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# Geographical diversity of *Armillaria mellea* s. s. based on phylogenetic analysis

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***Abstract:*** *Armillaria mellea* was once thought to be a morphologically variable species with a wide distribution and a very broad host range. The relatively recent development of an indirect assessment of sexual incompatibility in *Armillaria* has led to identification of biological species within *A. mellea* s. l. Partial intergenic spacer region (IGS) sequences of the ribosomal RNA (rRNA) operon have been determined for most of the Northern Hemisphere species of *Armillaria*, but not for *A. mellea* s. s. The aim of this study was to determine the phylogenetic relationships among isolates of *A. mellea* s. s. based on DNA sequences from the IGS as well as the internal transcribed spacer (ITS) regions. The IGS and the ITS sequence data indicate that *A. mellea* is highly variable but phylogenetically distant from the other species of *Armillaria*, and there are four separate groups of *A. mellea* distinguished by their geographical origin: Asia, western North America, eastern North America and Europe. The data suggest that *A. mellea* populations from different regions are genetically isolated and may be in the process of speciation.

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## INTRODUCTION

The species *Armillaria mellea* (Vahl.: Fr.) P. Kumm remains taxonomically controversial. The controversy has arisen due to the misinterpretation that it is a highly pleiomorphic species with a broad host range (Singer 1956). The name *A. mellea* has been arbitrarily applied to all species causing Armillaria root disease.

In the late 1970s, sexual compatibility tests showed that *A. mellea* is comprised of a relatively large number of sexual incompatibility groups (Korhonen 1978, Anderson and Ullrich 1979). These groups were referred to as Northern American biological species (NABS) (Anderson and Ullrich 1979) and European biological species (EBS) (Korhonen 1978). Subsequent studies showed that at least eight NABS and five EBS occur in North America and Europe, respectively (Korhonen 1978, Anderson and Ullrich 1979, Morrison et al 1985a, Termorshuizen and Arnolds 1987).

Numerous molecular techniques have been applied to the *Armillaria* taxonomy problem as an adjunct to morphology and sexual compatibility studies. Molecular approaches have included isozyme and protein electrophoresis (Morrison et al 1985b, Wahlström et al 1991, Bragaloni et al 1997); comparisons of restriction fragment length polymorphisms (RFLPs) of nuclear DNA (nDNA) as well as analysis of RFLPs derived from DNA sequences such as the ribosomal RNA (rRNA) operon and mitochondrial DNA (mtDNA) (Anderson et al 1987, 1989, Chillali et al 1998, Smith and Anderson 1989, Harrington and Wingfield 1995, White et al 1998). These methods have shown that molecular techniques can effectively be used to identify the biological species of *A. mellea* s. l.

Based on basidiome morphology and molecular data, *A. mellea* s. s. has been shown to be distinct from the other species of *Armillaria*. Morphological characters such as the honey colored caps, prominent annulus, robust appearance, uninucleate sub-hymenial tissue and lack of clamp connections at the base of the basidia set this species apart (Korhonen 1978, Mot-

ta and Korhonen 1986, Bérubé and Dessureault 1988). *Armillaria mellea* s. s. is further characterized by a 4 kb insertion in its ribosomal RNA operon (Anderson et al 1989). Similarly, Miller et al (1994) showed by DNA-DNA hybridization that *A. mellea* s. s. formed a homogeneous cluster separated from the other species of *Armillaria*.

Anderson and Stasovski (1992) determined the DNA sequence of the first intergenic spacer region (IGS-1) for most of the North American biological species of *Armillaria* except *A. mellea* s. s. and *A. tabescens*. Using RFLP of IGS-1 to differentiate between Northern Hemisphere species of *Armillaria*, Harrington and Wingfield (1995) noted distinct differences between the RFLP profiles of these two species and the other Northern Hemisphere *Armillaria* spp. The aim of the present study was to determine the phylogenetic relationships among *A. mellea* s. s. and other species of *Armillaria* using IGS sequence data. Sequences of the ITS region were also obtained for *A. mellea* isolates in order to determine whether the phylogeny inferred from the IGS data is supported by another region on the rRNA operon.

## MATERIALS AND METHODS

*Fungal isolates.*—Haploid and diploid isolates of *A. mellea* s. s. originated from North America, France, Britain, Hungary, Japan, South Korea and Iran (TABLE I). The isolates are from the collection of T.C. Harrington but are also available from the culture collection of the Tree Pathology Cooperative Programme (TPCP).

*DNA analysis.*—Isolates were grown in liquid MY (2% malt extract and 0.3% yeast extract) at 22 C in the dark for 2 wk. Mycelium was harvested by centrifugation (15 300 *g*, 20 min), lyophilized and ground to a fine powder in liquid nitrogen. One mL of extraction buffer (Raeder and Broda 1985) was added to approximately 0.5 g of the powdered mycelium, followed by further pulverization in the extraction buffer and incubation at 50 C for 20 min. Cell debris was precipitated by centrifugation (15 300 *g*, 10 min) and a phenol:chloroform (0.5 v/v) extraction was performed on the aqueous phase until a clean interphase was obtained. A final chloroform (0.5 v/v) extraction was done to remove excess phenol. Nucleic acid was precipitated with sodium acetate (3 M) and isopropanol. The precipitate was collected by centrifugation, washed with 70% ethanol, dried and dissolved in 50 µL sterile water. RNase A (0.01 mg/µL) (Boehringer and Mannheim, South Africa) was added and incubated with the nucleic acids at 37 C to remove contaminating RNA. The DNA was quantified by UV spectroscopy using a Beckman Du Series 7500 UV Spectrophotometer.

*PCR.* Polymerase chain reaction (PCR) used extracted DNA as template for amplification of the IGS-1 and ITS regions (Saiki et al 1988). The IGS-1 region between the large subunit (LSU) and the 5S gene of the rRNA operon was amplified with primer set P-1 (Hsiau 1996) and O-1 (Duchesne and Anderson 1990). PCR fragments for the ITS1 and ITS2 regions including the 5.8S gene between the small subunit (SSU) and large subunit (LSU) were obtained with the primer set ITS1 and ITS4 (White et al 1990).

The PCR reaction mixture included dNTPs (200 µM of each), $MgCl_2$ (2.65 mM), Expand HF buffer containing $MgCl_2$ (supplied with the enzyme), 0.375 µM of each primer, Expand® High Fidelity PCR System enzyme mix (2.6 U) (Boehringer Mannheim, Germany) and approximately 80 ng of template DNA. The PCR reaction mixtures were overlaid with mineral oil to prevent evaporation. Reaction conditions were an initial denaturation at 96 C for 1 min, followed by 10 cycles of annealing at 56 C for 30 s, elongation at 72 C for 2 min, ramp time 1.5 and denaturing at 94 C for 15 s. This was followed by 20 cycles of 56 C for 30 s, 72 C for 2 min with a time increase of 20 s per cycle, followed by 94 C for 15 s. A final elongation step was allowed for 5 min at 72 C. The PCR products were purified prior to sequencing using a QIAquick PCR Purification Kit (250) (QIAGEN, Germany).

*DNA sequencing.* DNA sequences were determined using an ABI PRISM® 377 DNA sequencer. Both DNA strands of the IGS region were sequenced using the primers P-1, O-1, MCO-2 (5′ CTT GAT ATC GGC CTT ATG G 3′) and MCO-2R (5′ CCA TAA GGC CGA TAT CAA G 3′). Sequencing primers MCO-2 and the reverse compliment MCO-2R anneal to a complimentary sequence in the middle of the IGS-1 region. The ITS region was sequenced in both directions with primers ITS1, ITS4, CS2 and CS3 (Visser et al 1995). The sequence reactions were carried out using an ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) according to the manufacturer's directions.

*Sequence analysis.* DNA sequences were manually aligned by inserting gaps and analyzed using PAUP* (version 4.0b2a., Swofford 1998). The alignment of IGS-1 and ITS sequences are deposited in TreeBASE as SN241 and SN308. Analysis was done using heuristic searches with random stepwise addition of sequences (100 replicates), TBR (Tree Bisection Reconnection) branch swapping, MULPAR effective, MaxTrees was set to auto-increase and branches collapsed if maximum branch length is zero. Insertions and deletions (indels) of more than one base were considered to be the result of a single evolutionary event (Saitou and Ueda 1994) and were, therefore, replaced with an additional single symbol. Gaps were treated as a fifth character (newstate) in the analysis with indels included and indels excluded. Ambiguously aligned sequence regions were excluded from the data matrix before analysis. Tree-length distribution of 100 randomly generated trees was evaluated to assess phylogenetic signal (Hillis and Huelsenbeck 1992). Changes that occur with high frequency were downweighted to reduce homoplasy by including all phylogenetically informative characters but applying successive weighting according to the mean consistency index of each character. The confidence levels of the branching points were determined by 1000 bootstrap replicates. IGS-1 and ITS phylogenetic trees generated were rooted with *A. ostoyae* and *A. sinapina* as outgroup taxa. IGS-1 sequence data for the out-

TABLE I. *Armillaria mellea* s. s. isolates used in this study

| Culture no. | Alternative No.[a] | Host | Origin | Collector | GenBank | |
|---|---|---|---|---|---|---|
| | | | | | IGS-1 | ITS |
| B176 | CMW3179, M1 | *Rosa* sp. | Cambs Co., England | Rishbeth, J. B. | AF162602 | AF163578 |
| B186 | CMW4603, BQ5F | unknown | Straiton, England | Gregory, S. | AF163604 | AF163582 |
| B927 | CMW3964 | *Quercus* sp. | Orinda, CA, USA | Bruns, T. D. | AF163608 | AF163595 |
| B929 | CMW3962, #3 Haploid sagn 8 | *Mayten boria* | Berkeley, CA, USA | Bruns, T. D. | AF163607 | AF163596 |
| B1217 | CMW4619, 216 | unknown | CA, USA | Raabe, B. | AF163609 | AF163597 |
| B1240 | CMW4624, AM1 | unknown | England | Sierra, A-P. | AF163606 | AF163580 |
| B282 | CMW4605, TCH-2-1 | *Betula populifolia* | Durham, NH, USA | Harrington, T.C. | AF163616 | AF163587 |
| B495 | CMW3155, NABS VI, 49-5 | *Acer saccharum* | Boston, MA, USA | Anderson, J. B. | AF163614 | AF163589 |
| B497 | CMW3956, NABS VI, 97-1 | *Acer rubrum* | Provincetown, MA, USA | Anderson, J. B. | AF163615 | AF163590 |
| B623 | CMW4609, PJZ-87-1A | unknown | Durham, NH, USA | Zambino, P. J. | AF163617 | AF163588 |
| B525 | CMW3957, KD1 | unknown | France | Guillaumin, J-J. | AF163600 | AF163585 |
| B527 | CMW4607, PM8 | unknown | France | Guillaumin, J-J. | AF163599 | AF163584 |
| B1212 | CMW4615, 94056/1 | unknown | Hungary | Szanto, M. | AF163605 | AF163581 |
| B1245 | CMW4627, D5 | *Prunus* sp. | France | unknown | AF163598 | AF163586 |
| B1247 | CMW4628, P-5580 | *Chamaecyparis* sp. | England | Sierra, A-P. | AF]63601 | AF163579 |
| B608 | CMW3966, AF | unknown | South Korea | Sung, J-M. | AF163611 | AF163591 |
| B916 | CMW4610, A-5 | unknown | South Korea | Sung, J-M. | AF163612 | AF163592 |
| B917 | CMW4611, A-2 | unknown | South Korea | Sung, J-M. | AF163613 | AF163593 |
| B731 | CMW3967, 1003" | *Chamaecyparis* sp. | Japan | Shaw, C. G. | AF163610 | AF163594 |
| B1205 | CMW4613, 86009/1 | unknown | Iran | Saber, M. | AF163606 | AF163583 |

[a] CMW refers to the culture collection of the Tree Pathology Co-operative Programme (TPCP). ATCC refers to the American Type Culture Collection and B numbers refer to the culture collection number of T.C. Harrington.

group taxa was obtained from Anderson and Stasovski (1992). The ITS sequences for *A. ostoyae* (B481, GenBank AF169645) and *A. sinapina* (B493, GenBank AF169646) were determined in our laboratory.

DNA sequences of most of the Northern Hemisphere species (Anderson and Stasovski 1992) were manually aligned to the *A. mellea* s. s. IGS-1 sequences. The data were separately analyzed with similar methodology to the DNA sequences for the *A. mellea* s. s. isolates alone. Intra- and interspecific mean distances for the IGS-1 data were calculated for *A. mellea* s. s. isolates and for most of the Northern Hemisphere *Armillaria* species (Anderson and Stasovski 1992) using PAUP*.

## RESULTS

*IGS sequence data.*—Both DNA strands of the IGS-1 region (39 bp upstream from the 5′ of the 5S gene were not included) and part of the LSU (98 bp) of the rRNA operon were sequenced. Alignment was accomplished by inserting gaps, which resulted in a total of 801 characters. Thirty two regions considered to be large indels were replaced by a single character, which led to a reduction of 121 characters. Two regions of 33 and 19 ambiguously aligned characters respectively, were excluded from the data set. Six hundred and twenty eight characters were used in the final analysis which included 55 uniformative and 198 informative characters. The estimated transition/transvertion ratio for the data set with indels included was 0.986 and 0.985 for the data set with indels excluded.

The mean distances among isolates within various *Armillaria* species were determined with the ambiguously aligned characters excluded but the indels included. The intraspecific mean distances within the species of *Armillaria* studied by Anderson and Stasovski (1992) ranged between 0 and 0.009, with the exception of two anamolous isolates, isolate 304 of *A. cepestipes* and isolate 337 of *A. ostoyae*, which had intraspecifc mean distances greater than 0.009. The mean distances among the *A. mellea* s. s. isolates were much higher, up to 0.193, but the greatest difference was between isolates from four broad geographic regions: Asia, western North America, eastern North America and Europe. The mean interregional distances among isolates of *A. mellea* s. s. ranged from 0.038 to 0.193. Distances among isolates of *A. mellea* s. s. from a given country in Europe ranged from 0 to 0.006, while isolates from different European countries had mean distances ranging from 0.004 to 0.041. One isolate from Britain (B1247) had mean distances ranging from 0.057 to 0.062 within the British group of isolates. The mean distances among the *A. mellea* s. s. isolates from eastern North America range from 0.006 to 0.029, while the mean distance among isolates from western North America ranged from 0.003 to 0.013. Isolates of *A. mellea* s. s. from Asia had mean distances ranging from 0 to 0.023.

Three most parsimonious phylogenetic trees of 554 steps were generated from a heuristic search with indels included in the IGS-1 data set for *A. mellea* s. s., *A. ostoyae* (300) and *A. sinapina* (205). These trees had similar topologies and differed only in their branch lengths. The consistency index (CI) and retention index (RI) values for these trees were 0.864 and 0.33, respectively.

In a second analysis, indels greater than two bases were substituted with a single character. Two most parsimonious phylogenetic trees of 338 steps were generated with CI and RI values of 0.876 and 0.936, respectively. Two most parsimonious trees were produced in a second heuristic search with characters reweighted by mean value of consistency index (FIG. 1).

In all the analyses, four major clades of *A. mellea* s. s. emerged. Each of the four groups was comprised of *A. mellea* s. s. isolates from the same geographical region. The European clade consisted of isolates from Britain, France, Hungary and Iran. Isolates of *A. mellea* from North America were separated into two well-supported clades, with isolates from eastern North America in one clade and those from western North America in the second clade. The Asian clade was represented by isolates from Japan and South Korea.

*ITS sequence data.*—The ITS1, ITS2 and 5.8S gene of the rRNA repeat was completely sequenced in both directions. The data set also included 6 bp of SSU 3′ and 36 bp of the LSU 5′. Two analyses were performed using ITS sequence data, one with indels included and the other with indels excluded. The ITS DNA sequences were aligned by inserting gaps, which resulted in a total alignment of 944 characters. Nineteen indels greater than two bases were removed from the data set and replaced with a single character leading to reduction in 100 characters. One region containing 22 ambiguously aligned characters was excluded from the data set. The final data set consisted of 822 characters that included 53 uninformative and 149 informative characters. The estimated transition/transvertion ratio for the ITS data set with indels included and indels excluded was 1.310 and 1.243, respectively.

The mean distances among the ITS sequences of the *A. mellea* s. s. isolates were determined with indels included in the aligned data set. Mean distances among isolates from Europe ranged from 0.01 to 0.013. Among isolates from eastern North America the mean distance ranged from 0.035 to 0.062. Among isolates from western North America the range was 0.011 to 0.022, and among those from Asia

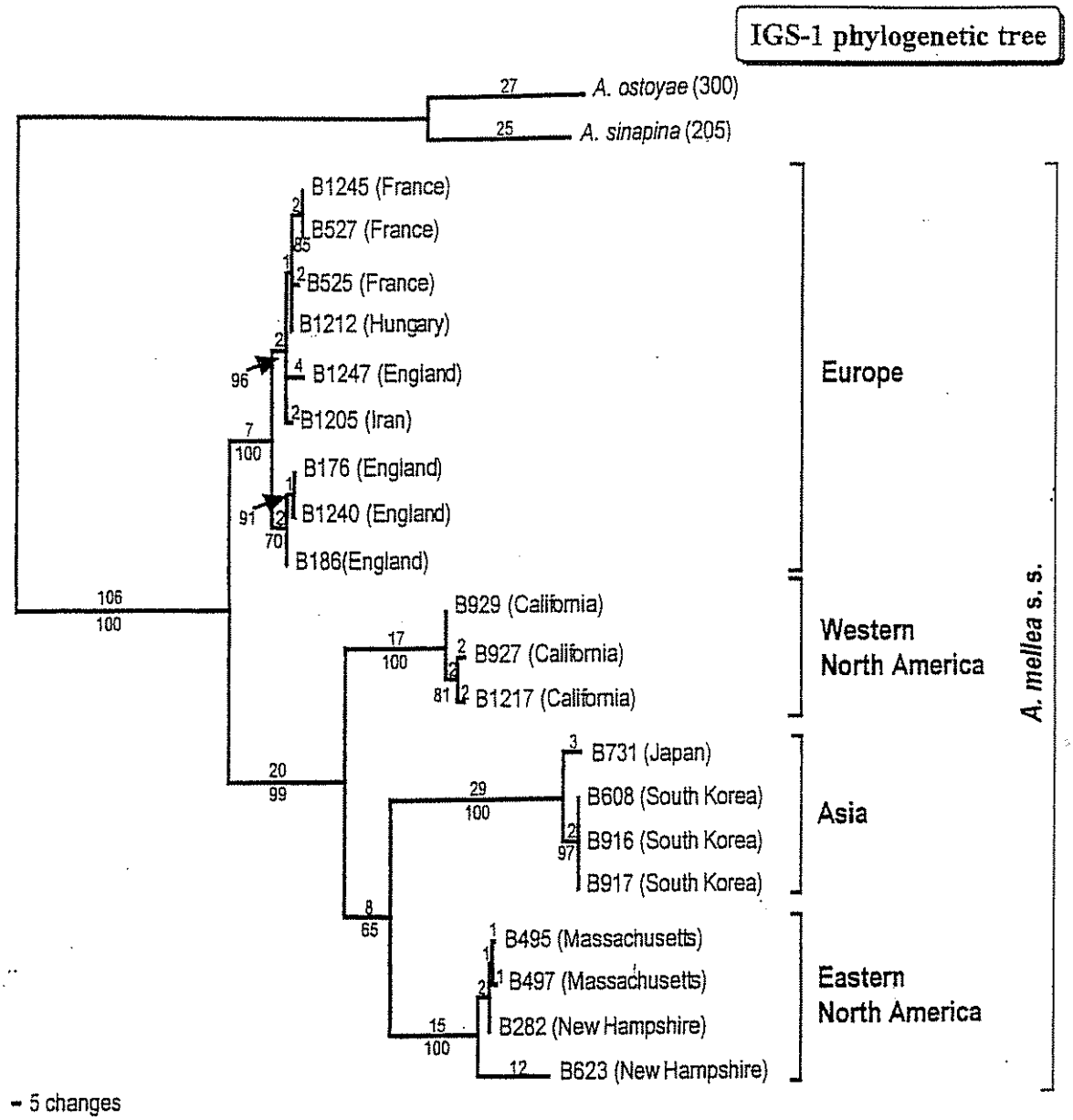


FIG. 1. One of the most parsimonious phylogenetic trees of *Armillaria mellea* isolates generated after a heuristic search from IGS-1 sequence data with indels excluded and characters reweighted. Bootstrap (1000 replicates) values are given below the branches and the number of base substitutions are indicated above the branches. Length of the tree = 296 steps, CI = 0.932, RI = 0.963 and g1 = −0.95.

the range was 0.004 to 0.014. The mean distances among the isolates from the four regions had a range from 0.083 to 0.226.

Sixty-four most parsimonious trees emerged from the search with ITS data set with indels included. The length of the trees was 469 steps and the CI = 0.819 and the RI = 0.899. This tree clearly separated four strongly supported (bootstrap values of 100%) clades: the Asian, European, western and eastern North American clades.

In a second analysis, with indels excluded from the ITS sequences, 114 most parsimonious trees of 311 steps were generated from a heuristic search. The CI and RI values were 0.775 and 0.853, respectively. Five most parsimonious trees were retained after a second heuristic search when characters were reweighted using the mean value of the consistency index (FIG. 2). As in the ITS tree with indels included, isolates from Europe, eastern North America, western North America and Asia grouped in four strongly supported clades, and the western North American clade grouped with the Asian clade.

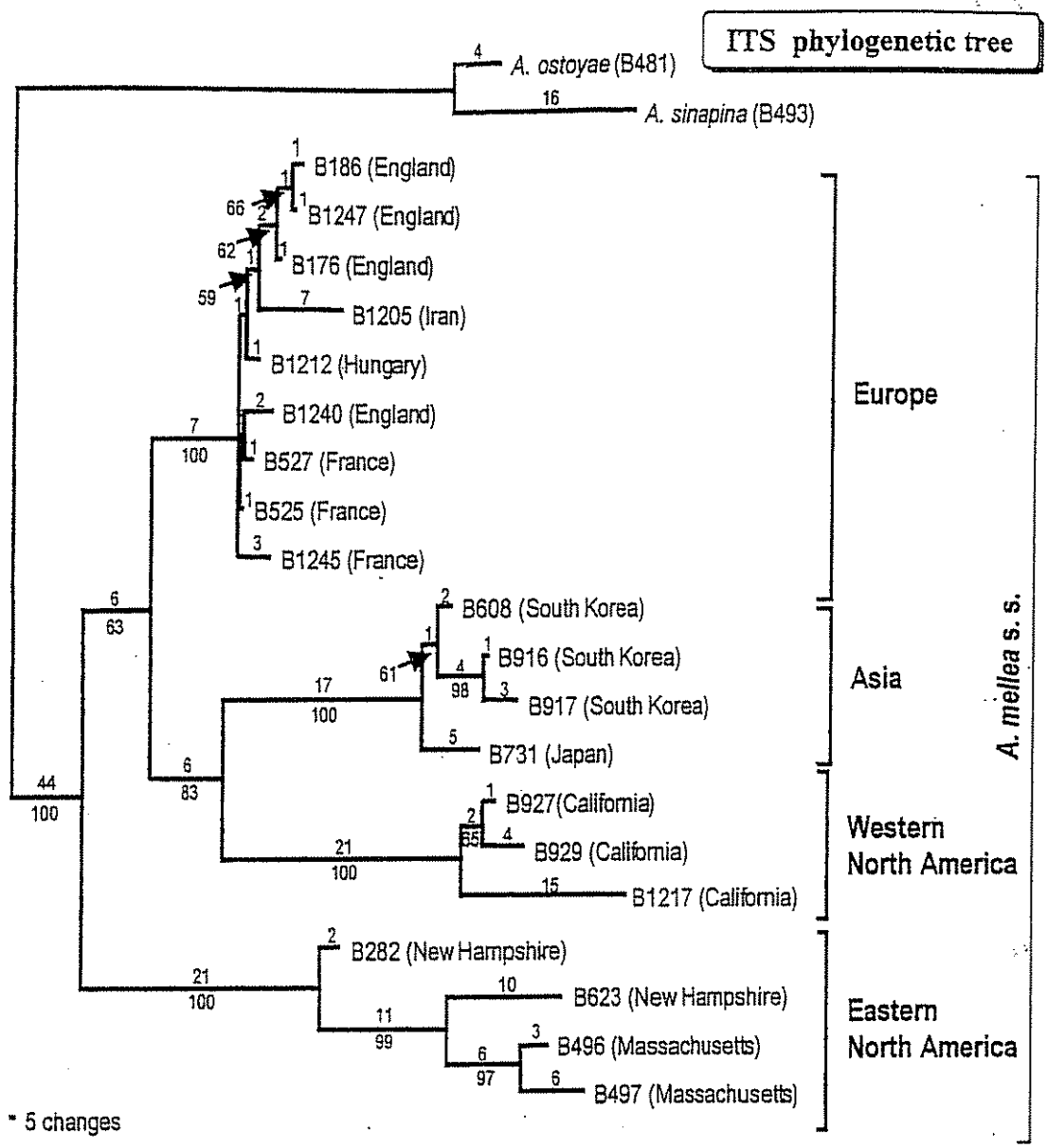


FIG. 2. One of the most parsimonious phylogenetic trees of *Armillaria mellea* isolates generated from ITS sequence data with indels excluded after a heuristic search with characters reweighted. Bootstrap (1000 replicates) values are indicated below the branches. Numbers above the branches indicate the number of base substitutions. Length of the tree = 241 steps, CI = 0.872, RI = 0.914 and g1 = −1.04.

## DISCUSSION

Phylograms for both the IGS and ITS regions separated *A. mellea* s. s. into four geographical lineages, but the relationship among the geographic lineages were not well resolved. The eastern and western North American isolates of *A. mellea* s. s. were well separated. The ITS sequences but not the IGS sequences grouped the California isolates closest to the Asian isolates.

This geographical differentiation based on ITS and IGS sequences is in contrast to interfertility based on diploid formation (Anderson et al 1980) and similarity in basidiocarp morphology between *A. mellea* s. s. from eastern North America and Europe (Motta and Korhonen 1986). Ota et al (1998) found that *A. mellea* s. s. isolates from Japan were compatible with *A. mellea* s. s. tester isolates from France but only partially compatible with *A. mellea* s. s. tester isolates from eastern North America. Anderson et al (1989) showed that isolates of *A. mellea* s. s. from Europe and eastern North America differed in their *Eco*RI, *Bam*HI and *Sal*I fragment patterns of the rRNA op-

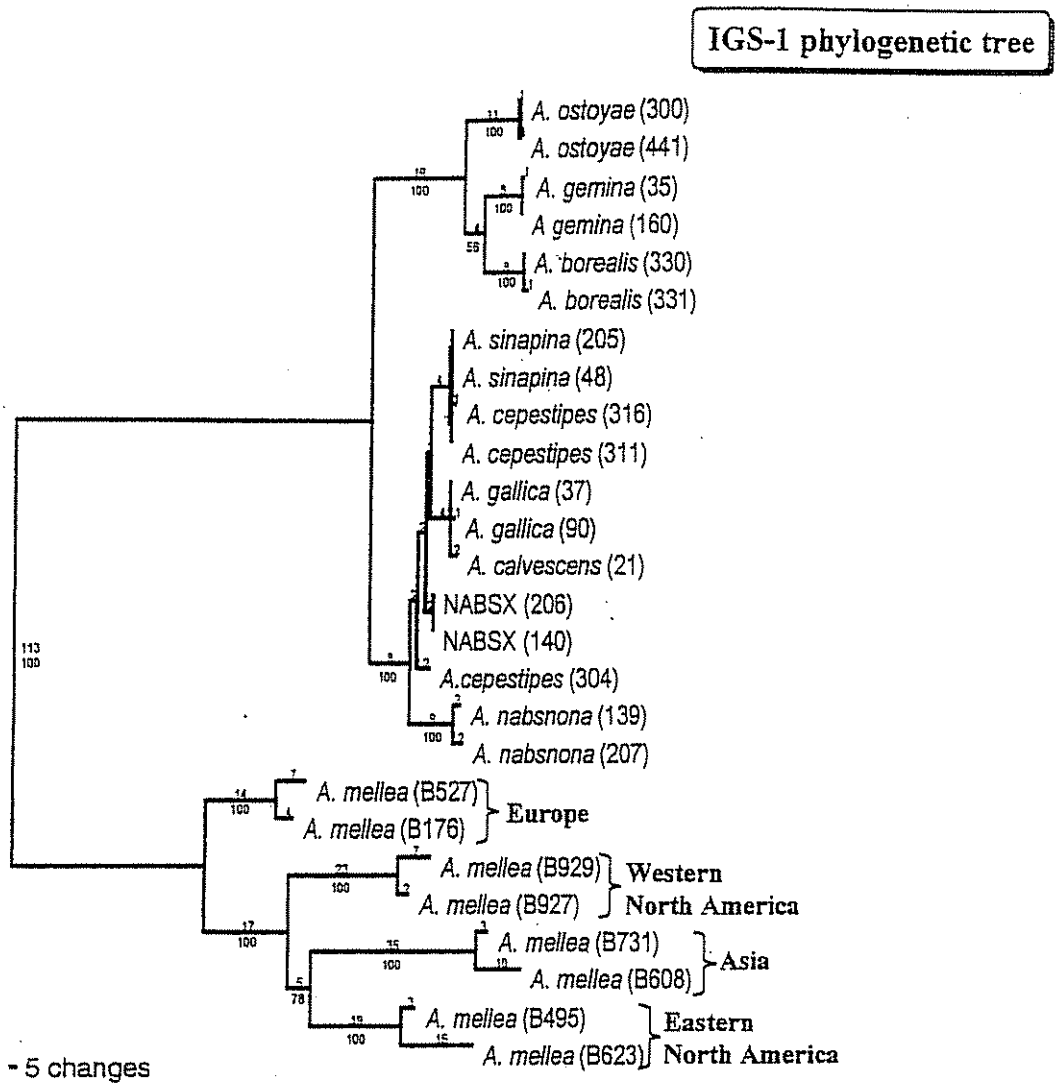


FIG. 3. One of the most parsimonious trees generated after a heuristic search from the IGS-1 DNA sequence data for most of the Northern Hemisphere biological species of *Armillaria* (data from Anderson and Stasovski 1992) and geographical representative *A. mellea* s. s. isolates.

eron. Thus, sexual compatibility and similar basidiocarp morphology have persisted in these geographic lineages.

Our analysis of IGS-1 sequence data showed that *A. mellea* s. s. forms a monophyletic group basal to the rest of the Northern Hemisphere biological species of *Armillaria* (data not shown). The phenogram derived from DNA-DNA hybridization similarities (Miller et al 1994), the dendrogram generated from ITS DNA sequence data (Chillali et al 1998), and the phylograms generated from anonymous DNA sequences obtained from different primer sets (Piercey-Normore et al 1998) also found *A. mellea* s. s. to be basal to the other annulated Northern Hemisphere species (FIG. 3).

The ITS region of the rRNA operon is known to contain lower genetic variability than the IGS region (Lee and Taylor 1992, Henrion et al 1994, Schulze et al 1997). Mean distances among *A. mellea* s. s. isolates based on ITS sequences were lower than those for the IGS region. Overall, the distances found within *A. mellea* s. s. were higher than intraspecific distances found in other *Armillaria* species. Distance analysis of the IGS sequence data gave mean distances that were smaller between sympatric isolates than between the allopatric isolates of *A. mellea* s. s., but even these within region differences were relatively high. This indicates that *A. mellea* s. s. is a more genetically diverse group than we originally suspected.

Allopatric isolates of *A. mellea* s. s. are highly divergent indicating that these four populations are genetically isolated. This divergence can be explained by hypothesizing that *A. mellea* s. s., as defined by intersterility tests and morphology, is in a process of

speciation, due to geographical barriers. This hypothesis implies that *A. mellea* s. s. is an ancient species that split from the other *Armillaria* long ago. The sequence data suggest that the eastern and western North American populations of *A. mellea* s. s. do not have a most recent common ancestor. Allopatric speciation results from an increased difference in genetic diversity between geographically isolated populations where ecological or physical barriers between the populations exist (Burnett 1983). Increase in genetic divergence, however, does not necessarily parallel development of intersterility barriers between isolated populations. Studies with basidiomycetes such as *Collybia dryophila* (Vilgalys 1991), *Heterobasidion annosum* (Garbelotto et al 1993) and *Pleurotus ostreatus* (Vilgalys and Sun 1994) showed that isolates from different geographical origins, that are interfertile with each other, differed at the genetic level. This correlates well with results from this study where it is shown that geographically separated interfertile isolates, identified as *A. mellea* s. s., differed at the genetic level.

This study has shown that *A. mellea* s. s. is a heterogeneous species that is distantly related to the other species of *Armillaria*. Based on RFLP profiles and sequence data, sympatric isolates could be grouped into clades that reflect distinct geographic lineages. The distances between allopatric isolates were much greater in both the ITS and the IGS sequences than those for the other species of *Armillaria*. Based on these observations it is suggested that the isolates represent biological entities in the process of speciation.

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