* sp. GZAAS 0006	*Ceratorhiza* sp.GZAAS 0006
	Ceratorhiza sp. GZAAS 0006	*Ceratorhiza* sp. GZAAS 0007	*Ceratorhiza* sp.GZAAS 0007
	Ceratorhiza sp. GZAAS 0007	*Ceratorhiza* sp. GZAAS 0008	*Ceratorhiza* sp.GZAAS 0008
	Ceratorhiza sp. GZAAS 0008		
C. appendiculata	*Epulorhiza* sp. GZAAS 0009	*Ceratorhiza* sp. GZAAS 0010	*Ceratorhiza* sp.GZAAS 0010
	Ceratorhiza sp. GZAAS 0010	*Ceratorhiza* sp. GZAAS 0011	*Ceratorhiza* sp.GZAAS 0011
	Ceratorhiza sp. GZAAS 0011	*Ceratorhiza* sp. GZAAS 0012	*Ceratorhiza* sp.GZAAS 0012
	Ceratorhiza sp. GZAAS 0012	*Monilioposis* sp. GZAAS 0013	*Monilioposis* sp.GZAAS 0013
	Monilioposis sp. GZAAS 0013	*Monilioposis* sp. GZAAS 0014	*Monilioposis* sp.GZAAS 0014
	Monilioposis sp. GZAAS 0014		
	Monilioposis sp. GZAAS 0015		

different orchid plants and different roots. It is therefore difficult to select pelotons from which living and culturable fungi can be isolated. In our technique, this problem is solved, as 1) pelotons with emerging hyphae can be selected from thousands of pelotons by incubating them in sterile distilled water, or 2 pelotons with emerging hyphae can be selected out from thousand of living pelotons by culturing them on artificial media disks 3) This is an economical method as 1 cm^2 PDA disks are used and less Petri dishes are required; 3-5 pelotons can be incubated on a 1 cm^2 medium disk. A 60 mm Petri dish holds 19 disks and 57-95 growing pelotons can be incubated.

Isolation and purification of several fungal taxa from a single peloton

Kristiansen *et al*. (2001b) first identified orchid mycorrhizae from single pelotons by molecular methods and confirmed that two different peloton-inhabiting fungi (*Tulasnella* and *Laccaria*) sometimes occurred together in a single peloton in cortex cells. However, it has not been previously possible to isolate several fungi from a single peloton. The technique presented in the paper provides an straightforward way to isolate all taxa from a single peloton. We found that *Epulorhiza* sp. GZAAS 0005 and *Ceratorhiza* sp. GZAAS 0006 were common mycorrhizal fungi in *Pleione yunnanensis*. Most mycorrhizae of these taxa existed singly within pelotons. However we also isolated them both from some pelotons using the purification method 2.

The implications and potential use of these techniques for further studies

The techniques presented here are a straightforward and workable method for isolating orchid mycorrhizae from the roots of wild orchids. However, in principle the technique can also be used to isolate endophytes of any plant organs with pelotons, such as protocorms, rhizomes, tubers and corms.

The technique can also be used to study dynamic changes in mycorrhizal communities. Mycorrhizal taxa from orchid roots can be confidently isolated during different seasons. The seasonal dynamic changes in the mycorrhizal communities and the environmental factors influencing fungal species could be analysed using this technique. The mycorrhizal taxa present at different growth periods could also be easily isolated. Knowledge of changes in mycorrhizal communities will provide more information concerning symbiotic relationships and allow mycorrhizae to be applied in the orchid industry.

Pelotons from which fungal hypae can be isolated can easily be identified and studied using these techniques. Fungi in pelotons readily grow out in the media because they readily obtain nutrition from the pelotons, orchid roots and endophytic bacteria. The fungi in pelotons can be isolated on artificial media and identified.

Unculturable fungi in pelotons can grow in sterile distilled water containing root extracts, but they cannot grow on artificial media. The hyphae of these unculturable taxa can be cut out and identified using molecular techniques (Kristiansen *et al*., 2001b).

Acknowledgements

This work was financed by Guizhou Elitist Fund (No:[2003] 200301) of China and Guizhou Natural Science Fund (No:[2005] 2020) of China. K.D. Hyde is thanked for correcting the manuscript

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