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Identification of *Armillaria* isolates from Bhutan based on DNA sequence comparisons

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Armillaria root rot is a serious disease in fir and mixed conifer forests of Bhutan, Eastern Himalayas. The species causing this disease have, however, never been identified. The aim of this study was to identify field isolates collected at four localities in Bhutan. Identification was based on RFLP analysis of the IGS-1 region, comparisons of ITS and IGS-1 sequence data with those available on GenBank, cladistic analyses and sexual compatibility studies. Isolates were found to reside in two distinct RFLP groups. RFLP group 1 isolates from *Pinus wallichiana* at Yusipang had RFLP profiles and IGS-1 sequences similar to those of *Armillaria mellea* ssp. *nipponica*. Although ITS sequence data are not available for *A. mellea* ssp. *nipponica*, sequences from this DNA region were most similar to the closely related *A. mellea* from Asia. The RFLP profile and IGS-1 sequences for RFLP group 2 isolates from *Abies densa* at Changaphug, *Tsuga dumosa* at Chimithanka as well as *Picea spinulosa* and *T. dumosa* in the Phobjikha valley were similar to those published for *Armillaria borealis*, *Armillaria cepistipes*, *Armillaria gemina* and *Armillaria ostoyae*. Distance analysis based on IGS-1 and ITS sequence data indicated that these isolates are closely related to *A. cepistipes*, *Armillaria gallica* and *Armillaria sinapina*. The isolates were, however, sexually incompatible with tester strains of *A. cepistipes*, *A. gallica* and *A. sinapina*. Although closely related to these species, they appear to represent a distinct taxon that will be referred to as Bhutanese phylogenetic species I (BPS I) until basidiocarps are found and the species can be described.

Keywords: *Armillaria mellea*, Armillaria root rot, Asia, Bhutan, biological species, Himalayas, IGS, ITS, phylogenetic species, RFLP

Introduction

Armillaria root rot is caused by various species of *Armillaria* (Tricholomataceae, Agaricales, Basidiomycetes). These fungi are pathogens occurring throughout temperate and most tropical regions of the world (Hood *et al*., 1991). *Armillaria* spp. survive as pathogens, saprobes or perthotrophs on woody trees and shrubs and tend not to show species-specific interactions with their hosts (Gregory *et al*., 1991; Termorshuizen, 2001). These survival strategies make *Armillaria* spp. serious pathogens capable of inflicting severe losses in forests and plantations. Historically, plant pathologists attributed armillaria root rot to the single species, *A. mellea*, based on the assumption that this is a highly pleomorphic species (Singer, 1956). This view changed with the adoption of a biological species concept for *Armillaria* and the subsequent identification of new biological species in Europe and North America (Korhonen, 1978; Ullrich & Anderson, 1978; Anderson & Ullrich, 1979). Based on morphological differences and sexual compatibility interactions, at least 36 species are now accepted in *Armillaria* (Volk & Burdsall, 1995).

A contemporary approach to the identification of *Armillaria* spp. has been to use DNA-based characteristics. Consequently, restriction fragment-length polymorphism (RFLP) profiles (Harrington & Wingfield, 1995) and DNA sequence data from the internal transcribed spacer (ITS) region (Coetzee *et al*., 2000, 2001), as well as the intergenic spacer region one (IGS-1) (Anderson & Stasovski, 1992) of the rRNA operon, have become available for most commonly known *Armillaria* spp. This has facilitated rapid

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identification of field isolates for which basidiocarps are not available.

The Kingdom of Bhutan is a small, landlocked country located in the Eastern Himalayas between China and India. The total area is 47 010 km^2, of which 64·2% is covered by forest (FAO, 2001). The dense forest cover of Bhutan is exceptional for southern and south-eastern Asia, which has generally been severely deforested. Forests are of immense socio-economic and ecological importance to Bhutan. Diseases affecting this natural resource therefore pose a great threat to the economic and social wellbeing of the country.

Very little is known regarding diseases in Bhutanese forests. Recent surveys have recorded a number of diseases, and have found armillaria root rot to be fairly common (Donaubauer, 1986, 1993; Nedomlel, 1994; Tshering & Chhetri, 2000; Chhetri & Gurung, 2002; Kirisits *et al*., 2002). Based on basidiocarp morphology, Nedomlel (1994) recorded the presence of *Armillaria ostoyae* in Bhutan. Apart from this record, virtually nothing is known regarding the identity of the *Armillaria* spp. causing root rot in conifer forests of this Himalayan country.

During the course of a survey of forest tree diseases in 2001 (Kirisits *et al*., 2002), typical symptoms and signs of armillaria root rot were found in various conifer forests in Bhutan. These included trees dying in patches, and white mycelial mats below the bark at the bases of dead and dying trees (Morrison *et al*., 1991). Rhizomorphs were also present in the soil and under the bark of dead and dying trees. Although basidiocarps were never encountered, it was possible to obtain diploid *Armillaria* isolates from dying trees. The aim of the present study was to identify field isolates from Bhutan using RFLP and DNA sequence data. Results from these DNA-based analyses were also evaluated using sexual compatibility tests with appropriate haploid tester strains.

Materials and methods

Collection sites

A total of 13 *Armillaria* isolates were collected from trees in fir and mixed conifer forests at four locations in Bhutan during July 2001 (Table 1). Collection sites included Changaphug, Yusipang and Chimithankha in the western part of the country (administration district Thimphu), and the Phobjikha valley in Central Bhutan (administration district Wangduephodrang) (Fig. 1). The high-altitude forests at Changaphug, which consist of Eastern Himalayan fir (*Abies densa*), suffered severely from a disease syndrome known as fir decline (Donaubauer, 1993). In the 1980s this syndrome resulted in the death of the majority of the trees at this site. This dramatic and widespread decline of fir in western Bhutan was thought to be caused primarily by prolonged drought, but various biotic agents, including *Armillaria* spp., were suggested to be involved as contributing factors (Donaubauer, 1986, 1987, 1993; Ciesla & Donaubauer, 1994). In the Phobjikha valley, isolates were collected in a stand of Eastern Himalayan spruce (*Picea spinulosa*) suffering from a local outbreak of the bark beetle *Ips schmutzenhoferi* (Schmutzenhofer, 1988; Kirisits *et al*., 2002). Obvious signs of armillaria root rot were present on spruce trees attacked by *I. schmutzenhoferi*. One isolate was also collected from Himalayan hemlock (*Tsuga dumosa*) at the same location. At Yusipang and Chimithankha, isolates were collected from Himalayan blue pine (*Pinus wallichiana*) and Himalayan hemlock, respectively. Armillaria root rot was not evident on living trees at the latter sites, but the isolates were included to gain a broader view of the occurrence and species composition of *Armillaria* in Bhutan.

Fungal isolation and cultivation

Isolates were obtained either from mycelial fans or from rhizomorphs found between the bark and the wood of dying trees or on stumps. Rhizomorphs from infected trees or stumps were surface sterilized in 96% ethanol for 1 min; small pieces from the inner parts were excised and placed on malt extract agar (MEA: 20 g L^{-1} malt extract, 16 g L^{-1} agar) or selective dichloran–benomyl–streptomycin (DBS) medium (Worrall, 1991). Isolation of samples from mycelial fans on infected trees, secondary isolations and maintenance of pure cultures followed the methods outlined by Coetzee *et al*. (2003). All isolates obtained from

Table 1 *Armillaria* isolates from Bhutan included in this study

Isolate number	Alternative number	Location in Bhutan	Host tree	RFLP group
CMW8081	Yus1	Yusipang	*Pinus wallichiana*	1
CMW8082	Yus2	Yusipang	*P. wallichiana*	1
CMW8084	Yus3	Yusipang	*P. wallichiana*	1
CMW8202	Yus4	Yusipang	*P. wallichiana*	1
CMW8095	Cha1	Changaphug	*Abies densa*	2
CMW8096	Cha2	Changaphug	*A. densa*	2
CMW10583	Phob2	Phobjikha valley	*Tsuga dumosa*	2
CMW10576	Phob3	Phobjikha valley	*Picea spinulosa*	2
CMW10577	Phob4	Phobjikha valley	*P. spinulosa*	2
CMW10578	Phob6	Phobjikha valley	*P. spinulosa*	2
CMW10579	Phob7	Phobjikha valley	*P. spinulosa*	2
CMW10581	Phob9	Phobjikha valley	*P. spinulosa*	2
CMW10582	Chim2	Chimithankha	*T. dumosa*	2

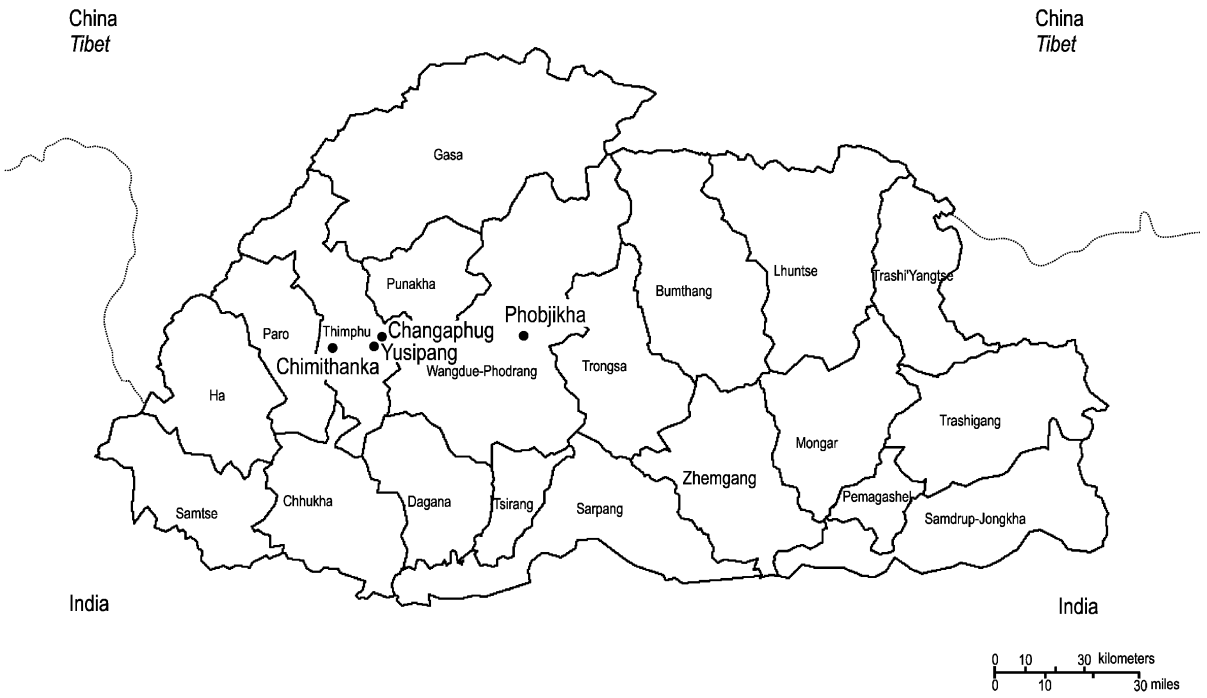

Figure 1 Map of Bhutan showing the administrative districts (dzongkhags) of the country and the four collection sites.

Bhutan are maintained in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI) (CMW), University of Pretoria, Pretoria, South Africa and the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria.

DNA extraction

Armillaria isolates were grown in liquid MY (10 g L^{-1} malt, 2 g L^{-1} yeast extract) medium at 24°C for 4 weeks in the dark. Mycelium was harvested using a sterile metal strainer, frozen at –70°C for 20 min and lyophilized. The freeze-dried mycelium was then ground to a fine powder in liquid nitrogen. DNA extraction from the powdered mycelium followed the method described by Coetzee *et al.* (2000).

Amplification of the ITS and IGS-1 regions

The ITS region (ITS1, 5·8S and ITS2) of the rRNA operon was amplified using primer set ITS1/ITS4 (White *et al.*, 1990). The IGS-1 region was amplified with primers CLR12R (Veldman *et al.*, 1981) and O-1 (Duchesne & Anderson, 1990). The PCR mixture and conditions for amplification of the ITS and IGS-1 regions were as described by Coetzee *et al.* (2003). Amplified ITS and IGS-1 PCR products were visualized on an agarose gel (10 g L^{-1} agar) stained with ethidium bromide under UV illumination.

RFLP analysis of the IGS-1 region

Restriction enzyme digestion of IGS-1 amplicons and separation of resulting fragments followed the procedure outlined by Coetzee *et al.* (2003). RFLP fragment sizes larger than 100 bp were determined with GELFRAG version 2·0·5 (National Center for Super Computing Applications, University of Illinois at Urbana Champaign, IL, USA). RFLP profiles obtained for the isolates were compared with those previously published for various *Armillaria* spp. from Asia, Europe and North America (Harrington & Wingfield, 1995; Schulze *et al.*, 1995; Banik *et al.*, 1996; Volk *et al.*, 1996; Coetzee, 1997; Chillali *et al.*, 1998; Frontz *et al.*, 1998; Terashima *et al.*, 1998; White *et al.*, 1998; Pérez-Sierra *et al.*, 1999; Coetzee *et al.*, 2000; Kim *et al.*, 2000, 2001).

DNA sequencing

DNA sequences of the ITS and IGS-1 regions were determined for a selection of isolates representing RFLP groups 1 and 2 (see Results). Two isolates were included from RFLP group 1 (Table 1). Isolates from RFLP group 2 were selected based on differences in origin and tree host species (Table 1). Thus one isolate was included from Changaphug (CMW8095) and Chimithanka (CMW 10582), respectively, and two isolates from Phobjikha valley (CMW10583 and CMW10581) that originated from different hosts. Isolate CMW10578 was also included in sequence analyses, as it had a RFLP profile different from the rest of the isolates in RFLP group 2. However, only sequences from the IGS-1 region were determined for this isolate.

DNA sequences for the ITS and IGS-1 regions were obtained using an ABI PRISM automated sequencer. PCR products were purified from unincorporated nucleotides and primer dimers prior to sequencing using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and eluted with 50 μL water. Sequence reactions were as described by Coetzee *et al*. (2003). The ITS region was sequenced in both directions using primers ITS1 and ITS4 as well as internal primers CS2B and CS3B (Coetzee *et al*., 2001). DNA sequences for the IGS-1 region were determined with primers CLR12R or P-1 (Hsiau, 1996), O-1 and primers MCO2 and MCO2R (Coetzee *et al*., 2000) that anneal to a region in the middle of the IGS-1 region.

Cloning of IGS-1 amplicons

IGS-1 PCR products from isolates that gave poor sequencing results were cloned into vector pCR®4-TOPO® after purification, as outlined above. Cloning reactions were done using a TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Carlsbad, CA, USA) with One Shot® TOP10 Chemically Competent *Escherichia coli* cells, following the manufacturer's directions. Positive inserts were verified by amplifying the IGS-1 directly from transformed *E. coli* cells. The PCR mixture included dNTPs (250 μM each), *Taq* polymerase (2·5 U) (Roche Diagnostics, Mannheim, Germany), PCR buffer with $MgCl_2$ (supplied by the manufacturer) and primers P-1 and O-1 (0·1 μM each). The final volume of the PCR reaction mix was brought to 50 μL with water. PCR conditions were as follows: 1 cycle at 95°C for 1 min (denaturation), 35 cycles of 60°C for 30 s (primer annealing), 70°C for 30 s (elongation) and 95°C for 30 s (denaturation). A final elongation step was allowed at 70°C for 7 min. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide. IGS-1 amplicons from 10 randomly chosen clones with positively transformed cells were digested with *Alu*I for each isolate, as described in the RFLP analysis section above. The selection of cloned IGS-1 amplicons for DNA sequencing, using the methods outlined above, was determined based on the differences in RFLP profiles. If all 10 cloned amplicons for the same isolate yielded identical profiles, two were selected for sequencing. If an isolate yielded two or more profiles, one amplicon was selected to represent each RFLP profile.

Sequence and phylogenetic analyses

ITS and IGS-1 sequences from representative isolates were compared with sequence data available on the National Center for Biotechnology Information (NCBI) databases using a nucleotide BLAST (Basic Local Alignment Search Tool) search. DNA sequences that showed a high similarity with the query sequence were downloaded and reanalysed using a Smith–Waterman local alignment algorithm (Smith & Waterman, 1981a, 1981b) within EMBOSS (Rice *et al*., 2000). This was followed by phylogenetic analyses to determine the relationship between the Bhutanese isolates and those *Armillaria* spp. with which they had high sequence similarity.

ITS and IGS-1 DNA sequences for representative isolates from Bhutan were aligned with sequences of various *Armillaria* spp. available on GenBank. Alignment was done with CLUSTAL X (Thompson *et al*., 1997) and manually corrected. Regions containing missing data were excluded from the 5′ and 3′ ends for all data sets. Phylogenetic analysis was based on distance methods using MEGA version 2·1 (Kumar *et al*., 2001). Distances were calculated based on pairwise deletion of gaps and missing data between taxa without correction of *P* values. Phylogenetic trees were generated using a neighbour-joining tree-building algorithm (Saitou & Nei, 1987). Confidence in branching points was determined by bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Sexual compatibility tests

Diploid isolates belonging to RFLP group 2 were paired with haploid tester strains of *Armillaria calvescens*, *Armillaria cepistipes*, *Armillaria gallica*, *Armillaria gemina*, *A. mellea* and *Armillaria sinapina* (Table 2) to confirm the

Table 2 *Armillaria* isolates used as testers in sexual compatibility tests

Species	Isolate number	Other numbers	Origin	Collector	Host
A. calvescens	CMW6893	PR-2, ss-2	USA	Banik MT	*Acer rubrum*
A. cepistipes	CMW6909	82-14-14	Canada	Morrison DJ	unknown
A. cepistipes	CMW6912	HHB–14868, ss-2	USA	Banik MT	*Alnus rubra*
A. cepistipes	CMW11262	IFFF 416, 92165	Finland	Korhonen K	*Salix caprea*
A. cepistipes	CMW11263	IFFF 417, 93288	Poland	Zólciak A	unknown
A. cepistipes	CMW11269	IFFF 441	unknown	unknown	unknown
A. gallica	CMW3169	B500, ATCC52114	USA	Anderson JB	unknown
A. gallica	CMW11272	IFFF 451	unknown	unknown	unknown
A. gemina	CMW3166	B735, AMP4B	USA	Worrall JJ	unknown
A. gemina	CMW3181	B485, ATCC52102	USA	Anderson JB	unknown
A. gemina	CMW6889	TJV 94-47, ss-2	USA	Banik MT	*Quercus velutina*
A. sinapina	CMW6894	HHB-14911, ss-9	USA	Banik MT	*Tsuga heterophylla*
A. mellea	CMW6901	IL-7, ss-3	USA	Banik MT	*Ulmus* sp.
A. mellea	CMW11271	IFFF 448	unknown	unknown	unknown

results of DNA-based identifications. Sexual compatibility tests were conducted on MEA medium (15 g L^{-1} Difco malt extract, 15 g L^{-1} Difco agar). Small (2 mm diameter) plugs from diploid Bhutanese cultures and haploid tester strains were placed 5 mm apart on the medium and incubated at 24°C in the dark. Mating reactions were evaluated after 4 and again after 6 weeks. Sexual compatibility tests were conducted at both FABI (for all tester strains) and IFFF (only for *A. cepistipes* and *A. gallica*).

Results

RFLP analyses of the IGS-1 region

All isolates from Bhutan resided in one of two groups based on their RFLP profiles (Table 1). These are referred to hereafter as RFLP group 1 and RFLP group 2 isolates. RFLP group 1 isolates yielded IGS-1 amplicons of *c.* 870 bp. The RFLP profile for this group had fragment sizes of 376 (374–379) and 166 (165–167) bp. This profile corresponded most closely to that of *A. mellea* ssp. *nipponica* from Japan (Terashima *et al.*, 1998).

RFLP group 2 isolates yielded IGS-1 amplicons of *c.* 910 bp. The RFLP fragment sizes for isolates in this group were 309 (305–316), 195 (189–199) and 139 (137–141) bp. Some variation was, however, observed among banding patterns for these isolates. The profile of isolate CMW10578 (Phob6), from the Phobjikha valley, differed slightly from that of the other isolates. RFLP fragment sizes for this isolate were 417, 313, 198 and 138 bp. A species name could not be assigned to isolates residing in RFLP group 2 because the banding patters were similar to those of four species: *Armillaria borealis*, *A. cepistipes*, *A. gemina* and *A. ostoyae* (Harrington & Wingfield, 1995; Pérez-Sierra *et al.*, 1999; Kim *et al.*, 2001).

Sequence analyses

RFLP group 1 isolates

IGS-1 DNA sequences for isolates CMW8082 and CMW8202 from Yusipang, residing in RFLP group 1, were most similar to those of *A. mellea* from Japan (AF163610) and South Korea (AF163613, AF163612 and AF163611) and *A. mellea* ssp. *nipponica* (D89922) (98%), as revealed by a BLAST search on GenBank and a Smith–Waterman local alignment. The highest ITS sequence identity for these Bhutanese isolates was with *A. mellea* (98–99%) from South Korea (AF163592, AF163593 and AF163591).

Phylogenetic trees generated from IGS-1 sequences (Fig. 2) placed isolates CMW8082 and CMW8202 in a strongly supported cluster that included *A. mellea sensu stricto* from Japan (AF163610) and South Korea (AF163611, AF162613) as well as *A. mellea* ssp. *nipponica* (100% bootstrap support). Neighbour-joining trees obtained from ITS sequences (Fig. 3) placed the two isolates in a strongly supported cluster (99% bootstrap support) that included isolates representing *A. mellea sensu stricto* from Japan (AF163594) and South Korea (AF163592 and AF163593).

RFLP group 2 isolates

The IGS-1 amplicons for representative isolates in RFLP group 2 could not be sequenced directly, and the fragments were subsequently cloned. Sequence heterogeneity within the IGS-1 repeat region of the rDNA was observed when comparing cloned IGS-1 amplicons from the same individual. IGS-1 sequence comparisons indicated the presence of one 4 bp indel and 31 nucleotide substitution sites. Loss of an *Alu*I restriction site was observed at positions 384–387 in sequence data from CMW10578 clone 3 and CMW10583 clone 4. An additional *Alu*I restriction site was observed at positions 773–776 for CMW10583 clone 8.

The highest IGS-1 sequence similarity for isolate CMW10583, from the Phobjikha valley, was with *A. cepistipes* (AF451083) (98%). ITS sequences for isolate CMW10583 had the highest identity with ITS sequences for *A. cepistipes* (AJ250053), *A. sinapina* (AF169646 and AY228346) and *A. gallica* (AY175808). DNA sequence similarities between these taxa and isolate CMW10583 were all 99% after a BLAST search on GenBank, but ranged from 99 to 99·8% using a Smith–Waterman search.

Neighbour-joining trees generated from the IGS-1 region grouped representative isolates (CMW8095, CMW10578, CMW10581 and CMW10583) from RFLP group 2 in a cluster (72% bootstrap support) (Fig. 4). Isolate CMW10578

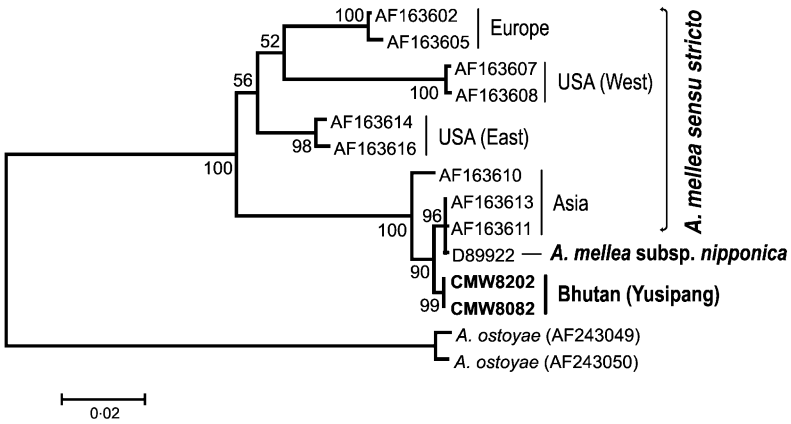

Figure 2 Neighbour-joining tree generated using IGS-1 sequence data (717 characters) from RFLP group 1. Numbers above and below the tree branches indicate the bootstrap support values for the branching nodes, respectively. The tree is rooted to *Armillaria ostoyae*. Scale bar: nucleotide substitutions per site. (GenBank accession numbers: CMW8082, AY554335; CMW8202, AY554334.)

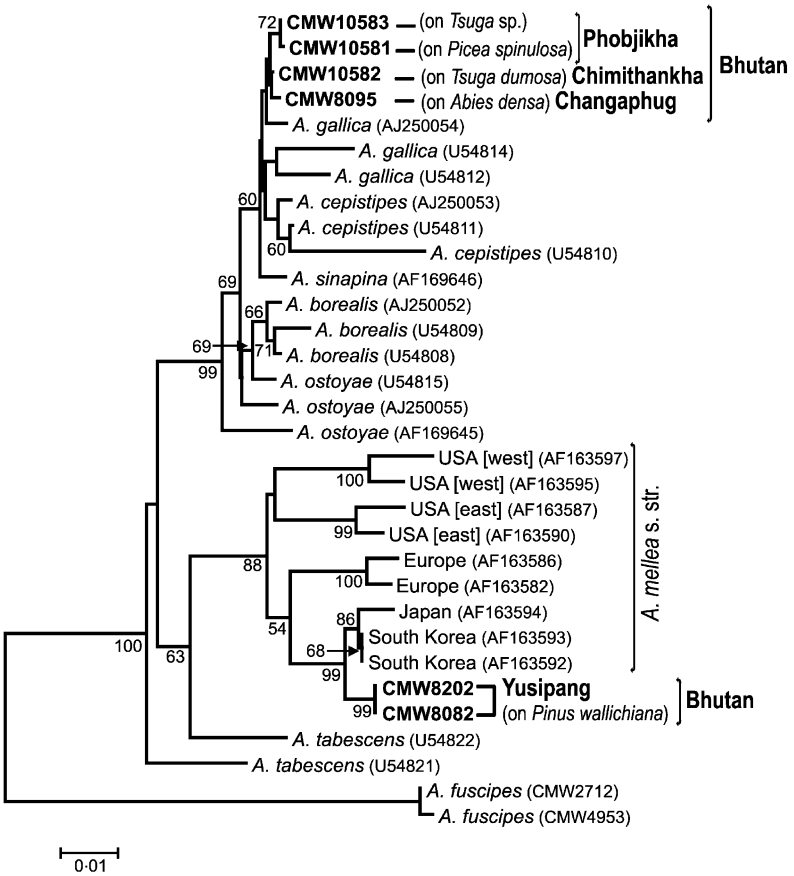

Figure 3 Neighbour-joining tree based on ITS sequence data (877 characters) for RFLP groups 1 and 2 from Bhutan. *Armillaria fuscipes* is used as the outgroup. (GenBank accession numbers: CMW8082, AY554333; CMW8202, AY554332; CMW8095, AY554330; CMW8096, AY554331; CMW10581, AY55429; CMW10583, AY554328.)

from Phobjika valley, which had a different RFLP pattern, grouped within this cluster. Neighbour-joining analyses placed the RFLP group 2 isolates within a major cluster that included AF451803 (92% bootstrap support) as well as AF451805 and AF451807 (55% bootstrap support) representing *A. cepistipes*. Neighbour-joining trees generated from the ITS data set placed isolates CMW10583, CMW10581, CMW10582 and CMW8095 from RFLP group 2 in a cluster that included *A. cepistipes* (U54811, U54810 and AJ250053), *A. gallica* (U54814, U54812 and AJ250054) and *A. sinapina* (AF169646) with 60% bootstrap support (Fig. 3).

Sexual compatibility tests

Haploid tester strains representing *A. calvescens*, *A. cepistipes*, *A. gallica* and *A. sinapina* were used for sexual compatibility tests because of close phylogenetic relationships with RFLP group 2 isolates. Tester strains of *A. mellea* and *A. gemina*, two species distantly related to the Bhutanese isolates, were included as negative controls. The haploid tester strains of these species retained their fluffy, white aerial mycelium when crossed with diploid isolates in RFLP group 2 (Fig. 5). Demarcation lines were also observed where mycelial growth from the different inocula interacted. These results indicate that the RFLP group 2 isolates from Bhutan are sexually incompatible with all tester strains included in this study.

Discussion

This study represents a first attempt to identify a collection of *Armillaria* isolates from Bhutan. As the isolates were from a variety of locations and hosts at different altitudes it was anticipated that a variety of *Armillaria* spp. would be found. RFLP analyses showed, however, that all isolates could be placed in one of two clearly distinct groups.

RFLP profiles of Bhutanese isolates from *P. wallichiana* at Yusipang (RFLP group 1) were similar to those previously published by Terashima *et al.* (1998) for the homothallic *A. mellea* ssp. *nipponica* from Japan. It was therefore suspected that RFLP group 1 isolates from Bhutan represent this subspecies of *A. mellea*. Phylogenetic analyses based on distance methods that incorporated IGS-1 and ITS sequence data were subsequently used to confirm this finding. Neighbour-joining trees generated in this study grouped the RFLP group 1 isolates in a strongly supported Asian *A. mellea* cluster, comprising isolates from Japan and Korea. Neighbour-joining trees generated from IGS-1 sequence data also included *A. mellea* ssp. *nipponica* in this cluster. The strongly supported grouping of this subspecies of *A. mellea* within the Asian cluster suggests that other isolates included in this cluster also represent *A. mellea* ssp. *nipponica*. Based on these findings, it is believed that the Bhutanese RFLP group 1 isolates belong to *A. mellea* ssp. *nipponica*.

Direct sequencing of the IGS-1 PCR products for representative isolates in RFLP group 2 was difficult, despite

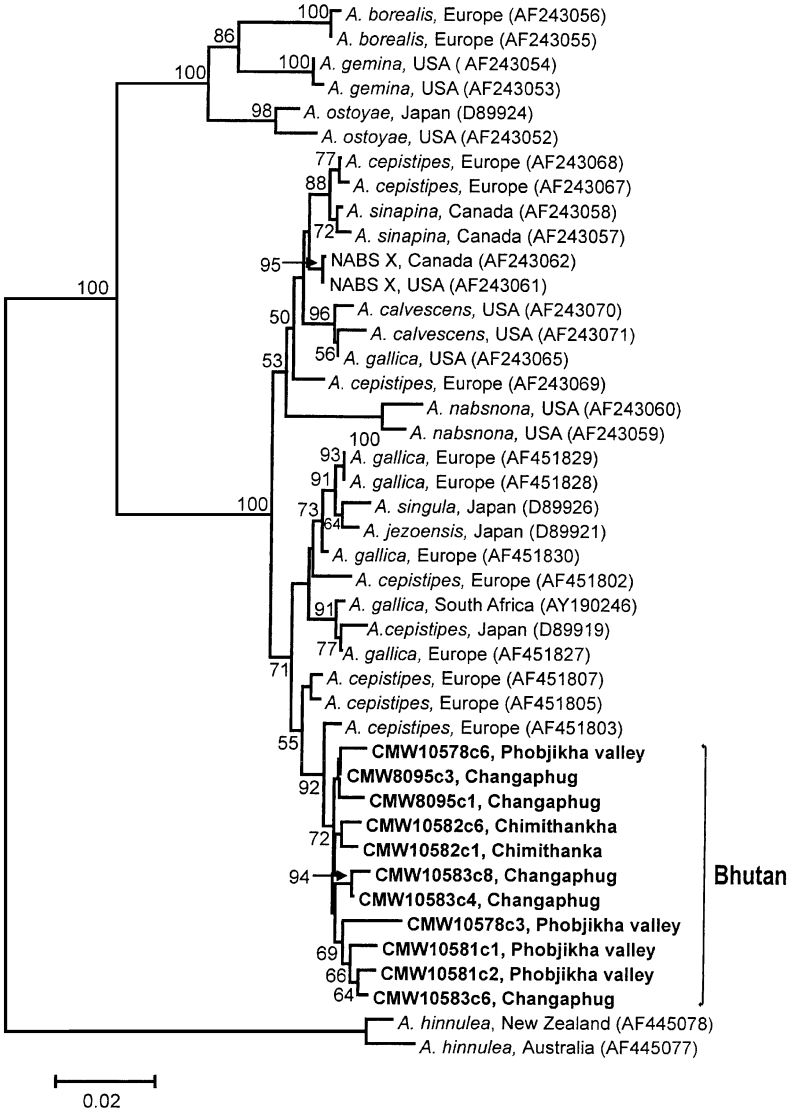

Figure 4 Neighbour-joining tree generated from IGS-1 sequence data (634 characters) for isolates from RFLP group 2. c-Numbers following the isolate number indicate the clone number for a specific isolate. Numbers above and below the tree branches indicate the bootstrap support values for the branching nodes, respectively. The outgroup taxon for this tree is *Armillaria hinnulea*. (GenBank accession numbers: CMW8095c1, AY624358; CMW8095c3, AY624359; CMW10578c3, AY624363; CMW10578c6, AY624364; CMW10581c1, AY624365; CMW10581c2, AY624366; CMW10582c1, AY624356; CMW10582c6, AY624357; CMW10583c4, AY624360; CMW10583c6, AY624361; CMW10583c8, AY624362.)

various attempts using different reaction conditions. The IGS-1 region forms part of the tandemly repeated rDNA multigene family (Long & Dawid, 1980). Sequences from a limited number of cloned IGS-1 fragments showed sequence heterogeneity among multicopies of this region; indicating intragenomic IGS-1 sequence variation within individuals. The limited data further indicated that the IGS-1 sequences could be separated into two nonorthologous (homologues that are not the result of speciation), intragenomic types based on the presence or absence of a 4 bp indel.

It was not possible to resolve fully the identity of isolates residing in RFLP group 2. This was firstly because their RFLP profiles resembled those of more than one *Armillaria* sp. Furthermore, overall there was poor statistical support for groupings based on phylogenetic analyses of ITS and IGS-1 sequences. However, it was clear that RFLP group 2 isolates are closely related to *A. cepistipes*, *A. sinapina* and *A. gallica*. The isolates were therefore considered to be part of the '*A. gallica* cluster' that includes *A. cepistipes*, *A. gallica*, *A. sinapina* and various other *Armillaria* spp. from the Northern Hemisphere (Korhonen, 1995). Species residing in this group are similar in having basidiocarps with a delicate annulus and a bulbous stipe base, thin cylindrical monopodially branching rhizomorphs, and saprophytic or weakly parasitic life cycles (Korhonen, 1995).

Isolates from Chimithankha, Changaphug and all but one of the isolates from Phobjikha valley had the same RFLP profile, and probably represent a single taxon. Isolate CMW10578 from *P. spinulosa* in the Phobjikha valley was the exception in having a RFLP profile slightly different from the rest of the RFLP group 2 isolates. Phylogenetic analyses, however, placed this isolate in a strongly supported cluster that included representative isolates from the same region and host. Despite RFLP and IGS-1 sequence variation, this isolate (CMW10578) is therefore thought to represent the same species as others in RFLP group 2.

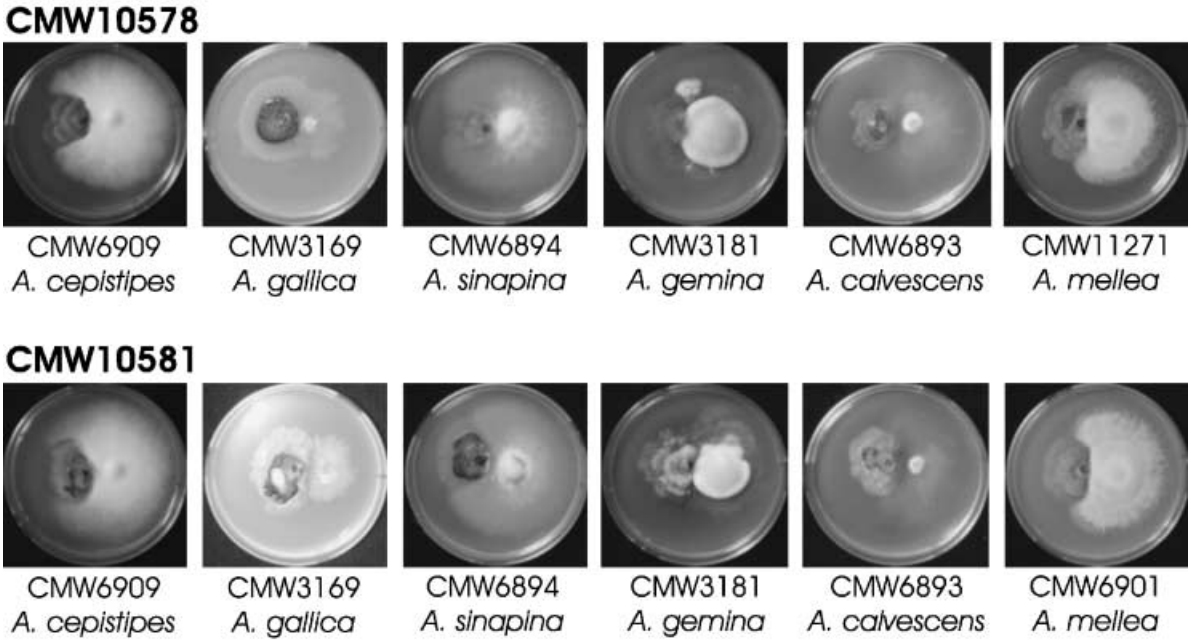

Figure 5 Two examples of results obtained after sexual compatibility tests between diploid RFLP group 2 isolates of *Armillaria* from Bhutan (left inoculum) and haploid tester strains (right inoculum).

Representative isolates in RFLP group 2 could not be identified based on mating tests. These isolates were clearly intersterile with those species (*A. cepistipes*, *A. gallica* and *A. sinapina*) phylogenetically most closely related to them. However, it is known that haploid tester isolates may become degenerate over time, in which case their culture morphology becomes depressed and moist and they lose their ability to mate with other haploid or diploid isolates *in vitro* (Guillaumin *et al*., 1991). There are thus three possible scenarios regarding the identity of the RFLP group 2 isolates. The first is that they belong to one of the phylogenetically closely related biological species mentioned above, but that mating tests failed to reveal this identity because the tester isolates had degenerated. This scenario is unlikely because the primary haploid tester isolates used in this study had white, fluffy aerial mycelia typical of nondegenerate isolates. The second scenario is that isolates represent one of the Indian (Himalayan) *Armillaria* spp. (Chandra & Watling, 1981) for which neither DNA sequence data nor reference cultures or tester strains for matings are available. The remaining scenario is that the isolates in RFLP group 2 represent an undescribed taxon. Until basidiocarps linked to this group of isolates can be collected and examined, their exact identity cannot be resolved. For the present, these will be referred to as Bhutanese phylogenetic species I (BPS I).

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