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
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 Chimithanka 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 Chimithanka, 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>
<thead>
<tr>
<th>Isolate number</th>
<th>Alternative number</th>
<th>Location in Bhutan</th>
<th>Host tree</th>
<th>RFLP group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMW8081</td>
<td>Yus1</td>
<td>Yusipang</td>
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<td>Yusipang</td>
<td>P. wallichiana</td>
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<tr>
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<tr>
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<td>Yus4</td>
<td>Yusipang</td>
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<td>Abies densa</td>
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<tr>
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<td>A. densa</td>
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<tr>
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<td>Phob2</td>
<td>Phobjikha valley</td>
<td>Tsuga dumosa</td>
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<td>Picea spinulosa</td>
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<td>Phobjikha valley</td>
<td>P. spinulosa</td>
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<td>P. spinulosa</td>
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<td>Chim2</td>
<td>Chimithanka</td>
<td>T. dumosa</td>
<td>2</td>
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</table>
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, 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 MgCl₂, (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 AluI 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 from 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>
<thead>
<tr>
<th>Table 2</th>
<th>Armillaria isolates used as testers in sexual compatibility tests</th>
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<tr>
<td>Species</td>
<td>Isolate number</td>
</tr>
<tr>
<td>A. calvescens</td>
<td>CMW6893</td>
</tr>
<tr>
<td>A. cepistipes</td>
<td>CMW6909</td>
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<td>CMW11263</td>
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<tr>
<td>A. gallica</td>
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<td>A. gallica</td>
<td>CMW11272</td>
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<td>A. gemina</td>
<td>CMW3166</td>
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<tr>
<td>A. gemina</td>
<td>CMW3181</td>
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<td>A. sinapina</td>
<td>CMW6889</td>
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<td>A. mellea</td>
<td>CMW6894</td>
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<td>CMW6901</td>
</tr>
<tr>
<td>A. mellea</td>
<td>CMW11271</td>
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</table>
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 \(A. \textit{ostoyae}\) and \(A. \textit{gallica}\)).

**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 \(A. \textit{mellea}\) ssp. \(\textit{nipponica}\) 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 patterns were similar to those of four species: \(A. \textit{borealis}\), \(A. \textit{cepistipes}\), \(A. \textit{gemma}\) and \(A. \textit{ostoyae}\) (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 \(A. \textit{mellea}\) from Japan (AF163610) and South Korea (AF163592, AF163593 and AF163591) and \(A. \textit{mellea}\) ssp. \(\textit{nipponica}\) (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 \(A. \textit{mellea}\) (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 \(A. \textit{mellea}\) ssp. \textit{stricto} from Japan (AF163610) and South Korea (AF163611, AF162613) as well as \(A. \textit{mellea}\) ssp. \(\textit{nipponica}\) (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 \(A. \textit{mellea}\) ssp. \textit{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 substitutions per site. The highest IGS-1 sequence similarity for isolate CMW10583 had the highest identity with ITS sequences from Japan (AF451083) (98%). ITS sequences for isolate CMW10583 had the highest identity with ITS sequences for \(A. \textit{cepistipes}\) (AJ250053), \(A. \textit{sinapina}\) (AF169646 and AY228346) and \(A. \textit{gallica}\) (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
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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 (U54810 and AJ250054), 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. geminea, 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
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 multicoys 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 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 hinnulae. (GenBank accession numbers: CMW8095c1, AY624358; CMW8095c3, AY624359; CMW10578c3, AY624363; CMW10578c6, AY624364; CMW10581c1, AY624365; CMW10581c2, AY624366; CMW10582c1, AY624357; CMW10583c4, AY624360; CMW10583c6, AY624361; CMW10583c8, AY624362.)
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).

Acknowledgements
We thank the Conifer Research and Training Partnership (CORET) (http://woek.boku.ac.at/coret), funded by the Austrian Development Co-operation (Austrian Ministry of Foreign Affairs) and the Royal Government of Bhutan, for the opportunity and partial funding to undertake this study. Georg Gratzer, Gerhard Glatzel, Lungten Norbu, Kinzang Wangdi, Phuntsho Namgyel and Purna B. Chhetri helped to organize the stay of Thomas Kirisits and Michael Wingfield in Bhutan in July 2001, during which the Armillaria isolates were collected. We also acknowledge the members of the Tree Protection Co-operative Programme (TPCP), the National Research Foundation (NRF) of South Africa, the THRIP initiative of the Department of Trade and Industry, South Africa and the Mellon Foundation for financial support. Furthermore, we are grateful to Mark Banik (Centre for Forest Mycology Research, Forest Products Laboratory, USDA Forest Service, Madison, WI, USA), Dr Kari Korhonen (Finnish Forest Research Institute, Vantaa, Finland) and Dr Ottmar Holdenrieder (Federal Institute of Technology, Department of Forest and Wood Sciences, Zürich, Switzerland) who provided us with tester strains used in this study. Thanks are also due to the staff of the Renewable Natural Resources Research Centre (RNRC), Yusipang, Thimphu, the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria and the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria for their technical assistance in the field and laboratory.

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