Sequence-based identification and ecology of *Armillaria* species on conifers in Japan

（日本の針葉樹に産するナラタケ属菌のDNA配列による識別と生態）

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Contents

Introduction

The genus *Armillaria* (Fr.:Fr.) Staude is distributed in the northern and southern hemispheres and includes pathogens causing root rot in a wide variety of trees and shrubs (Hood et al 1991).

For almost 100 years following the publication of the first monograph on this disease (Hartig 1874), many forest pathologists referred to the cause of this disease as a single fungal species "*Armillaria mellea*". This recognition made literature on forest pathology extremely confusing. The fungus was considered to have a polymorphic fruit body, a wide host range and also wide distribution. Moreover, various pathogenic roles were described: an aggressive killer of healthy trees, a secondary pathogen of stressed trees, and a saprotroph of dead trees (Wargo and Shaw 1985).

Other fungal taxonomists, on the other hand, had described a much larger number of different species than the number now accepted in the genus (Volk and Burdsall 1995). Because of the difficulty with studying the fruit body morphology using traditional characters, and the extremely variable generic concept, the taxonomy of *Armillaria* became complicated and confusing (Volk and Burdsall 1995).

This controversy has been largely resolved by the introduction of the "biological species concept" to the taxonomy of *Armillaria* by Ullrich and Anderson (1978) and Korhonen (1978), and by subsequent taxonomical re-examination in the northern hemisphere. Most of the biological species are now also equated with morphological species (Volk and Burdsall 1995).

With the new species concept, forest pathologists have begun to describe the ecology of each of the *Armillaria* species (Guillaumin et al 1993, Tsopelas 1999, Keča et al 2009). At the same time,

molecular techniques have been developed for rapid and reliable identification of fungal species (Kim et al 2006 and references therein). This type of technological innovation that can treat many samples at a time is of great importance for ecological studies on the genus *Armillaria*, because *Armillaria* species are often sympatric in the same forest stand (Prospero et al 2003, Keća et al 2009), and collected frequently as mycelial mats or rhizomorphs rather than as fruit bodies.

In Japan, Armillaria root disease affects many tree species (The Phytopathological Society of Japan 2000). In particular, the damage inflicted on plantation conifers has commercial importance. Approximately 10 different biological species of *Armillaria* have been reported in Japan (Ota et al 1998b). However, very few studies have examined the host specificity of *Armillaria* species on conifers. Information of ecology, including pathogenicity and host specificity, of each species is indispensable for control of Armillaria root disease. This study aims to evaluate three molecular markers for species identification of *Armillaria*, and analyze the ecology of Japanese *Armillaria* species on conifers using the most effective marker. In chapter 1, studies on the species concepts and ecology of *Armillaria* is reviewed. In chapter 2, species identification methods using the internal transcribed spacer (ITS), intergenic spacer (IGS), and the translation elongation factor-1α (EF-1α) gene are evaluated. In chapter 3, pathogenic roles of Japanese *Armillaria* species are discussed based on field observation with identification using the EF-1α gene.

1 Literature review: Species concept and ecology of *Armillaria*

1-1 History of *Armillaria* taxonomy

Morphological species

In the binomial nomenclature, taxonomic position of a species is directly affected by the generic concept. The nomenclature and taxonomy of the genus *Armillaria* was long surrounded by confusion, resulting in difficulties in describing and naming individual species. Volk and Burdsall (1995) listed at least 274 species and varieties that have been placed in the genus *Armillaria* (or in *Armillariella* Karst., its obligate synonym), whereas approximately 40 species are accepted in the modern description of the genus. They also listed 44 genera in which the species that were once placed in the genera *Armillaria* or *Armillariella* should now be accommodated. On the other hand, some authors moved many species from *Armillaria* to other genera. Notably, the present-day type species of *Armillaria* was moved to the genera *Clitocybe* and *Lepiota* (Volk and Burdsall 1995). One reason for this confusion was the extremely variable generic concept, which arose from the recognition of different type species (Watling et al 1982, Volk and Burdsall 1995).

The first record of *Armillaria* species was probably in 18th century (Watling et al 1991). However, several taxa now assigned to *Armillaria* in its strict sense could not be recognized unequivocally until the later classical authors began to describe the larger fungi. According to Watling et al (1982) and Volk and Burdsall (1995), Fries established the name tribus *Armillaria* in genus *Agaricus* in 1819, and later treated it in the Systema Mycologicum in 1821, but in subsequent publications, Fries was uncertain as to which species to include, or even whether to consider

Armillaria as a tribe[1] (Fries 1819, 1821). In 1825, Fries abandoned the tribe *Armillaria*, placing most of the species in the tribe *Lepiota* of *Agaricus* (Fries 1825). In 1838, Fries again recognized the tribe *Armillaria* of *Agaricus*, including 24 species (Fries 1838). However, in 1854 and 1857, Fries again abandoned the tribe *Armillaria* and distributed the species in the tribes *Tricholoma* and *Clitocybe* (Fries 1854, 1857). Nevertheless, in 1874 Fries again recognized the tribe *Armillaria*, including 30 species, even after several authors had accepted *Armillaria* as a distinct genus (Fries 1874, Volk and Burdsall 1995).

Several taxonomists assigned the generic rank to *Armillaria* independently, and nomenclatural problems and confusion have arisen out of the variously accepted validations of *Armillaria* at the generic level and the interrelated typifications of the names. Watling et al (1982) argued that Staude was the first to raise *Armillaria* to the generic rank in 1857, and proposed the name "*Armillaria* (Fr.: Fr.) Staude," selecting *Agaricus melleus* Vahl:Fr. (=*Armillaria mellea* (Vahl: Fr.) P. Kumm.) as the type species (Staude 1857). This proposal is now widely accepted (Watling et al 1991, Volk and Burdsall 1995).

Karsten (1881) raised the genus *Armillariella*, with the type species presumably being *Armillariella mellea* (Vahl:Fr.) Karsten. The genus *Armillariella* gained wide acceptance. Today, this genus is considered an obligate synonym of *Armillaria* (Watling et al 1991, Volk and Burdsall 1995). It should be noted here that the controversy surrounding *Armillaria* and *Armillariella* only concerned the names of the genera, not the circumscription of the generic concepts (Volk and Burdsall 1995).

[1] "Tribes (tribus)" in Fries's "Systema mycologicum (1821-1832)" are subdivisions of genera (McNeill et al 2006).

However, according to Watling et al (1982), Singer accepted both *Armillariella* and *Armillaria* in 1936 (Singer 1936). He recognized *Armillaria* as a genus of ectomycorrhizal species and selected *Agaricus luteovirens* Alb. & Schw. :Fr. as the type species. This species was not originally included in Fries' tribe and this typification should not be accepted. In 1957, *Ag. luteovirens* was accommodated in the genus *Floccularia* by Pouzar (Pouzar 1957, Watling et al 1982, Volk and Burdsall 1995). Watling et al (1982) listed names of six species that have ever been typified to *Armillaria* or *Armillariella*, and they rejected all but *Ag. melleus*.

Another typonym of *Armillaria* is *Polymyces* Earle. On the other hand, *Rhizomorpha fragilis* Roth, is apparently a rhizomorph of *Armillaria* (Hartig 1874), although it is not possible at this time to correlate species of *Rhizomorpha* with particular *Armillaria* species (Volk and Burdsall 1995).

By the end of the 20th century, nomenclature and typification of *Armillaria* had been re-arranged, and previously reported species that should be included in *Armillaria* had been confirmed (Watling et al 1982, Volk and Burdsall 1995). Volk and Burdsall (1995) made comments on the generic characters of *Armillaria* as follows:

"…the modern concept of the genus *Armillaria* (Fr.:Fr.) Staude includes tricholomatoid Basidiomycotina with basidiomata usually emerging from black rhizomorphs, with adnate to decurrent gills bearing basidia with pale, nonamyloid, non-dextrinoid basidiospores. The nutritional status is saprophytic to parasitic (wood decay or root rot fungi) and generally not mycorrhizal."

Further, in relation to *Clitocybe*, Watling et al (1982) argued that the presence of a veil (not a

ring) on the fruit body is the defining characteristic of *Armillaria*. The salient characteristics of *Armillaria* listed by Watling et al (1991) are presented in Table 1-1.

Provided that the modern generic concept had been confirmed, species delimitation in *Armillaria* still needed rearrangement. Some authors, including Romagnesi (1970, 1973), published comprehensive taxonomic studies on *Armillaria,* indicating that the genus could be divided into a larger number of species, any of which are probably native to different geographical regions or associated with particular vegetation types and therefore with different significance for plant pathology (Watling et al 1982). However, because of the difficulties in studying the fruit body using traditional characters, forest pathologists had considered the pathogen of Armillaria root disease as a single species "*Armillaria mellea*," with variable characteristics (Watling et al 1991, Volk and Burdsall 1995). Such tradition kept the species in the genus ambiguous and controversial.

Biological species

In the 1970s, annulate *Armillaria* proved to be a complex of several biological species (Korhonen 1978, Ullrich and Anderson 1978). According to Mayr's definition, biological species are "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1982). Mayr's idea changed the species concept from a rank in the taxonomic hierarchy to a reproductive unit, integrating systematics under the influence of evolutionary biology (de Queiroz 2005).

Hintikka (1973) in Europe developed a technique for determining mating types in *Armillaria*. Unlike other basidiomycete fungi, a hyphal cell of *Armillaria* has one diploid nucleus instead of

two haploid nuclei and lacks clamp connection (Guillaumin et al 1991). Thus, the number of nucleus per cell or formation of clamp connections cannot be used as a criterion for mating success. Instead, Hintikka (1973) observed changes in culture morphology. Cultures of a single-spore (haploid) isolate have a white, fluffy appearance, whereas in pairings, the morphology changes to dark and crustose. Because the latter morphology was identical in the tissue (diploid) isolates, Hintikka (1973) considered the change in culture morphology in pairings as a sign of a fully compatible reaction. He found tetra-polar mating system (= bifactorial heterothallism) by pairing single-spore isolates derived from a single fruit body. That is, alleles on two loci determine the mating system, and no other combinations of haploid isolates with different alleles on each of the two loci can be fully compatible.

Hintikka's interpretation was also confirmed by Ullrich and Anderson (1978) in North America using pairings of auxotrophic haploid isolates. Furthermore, by pairing single-spore isolates derived from different fruit bodies, they found intersterility among strains of annulate *Armillaria*. Korhonen (1978) also reported five intersterility groups (i.e., biological species) in European annulate *Armillaria*. The biological species in Europe have been confirmed to correspond to taxonomic species: their characteristics are sufficiently different to be described as distinct species (Guillaumin et al 1991). Once the relationships between morphological traits and the biological species were established, the delimitation of species in *Armillaria* was greatly facilitated by sexual compatibility tests (Harrington and Rizzo 1999). Biological species of *Armillaria* have been reported in Europe, North America, Australasia and Asia (Guillaumin et al 1991, Sung et al 1994, Ota et al 1998b, Asef et al 2003, Qin et al 2007). These species are listed in Table 1-2.

Nine biological species of annulate *Armillaria* have been reported in North America (North

American Biological Species; NABS). Eight of them have been described taxonomically, and four of the eight were conspecific with European species (Guillaumin et al 1991, Banik and Burdsall 1998). European and North American populations belonging to *A. mellea*, *Armillaria gallica* Marxmüller & Romagn. and *Armillaria cepistipes* Velen. were highly compatible within the species. However, only partial compatibility was observed between European and North American isolates of *Armillaria ostoyae* (Romagn.) Herink (also described as *Armillaria solidipes* Peck (Burdsall and Volk (2008)). Interestingly, partial compatibility at a lower level was also observed between different allopatric intersterility groups: *A. cepistipes* in Europe is partially compatible with North American *Armillaria sinapina* Bérubé & Dessur. and taxonomically undescribed NABS X (Guillaumin et al 1991, Banik and Burdsall 1998). Partial compatibility between these biological species may be associated with recent speciation or with taxa in the process of speciation (Boidin 1986).

In addition, two species of exannulate *Armillaria* have been reported in Europe, and one in North America (Guillaumin et al 1991). They should be included in biological species of *Armillaria* in each region (Korhonen 1987).

In Japan, eight biological species of annulate and exannulate *Armillaria* have been reported from Hokkaido in the north to Kyushu in the south (Nagasawa 1991, Ota et al 1998b, 2009), and independently, six biological species have been reported in Hokkaido (Cha et al 1992, 1994, Cha and Igarashi 1995). Four of them have been identified conspecific, and in total, 10 biological species have been reported in this region. All but one have been described as taxonomic species. The undescribed biological species is called "Nagasawa's E (Nag. E)." Seven of 10 have proved to be compatible with either European or North American biological species. One biological species that is compatible with European and North American *A. mellea* proved to be

non-heterothallic (Ota et al 1998a) and named "*Armillaria mellea* subsp. *nipponica* Cha & Igarashi" (Cha and Igarashi 1995), whereas the European and the North American counterpart species is heterothallic. No counterpart species have been found for three biological species.

In Korea, four biological species have been reported to be compatible with either European or North American biological species (Sung et al 1994). In China, 14 biological species have been reported, six of which have shown compatibility with either European or North American biological species (Qin et al 2008). One biological species have been identified as heterothallic *A. mellea*, and another biological species that have shown a homothallic life cycle demonstrated partial compatibility with *A. mellea* in China, Europe and North America. Qin et al (2008) discussed that this homothallic species should be a distinct species, not a subspecies of *A. mellea*. In Iran, six biological species have been reported, four of which have been compatible with European biological species (Asef et al 2003).

In the southern hemisphere, five biological species in Australia and three in New Zealand have been reported. Five Australian biological species have been described taxonomically. Two of the New Zealander biological species have been described taxonomically, one of which is compatible with an Australian one and considered conspecific with it. The other biological and taxonomic species is considered distinct from Australian and the northern hemisphere species. The third New Zealander biological species, although tentatively identified conspecific with Australian *Armillaria hinnulea* Kile & Watling by fruit body morphology, proved to be incompatible with the Australian species. The third biological species in New Zealand has been considered as a distinct species (Kile and Watling 1988).

Molecular phylogenetic analysis

Hennig (1966), who developed the methods of phylogenetic systematics which provided a rigorous framework for testing hypotheses of evolutionary relatedness (McCravy 2008), considered species situated at the boundary of reticulate (tokogenetic) and hierarchical (phylogenetic) systems. Although phylogenetic species concept now represents diverse set of species concepts, it is common that they are outgrowth of a need for an operational and process-free definition of species, as the smallest biological entities (i.e. lineages) that are diagnosable and/or monophyletic (Mayden 1997).

Recently, DNA sequencing techniques, which enabled direct access to genotypic information from sampled individuals, have developed phylogenetic analyses to identify individuals, populations and species. Introduction of molecular (DNA sequence-based) phylogenetic analysis, as well as biological species concept, added an evolutionary aspect to the taxonomic studies of fungi. Because not rigorous frameworks, but a practical view for species concepts and the resulting grouping are concerned, the term "phylogenetic species" is used for "DNA sequence-based phylogenetic species" in the present text.

In identification of fungal species, phylogenetic species recognition has advantages compared to morphological and biological species recognition. The simple morphology of fungi limits the number of potential characters available (Harrington and Rizzo 1999). Moreover, changes in gene sequences occur and can be recognized before changes have occurred in morphology or mating behavior (Taylor et al 2000). In addition, biological species recognition (i.e. mating test) cannot be applied to asexual or homothallic species, and do not function appropriately in the cases of sexually compatible isolates that represent the geographically separated lineages (Harrington and Rizzo 1999). Thus, phylogenetic species recognition can discover cryptic species in a

morphological or biological species. *Candida albicans* (C.P. Robin) Berkhout (Sullivan et al 1995), *Botryotinia fuckeliana* (de Bary) Whetzel (mitosporic state: *Botrytis cinerea* Pers.:Fr.) (Giraud et al 1997), and *Lentinula* Earle (Hibbett et al 1995, Hibbett and Donoghue 1996) represent cases where molecular phylogeny has helped to discover cryptic phylogenetic species in biological and morphological species.

However, different genetic markers produce each different phylogenetic tree, and some genetic markers can even identify individuals. Consequently, phylogenetic species recognition may split species into smaller and arbitrary groups. Some authors proposed that concordance of more than one gene genealogy can avoid the subjectivity of determining the limits of a species (Taylor et al 2000). This method is effective when species should be delimited only by molecular phylogeny.

Fungal molecular phylogeny has historically been based on analysis of the ribosomal RNA gene cluster (ribosomal DNA cluster: rDNA cluster; Wu et al 1983, Specht et al 1984) and mitochondrial DNA (Specht et al 1983). The fungal rDNA cluster, in common with other eukaryotic organisms, is a multiply repeated cluster that comprises the genes for the small ribosomal subunit (SSU), the large ribosomal subunit (LSU), and the gene for the 5.8S subunit. The 5.8S subunit gene is located between the SSU and LSU, and the three genes are separated by two internally transcribed spacers (ITS). The individual gene clusters are separated by intergenic spacers (IGS). These regions have been extensively analyzed for molecular taxonomy (Bridge et al 2005, Bruns and Shefferson 2004). The structural ribosomal genes, the genes for SSU, LSU and 5.8S subunit, have been very popular for higher-level systematics, whereas ITS shows variation around the species level. Usually multiple copies of identical sequences of rDNA are maintained by concerted evolution (Hoelzel and Dover 1991). Multi-copy arrangement and highly conserved priming sites of ITS make it easy to amplify from virtually all fungi, even when

the material is marginal in quantity or quality. The accumulation of sequence data available for ITS has enhanced its value. For these reasons it has been an important locus for phylogenetics and especially ecology.

However, ITS has some serious deficiencies for a robust estimate of the phylogeny. Frequent indels (insertion and (or) deletions) of spacer regions make alignment difficult, and finally arbitrary, as divergence increases. An additional problem is that very closely related species may not have accumulated many differences within the ITS; thus, an analysis based only on ITS may fail to separate all species (Bruns and Shefferson 2004). These deficiencies are also the case with IGS (Hanna et al 2007, Harrington and Wingfield 1995, McLaughlin and Hsiang 2010).

Because of these problems, protein-coding loci have begun to be used for phylogenetic studies in fungi (Bruns and Shefferson 2004). One of the primary advantages of protein coding sequences is that they are easy to align. This ease of alignment is due to the fact that they are constrained by reading frames, and thus indels are much less common. However, protein coding sequences are not as conserved at the nucleotide level as structural RNA genes. This is because it is the translated gene product rather than the gene that is primarily under selection. This difference is useful at lower taxonomic levels, because it means that third-base positions and sites within introns, which are typically under little selection, often provide informative characters among recently diverged taxa. Genes for β-tubulin (Schardl et al 1997), elongation factor-1α (EF-1α) (O'Donnell et al 1998), ribosomal polymerase B (Liu et al 1999), and mitochondrial ATPase 6 (Kretzer and Bruns 1999) have been frequently analyzed. Some of the protein-coding genes plus rDNA have been frequently used to phylogenetic analysis based on genealogical concordance (examples in "Deep hypha issue" of Mycologia 98 (6), 2007, which contains 21 phylogenetic studies). However, in identification of samples, use of a single DNA region would be labor- and

resource saving.

In the late 1980's, phylogenetic studies of *Armillaria* using mitochondrial and nuclear DNA were published (Anderson et al 1987, Jahnke et al 1987, Anderson et al 1989, Smith and Anderson 1989). These works aimed to reveal phylogenetic relationships among the taxonomic and biological species of *Armillaria*. Because mitochondrial DNA produced extremely high divergence, it was judged unsuitable for phylogenetic study (Anderson et al 1987, Smith and Anderson 1989). Anderson et al (1989) divided the European and the North American *Armillaria* species into six classes based on restriction fragment length polymorphisms (RFLPs) of rDNA. *Armillaria ostoyae*, *Armillaria gemina* Bérubé & Dessur., *Armillaria borealis* Marxmüller & Korhonen and *A. mellea* were situated in each different class, whereas two classes included more than one species: *Armillaria calvescens* Bérubé & Dessur.-*A. gallica* class, and *A. sinapina*-*Armillaria nabsnona* Volk & Burdsall-NABS X class. This scheme indicates that the rDNA sequences of the species in the same class have high similarity. In addition, the classes of *A. ostoyae*, *A. gemina* and *A. borealis* composed a larger group, indicating a phylogenetically close relationship among the species.

Ribosomal DNA, including ITS, IGS, 5.8S and LSU have been sequenced for phylogenetic analysis (Anderson and Stasovski 1992, Chillali et al 1998a, b, Terashima et al 1998a, Kim et al 2006, Hanna et al 2007, Coetzee et al 2000, 2001, 2005a, b, Pérez-Sierra et al 2004). Phylogenetic relationships within and among European and North American *Armillaria* species based on the ITS and IGS regions of rDNA showed roughly the similar structure to the scheme proposed by Anderson et al (1989). Phylogenetic analysis based on these rDNA could not reliably distinguish closely related species *A. calvescens*, *A. gallica*, *A. sinapina* and *A. cepistipes* in North America (Kim et al 2006). Techniques using polymerase chain reaction (PCR) with

species-specific primers and RFLPs of the ITS and IGS regions have been developed for identification of species (Harrington and Wingfield 1995, Schulze et al 1995, Banik et al 1996, Volk et al 1996, Chillali et al 1998a, b, Frontz et al 1998, Terashima et al 1998b, White et al 1998, Pérez-Sierra et al 1999, Fukuda et al 2003, Sicoli et al 2003, Lochman et al 2004a, Matsushita and Suzuki 2005, Schnabel et al 2005, Keča et al 2006, Prodorutti et al 2009, McLaughlin and Hsiang 2010). European isolates including those of *Armillaria tabescens* (Scop.) Emel and *Armillaria ectypa* (Fr.) Emel, which were phylogenetically distinct and distant from other *Armillaria* species, could be successfully identified by these methods (Schulze et al 1995, Chillali et al 1998a, b, Pérez-Sierra et al 1999, Lochman et al 2004a, Keča et al 2006). However, some of the North American isolates could not be identified because of the high sequence similarity between *A. calvescens* and *A. gallica* (Harrington and Wingfield 1995, McLaughlin and Hsiang 2010). If there is possibility that isolates from allopatric populations are included in the samples, interpretation of the results of these techniques will be difficult (Prodorutti et al 2009).

Phylogenetic analysis of *Armillaria* species in Australia and New Zealand based on rDNA-ITS sequences revealed that isolates of Australian and New Zealander species were phylogenetically distant from those of European and North American species except for *A. hinnulea*. The result indicated that most of the Australian and New Zealander species were phylogenetically distinct, but this technique could not separate *Armillaria fumosa* Kile & Watling and *Armillaria pallidula* Kile & Watling (Coetzee et al 2001).

In Japan, Terashima et al (1998a) analyzed sequences of rDNA-IGS of isolates belonging to *A. ostoyae*, *A. gallica*, *Armillaria jezoensis* Cha & Igarashi, *A. sinapina*, *Armillaria singula* Cha & Igarashi and *A. mellea* from Hokkaido, *A. cepistipes* from Honshu, and those of European and North American isolates of the genus. The phylogenetic tree obtained in the study divided isolates

into two major clades: one was composed of *A. ostoyae*, *A. borealis* and *A. gemina*, and the other was composed of the rest of the Japanese species used in the study, *A. cepistipes* and *A. gallica* from Europe, *A. sinapina*, *A. gallica*, *A. calvescens*, *A. nabsnona* and NABS X from North America. In each clade, isolates belonging to the same species were distributed in different subclades. However, isolates belonging to abovementioned species from Hokkaido were successfully identified by a technique using RFLPs of rDNA-IGS except *A. jezoensis* and *A. gallica* (Terashima et al 1998b).

Fukuda et al (2003) applied a technique based on the RFLPs of rDNA-IGS to identification of *Armillaria* isolated from the fungal symbiont genera *Wynnea* Berk. & M.A. Curtis and *Entoloma* (Fr.) P. Kumm. They confirmed utility of this technique with known isolates collected from Honshu belonging to *A. gallica*, *A. nabsnona*, *A. ostoyae*, *A. cepistipes*, *A. mellea* and Nag. E, and successfully identified isolates in question as *A. mellea*, *A. cepistipes* and Nag. E. Matsushita and Suzuki (2005) also developed a technique based on the RFLPs of rDNA-IGS with three restriction enzymes to identify isolates belonging to abovementioned six species plus *A. tabescens*. With the technique, 70 Japanese isolates were successfully identified. Nevertheless, they were cautious about applying this technique to European and North American isolates, because considerable difference in the rDNA-IGS region between the allopatric isolates of the same species would result in different RFLP patterns. Sekizaki et al (2008) identified *Armillaria* isolates from achlorophyllous plant symbiont *Gastrodia* R. Br. using analysis on the sequences and RFLPs of the ITS and IGS regions of rDNA and mating tests, and concluded that the isolates in question were *A. cepistipes*.

Other DNA regions than rDNA have also been utilized for more resolved phylogenetic studies. Piercey-Normore et al (1998) combined sequence data of four anonymous DNA regions to obtain

a phylogenetic tree of North American *Armillaria* species. Maphosa et al (2006) and Antonín et al (2009) utilized translation EF-1α gene to discuss phylogenetic relationship among *Armillaria* species. Amplified fragment length polymorphisms (AFLP) and microsatellite markers have been applied for population-level analysis (Langrell et al 2001, Kim et al 2006, Terashima et al 2006, Baumgartner et al 2009, Prospero et al 2010). Analysis on rDNA sequences in some cases reveals relationships among allopatric populations within a species (Coetzee et al 2000, 2001, Hanna et al 2007).

1-2 Practical techniques for species identification

In an ecological research of *Armillaria*, a large number of samples, such as fruit bodies, rhizomorphs, mycelial mats, and rotted wood require species identification. Therefore, a rapid and reliable diagnostic method that can be applied to many diploid samples at a time is preferred. In this respect, taxonomy based on fruit body morphology and mating tests have some disadvantages.

Identification by fruit body morphology

Because fruit bodies are seasonal and short-lived, constant sampling is not realistic. Even if they are available, some characters of fruit bodies are transient. For example, scales and rings may disappear from old fruit bodies. The color and shape of fruit bodies have considerable intraspecific variation and influenced by environmental conditions. Moreover, as mentioned above, some of the sympatric biological species of *Armillaria* have been reported to be difficult to distinguish from each other by fruit body morphology (Guillaumin et al 1991).

Identification by mating tests

The results of a mating test does not affected by the environmental conditions of the sampling sites or intraspecific variation of fruit body morphology. However, mating tests have two critical disadvantages: this method is labor- and time consuming and the results are sometimes ambiguous.

Isolation, subculturing, and mating experiments usually require more than five weeks in total. Moreover, diploid-haploid mating tests take longer (Guillaumin et al 1991).

Ambiguous changes in colony morphology frequently hamper interpretation of results. Stock cultures can lose mating ability after long-term storage, and this senescence may lead to ambiguous results in mating tests. Mating tests for species identification require a set of haploid voucher strains of a known biological species, at least from within and around the sampled area. Because of the possibility that the tester and the unknown haploid isolates are conspecific but incompatible in that they have identical mating alleles, at least two different testers must be used for each species. To keep them fresh, continuous collection of haploid isolates of each species is required.

Diploid-haploid pairings may also result in ambiguity (Guillaumin et al 1991). This can be critical, because, as mentioned above, constant collection of fruit bodies is not realistic, and diploid samples are much easier to obtain.

Mating reaction cannot be expected for homothallic species. In such cases, somatic incompatibility tests have been adapted (Mohammed et al 1994, Abomo-Ndongo and Guillaumin

1997, Ota et al 2005). When two isolates belonging to different biological species are paired, a dark demarcation line (black line) emerges between the two mycelia, whereas no pigmented line emerges between isolates belonging to the same biological species (Mallett and Hiratsuka 1986). However, this phenomenon tends to be ambiguous (Abomo-Ndongo and Guillaumin 1997, Ota et al 2005).

DNA sequence-based identification

DNA sequence-based techniques are rapid and suitable for treating a large number of samples. Moreover, direct PCR may be applicable to crude samples without isolation and culturing (Lochman et al 2004b). Sequence data of voucher strains required for analysis is occasionally available from public databanks although it depends on the target sequence region.

Sequence-based phylogenetic analysis is especially useful to treat diploid isolates, because a result of a pairing test using diploid isolates tends ambiguous and difficult to interpret. It is also the case with homothallic species (Pérez-Sierra et al 2004, Coetzee et al 2005a, Ota et al 2005).

However, as mentioned above, phylogenetic trees differ depending on genetic markers used in the analysis. In identification of samples, the choice of genetic markers is the key to obtain the result that meets the purpose.

As previously mentioned, DNA sequence-based identification technique conventionally targets conserved regions of rDNA, particularly the ITS and/or the IGS regions. However, species identification using rDNA sequences has some difficulties, and fails to distinguish species in some cases. According to Bruns and Shefferson (2004), the most important deficiency of rDNA

region is frequent indels that make alignments less reliable. Heterogeneity of the sequences is also the problem. The ITS and IGS regions are parts of tandemly repeated rDNA sequences (Long and Dawid 1980). Usually multiple copies of identical sequences of rDNA are maintained by concerted evolution (Hoelzel and Dover 1991). However divergent copies can coexist within an rDNA repeat (Iraçabal and Labarère 1994) and the heterogeneity of the IGS region, which is indicative of variable multicopies in an rDNA array, has been reported in *Armillaria* species (Coetzee et al 2005b, Schnabel et al 2005, Hanna et al 2007). In such cases, direct sequencing of PCR products is often difficult and cloning is required before sequencing.

Heterogeneity (dimorphism in this case) of the ITS and IGS regions of rDNA in diploid isolates also has been described in *Armillaria* species, which suggests heterozygosity as well as the existence of variable multicopies in an rDNA array (Banik et al 1996; Volk et al 1996; White et al 1998; Pérez-Sierra et al 1999; Kim et al 2000; Dunne et al 2002; Smith-White et al 2002; Lochman et al 2004a, b; Schnabel et al 2005; Keča et al 2006). In addition, interspecific hybrid RFLP and sequence patterns have been discovered in the IGS and ITS regions of rDNA in two *Armillaria* species (Kim et al 2006, Antonín et al 2009, McLaughlin and Hsiang 2010). These phenomena make it difficult to obtain clear results from rDNA analysis.

Furthermore, in some cases, very small difference is found between DNA sequences of isolates belonging to different biological species (Anderson and Stasovski 1992, Harrington and Wingfield 1995, Coetzee et al 2001, Antonín et al 2009, Prodorutti et al 2009, McLaughlin and Hsiang 2010). In such cases, identification based on sequences of such DNA regions may fail to distinguish species.

However, it is evident that different regions of DNA in fungi can show considerably different

rates of evolution (Bruns et al 1991), and phylogenetic analysis using different gene region, or a combination of different gene regions may give more resolved results (Piercey-Normore et al 1998, Kim et al 2006, Maphosa et al 2006, Antonín et al 2009).

Recently, EF-1α gene, which encodes an essential part of the protein translation machinery, was shown to have high phylogenetic utility (Geiser 2004). Subsequently, this gene has been used widely for taxonomic and phylogenetic analysis of fungal species, including *Fusarium* Link, *Aspergillus* P. Micheli: Link and Mucorales species (Balajee et al 2009). Unlike rDNA, EF-1α is usually present as a single copy gene in most fungal genomes (except the basal lineages with an EF-1α-like gene instead of, or with, EF-1α gene (Baldauf 1999, James et al 2006)).

Maphosa et al (2006) used EF-1α for a phylogenetic study of *Armillaria* and established the relationships between *Armillaria* species from the northern and southern hemispheres. Antonín et al (2009) used this gene to elucidate the relationship between the two closely related species of this genus: *A. gallica* and *A. cepistipes*.

Conflicts among the species identified by the three methods

Taxonomic species identified by fruit body morphology, biological species identified by mating tests and lineages inferred by phylogenetic analysis generally overlap but are different in some cases. For example, fruit body morphology of some of the sympatric biological species of *Armillaria* have been reported to be difficult to distinguish from each other (cf. *A. cepistipes* and *A. gallica* in Europe, *A. ostoyae* and *A. gemina* in North America (Guillaumin et al 1991)). Sequence-based analysis also in some cases failed to distinguish biological and morphological species. As previously mentioned, very small difference found between rDNA-IGS sequences of

isolates belonging to *A. gallica* and *A. calvescens* did not reflect in patterns of RFLPs (Anderson and Stasovski 1992, Harrington and Wingfield 1995, McLaughlin and Hsiang 2010). Similarly, *A. fumosa* and *A. pallidula* could not be separated based on rDNA-ITS sequences (Coetzee et al 2001).

Phylogeny contributes taxonomy by supporting morphological or biological species recognition when the former is consistent with the latter. For instance, Pildain et al (2009, 2010) revealed four lineages in South American *Armillaria* based on the sequences of the ITS and LSU of rDNA and linked them to previously described taxa based on morphology. However, in the study of species delimitation of *Armillaria*, the role of phylogeny is not confirmed when phylogenetic species recognition conflicts with morphological or biological species recognition. Based on the analysis on rDNA and physiological characteristics of the isolates, Otieno et al (2003) and Coetzee et al (2005a) argued that the two distinct species are included in a single somatic incompatibility group that corresponds to African *Armillaria fuscipes* Petch., whereas Pérez-Sierra et al (2004) withheld to divide a somatic incompatibility group that correspond to African *Armillaria heimii* Pegler into two species in that they found fertility and only little difference of fruit body morphology between the two groups despite the result of the analysis on rDNA and AFLPs.

The primary interest of this study is fungal ecology, and for this purpose species concept should be a means of communication among biologists. Species concept commonly recognized by those who studies *Armillaria*, at least for now, would be morphological and biological species concept. From this view point, in the next chapter, sequence data of the EF-1α gene from Japanese *Armillaria* isolates are analyzed and the utility of this gene in identifying different species is compared with those of rDNA-IGS and ITS sequences.

Species concept should serve as a hypothesis for testing modes of speciation and evolution (Harrington and Rizzo 1999). Many agree that the process of speciation is a population-level phenomenon. Isolates in well diverged, distinct clades within a species can be assumed to represent populations, and ecological and physiological study of such populations may reveal modes of speciation and evolution.

1-3 Ecological studies of *Armillaria* species in the northern hemisphere

Distribution, host species and pathogenic role of *Armillaria* species in the northern hemisphere have been studied extensively in Europe and North America (Kile et al 1991). Because many of the counterpart species[2] of Japanese *Armillaria* have been reported in northern hemisphere, ecological studies of *Armillaria* species in that region are reviewed in this section.

Distribution

In Europe, *Armillaria* species distribute from Scandinavia to Greece and Spain, and reach North Africa (Korhonen 1978, Guillaumin et al 1993, Tsopelas 1999, Aguín-Casal et al 2004, Marxmüller and Guillaumin 2005). Although the distribution of each *Armillaria* species largely overlaps, it exhibits the following order from the north to the south: *Armillaria borealis* Marxmüller & Korhonen, *A. cepistipes*, *A. ostoyae*, *A. gallica*, *A. mellea* and *A. tabescens* (Marxmüller and Guillaumin 2005, Figure 1-1). Generally, vertical distribution is also in the same order (Guillaumin et al 1993, Tsopelas 1999, Keča et al 2009). *Armillaria ectypa* is reported on peat bogs in wet lands in high latitude (Ohenoja 2006).

[2] "Counterpart species" in this paper is used for allopatric populations or populations that have different sexual systems in the same taxonomical species.

This distribution pattern correlates the thermal property of the species. Guillaumin et al (1989) reported that the optimum growth temperature of *A. borealis*, *A. cepistipes* and *A. ostoyae* was 22C and that of *A. gallica* and *A. mellea* was 25C. Rishbeth (1986) reported that the optimum growth of *A. mellea*, *A. ostoyae*, *A. gallica* and *A. tabescens* in vitro was observed at 25C. However, the growth rate of *A. tabescens* declined most gradually toward 28C, showing thermophilic property of the species.

In North America, *A. sinapina*, *A. ostoyae*, *A. gallica* and *A. mellea* have wide distribution. As is the case in Europe, these species are distributed roughly from the north to the south in this order. *A. sinapina* is distributed widely in Canada and the northern part of USA (Anderson 1986, Mallett 1990, Harrington and Rizzo 1993, Banik et al 1995, 1996, Frontz et al 1998, Klopfenstein et al 2009). *Armillaria ostoyae* is widely distributed in Canada, northwestern, interior south-western, north-central and north-eastern USA (Mallett 1990, Hanna et al 2007). It has been also found in Mexico (Shaw 1989). *Armillaria gallica* is widely distributed in southern part of Canada and USA (Morrison et al 1985, Motta and Korhonen 1986, Harrington and Rizzo 1993, Banik 1995, 1996, Dumas 1988, Bruhn et al 2000, McLaughlin 2001, Baumgartner and Rizzo 2001). *Armillaria mellea* is reported from south eastern Canada to Deep South and California in USA (Bérubé and Dessureault 1988, Bruhn et al 2000, McLaughlin 2001, Schnabel et al 2005, Baumgartner et al 2010). Other species have relatively restricted distribution. *Armillaria cepistipes* has been reported in British Columbia and Washington (Morrison et al 1985, Banik et al 1996). *Armillaria gemina* and *A. calvescens* have northeastern distribution in North America (Harrington and Rizzo 1993, Proffer et al 1987, Ellis et al 2007).

In Japan, *A. sinapina*, *A. jezoensis* and *A. singula* have been reported in Hokkaido (Cha et al

1994). *Armillaria ostoyae*, *A. cepistipes*, *A. nabsnona* and Nag. E have been found from Hokkaido and Honshu, but not from Kyushu (Cha et al 1992, Ota et al 1998b, 2009, Fukuda et al 2003, Sekizaki et al 2008). *Armillaria gallica* and *A. mellea* have been found from Hokkaido, Honshu and Kyushu (Cha et al 1992, Cha and Igarashi 1995, Ota et al 1998b, 2009). *Armillaria. tabescens* has been found in Honshu and Kyushu (Ota et al 1998b). In addition, *A. ectypa*, homothallic and presumably non-wood-inhabiting fungus in wet land has been found from Aomori and Oze in Honshu (Kudo and Nagasawa 2003, Ota et al 2005), and *A. fuscipes*, identified by fruitbody morphology, has been reported in Amami, south of Kyushu (Ota et al 2011). Distribution of *A. sinapina*, *A. ostoyae*, *A. cepistipes*, *A. gallica*, *A. mellea* and *A. tabescens* in Japan exhibit roughly the parallel order of the distribution of counterpart species. Habitat of Japanese *A. ectypa* is also similar to that of the European counterpart.

Host Specificity and Pathogenicity

In Mediterranean Europe and California in North America, *A. mellea* is recognized as the dominant *Armillaria*, and although it attacks both hardwoods and conifers, it is not widely associated with disease in forest or plantation conifers: it has been considered as an aggressive pathogen of hardwood species in orchards and gardens (Gregory et al 1991, Guillaumin et al 1993, Baumgartner and Rizzo 2001).

In Europe and North America, *A. ostoyae* is considered a pathogen of conifers and its virulence varies among isolates (reviewed by Gregory et al 1991 and Hanna et al 2007). However, field observations have suggested that this species is capable of attacking hardwoods, at least as a secondary pathogen, and utilizing hardwoods as food base (Gregory et al 1991, Guillaumin et al 1993).

Armillaria cepistipes and *A. sinapina* have been considered as weak pathogens on conifers (Gregory et al 1991, Guillaumin et al 1993, Morrison 2004, Prospero et al 2004). In North America, *A. sinapina* is found both on hardwoods and conifers (Mallett 1990, Dettman and van der Kamp 2001).

In Europe and North America, *A. gallica* is categorized as a weak pathogen frequently found on hardwood species and infrequently on conifers (Rishbeth 1982, Morrison et al 1985, Gregory 1989, Guillaumin et al 1993).

Armillaria nabsnona in North America is a weak pathogen on hardwood species and has limited pathogenicity on young conifers (Gregory et al 1991, Volk et al 1996).

In Europe, *A. tabescens* has been reported to be surprisingly aggressive to some hosts including introduced Eucalyptus and opportunist parasite on oaks. But in most cases it has been regarded as a saprobe on the stumps of oak species (Guillaumin et al 1993). On the other hand, in North America, a fungus referred to as *A. tabescens* is known to attack and kill wide range of hosts including pine (Sinclair and Lyon 2005). Some authors are suspicious that the two allopatric populations belong to different species (Kile et al 1994, Antonín et al 2006). Japanese isolates of *A. tabescens* have been reported to be compatible with European isolates of the species, but not compatible with North American ones (Ota et al 1988b).

Armillaria borealis has been regarded as a weak pathogen, although its association with butt rot of *Picea abies* (L.) H. Karst. has been observed (Gregory et al 1991). *Armillaria calvescens* and *A. gemina* have been found frequently from hardwoods, considered to be weak pathogens that

attacks mostly stressed trees (Bérubé and Dessureault 1989, Rizzo and Harrington 1993). NABS X has been found both on conifers and hardwoods, but pathogenic property of the species has not been clear (Morrison et al 1985).

Very few studies in Japan have examined host specificity of *Armillaria* species. Terashita and Sawaguchi (1991) and Matsushita (2002) reported a severe damage on pines caused by *A. ostoyae* in Aomori Prefecture. Matsushita (2002) also revealed pathogenicity of *A. ostoyae* isolates on pine seedlings by inoculation tests. As for *A. tabescens*, probably because the fungus is identified easily by fruit body morphology, there are several reports. Fujii and Hatamoto (1974), Kaneko and Ogawa (1998), Sato and Suzuki (2002), Hasegawa (2005) and Onozato et al (2008) reported that *A. tabescens* attacked various kind of trees in gardens in Honshu and Kyushu.

Armillaria root disease on conifers in Japan

In Japan, *Armillaria* species are considered as pathogens of many orchard, garden and forest trees, including conifers (The Phytopathological Society of Japan 2000). Coniferous plantations provide most of the timber produced in Japan (Forestry Agency 2009a, b), where afforestation of conifers after clear-cutting of indigenous broad-leaved forests has rapidly expanded since the 1950s. In parallel, reports of Armillaria root disease on conifers have increased through the 1950s and 1960s (reviewed by Ono 1970). Current forest plantations, mostly conifer, cover approximately 10 million ha, accounting for 40% of the total forest area in Japan (Forestry Agency 2009a, b).

Important plantation species in Japan are *Cryptomeria japonica* (L. f.) D. Don, *Chamaecyparis obtusa* (Sieb. et Zucc.) Endl., *Pinus densiflora* Sieb. et Zucc., *Larix kaempferi* (Lamb.) Carr.,

Abies sachalinensis (Fr. Schmidt) Mast. and *Picea jezoensis* (Sieb. et Zucc.) Carr. (Forestry Agency 2009b).

Armillaria root disease is damaging all of these species (reviewed by Matsushita 2002). In spite of the economic importance of this disease, very few studies in Japan have examined host specificity of *Armillaria* species in conifers (Terashita and Sawaguchi 1991, Matsushita 2002). The difficulties with species identification within *Armillaria* have impeded such ecological studies.

Chapter 3 focuses on *Armillaria* isolates obtained from conifers in Japan, and species are identified using mating tests and the EF-1α sequence data. On the basis of the records collected, including those of host species, host condition and geographical information, characteristics and the potential pathogenicity of the *Armillaria* species on conifers are discussed.

Table 1-1. The salient characteristics of *Armillaria**.

Habit — clitocyboid with slightly sinuate, adnexed, subdecurrent or decurrent gills; bivelangiocarpic or metavelangiocarpic development in annulate species, apparently monovelangiocarpic development in exannulate species; solitary, gregarious, or caespitose.

Pileus — fleshy, thinning towards margin, expallant, hygrophanous or not; color variable yellow-brown, yellow-olivaceous, ochraceous, rusty-tawny, umber, cigar brown, less commonly buff or clay pink, sometimes ivory, pallid, or even mouse gray; surface glabrous, scurfy, squamulose, squamules darker than ground color, sometimes restricted to disc; glabrescent as scales are lost; dry or becoming viscid to distinctly viscid, in some species almost glutinous.

Stipe — central, fibrous-fleshy, not characteristically cartilaginous; often becoming hollow and the outermost layers splitting and curling back to expose flesh; more or less annulate with floccose-membranous to arachnoid veil; often arising from sheets of white mycelia or from well-differentiated black rhizomorphs, and/ or, associated with plaques of thin, black, tough tissue.

Lamellae — close to sub distant; moderately thick; nearly white, ivory, or cream-color at first but frequently becoming spotted with cinnamon-buff, rusty-tawny, or sometimes, particularly with age, with a tinge of purple or distinctly pink; sinuate; adnexed to deeply decurrent.

Flesh — of pileus pale and of stipe white at first, becoming as dark as umber or Vandyke brown downwards and sometimes tinted red or bluish at base where colonized by pigment-producing bacteria or nectriaceous fungi.

Spore-print — white to cream-color darkening slightly on drying, and in herbarium material.

Basidia — 4-spored, sometimes 2-spored; thin-walled; with or without a basal clamp-connection; hyaline; smooth-walled in aqueous alkali solutions or if thick-walled [= crassobasidia (Chandra and Watling 1983)] then appearing silvery or glassy, and/ or, becoming ochraceous or fulvous.

Basidiospores — ellipsoid; inamyloid; hyaline, yellowish cream-color or ochraceous in aqueous alkali solutions; weakly cyanophilic; thin to moderately thick-walled; smooth or slightly verruculose or rugulose with broad, blunt usually prominent apiculus; lacking germ-pore or apical differentiation (thinning or thickening).

Cheilocystidia — present or absent, often inconspicuous; variable in shape sometimes catenulate-septate; thin-walled or becoming slightly thick-walled with age sometimes with apical prolongation and with or without basal clamp-connection; smooth; hyaline to honey-colored in aqueous alkali solutions.

Table 1-1. Continued.

Pleurocystidia — absent or, if present, thin-walled; poorly differentiated and rarely visible above the level of the basidia.

Pileipellis — an irregular, disrupted trichodermium consisting of (i) an irregular, easily destroyed *suprapellis* composed of groups of fulvous or cinnamon, subparallel, ascendant, loosely to strongly adhering hyphae intermixed with broad, frequently encrusted hyphae (which form the scales), often with clamp-connections; ascendant hyphae becoming repent to form a rather amorphous adnate layer; (ii) *mediopellis* - of parallel to subparallel hyphae forming a cutis that may or may not gelatinize but sooner or later becomes the outermost layer; and (iii) *subpellis* - a compact hyphal layer.

Stipitipellis — parallel hyphae overlain by more or less strongly developed, irregular, filamentous velar remnants; in parts of stipe free from velar material showing development of cylindric to elongate clavate or lageniform caulocystidia.

Pileus and stipe trama - monomitic; hyphae inamyloid, generally lacking clamp connections.

Hymenophoral trama - bilateral at first and remaining so or becoming regular with age although always demonstrating some divergent arrangement; constitutive hyphae generally lacking clamp-connections; inamyloid.

Vegetative growth - variable on agar media but typically reddish-brown crustose surface mycelium; usually slow growing; with or without tufts of cinnamon aerial mycelium; with or without reddish-brown rhizomorphs or with white to cream-color rhizomorphs embedded in the medium with emergent reddish-brown tips; rhizomorphs branch monopodially, dichotomously, or irregularly; vegetative mycelium often bioluminescent; cells uni- or multinucleate; nuclei apparently diploid.

Rhizomorphs — mycelial aggregations with a melanized outer layer and pale, apical growing tip; produced in culture and from infected lignicolous material.

Single basidiospore isolates — from heterothallic species typically slow growing; producing white, fluffy to cottony mycelium, sometimes with areas of brown or reddish; with or without sparse rhizomorph development; nuclei haploid.

Compatibility system - bifactorial; heterothallic with multiple alleles at the incompatibility loci; some species possibly homothallic.

*Collectively, these characters define the genus, and variations among them define species (Watling et al 1991).

Table 1-2. Biological species of *Armillaria*.

Biological species	Taxonomic name	Remarks
Europe		
Korhonen's biological species (KBS) A	*Armillaria borealis* Marxmüller & Korhonen	
KBS B	*Armillaria cepistipes* Velen.	
KBS C	*Armillaria ostoyae* (Romagn.) Herink	syn. *Armillaria solidipes* Peck
KBS D	*Armillaria mellea* (Vahl:Fr.) Kumm.	heterothallic
KBS E	*Armillaria gallica* Marxmüller & Romagn.	
Armillaria tabescens	*Armillaria tabescens* (Scop.) Emel	exannulate
Armillaria ectypa	*Armillaria ectypa* (Fr.) Emel	exannulate
North America		
North American biological species (NABS) I	*A. ostoyae*	
NABS II	*Armillaria gemina* Bérubé & Dessur.	
NABS III	*Armillaria calvescens* Bérubé & Dessur.	
NABS V	*Armillaria sinapina* Bérubé & Dessur.	partially compatible with European *A. cepistipes*
NABS VI	*A. mellea*	heterothallic
NABS VII	*A. gallica*	
NABS IX	*Armillaria nabsnona* Volk & Burdsall	
NABS X		partially compatible with European *A. cepistipes*
NABS XI	*A. cepistipes*	
A. tabescens?	*A. tabescens*?	may not be conspecific with European *A. tabescens*

Table 1-2. Continued.

Biological species	Taxonomic name	Remarks
Iran		
Iranian intersterility groups (IISG) 1		
IISG 2	*A. mellea*	heterothallic
IISG 3	*A. cepistipes*	
IISG 4	*A. borealis*	
IISG 5		
IISG 6		
Korea		
A. gallica	*A. gallica*	
A. ostoyae	*A. ostoyae*	
A. tabescens	*A. tabescens*	
A. mellea	*A. mellea*	
Japan		
Nagasawa's biological species (Nag.) A	*A. gallica*	
Nag. B	*A. nabsnona*	
Nag. C	*A. ostoyae*	
Nag. D	*A. cepistipes*	
Nag. E		
Nag. AM	*A. mellea*	nonheterothallic
A. sinapina	*A. sinapina*	
Armillaria jezoensis	*Armillaria jezoensis* Cha & Igarashi	
Armillaria singula	*Armillaria singula* Cha & Igarashi	
A. tabescens	*A. tabescens*	compatible with European *A. tabescens*
A. ectypa	*A. ectypa*	

Table 1-2. Continued.

Biological species	Taxonomic name	Remarks
China		
Chinese biological species (CBS) A	*A. sinapina*	
CBS B	*A. gallica*	heterothallic
CBS C		
CBS D	*A. ostoyae*	
CBS F		
CBS G		homothallic, partially compatible with *A. mellea*
CBS H		
CBS I	*A. tabescens*	compatible with European *A. tabescens*
CBS J		
CBS K	*A. mellea*	
CBS L		
CBS M	*A. borealis*	
CBS N		
CBS O		
Australia		
Armillaria novae-zelandiae	*Armillaria novae-zelandiae* (G.Stev.) Herink	
Armillaria fumosa	*Armillaria fumosa* Kile & Watling	
Armillaria hinnulea	*Armillaria hinnulea* Kile & Watling	
Armillaria luteobubalina	*Armillaria luteobubalina* Kile & Watling	
Armillaria pallidula	*Armillaria pallidula* Kile & Watling	
New Zealand		
Armillaria novae-zelandiaea	*Armillaria novae-zelandiaea*	
Armillaria limonea	*Armillaria limonea* (G.Stev.) Boesew.	
A. hinnulea	*A. hinnulea*	
unnamed biological species		morphologically resembles to *A. hinnulea*

Table 1-2. Continued.

Biological species	Taxonomic name	Remarks
Africa*		
Somatic incompatibility (SI) group 1	*A. mellea*	homothallic
SI group 2	*Armillaria heimii* Pegler?	homothallic type and bipolar-heterothallic type
SI group 3		
SI group 4		

* Mohammed et al 1994

(Guillaumin et al 1991, Asef et al 2003, Sung et al 1994, Cha et al 1994, Ota et al 1998b, Qin et al 2007, Kile and Watling 1988, Mohammed et al 1994)

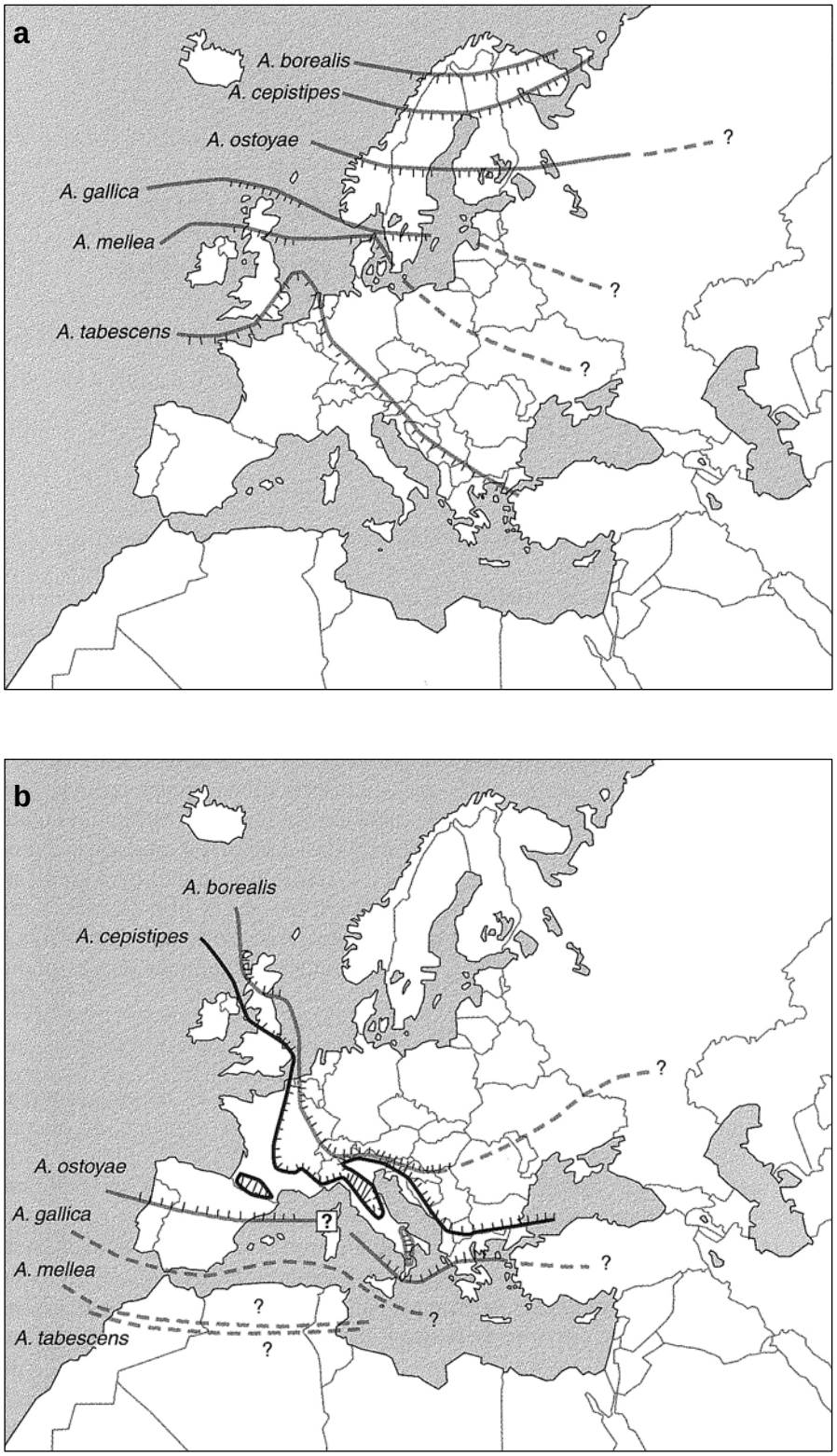


Figure 1-1. Distribution of *Armillaria* species in Europe. a) Northern limit; b) Southern limit (Marxmüller and Guillaumin 2005).

2 Sequence-based identification of Japanese *Armillaria* species using the translation elongation factor-1α gene

2-1 Objectives

The objectives of this study are (i) to analyze the sequences of the EF-1α gene of Japanese *Armillaria* isolates and (ii) to compare the usefulness for identification of species between these sequences and the IGS and ITS sequences of rDNA.

2-2 Materials and methods

Isolates

The species studied included *A. mellea*, *A. ostoyae*, *A. nabsnona*, *A. cepistipes*, *A. gallica*, *A. sinapina*, *A. tabescens* and Nag. E. A total of 49 *Armillaria* isolates collected in Japan were used in this study (Table 2-1). At least three isolates of each species were chosen. Most of the isolates were obtained from single spores. They have been deposited at the National Institute of Agrobiological Sciences (NIAS) Genebank, Forestry and Forest Products Research Institute (FFPRI) culture bank and/or the culture bank of the Microbial Ecology Lab, FFPRI. Voucher specimens were deposited at the Mycological Herbarium of FFPRI (TFM).

The biological species of each isolate was identified by haploid-haploid or haploid-diploid pairing tests (Guillaumin et al 1991). The isolates of *A. singula* and *A. jezoensis* were not included in the present study because they were not available as field samples or in culture collections.

DNA extraction

Isolates were cultured on cellophane membranes placed on potato dextrose agar (PDA; Eiken Chemical, Tokyo, Japan) plates at 25 C for 1–2 wk. Mycelia were harvested from each plate by scratching the surface of the membranes with a sterilized micro spatula and placed in 1.5 mL tubes. The mycelia were frozen at −80 C for more than 30 min and lyophilized. Freeze-dried mycelia then were ground to a fine powder with a sterile pipette tip.

DNA extraction was performed as described by Kikuchi et al (2009) with some modifications. FG1, FG2 and FG3 solutions from the fungal DNA extraction kit (Omega, Norcross, Georgia) were used respectively for lysis, neutralization and binding. Genomic DNA was bound to a 96-well glass-fiber filter (Pall, East Hills, New York). After washing twice with 80% ethanol, DNA was eluted with 50 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) preheated at 65 C.

PCR amplification

The IGS-1 and ITS regions of rDNA were amplified respectively with PCR primer pairs LR12R/O-1 (Harrington and Wingfield 1995) and ITS1-F/ITS4-B (a location map and oligonucleotide sequences of these primers can be found at http://www.biology.duke.edu/fungi/mycolab/primers.htm).
Primers EF595F and EF1160R (Maphosa et al 2006) were used for the amplification of the EF-1α gene. PCR amplifications were carried out in 30 mL reaction mixtures containing 15 mL GoTaq Green Master Mix (Promega, Madison, Wisconsin), 0.5 mM of each primer and 1 mL of each

DNA extract. Cycling conditions were 94 C for 1 min, then 30 cycles at 94 C for 30 s, 53 C for 30 s, and 72 C for 1 min. The PCR products were electrophoresed on 1% agarose gels, and the bands were visualized by ethidium bromide and UV illumination.

DNA sequencing

PCR products were purified before sequencing with a MinElute 96 UF PCR purification plate (QIAGEN, Hilden, Germany). DNA sequencing was performed with the BigDye Terminator 3.1 kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). To obtain entire sequences of the amplified IGS regions two internal primers (ArmIGSin1f: GCACTCSCRACAGCATGT and ArmIGSin1r: ACATGCTGTYGSGAGTGC) were designed and used as sequencing primers. The sequences have been deposited in the DNA Data Bank of Japan (DDBJ; accession numbers are AB510759–AB510900).

Phylogenetic analysis and computer-simulated RFLP

Phylogenetic analyses were performed on the Phylogeny.fr platform (Dereeper et al 2008, 2010) and comprised the following steps. DNA sequences obtained from the isolates (Table 2-1) and the additional sequences available in GenBank (accession numbers in Figures 2-5, 2-6, 2-7, 2-8, 2-11 and 2-12) were aligned with the Muscle 3. 7 program (Edgar 2004) in the default mode. Neighbor-joining (NJ) phylogenetic trees were constructed with the NEIGHBOR program with 1000 bootstrap replicates (Gascuel 1997). The distances were calculated with FastDist (Elias and Lagergren 2007). The Kimura 2- parameter (K2P) substitution model was selected for the analysis (Kimura 1980). Maximum-likelihood (ML) trees were constructed with the PhyML 3.0 program (Guindon and Gascuel 2003). The general time-reversible model (GTR model) was used

as the substitution model with an estimated proportion of invariant sites (0.542, 0.573 and 0.460 for the ITS and IGS regions and EF-1α gene in Japanese isolates, respectively) and four gamma distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (1.388, 98.561 and 0.751 for the ITS and IGS regions and EF-1α gene in Japanese isolates, respectively). The reliability value of each internal branch was assessed with the aLRT test (SH-Like) (Anisimova and Gascuel 2006). This test is based on an approximation of the standard likelihood ratio test and is much faster to compute than the usual bootstrap procedure. Both methods output the same trees, and branch supports generally are highly correlated (Dereeper et al 2008). The graphical representation and editing of the phylogenetic tree were achieved with TreeDyn (Chevenet et al 2006). Sequence alignments were deposited at TreeBase under accession Nos. M5105–M5108 and at the following site: http://cse.ffpri.affrc.go.jp/haseg/thesis/alignments.html

Computer-simulated RFLP were generated with the IGS region of rDNA, amplified with the primers described above with DistinctiEnz software (http://www.bioinformatics.org/). The entire sequences including PCR primer-sites were used in this analysis to reflect fragments that would occur in a real RFLP. Digestion with the restriction enzymes AluI, Hae III, Hinf I and MspI (Terashima et al 1998b, Fukuda et al 2003, Matsushita and Suzuki 2005, Sekizaki et al 2008) was simulated. Fragments smaller than100 bp were not considered when grouping the RFLP patterns because these fragments were difficult to identify on real gel plates (Terashima et al 1998b, Matsushita and Suzuki 2005). Fragments between which there was a difference of less than 10 bp were considered identical for the same reason.

2-3 RESULTS

PCR amplification and sequencing

For most species PCR amplification of the ITS region yielded a single strong band of about 1 kbp. PCR products of *A. mellea* and *A. tabescens* gave slightly smaller bands on the agarose gel. Most of amplicons of the IGS-1 region were around 1 kbp, but those from *A. mellea* and *A. tabescens* were slightly smaller (about 0.9 kbp). The amplification of the EF-1α region yielded a strong band of expected size (about 0.6 kbp) (Maphosa et al 2006) for all species.

PCR products were purified and directly used for sequencing. Sequence analysis revealed that the IGS regions of five isolates (NA17, NB3, 96-8-1, ND11, 96-19-1) were heterogeneous, making it difficult to accurately determine their DNA sequences; hence these sequences were not used for further analysis. The amplicons of EF-1α from four *A. mellea* isolates (A-10, A-12, 89-07, 97-6) also showed heterogeneity. This heterogeneity was likely to have originated from two fragments with slightly different lengths. Apparently this difference arose because of a single small gap, and so the sequences could be determined with manual adjustments and the longer sequence was used for the following analysis. In total 49 ITS sequences, 44 IGS-1 sequences and 49 EF-1α gene sequences were obtained and used.

Computer-simulated RFLP

Full-length DNA sequences of the amplified products of the IGS region were subjected to the computer-simulated RFLP analysis (Table 2-2). Each restriction enzyme yielded at least nine fragment patterns. A single restriction enzyme yielded one to three RFLP patterns for a species. The isolates of three species had the same RFLP patterns by digestion with two restriction enzymes respectively. The species to which most isolates belonged could be identified with the

RFLP patterns generated by a combination of restriction enzymes. However five isolates of *A. cepistipes* and two isolates of *A. sinapina* had identical RFLP patterns for all restriction enzymes and these isolates could not be distinguished.

Phylogenetic analyses

The sequences of three DNA regions, the ITS and IGS regions and the EF-1α gene were analyzed. The total number of characters included in the data matrix for ITS, IGS and EF-1α were respectively 870, 922 and 463 after alignment by inserting gaps. The parameters in ML analysis on each DNA region were indicated in Figures 2-1, 2-3, 2-5. 2-7, 2-9 and 2-11.

A similar structure was observed between the ML and NJ trees of the ITS region of Japanese isolates (Figures 2-1, 2-2). Each tree contained three major clades: the most distant clade was formed by isolates of *A. mellea*, the second clade consisted of isolates of *A. tabescens*, and all the remaining isolates that were analyzed were included in the third clade. In the ML tree, isolates of *A. ostoyae* appeared at the base of the third clade, whereas they composed a distinct subclade in the NJ tree. In both trees, isolates of *A. nabsnona* and Nag. E formed a distinct subclade, and the former were situated basal to the latter. The isolates of *A. gallica*, together with those of *A. sinapina* and *A. cepistipes*, were placed in a sister clade of *A. nabsnona*-Nag. E clade, except for one *A. sinapina* isolate (05-46-1). The isolates of these three species could not be differentiated from each other. Reliability values (aLRT values) in the ML tree and bootstrap values in the NJ tree strongly supported *A. mellea* clade, *A. tabescens* clade and Nag. E clade (99–100%).

The ML and NJ trees of the IGS-1 region had the following points in common.(Figures 2-3, 2-4) The most distant clade consisted of isolates of *A. mellea*, and the second clade was formed

by isolates of *A. tabescens*. Isolates of *A. ostoyae*, *A. nabsnona* and Nag. E appeared in distinct clades of each species. Reliability values in the ML tree and bootstrap values in the NJ tree strongly supported these clades (87–100%). In addition, isolates of *A. gallica*, *A. cepistipes* and *A. sinapina* were situated together (except for an isolate of *A. sinapina* (05-46-1) in both trees and that of *A. gallica* (NA4) in the ML tree) and could not be differentiated from each other.

Trees with ITS and IGS data of Japanese and some overseas *Armillaria* isolates were also generated. In the resulting trees the overseas isolates were situated in the same or adjacent clades of their counterpart species (Figures 2-5, 2-6, 2-7 and 2-8).

These ITS and IGS trees indicated that among the *Armillaria* isolates used in this study, those of *A. mellea*, *A. tabescens*, and Nag. E formed distinct clades of each species. In some cases isolates of *A. ostoyae* and *A. nabsnona* did not form each distinct clade and were situated basal to the parent clades of other species' clades, but they did not share their positions with isolates of other species. Isolates of the remaining three species, *A. gallica*, *A. cepistipes* and *A. sinapina* appeared in the same or adjacent clade and did not form distinct clades of each species.

The ML and NJ trees based on the EF-1α gene of Japanese *Armillaria* isolates were shown in Figures 2-9, 2-10. In both trees, the most distant clade comprised isolates of *A. tabescens*. In the MLtree, the second and the third distant clades were formed by isolates of Nag. E and *A. nabsnona*, respectively. The forth clade contained two subclades. The isolates of *A. gallica* and *A. mellea* were situated in the first subclade, and the isolates of *A. mellea* further formed a distinct clade. The second subclade was separated further into three sub-subclades, the *A. ostoyae* clade, *A. sinapina* clade and *A. cepistipes* clade. The latter two formed sister clades. *Armillaria tabescens*, Nag. E, *A. nabsnona*, *A. mellea*, *A. ostoyae*, *A. sinapina* and *A. cepistipes* formed the

clades that were strongly supported by reliability values (88–100%). In the NJ tree, the second major clade was divided into two subclades. The first subclade was composed of *A. ostoyae* clade, *A. sinapina* clade and *A. cepistipes* clade. The second subclade included *A. mellea* clade, *A. gallica* clade, *A. nabsnona* clade and Nag. E clade. Each species formed a distinct clade that was supported by high reliability values (96-100%) except for *A. gallica* (63%).

The ML and NJ trees were constructed with the EF-1α gene of Japanese and foreign isolates (Figures 2-11, 2-12). A similar topographic trend was observed in other trees (Figures 2-5, 2-6, 2-7 and 2-8.)

2-4 DISCUSSION

The sequences of the IGS and ITS regions of rDNA and the EF-1α gene in Japanese *Armillaria* species were determined. This is the first comprehensive study on the phylogeny of the genus in Japan.

In this study 49 isolates of seven described and one undescribed *Armillaria* species were analyzed. *Armillaria* jezoensis and *A. singula*, which have been described from Hokkaido (Cha et al 1994), could not be included in the analysis because no isolate or specimen was available. Extensive collections by Ota et al (1998b, 2009) identified a number of isolates of the eight species but could not identify the other two in Hokkaido or other areas of Japan. Thus, the eight species in this study may be common wood inhabiting *Armillaria* species and potential pathogens of trees and shrubs in Japan, and that *A. jezoensis* and *A. singula* may be rare.

The biological species Nag. E has not been described yet, but is considered to be new one on the

basis of morphological observations (Nagasawa 1991) and mating tests with European, North American and Japanese species except *A. jezoensis* and *A. singula* (Ota et al 1998b). In all phylogenetic trees generated in this study, isolates of Nag. E formed an independent clade. This strongly supports the hypothesis that Nag. E is a distinct species. Another possibility that Nag. E is synonymous with *A. jezoensis* or *A. singula* can be eliminated by both morphological and ecological characteristics of these species: Nag. E can be clearly differentiated morphologically from *A. jezoensis* and *A. singula* by its pileus covered with fine scales and its stipe that has the same or finer scales at least when young (Nagasawa 1991, Ota et al 2009). In ecological aspects Nag. E occurs twice a year in Hokkaido (Ota et al 2009) while *A. jezoensis* and *A. singula* occur once a year (Cha et al 1994). Furthermore, although basidiocarps of Nag. E grow most commonly in large groups (Ota et al 2009), those of *A. jezoensis* and *A. singula* are caespitose (in groups) to solitary and solitary, respectively(Cha et al 1994).

Our sequence analyses suggested that the EF-1α gene is more useful for species identification than rDNA, which is commonly used for molecular identification and phylogenetic study of the genus *Armillaria* in Japan. The topology of the phylogenetic trees constructed with sequence data of the ITS and IGS regions of rDNA and the EF-1α gene was roughly similar each other. That is, *A. tabescens* and *A. mellea* were located in the most divergent positions in genus *Armillaria* and *A. nabsnona*, Nag. E, and *A. ostoyae* each formed a species-specific clade in all trees.

However, some topological differences were found among trees on a finer scale. In the trees generated from the data of the IGS and ITS regions of rDNA, the Japanese isolates of the three species, *A. gallica*, *A. sinapina* and *A. cepistipes*, were not separated. This result is consistent with the prior work on *Armillaria* isolates from the northern hemisphere (Harrington and Wingfield 1995; Chillali et al 1998a, b; Terashima et al 1998a; White et al 1998; Kim et al 2000,

2006; Sicoli et al 2003, McLaughlin and Hsiang 2010), indicating that the resolution of the methods for identifying species of *Armillaria* was insufficient. In contrast, in the trees constructed with the EF-1α data, all species, including the above three, each formed a distinct clade. In particular, it is important that *A. cepistipes* and *A. sinapina* were clearly separated. *Armillaria cepistipes* has been reported in Europe (Korhonen 1978, Romagnesi and Marxmüller 1983) and North America where it is called North American biological species XI (Morrison et al 1985, Banik and Burdsall 1998), and *A. sinapina* has been described in North America (Bérubé and Dessureault 1988). These two species are considered to be closely related because of their partial interfertility (Anderson et al 1980, Banik and Burdsall 1998). In the present study EF-1α data showed that the Japanese isolates of *A. cepistipes* and *A. sinapina* belong to different groups, demonstrating the high resolution of this method for identifying species of Japanese *Armillaria*.

Most of the Japanese isolates formed species-specific clades with high branch support values in the ML and NJ tree with EF-1α data. Isolates of *A. gallica* was the only exception in that the branch support of their clade in the NJ tree was less than 70% (63%). On the other hand, in the ML tree, isolates of *A. gallica* did not solely form a distinct clade but were situated basal in the parent clade of *A. mellea*. Thus, the two analysis can be considered to work almost equivalently in the identification of Japanese *Armillaria* species using EF-1α sequences.

The IGS region of five haploid isolates could not be sequenced by a direct sequencing technique probably because of the intragenomic variation of this region. This phenomenon may be caused by variable multicopies in an rDNA array (Coetzee et al 2005b, Schnabel et al 2005, Hanna et al 2007). In such cases, direct sequencing of PCR products is often difficult and cloning is required before sequencing.

However, heterogeneity was observed in the EF-1α gene of some isolates of *A. mellea*. Japanese isolates of this species are non-heterothallic (Ota et al 1998a). Therefore all isolates of this taxon in this study are diploid and some of them could be heterozygotes. This could explain the heterogeneity found in this study, and this observation might provide a clue to the genetics of this non-heterothallic taxon; its life cycle has the transient haploid stage limited to the postmeiosis in basidia (Ota et al 1998a).

By contrast one of the obvious benefits to the use of the ITS and IGS region is that these regions are relatively easy to amplify due to large copy numbers and in many cases it is possible to amplify these regions directly from decayed wood, mycelial fans, rhizomorph tissues and fungal cultures without DNA extraction. Although EF-1α genes could be amplified as readily as the ITS and IGS regions in this study, it might be a challenge to amplify the gene from samples under aggravated conditions, such as aging herbarium specimens or crude homogenized solutions without DNA extraction.

PCR-RFLP analysis of the IGS region has been used to identify *Armillaria* species (Harrington and Wingfield 1995, Banik et al 1996, Volk et al 1996, Chillali et al 1998a, Terashima et al 1998b, White et al 1998, Pérez-Sierra et al 1999, Kim et al 2000, Fukuda et al 2003, Coetzee et al 2005a, b, Matsushita and Suzuki 2005, Keča et al 2006, Sekizaki et al 2008, Keča et al 2009, McLaughlin and Hsiang 2010). In this study the RFLP patterns of the sequence data of the IGS region could be used to distinguish most taxa of the Japanese *Armillaria* isolates that were analyzed. However *A. cepistipes* and *A. sinapina* could not be distinguished from each other. Furthermore, up to three banding patterns were obtained with each restriction enzyme for isolates of the same species, implying that more latent patterns exist. These results suggest that the IGS-RFLP method has a limitation on the use for the identification of the *Armillaria* species in

Japan.

Phylogenetic trees constructed only with the sequence data of the EF-1α gene in Japanese *Armillaria* isolates and with those of the Eurasian and North American counterparts together with Japanese ones showed largely similar topologies each other (Figures 2-9, 2-10, 2-11 and 2-12). In these trees, isolates of the same species were placed in the same or in adjacent clades. However, some points remain unclear. Although European *A. cepistipes* (EU251392, EU251393 and EU251396) formed a sister clade with the Japanese counterparts, North American *A. cepistipes* (DQ435630) was distant from Japanese *A. cepistipes* and close to Japanese *A. nabsnona*. In addition North American *A. nabsnona* (DQ435631) appeared closer to Japanese *A. gallica* than to Japanese *A. nabsnona* in the ML tree. These inconsistencies might be caused by a simple human error, such as incorrect species names being applied to some cultures in the database. Nevertheless, explanation of the genetic relationships among these lineages require further taxonomic and phylogenetic studies with a larger number of samples.

In the present study, sequence analyses were performed on the IGS and ITS regions of rDNA and the EF-1α gene in the common wood-inhabiting Japanese *Armillaria* species. Analysis of the EF-1α gene sequences successfully distinguished among all the species that were analyzed, including the closely related species *A. cepistipes* and *A. sinapina*. Species identification in the genus *Armillaria* based on the EF-1α gene is an innovative method that gives clear results by direct sequencing, and it will be a great help in the ecological and epidemiological studies of *Armillaria*, which require the identification of a number of field samples.

Table 2-1. *Armillaria* isolates used in this study

Species	Isolate no.	Alternative culture collection no.	Derivation	Location	Specimen No.	GenBank accession numbers		
						ITS	IGS	EF-1α
A. tabescens	96-1-8	MAFF420667, WD2607	Single spore	Ibaraki	TFM27084	AB510867	AB510823	AB510804
A. tabescens	96-3-3	FFPRI420858, WD2585	Single spore	Tokyo		AB510868	AB510824	AB510805
A. tabescens	2002-06-03		Single spore	Ibaraki		AB510892	AB510844	AB510806
A. tabescens	2006-20-01		Single spore	Gunma	TFM27098	AB510887	AB510839	AB510807
Nag.E	NE4[ab]		Single spore	Tottori		AB510874	AB510828	AB510771
Nag.E	94-2-1[f]	MAFF420665, WD2605	Single spore	Nagano	TFM27096	AB510888	AB510840	AB510768
Nag.E	94-35-01		Single spore	Yamagata	TFM27099	AB510891	AB510843	AB510770
Nag.E	94-43-08		Single spore	Fukushima		AB510895	AB510846	AB510772
Nag.E	96-37-1	FFPRI420859, WD2586	Single spore	Kanagawa	TFM26893	AB510893	AB510845	AB510769
Nag.E	2000-23-02		Single spore	Aomori	TFM27100	AB510879	AB510832	AB510773
Nag.E	2003-71-13		Single spore	Ibaraki		AB510871	AB510826	AB510767
A. gallica	NA4[abe]	TMIC 31063	Single spore	Fukushima		AB510881	AB510834	AB510761
A. gallica	NA13[abde]	TMIC 31525	Single spore	Aomori		AB510890	AB510842	AB510760
A. gallica	NA17[ae]		Fruitbody	Tottori		AB510872	[g]	AB510759
A. nabsnona	NB3[abde]	TMIC 30987	Single spore	Tottori		AB510894	[g]	AB510763
A. nabsnona	NB4[abde]		Single spore	Aomori		AB510900	AB510851	AB510764
A. nabsnona	00-3-1[f]	MAFF420659, WD2599	Single spore	Aomori	TFM27089	AB510899	AB510850	AB510766
A. nabsnona	00-4-4	MAFF420730, WD2613	Single spore	Kanagawa	TFM27094	AB510876	AB510830	AB510762
A. nabsnona	00-16-4	FFPRI420860, WD2587	Single spore	Ibaraki		AB510883	AB510835	AB510765
A. mellea	A-10		Mycerial mat	Tokyo		AB510860	AB510816	AB510800
A. mellea	A-12		Wood	Wakayama		AB510864	AB510820	AB510801
A. mellea	89-07	FFPRI420861, WD2588	Mycerial mat	Miyazaki		AB510852	AB510808	AB510796
A. mellea	94-5[c]		-	Tokyo		AB510880	AB510833	AB510802
A. mellea	94-7	MAFF420656, WD2596	Fruitbody	Tokyo	TFM27097	AB510863	AB510819	AB510799
A. mellea	94-10-1		Single spore	Kanagawa		AB510857	AB510813	AB510798
A. mellea	94-68		Mycerial mat	Iwate		AB510858	AB510814	AB510803

Table 2-1. Continued

Species	Isolate no.	Alternative culture collection no.	Derivation	Location	Specimen No.	GenBank accession numbers		
						ITS	IGS	EF-1α
A. mellea	97-6		Fruitbody	Ibaraki		AB510856	AB510812	AB510797
A. sinapina	96-7-1[bf]	MAFF420663, WD2603	Single spore	Hokkaido	TFM27085	AB510873	AB510827	AB510774
A. sinapina	96-8-1[f]		Single spore	Hokkaido	TFM26872	AB510878	[g]	AB510777
A. sinapina	05-7-1		Single spore	Hokkaido	TFM26986	AB510886	AB510838	AB510775
A. sinapina	05-13-2[f]	FFPRI420862, WD2589	Single spore	Hokkaido	TFM26991	AB510884	AB510836	AB510776
A. sinapina	05-46-1[f]		Single spore	Hokkaido	TFM27021	AB510889	AB510841	AB510778
A. cepistipes	ND1[abde]	TMIC 30968	Single spore	Aomori		AB510885	AB510837	AB510792
A. cepistipes	ND11[abe]	TMIC 31069	Single spore	Aomori		AB510869	[g]	AB510787
A. cepistipes	90-10-12		Single spore	Niigata		AB510862	AB510818	AB510790
A. cepistipes	94-33-01	FFPRI420864, WD2591	Single spore	Yamagata		AB510866	AB510822	AB510789
A. cepistipes	94-39-04	FFPRI420863, WD2590	Single spore	Yamagata	TFM27101	AB510853	AB510809	AB510786
A. cepistipes	94-46-01		Single spore	Fukushima		AB510898	AB510849	AB510793
A. cepistipes	96-19-1		Single spore	Hokkaido		AB510882	[g]	AB510791
A. cepistipes	2000-07-04		Single spore	Aomori		AB510870	AB510825	AB510788
A. cepistipes	2000-08-14		Single spore	Aomori		AB510875	AB510829	AB510794
A. cepistipes	04-16-1[f]		Single spore	Hokkaido	TFM26966	AB510877	AB510831	AB510795
A. ostoyae	NC8[ae]	TMIC 31491	Single spore	Aomori		AB510897	AB510848	AB510782
A. ostoyae	88-01-19		Single spore	Aomori		AB510859	AB510815	AB510784
A. ostoyae	89-03B-09		Single spore	Hokkaido		AB510861	AB510817	AB510780
A. ostoyae	91-01-10		Single spore	Hokkaido		AB510865	AB510821	AB510785
A. ostoyae	94-8-2	MAFF420661, WD2601	Single spore	Aomori		AB510855	AB510811	AB510783
A. ostoyae	94-75-07	FFPRI420865, WD2592	Single spore	Iwate	TFM27102	AB510854	AB510810	AB510779
A. ostoyae	2002-66-03		Single spore	Tochigi		AB510896	AB510847	AB510781

[a] Isolates supplied by Dr. E. Nagasawa (Tottori Mycological Institute).

[b–f] Isolates identified in previous studies ([b] Ota et al 1998, [c] Ota et al 2000, [d] Fukuda et al 2003, [e] Matsushita and Suzuki 2005, [f] Ota et al 2009)

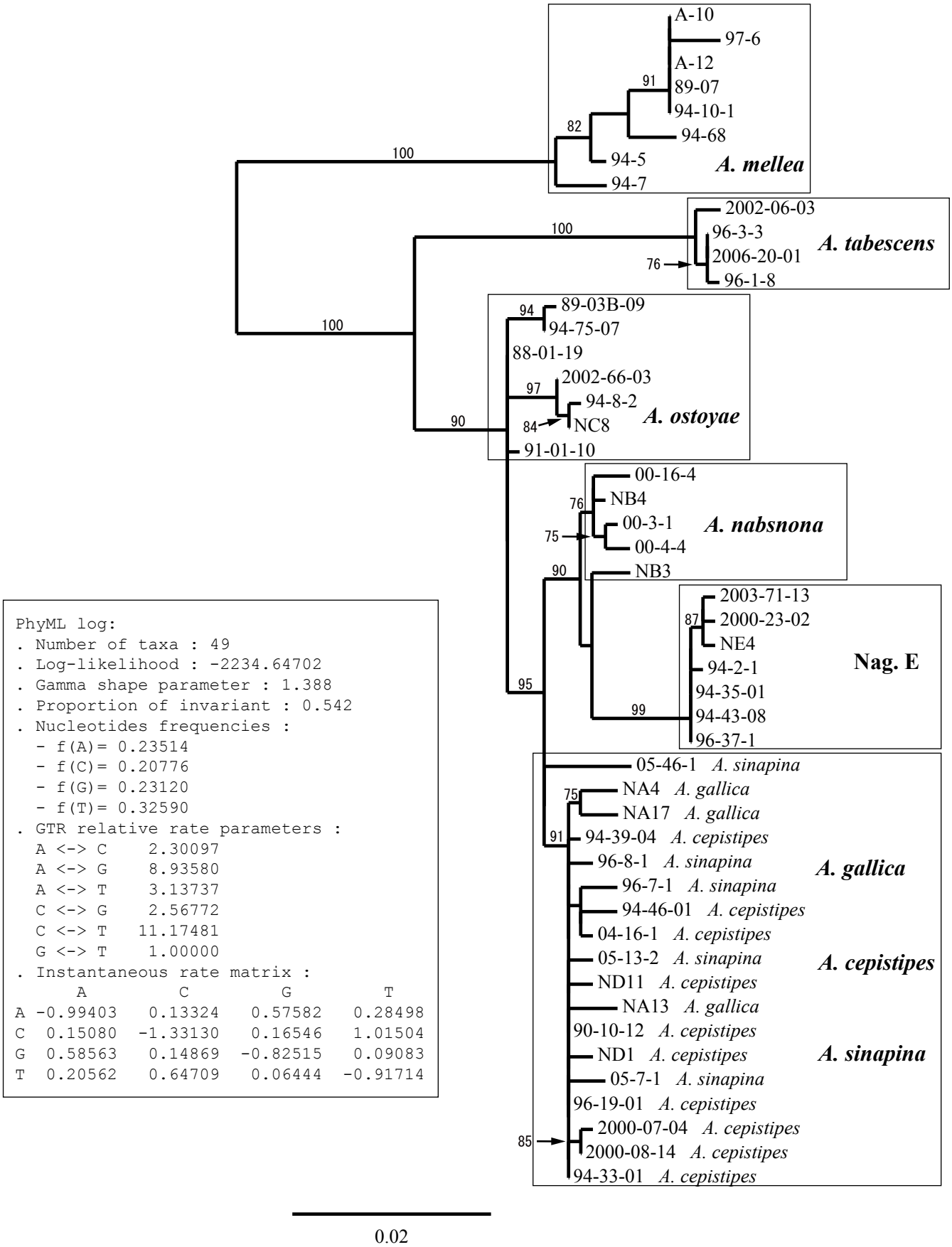
[g] The IGS regions were heterogeneous and the DNA sequences could not be determined accurately.

Table 2-2. Expected restriction fragment sizes of the IGS-1 region of Japanese *Armillaria* species

Species	Isolate no.	*Alu* I		*Hae* III		*Hinf* I		*Msp* I	
		Type	Fragment size (bp)	Type	Fragment size	Type	Fragment size	Type	Fragment size
A. tabescens	96-1-8	a	234, 422	a	138, 277, 334	a	154, 666	a	335, 508
	96-3-3	a	234, 422	a	138, 277, 334	a	154, 666	a	335, 508
	2006-20-01	a	233, 422	a	138, 276, 334	a	154, 665	a	335, 507
	2002-06-03	b	221, 422	b	138, 250, 334	b	154, 639	b	335, 481
A. mellea	A-10	c	162, 163, 307	c	163, 256, 336	c	155, 238, 310	c	114, 337, 396
	A-12	c	162, 163, 307	c	163, 256, 336	c	155, 238, 310	c	114, 337, 396
	89-07	c	162, 163, 307	c	163, 256, 336	c	155, 238, 310	c	114, 337, 396
	94-10-1	c	162, 163, 307	c	163, 256, 336	c	155, 238, 310	c	114, 337, 396
	94-68	c	162, 163, 308	c	163, 256, 337	c	155, 239, 310	c	114, 338, 396
	97-6	c	162, 163, 307	c	163, 256, 336	c	155, 238, 310	c	114, 337, 396
	94-7	c	162, 163, 308	c	169, 256, 337	d	155, 245, 310	c	114, 338, 402
	94-5	d	162, 163, 379	c	169, 256, 342	d	155, 250, 310	c	114, 343, 402
A. nabsnona	NB4	e	135, 194, 308	d	191, 273, 330	e	154, 197, 459	d	114, 331, 444
	00-3-1	e	135, 193, 308	d	190, 274, 330	e	154, 197, 458	d	113, 331, 445
	00-4-4	e	135, 194, 308	d	191, 274, 330	e	154, 197, 459	d	114, 331, 445
	00-16-4	e	135, 194, 308	d	191, 274, 330	e	154, 197, 459	d	114, 331, 445
A. ostoyae	NC8	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
	88-01-19	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
	89-03B-09	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
	91-01-10	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
	94-8-2	e	135, 193, 308	e	114, 190, 208, 330	f	151, 154, 250, 329	e	331, 576
	94-75-07	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
	2002-66-03	e	135, 194, 308	e	114, 191, 208, 330	f	151, 154, 250, 330	e	331, 577
Nag.E	NE4	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471
	94-2-1	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471
	94-35-01	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471
	94-43-08	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471
	96-37-1	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471

Table 2-2. Continued

Species	Isolate no.	*Alu* I		*Hae* III		*Hinf* I		*Msp* I	
		Type	Fragment size (bp)	Type	Fragment size	Type	Fragment size	Type	Fragment size
Nag.E	2000-23-02	f	135, 193, 405	f	330, 520	g	154, 197, 481	f	114, 331, 470
	2003-71-13	f	135, 194, 405	f	330, 521	g	154, 197, 482	f	114, 331, 471
A. gallica	NA4	f	135, 194, 401	g	190, 211, 330	h	154, 197, 326	e	331, 579
	NA13	g	182, 194, 399	g	191, 211, 328	h	154, 195, 327	e	329, 580
A. sinapina	05-7-1	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	05-13-2	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	96-7-1	h	182, 233, 401	h	179, 221, 330	h	154, 197, 327	g	114, 331, 421
	05-46-1	e	135, 193, 308	e	114, 191, 211, 330	g	154, 197, 480	f	113, 331, 466
A. cepistipes	ND1	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	90-10-12	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	94-46-01	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	2000-07-04	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	2000-08-14	h	182, 233, 401	g	191, 211, 330	h	154, 197, 327	g	114, 331, 421
	94-39-04	i	181, 233, 308	g	191, 211, 330	i	136, 154, 197, 327	g	114, 331, 420
	94-33-01	j	152, 233, 401	g	191, 211, 330	j	154, 197, 297	h	114, 331, 391
	04-16-1	k	182, 233, 411	i	191, 221, 330	h	154, 197, 327	i	114, 331, 431




```
PhyML log:
. Number of taxa : 49
. Log-likelihood : -2234.64702
. Gamma shape parameter : 1.388
. Proportion of invariant : 0.542
. Nucleotides frequencies :
  - f(A)= 0.23514
  - f(C)= 0.20776
  - f(G)= 0.23120
  - f(T)= 0.32590
. GTR relative rate parameters :
  A <-> C    2.30097
  A <-> G    8.93580
  A <-> T    3.13737
  C <-> G    2.56772
  C <-> T   11.17481
  G <-> T    1.00000
. Instantaneous rate matrix :
      A         C         G         T
A -0.99403   0.13324   0.57582   0.28498
C  0.15080  -1.33130   0.16546   1.01504
G  0.58563   0.14869  -0.82515   0.09083
T  0.20562   0.64709   0.06444  -0.91714
```

Figure 2-1. The maximum-likelihood tree generated with the ITS sequences in Japanese *Armillaria* species. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the left box.

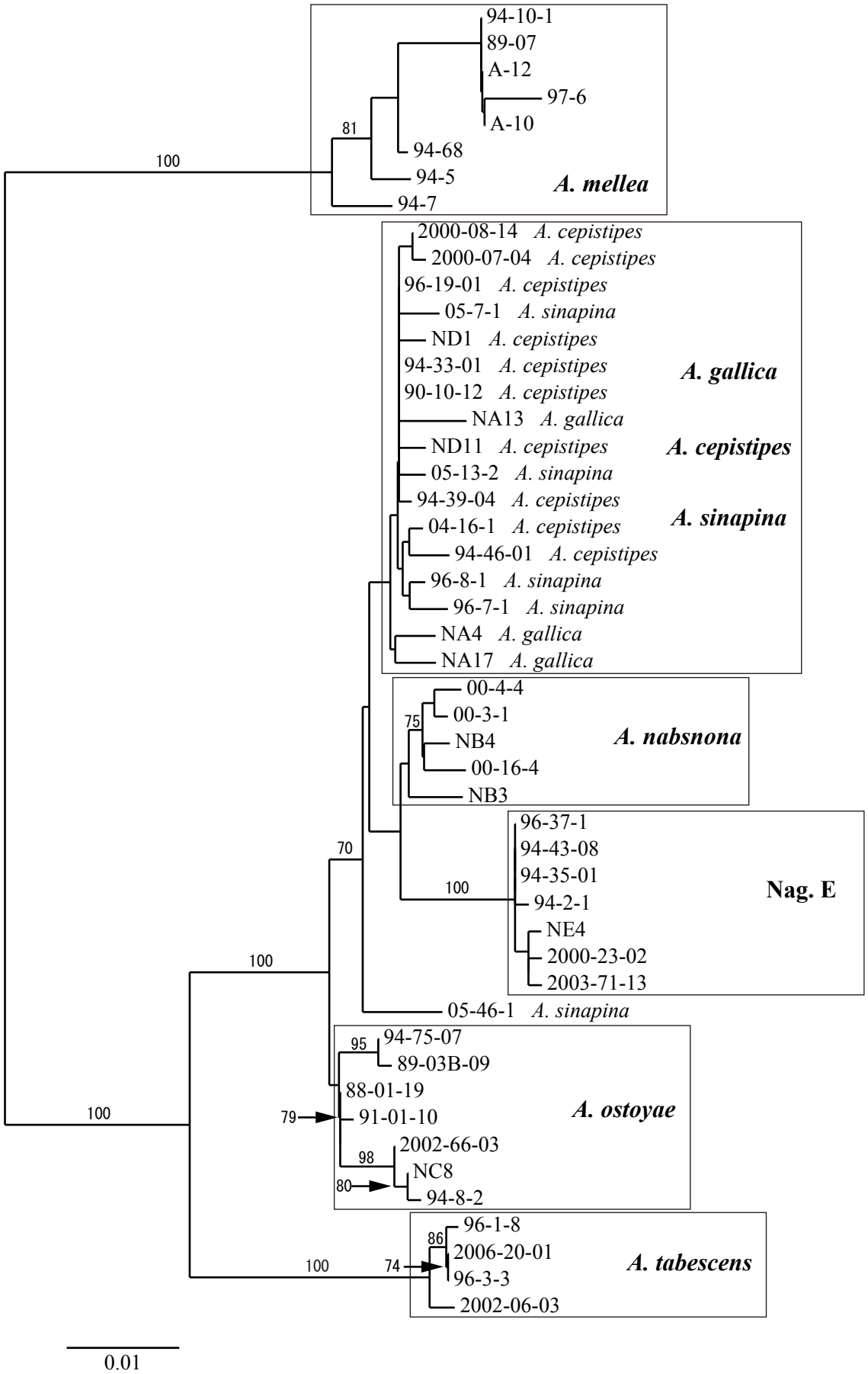


Figure 2-2. The neighbor-joining tree generated with the ITS sequences in Japanese *Armillaria* species. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

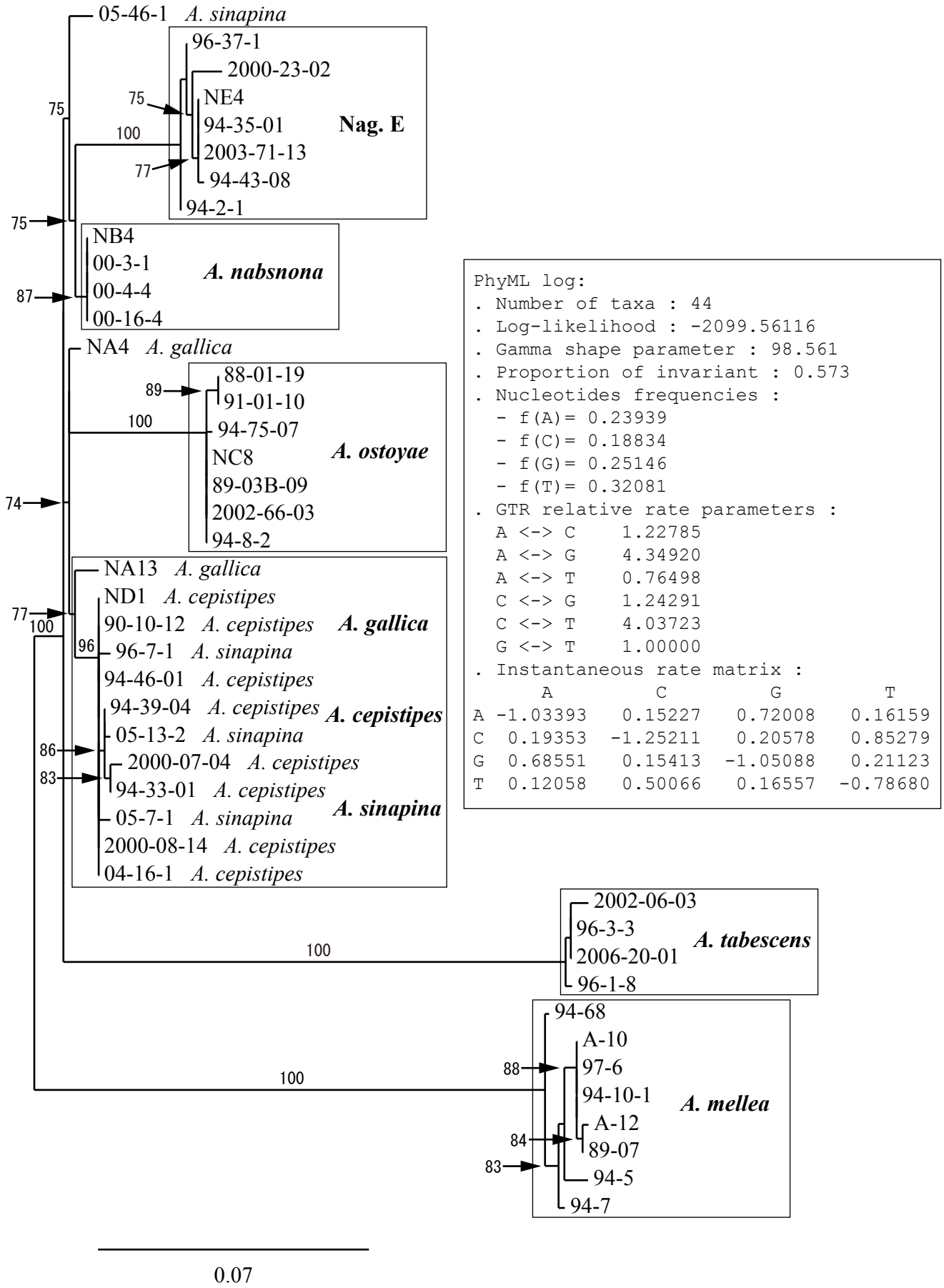


Figure 2-3. The maximum-likelihood tree generated with the IGS-1 sequences in Japanese *Armillaria* species. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the right box.

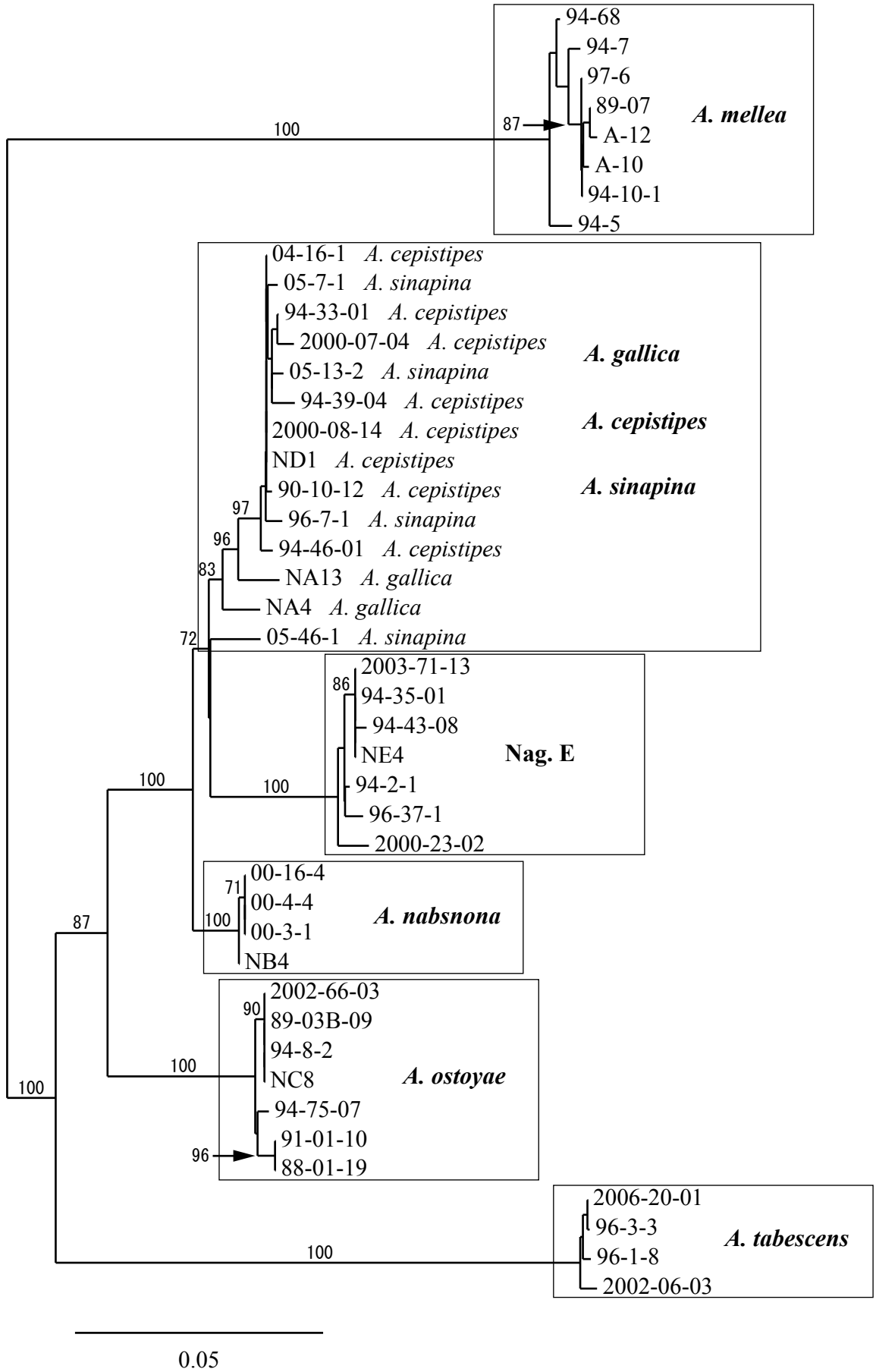
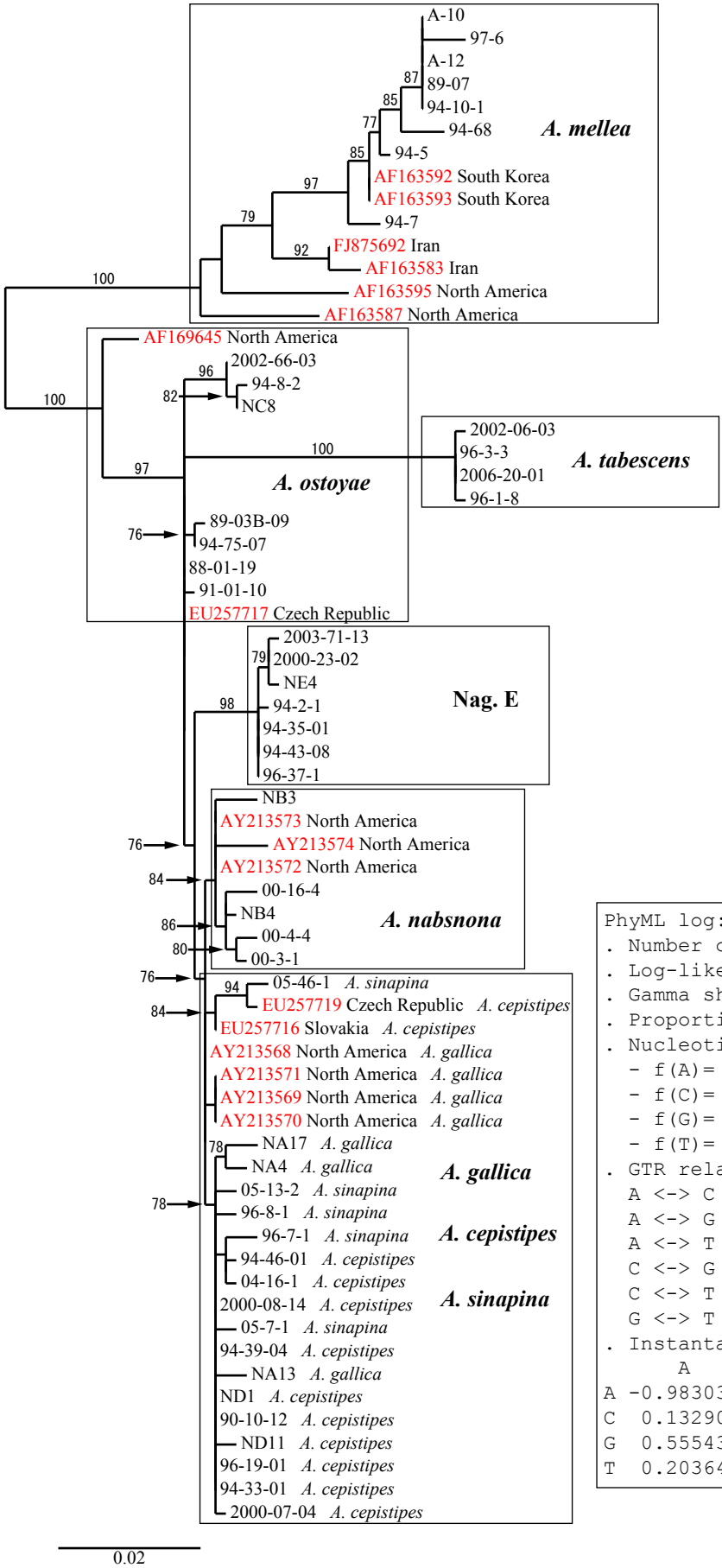


Figure 2-4. The neighbor-joining tree generated with the IGS-1 sequences in Japanese *Armillaria* species. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.




```
PhyML log:
. Number of taxa : 66
. Log-likelihood : -2205.83228
. Gamma shape parameter : 0.716
. Proportion of invariant : 0.434
. Nucleotides frequencies :
  - f(A)= 0.22770
  - f(C)= 0.20586
  - f(G)= 0.23060
  - f(T)= 0.33584
. GTR relative rate parameters :
  A <-> C    1.61635
  A <-> G    6.75502
  A <-> T    2.47666
  C <-> G    3.02527
  C <-> T    7.86446
  G <-> T    1.00000
. Instantaneous rate matrix :
       A          C          G          T
A -0.98303   0.12015   0.56251   0.30036
C  0.13290  -1.33860   0.25192   0.95377
G  0.55543   0.22489  -0.90159   0.12128
T  0.20364   0.58462   0.08327  -0.87153
```

Figure 2-5. The maximum-likelihood tree generated with the ITS sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the right box.

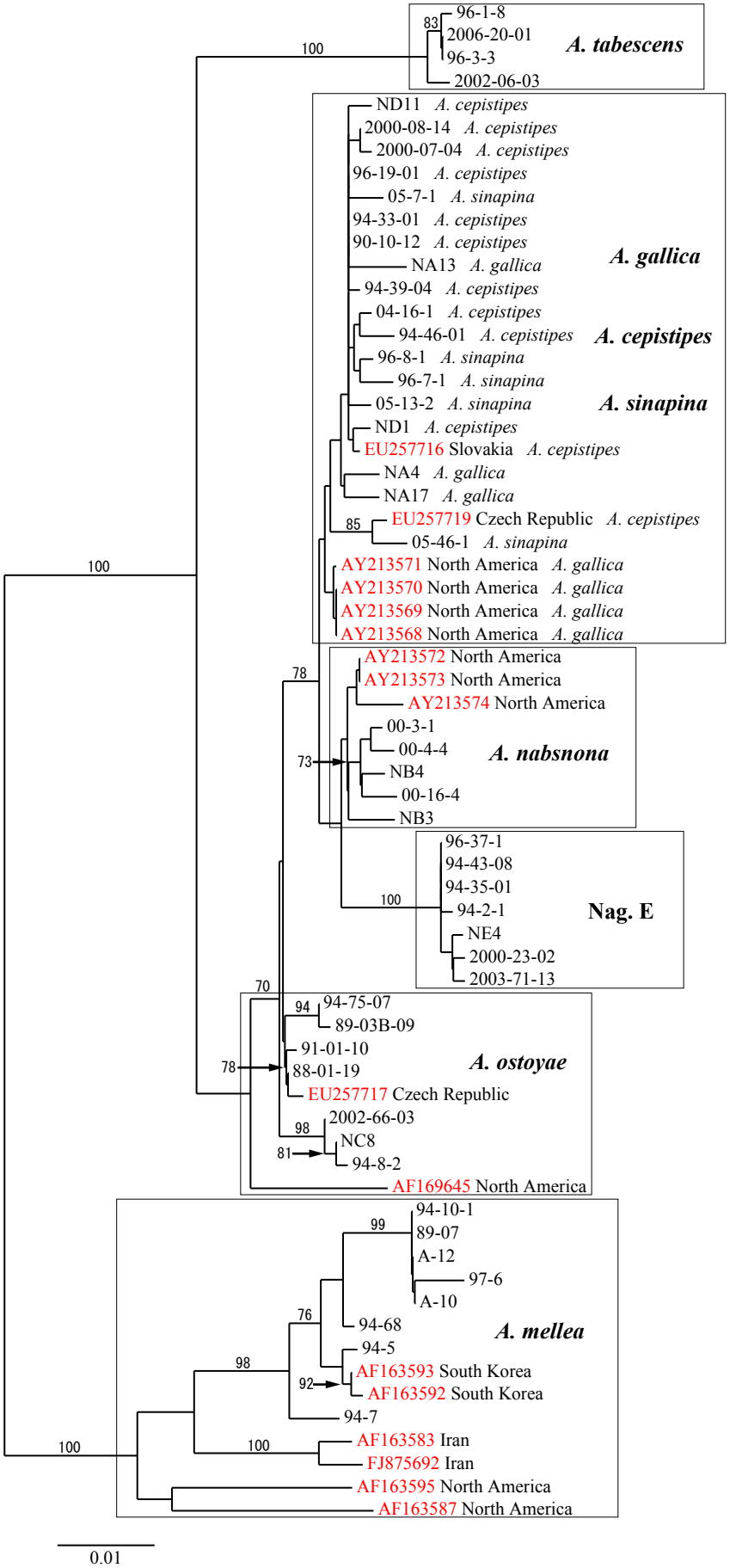


Figure 2-6. The neighbor-joining tree generated with the ITS sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

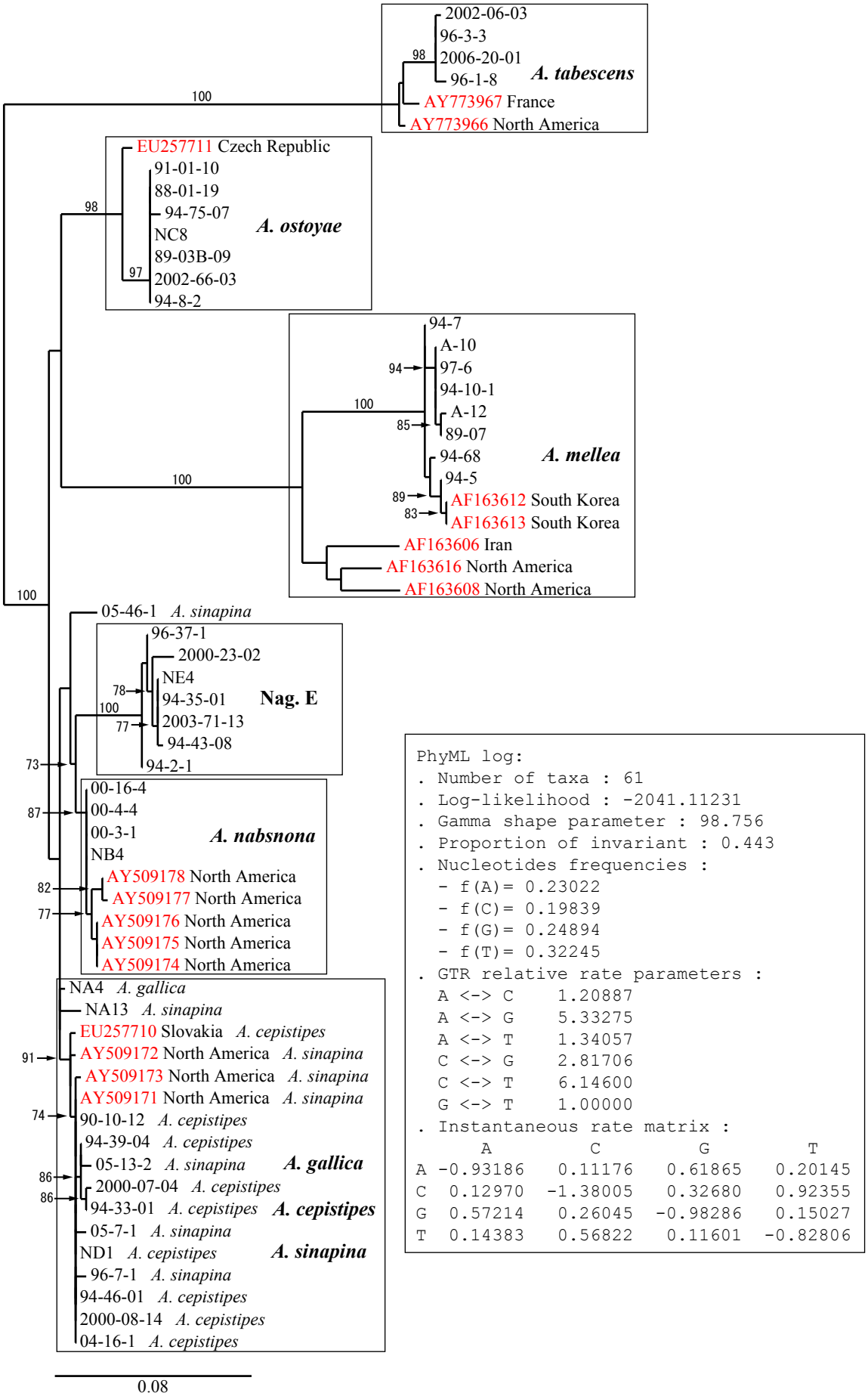


Figure 2-7. The maximum-likelihood tree generated with the IGS-1 sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the right box.

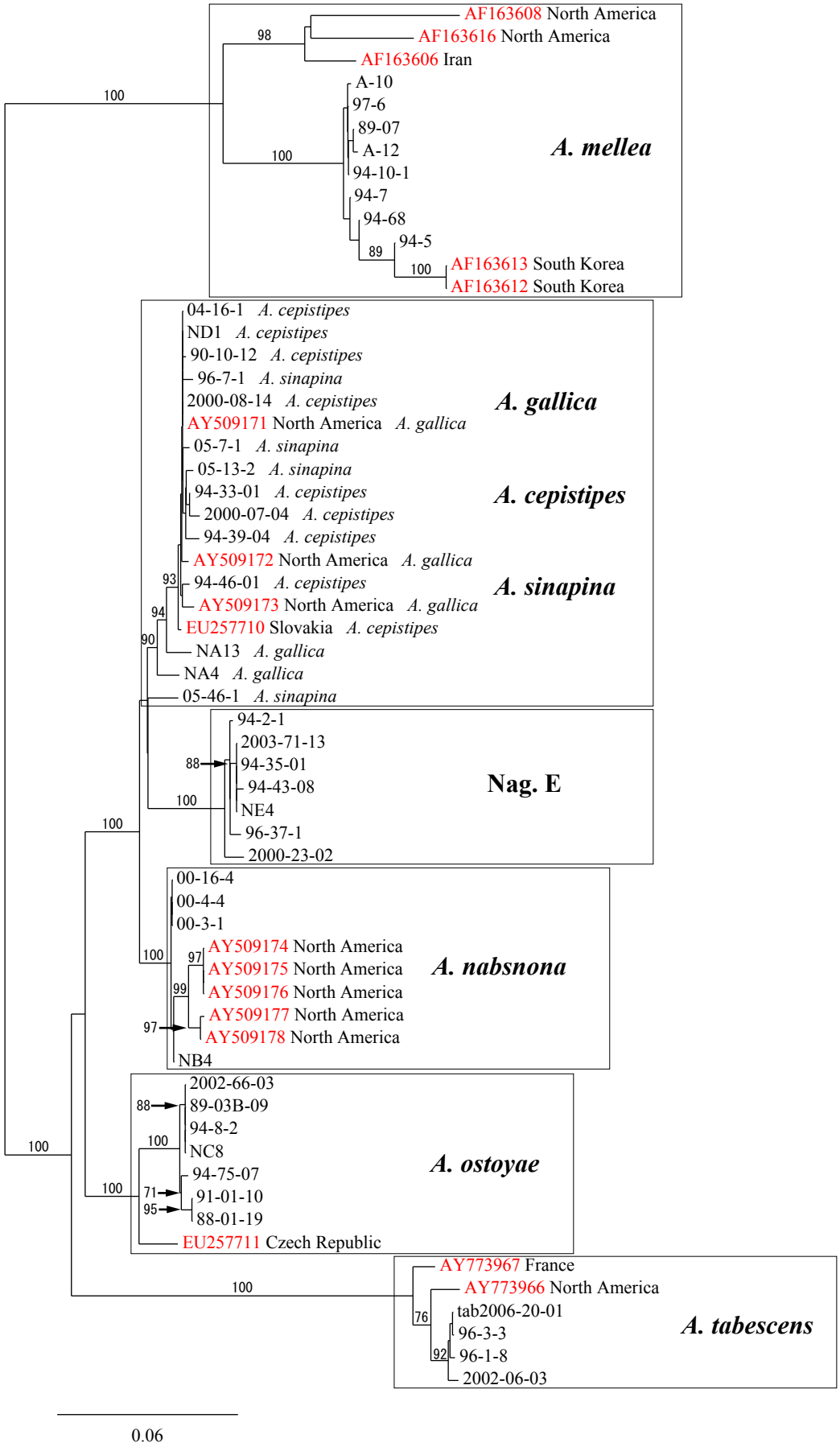


Figure 2-8. The neighbor-joining tree generated with the IGS-1 sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

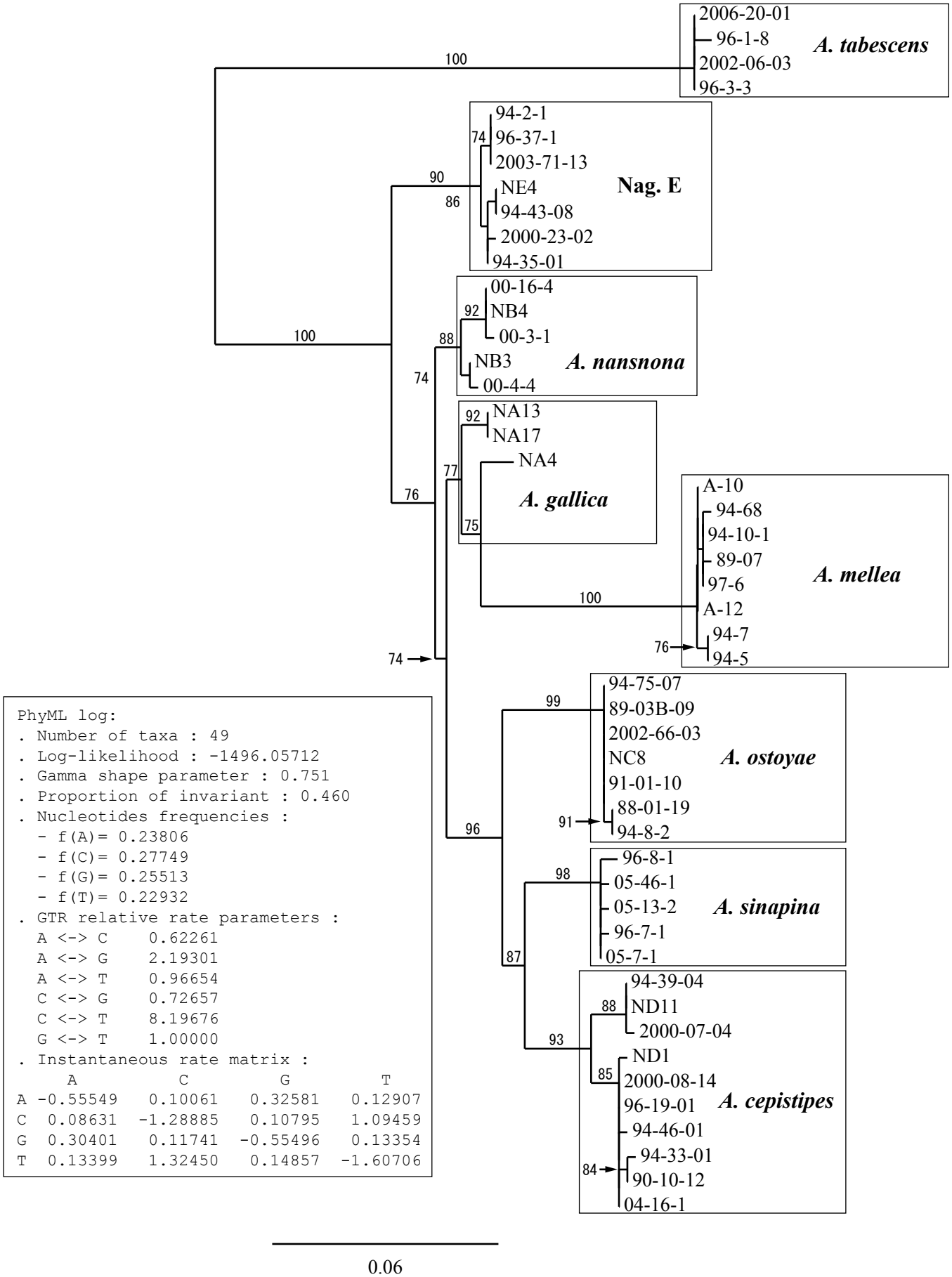


Figure 2-9. The maximum-likelihood tree generated with the EF-1α sequences in Japanese *Armillaria* species. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the left box.

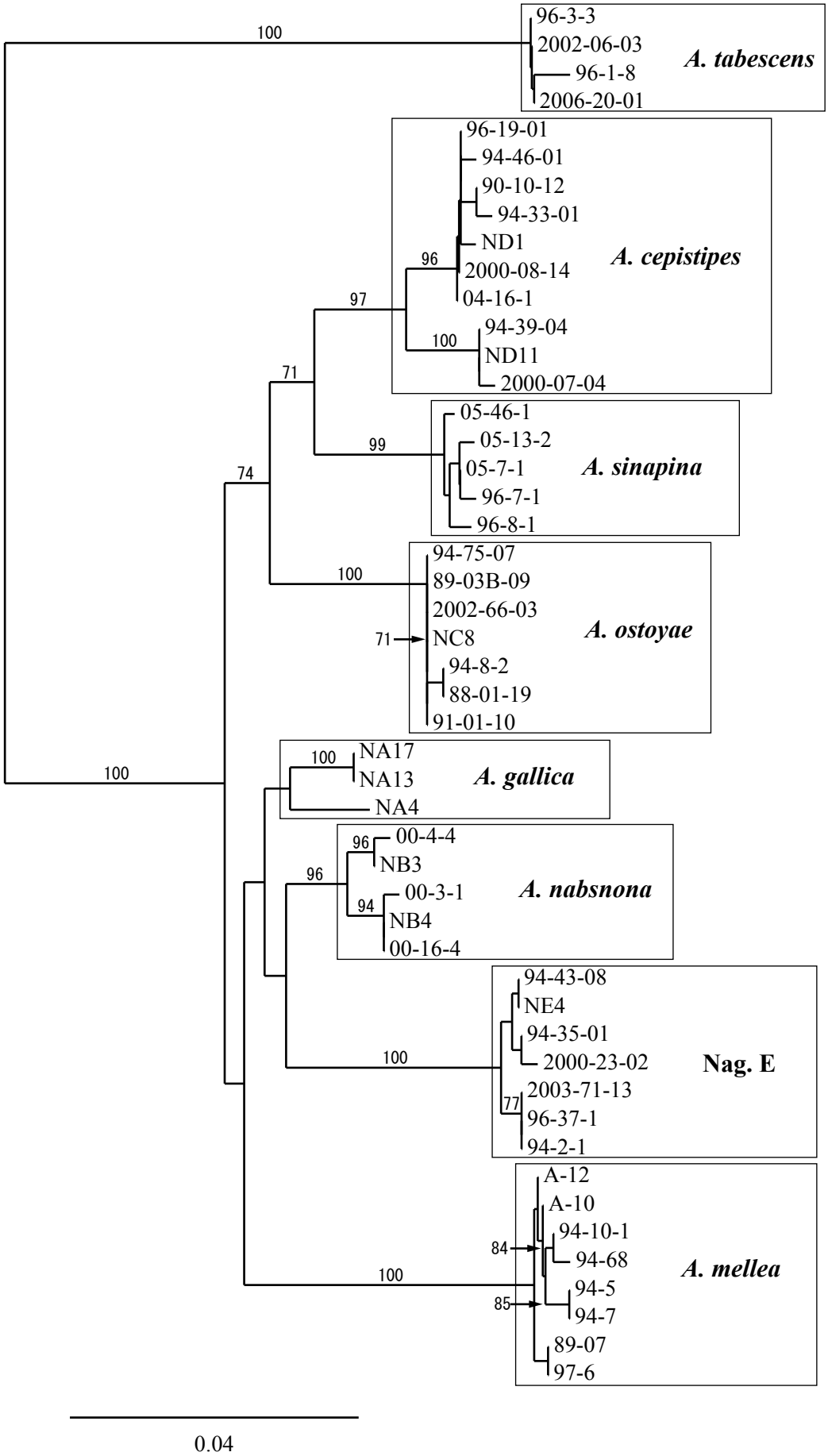


Figure 2-10. The neighbor-joining tree generated with the EF-1α sequences in Japanese *Armillaria* species. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

```
PhyML log:
. Number of taxa : 75
. Log-likelihood : -2224.61166
. Gamma shape parameter : 0.600
. Proportion of invariant : 0.337
. Nucleotides frequencies :
  - f(A)= 0.23843
  - f(C)= 0.27707
  - f(G)= 0.25518
  - f(T)= 0.22932
. GTR relative rate parameters :
  A <-> C    0.69478
  A <-> G    2.20471
  A <-> T    0.86649
  C <-> G    0.61030
  C <-> T    6.13291
  G <-> T    1.00000
. Instantaneous rate matrix :
      A          C          G          T
A -0.66351   0.13392   0.39137   0.13823
C  0.11524  -1.20192   0.10834   0.97834
G  0.36568   0.11763  -0.64284   0.15952
T  0.14372   1.18210   0.17751  -1.50333
```

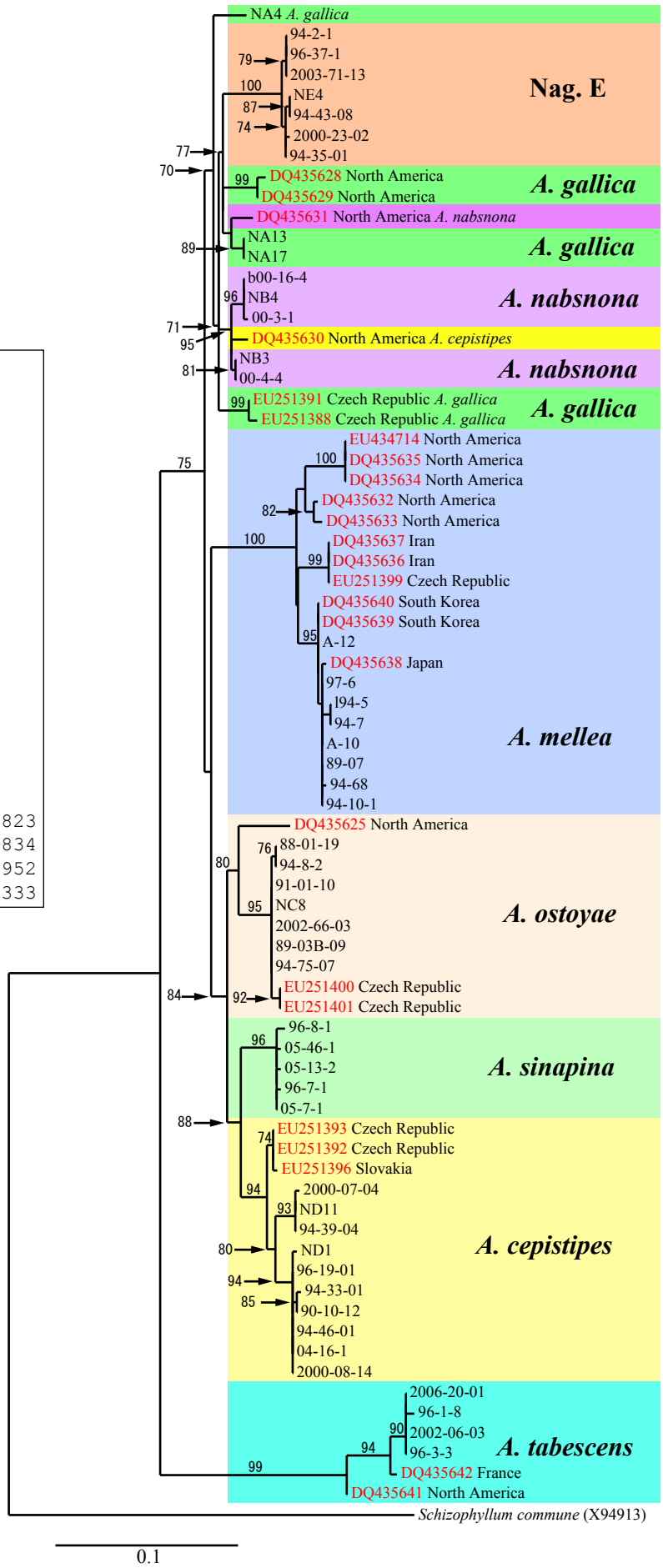


Figure 2-11. The maximum-likelihood tree generated with the EF-1α sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. The *Schizophyllum commune* sequence was used as an outgroup sequence. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences. Parameters of the analysis are shown in the left box.

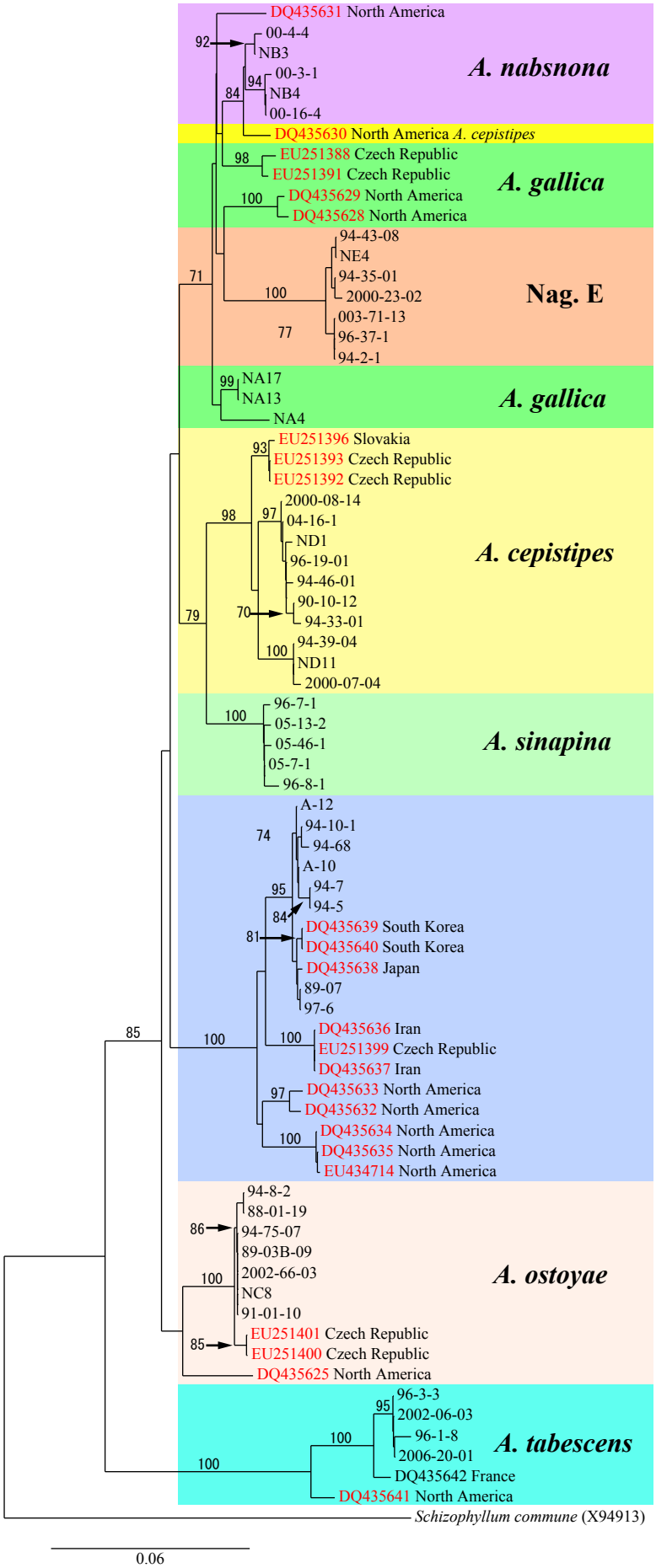


Figure 2-12. The neighbor-joining tree generated with the EF-1α sequences of *Armillaria* isolates from Japan and other locations in the northern hemisphere. GenBank accession numbers of overseas isolates are indicated in red letters. The *Schizophyllum commune* sequence was used as an outgroup sequence. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

3 Ecology of *Armillaria* species on conifers in Japan

3-1 Objectives

The objective of this study is to describe ecology of each *Armillaria* species in Japan using EF-1α for species identification. On the basis of the records on collection, including host species, host condition and geographical information, the ecological characteristics of each *Armillaria* species and its potential pathogenicity to conifers are discussed.

3-2 Materials and methods

Fungal collection

Armillaria isolates were collected using information on the occurrence of Armillaria root disease provided by local researchers and by surveys in apparently healthy forests. Collections were carried out over a 30-year period from 1976 to 2007. Isolates studied included stock cultures in the Laboratory of Forest Plants and Forest Health in the Department of Forest Science, the University of Tokyo, the Forest Pathology Laboratory and Microbial Ecology Laboratory in FFPRI. Fifty-nine collection sites were distributed from Hokkaido in the north to Kyushu in the south and covered a variety of artificial and natural conifer stands in Japan.

Host records and isolate pathogenicity

Isolate pathogenicities were categorized into classes developed by Gregory (1989), Guillaumin et al (1993) and Keča et al (2009), with some modifications:

(a) Isolates from mycelium or a fruit body in／on a living tree showing no obvious predisposing factor for infection.

(a') Isolates from mycelium or a fruit body in／on a tree that had been killed in the previous year.

(b) Isolates from mycelium or a fruit body in／on a living tree stressed by some factor other than *Armillaria*.

(c) Isolates from mycelium or a fruit body in／on decayed heart wood of a living tree showing no symptoms.

(d) Isolates from mycelium, rhizomorph or a fruit body on a tree or a stump that had been killed more than 1 year ago, on a wind-thrown tree or on wood debris.

Case (a) corresponds to Class 1a in Guillaumin et al (1993), and case (a') is regarded as a disease progression of (a). In these cases, the isolates were classified as primary parasites. Case (b) corresponds to Guillaumin's Class 1b, and isolates were classified as opportunists. Case (c) corresponds to Guillaumin's Class 1c. Isolates in cases (c) and (d) were classified as saprotrophs.

Fungal isolation

Cultures were obtained from mycelial mats, decayed wood, rhizomorphs and fruit bodies. Half-strength potato dextrose agar medium [half-strength PDA: half concentration of PDA (Eiken, Tokyo, Japan) plus 0.75% agar (Wako, Osaka, Japan); final concentrations of potato extract, dextrose and agar were 0.2, 1.0 and 1.5%, respectively] containing 0.03% streptomycin sulphate (Meiji Seika, Tokyo, Japan) was used for tissue isolations. Rhizomorphs were cleaned of adhering soil with tap water and immersed first in 70% ethanol and then in 1% NaOCl for ca. 1 min and finally rinsed in sterilized water before plating on medium. Approximately 1-cm-long fragments of a fruit body, decayed wood or mycelial mat in infected phloem were excised from the freshly cut surface and placed on the medium.

Spores were allowed to germinate on 1.5% water agar medium containing 0.03% of streptomycin sulphate, and single spores were transferred to a half-strength PDA plate without antibiotics under a microscope.

Cultures were incubated at room temperature for 2 weeks, and emerging hyphae were transferred to glass tubes containing half-strength PDA without antibiotics and stored at 10 C. The isolates examined were deposited in the Microbial Ecology Laboratory, FFPRI, NIAS Genebank and the FFPRI culture bank.

Identification of *Armillaria* species

The biological species of each isolate was determined using haploid–haploid or haploid–diploid mating tests (Guillaumin et al 1991) with tester strains of the eight Japanese *Armillaria* species. The EF-1α gene of each isolate was sequenced, and phylogenetic analysis was performed as described in chapter 2. The sequences were deposited in the DNA Data Bank of Japan (DDBJ; for accession numbers, see Table 3-1). Isolates previously characterized by both mating tests and phylogenetic analysis based on EF-1α were also used as reference isolates for the eight Japanese *Armillaria* species (identified by accession numbers in Figure 3-1, 3-2).

Construction of collection site map

Collection sites of *Armillaria* isolates were plotted on the Kira's s warmth index (WI) map to show the thermal preference for each *Armillaria* species in Japan. WI is defined as the annual sum of positive differences between monthly mean temperature and +5 C (Kira 1949, 1977,

1991). WI was calculated using 30-year monthly mean temperatures for the period 1971–2000 for each 1 km x 1 km grid of land surface on the Japanese islands (Japan Meteorological Agency 2002) and plotted on a map.

Warmth index is based on the idea that the sequence of forest formations follows a thermal gradient under sufficiently moist climates (Kira 1991). WI represents the total amount of heat available for the growth of plants. Following correlations between WI and forest types have been reported: 180–240 for subtropical evergreen forest, 85–180 for warm-temperate evergreen forest, 45–85 for cool-temperate deciduous forest and 15–45 for subarctic／subalpine coniferous forest (Kira 1991). WI has been used with success for studies of temperate and subtropical vegetation in East Asia (Kira 1977, 1991, Itow 1988), Mediterranean-type vegetation in Europe and Australia (Federici and Pignatti 1991) and boreal vegetation in the Russian Far East (Grishin 1995). It has also been adapted to modeling effects of climatic change on vegetation (Matsui et al 2004, Casalegno et al 2010).

3-3 Results and discussion

Identification of the *Armillaria* isolates

In total, 65 *Armillaria* isolates were collected from the following 19 conifers in Japan: *Cryptomeria. japonica* (*C. japonica*), *Chamaecyparis. obtusa* (*Ch. obtusa*), *Pinus densiflora* (*P. densiflora*), *Larix kaempferi* (*L. kaempferi*), *Abies sachalinensis* (*Ab. sachalinensis*), *Picea jezoensis* (*Pi. jezoensis*), *Pinus palstlis* Mill., *Pinus thunbergii* Parl., *Abies firma* Sieb. et Zucc., *Abies homolepis* Sieb. et Zucc., *Picea abies* (L.) Karst., *Picea glehnii* (Fr. Schmidt) Mast., *Tsuga diversifolia* (Maxim.) Mast. (*T. diversifolia*), *Picea koyamae* Shiras., *Abies mariesii* Mast., *Pinus*

sylvestris L., *Picea polita* (Sieb. et Zucc.) Carr., *Abies veitchii* Lindl. and *Cedrus deodara* (Roxb.) G. Don ex Loudon (*Ce. deodara*)(Table 3-1). Isolates were identified to species level using mating tests and DNA sequence data (Table 3-1). Most of the isolates were identified with the mating tests, although some were difficult to identify because of ambiguous results. Ambiguity in mating tests was more common in haploid–diploid pairings, making interpretations unreliable. Sequence analysis of the EF-1α gene generated a phylogenetic tree with eight clades that were supported by high reliability values in the ML tree (Figure 3-1) and bootstrap values in the NJ tree (Figure 3-2). Each reference isolate of the eight Japanese *Armillaria* species was allocated to one of the eight clades. Thus, the eight clades corresponded to eight Japanese *Armillaria* species. Every isolate, including the reference isolates, fell into one of the eight clades.

Analysis of the EF-1α region is a powerful tool for identification of Japanese *Armillaria* species. In this study, this technique successfully identified all of the isolates, indicating its high utility.

Distribution and host range

Seven *Armillaria* species were isolated from conifers (Table 3-1); their collection sites are shown in Figure 3-3. *Armillaria ostoyae* was collected most frequently (22 isolates), followed by *A. cepistipes* and *A. mellea* (14 isolates each), and *A. sinapina* (11 isolates) (Figure 3-4). These four species are likely the major *Armillaria* species occurring on conifers in Japan.

Isolates of *A. mellea* were collected from Hokkaido in the north to Kyushu in the south (Figure 3-3), and the WI values of the collection sites ranged from 55-145 (Figure 3-5), showing that this species has more southern distribution and is relatively thermophilic among Japanese *Armillaria* species in this study. *Armillaria mellea* is recognized as the dominant *Armillaria* in

Mediterranean region in Europe and California in North America. These are the southernmost areas in each regions and this species is regarded as more thermophilic species (Guillaumin et al 1993, Baumgartner and Rizzo 2001, Marxmüller and Guillaumin 2005,).

The WI values of *A. cepistipes* and *A. ostoyae* collection sites were between 25 and 115 and between 10 and 100, respectively (Figures 3-3, 3-5). Hence, these species have similar thermal preferences and are less thermophilic than *A. mellea*. In Europe, *A. ostoyae* and *A. cepistipes* tend to occur at higher latitude or altitude than *A. mellea* (Tsopelas 1999, Marxmüller and Guillaumin 2005, Keča et al 2009), indicating a very similar order of thermal preferences in these three species between Europe and Japan.

Collection sites of *A. sinapina* were in high latitude (Hokkaido) or altitude (Honshu) (Figure 3-3). This is the first report of *A. sinapina* from areas south of Hokkaido in Japan. Collection sites of *A. sinapina* were in cool regions (WI: 10–70, Figure 3-5). Similarly, in North America, *A. sinapina* has been reported frequently from the northern part of the distribution area of *Armillaria*, including boreal and subalpine forests in Canada (Morrison et al 1985, Dumas 1988, Shaw and Loopstra 1988, Mallett 1990, Blodgett and Worrall 1992, Harrington and Rizzo 1993, Banik et al 1995, 1996, McLaughlin 2001). Also in China, *A. sinapina* was reported from the northern part of the collection sites of the genus (Qin et al 2007). This information suggests that *A. sinapina* is less thermophilic in the genus.

The hosts of *Armillaria* species in this study are shown in Table 3-1and Figure 3-6. *Armillaria mellea* was frequently collected from *Ch. obtusa*, which was an almost exclusive substrate for this species in the present result (Figure 3-6). Collection sites of *A. mellea* indicated a species preference for relatively high temperatures (WI: 55-145, Figures 3-2, 3-5). This preference may

provide opportunities for *A. mellea* to encounter *Ch. obtusa*, which is relatively thermophilic in relation to other conifers listed in Table 3-1: WI of *Ch. obtusa* distribution ranges from 80 to 140 (Kira 1949, Figure 3-7). However, another relatively thermophilic tree and the most popular plantation conifer, *C. japonica*, was not identified as a host for *A. mellea*: WI of *C. japonica* distribution ranges from 75 to 140 (Kira 1949, Figure 3-7). Thus, additional factors likely determine the host preference of *A. mellea*. In contrast, *A. mellea* is frequently isolated from *C. japonica* in the Azores Islands of Portugal, where three *Armillaria* species, *A. mellea*, *A. gallica* and *A. tabescens* were detected and *A. mellea* was the exclusive host of *C. japonica* (Bragança et al 2004). In other regions of Europe, various species in the Pinaceae can serve as hosts, although members of the Cupressaceae are more frequently attacked by *A. mellea* than members of the Pinaceae (Guillaumin et al 1993). Further study is needed to determine the mechanisms of the host preference of Japanese *A. mellea*.

Armillaria cepistipes and *A. ostoyae* were collected from many conifer species, indicating that these two species can utilize a variety of coniferous substrates (Table 3-1, Figure 3-6). *Armillaria ostoyae* was frequently collected from members of the Pinaceae. This is also the case in Europe (Guillaumin et al 1993, Tsopelas 1999, Żółciak 2007, Keča et al 2009), North America (Anderson and Ullrich 1979, Dumas 1988, Blodgett and Worrall 1992, Banik et al 1995, 1996) and Korea (Sung et al 1991). *Armillaria cepistipes* was collected frequently from *C. japonica*. In Europe, *A. cepistipes* has been found in association with members of the Pinaceae (Korhonen 1978, Gregory 1989, Tsopelas 1999, Prospero et al 2003, Bendel et al 2006, Keča et al 2009) and also with a juniper (Keča et al 2009). Substrate preference of Japanese *A. cepistipes* indicates its adaptation to Japanese vegetation.

Armillaria sinapina was collected primarily from *Ab. veitchii* and *Ab. mariesii* and also from *L.*

kaempferi and *Ab. sachalinensis* (Table 3-1, Figure 3-6). The forests of these four host trees were located in the cool-temperate zone: WI of each distribution ranges from 15 to 45 for *Ab. veitchii* and *Ab. mariesii*, from 15 to 75 for *L. kaempferi* and from 15 to 60 for *Ab. sachalinensis* (Kira 1949, Figure 3-7), and *Ab. veitchii* and *Ab. mariesii* are subalpine forest species in Honshu. *Armillaria sinapina* is considered to prefer subalpine *Abies* species as coniferous substrate. In North America, the counterpart of this species has been found on boreal and coastal forest species of *Abies*, *Picea*, *Pinus* and *Tsuga* (Mallett 1990, Banik et al 1996). It indicates that the species in North America can utilize various genera as its substrate, and host preference of Japanese species needs further study, for it may utilize other genera in boreal and highland forests.

Only two isolates of Nag. E, one of *A. nabsnona* and one of *A. tabescens* were collected from conifers. This is the first collection of *A. nabsnona* from a conifer in Japan. No isolates of *A. gallica* were collected in this study. The main substrate of these four species is hardwood (Ota et al 1998b, 2009), which explains the infrequency of collections from conifers in this study. The counterpart species of *A. nabsnona* in North America and of *A. tabescens* in Europe are also reported to be hardwood-inhabiting species (Guillaumin et al 1993, Volk et al 1996, Antonín et al 2006).

Pathogenicity

Classes of pathogenicity associated with *Armillaria* species are shown in Table 3-2. *Armillaria mellea* was frequently recorded as a primary parasite, and 10 of 14 records in this pathogenicity category were from plantation-grown *Ch. obtusa* (Tables 3-1, 3-2). These findings are consistent with inoculation tests showing high virulence of *A. mellea* on *Ch. obtusa* (Hasegawa 1998). In Mediterranean Europe and California in North America, *A. mellea* is recognized as the dominant

Armillaria, and although it attacks both hardwoods and conifers, it is not widely associated with disease on conifers in forest and plantation (Guillaumin et al 1993, Baumgartner and Rizzo 2001). One reason for the high incidence of *A. mellea*-associated disease in Japan may be that plantations of the susceptible species *Ch. obtusa* are distributed consistently with the fungus. As in Europe and North America, *A. mellea* is also regarded as an important pathogen of hardwood species, especially orchard trees in Japan (Kishi 1998).

Armillaria ostoyae was frequently recorded as a primary parasite on conifers, including *Ch. obtusa* and *P. densiflora* (Tables 3-1, 3-2, Figure 3-6). Terashita and Sawaguchi (1991) reported that *A. ostoyae* was a causal pathogen in severely damaged plantations of *P. densiflora*. By contrast, six *A. ostoyae* isolates were recorded as saprotrophs in the present study. Collection of saprotrophic phase does not always mean that the fungus is less pathogenic, but the ratio of saprotrophic collection in relation to the parasitic collection suggests its pathogenicity. In previous inoculation tests, *A. ostoyae* exhibited moderate to high virulence on *Ch. obtusa* (Hasegawa 1998) and *P. densiflora* (Inagawa et al 2002, Matsushita 2002), which is consistent with the findings in the present study. In Europe and North America, *A. ostoyae* is also considered a pathogen of conifers, although its virulence varies among isolates (reviewed by Gregory et al 1991 and Hanna et al 2007).

Two isolates of *A. cepistipes* were recorded as primary parasites, indicating that this fungus is somewhat pathogenic to conifers (Table 3-2). However, it was also collected once as an opportunist and nine times as a saprotroph. Five of nine isolates were collected as saprotrophs from thinning stumps in plantations. Hence, Japanese *A. cepistipes* is saprotrophic on conifers in many situations, but it can be occasionally pathogenic. Field observations (Gregory et al 1991, Guillaumin et al 1993) and inoculation tests (Morrison 2004, Prospero et al 2004) showed that

the counterpart of this species in Europe and North America is a weak pathogen as a whole.

Armillaria sinapina was not recorded as a primary parasite or an opportunist, indicating that this fungus is non-pathogenic on conifers (Table 3-2). *Armillaria sinapina* is considered adapted to subalpine natural forests in Japan, within which it causes few visible destructive effects. In North America, *A. sinapina* is regarded as a weak pathogen from field observations and inoculation tests suggested that it has low virulence towards some conifers, but it was more virulent than *A. ostoyae* in one exceptional case (Gregory et al 1991). In the present study, one isolate was found as a saprotroph on a living tree (without symptoms) suggesting that *A. sinapina* is able to enter living tissue. Pathogenicity towards Japanese conifers has yet to be determined.

One isolate of *A. tabescens* was collected as a primary parasite from a *Ce. deodara* planted in a garden (Tables 3-1, 3-2). This fact indicates that the fungus can make damage on coniferous species. Since *A. tabescens* was reported as a pathogen of peach orchards in 1974 by Fujii and Hatamoto, reports of disease caused by this fungus have been increasing in managed conditions, including gardens and boulevards in Japan (Kaneko and Ogawa 1998, Sato and Suzuki 2002, Hasegawa 2005, Onozato et al 2008). The conifers *Podocarpus macrophyllus* var. *maki* Sieb. (Sato and Suzuki 2002) and *Cunninghamia lanceolata* (Lamb.) Hook. (Kaneko and Ogawa 1998) have been killed by *A. tabescens*. Fruit bodies of *A. tabescens* were previously observed on the living trunk of *C. japonica* (Sugiyama 1992). Inoculation tests with *A. tabescens* caused infection on a *P. densiflora* seedling and mortality of a flowering cherry tree (Inagawa and Suzuki 2000, Onozato et al 2009). These reports indicate that this fungus is a pathogen of both hardwoods and conifers. In Europe, *A. tabescens* has been reported to be surprisingly aggressive to some hosts including introduced Eucalyptus, but in most cases it has been regarded as a saprobe on the stumps of oak species (Guillaumin et al 1993). In North America, a fungus referred to as *A.*

tabescens is known to attack and kill wide range of hosts including pine (Sinclair and Lyon 2005). Some authors are suspicious that the two allopatric populations belong to different species (Kile et al 1994, Antonín et al 2006). Although Japanese isolates of *A. tabescens* have been reported to be compatible with European isolates of the species, but not compatible with North American ones (Ota et al 1988b), pathogenic property of Japanese *A. tabescens* is more close to that of North American population. Further study is needed to clarify the relationships among these populations, with taxonomic analysis and inoculation tests to clarify pathogenicity on different hosts.

Nagasawa's E and *A. nabsnona* were collected from old stumps and are considered to be saprotrophs, although this designation is based on a few isolates (Table 3-2). *Armillaria nabsnona* in North America is a weak pathogen on hardwood species and has limited pathogenicity on young conifers (Gregory et al 1991), and it is consistent with the result in the present study.

Armillaria gallica was not collected from conifers in this study. However, *A. gallica* was previously reported on *P. densiflora* and *Ab. sachalinensis* in Japan (Ota et al 1998b). These records do not include information on the host status; pathogenicity of *A. gallica* on conifers in Japan remains unclear. In Europe and North America, this species is categorized as a weak pathogen frequently found on hardwood species and infrequently on conifers (Rishbeth 1982, Morrison et al 1985, Gregory 1989, Guillaumin et al 1993).

Table 3-1. Isolates of *Armillaria* collected from conifers in Japan.

Isolate no.[a]	Species determined by EF-1α	Mating Test[b]	Derivation[c]	Host species[d]	Class of host[e]	Type of forest[f]	Location	GenBank accession number
AS-1	*A. mellea*	+	NR	*Ch. obtusa*	a	p	Oita	AB539508
AS-2	*A. mellea*	+	NR	*Ch. obtusa*	a	p	Nagasaki	AB539506
A-10*	*A. mellea*	+	m	*P. palstlis*	NR	p	Tokyo	AB510800
A-12*	*A. mellea*	+	w	*Ch. obtusa*	a'	p	Wakayama	AB510801
89-07*	*A. mellea*	+	m	*Ch. obtusa*	a'	p	Miyazaki	AB510796
92-41	*A. mellea*	+	m	*Ch. obtusa*	a	p	Yamanashi	AB539510
93-43	*A. mellea*	+	f	*Ch. obtusa*	d	p	Tokyo	AB539511
94-7*	*A. mellea*	NT	f	*Ch. obtusa*	a'	p	Tokyo	AB510799
94-10-1*	*A. mellea*	NT	s	*P. thunbergii*	d	p	Kanagawa	AB510798
94-65	*A. mellea*	+	f	*Ch. obtusa*	a'	p	Iwate	AB539513
94-68*	*A. mellea*	+	m	*Ch. obtusa*	a'	p	Iwate	AB510803
97-29	*A. mellea*	NT	f	*Ch. obtusa*	a'	p	Fukushima	AB539512
2003-73	*A. mellea*	-	m	*Ch. obtusa*	a'	p	Toyama	AB539507
05-100-2	*A. mellea*	NT	s	conifer	NR	p	Hokkaido	AB539509
A-14	*A. cepistipes*	-	m	*Pi. koyamae*	NR	p	Tokyo	AB539499
90-10-12*	*A. cepistipes*	+	s	*Pi. abies*	d	p	Niigata	AB510790
92-19	*A. cepistipes*	+	f	*Ch. obtusa*	d	p	Gunma	AB539498
94-31	*A. cepistipes*	+	f	*C. japonica*	d	p	Yamagata	AB539497
94-33-01*	*A. cepistipes*	+	s	*C. japonica*	d	p	Yamagata	AB510789
94-39-04*	*A. cepistipes*	+	s	*C. japonica*	d	p	Yamagata	AB510786
01-1	*A. cepistipes*	-	f	*Ab. mariesii*	NR	n	Ishikawa	AB539500
01-11	*A. cepistipes*	+	f	*C. japonica*	d	p	Ishikawa	AB539505
2000-49	*A. cepistipes*	-	m	*Ab. firma*	d	n	Shizuoka	AB539504
2000-51	*A. cepistipes*	-	r	*P. sylvestris*	a'	p	Hokkaido	AB539502
2002-01	*A. cepistipes*	+	r	*Pi. polita*	d	n	Yamanashi	AB539503
2002-02	*A. cepistipes*	+	r	*Pi. polita*	a'	n	Yamanashi	AB539501
2002-04	*A. cepistipes*	-	r	*C. japonica*	b	p	Toyama	AB539496
2003-01	*A. cepistipes*	-	w	*C. japonica*	d	p	Toyama	AB539495
88-01-19*	*A. ostoyae*	+	s	*P. densiflora*	NR	p	Aomori	AB510784
89-03B-09*	*A. ostoyae*	+	s	*Ab. sachalinensis*	d	p	Hokkaido	AB510780
90-03	*A. ostoyae*	+	m	*Ch. obtusa*	a'	p	Saitama	AB539471
91-01-10*	*A. ostoyae*	+	s	*Ab. sachalinensis*	d	p	Hokkaido	AB510785
92-37	*A. ostoyae*	+	m	*Ch. obtusa*	d	p	Yamanashi	AB539467
93-33	*A. ostoyae*	+	m	*P. densiflora*	a'	p	Aomori	AB539466
94-8-2*	*A. ostoyae*	+	s	*P. densiflora*	NR	p	Aomori	AB510783
94-72	*A. ostoyae*	+	f	*Ch. obtusa*	a'	p	Iwate	AB539469
94-75-07*	*A. ostoyae*	+	s	*P. densiflora*	d	p	Iwate	AB510779

Table 3-1. Continued.

Isolate no.[a]	Species determined by EF-1α	Mating Test[b]	Derivation[c]	Host species[d]	Class of host[e]	Type of forest[f]	Location	GenBank accession number
96-25	*A. ostoyae*	+	f	*Ab. sachalinensis*	NR	p	Hokkaido	AB539464
96-28a-1	*A. ostoyae*	+	s	*Ab. sachalinensis*	NR	NR	Hokkaido	AB539468
96-40	*A. ostoyae*	+	f	*Ab. firma*	NR	n	Kanagawa	AB539478
97-9	*A. ostoyae*	-	f	*Pi. jezoensis*	NR	NR	Hokkaido	AB539465
00-10	*A. ostoyae*	+	f	*A. homolepis*	d	n	Mie	AB539463
02-9	*A. ostoyae*	+	f	*L. kaempferi*	NR	p	Nagano	AB539474
02-12	*A. ostoyae*	+	f	*L. kaempferi*	NR	p	Ishikawa	AB539477
2002-50-01	*A. ostoyae*	-	s	*Pi. abies*	a	p	Niigata	AB539473
04-7	*A. ostoyae*	+	f	conifer	NR	NR	Hokkaido	AB539472
05-18	*A. ostoyae*	+	f	*Ab. sachalinensis*	NR	n	Hokkaido	AB539476
05-82	*A. ostoyae*	NT	f	*Ab. sachalinensis*	NR	p	Hokkaido	AB539479
05-101	*A. ostoyae*	+	s	*Pi. glehnii*	NR	p	Hokkaido	AB539475
2007-11	*A. ostoyae*	NT	f	*T. diversifolia*	c	n	Gunma	AB539470
89-09	*A. sinapina*	+	r	*Ab. veitchii*	d	n	Nagano	AB539486
89-10	*A. sinapina*	+	r	*Ab. veitchii*	d	n	Nagano	AB539487
90-02	*A. sinapina*	+	r	*L. kaempferi*	d	p	Saitama	AB539488
90-07	*A. sinapina*	+	f	*Ab. veitchii*	d	n	Nagano	AB539492
92-02	*A. sinapina*	+	r	*Ab. veitchii*	d	n	Yamanashi	AB539484
93-48	*A. sinapina*	+	r	*L. kaempferi*	d	p	Tochigi	AB539489
96-05-08	*A. sinapina*	NT	s	*Ab. veitchii*	d	n	Gunma	AB539490
96-21	*A. sinapina*	+	f	*Ab. mariesii*	c	n	Iwate	AB539491
2002-18	*A. sinapina*	NT	f	*Ab. veitchii*	d	n	Gunma	AB539485
2002-65	*A. sinapina*	+	r	*Ab. veitchii*	d	p	Yamanashi	AB539493
05-21	*A. sinapina*	+	f	*Pi. jezoensis*	NR	n	Hokkaido	AB539494
94-20	*A. nabsnona*	+	f	*C. japonica*	d	p	Iwate	AB539482
95-28	Nag. E	NT	f	*L. kaempferi*	d	p	Iwate	AB539481
2002-49	Nag. E	NT	f	*Pi. abies*	d	p	Niigata	AB539480
02-26	*A. tabescens*	+	m	*Ce. deodara*	a'	p	Ibaraki	AB539514

[a] Isolates with asterisks were analyzed by HASEGAWA et al. (2010) and function here as reference isolates in the phylogenic tree of the translation elongation factor-1α (EF-1α) (Figure 3-1).

[b] +, matches EF-1α result; -, ambiguous; NT, not tested

[c] f, fruit-body; m, mycelial mat; r, rhizomorph; s, single spore; w, decayed wood; NR, no record.

[d] Host species: *Ab. firma* = *Abies firma*; *Ab. homolepis* = *Abies homolepis*; *Ab. mariesii* = *Abies mariesii*; *Ab. sachalinensis* = *Abies sachalinensis*; *Ab. veitchii* = *Abies veitchii*; *Ce. deodara* = *Cedrus deodara*; *Ch. obtusa* = *Chamaecyparis obtusa*; *C. japonica* = *Cryptomeria japonica*; *L. kaempferi* = *Larix kaempferi*; *Pi. abies* = *Picea abies*; *Pi. glehnii* = *Picea glehnii*; *Pi. jezoensis* = *Picea jezoensis*; *Pi.*

Table 3-1. Continued.

koyamae = *Picea koyamae*; *Pi. polita* = *Picea polita*; *P. densiflora* = *Pinus densiflora*; *P. palstlis* = *Pinus palstlis*; *P. sylvestris* = *Pinus sylvestris*; *P. thunbergii* = *Pinus thunbergii*; *T. diversifolia* = *Tsuga diversifolia.*

[e] Explanation for each class is given in Material and Methods; NR, no record.

[f] n, natural; p, planted; NR, no record.

Pinus thunbergii is extensively planted along seashores as a windbreaker, whereas *P. densiflora* in mountains for timber production.

Abies firma and *Ab. homolepis* are components of natural coniferous forests in areas warmer than the distribution ranges of *Ab. veitchii*, *Ab. mariesii* and *T. diversifolia*.

Abies sachalinensis, *Pi. jezoensis* and *Pi. glehnii* are components of natural coniferous forests in Hokkaido, and *Ab. sachalinensis* is also planted regularly for silviculture.

Picea koyamae and *Pi. polita* are rare species occurring in limited areas on Shikoku and Honshu.

Pinus palstlis, *P. sylvestris*, *Ce. deodara* and *Pi. abies* are exotic species rarely used for silvicultural purpose. *Ce. deodara* is frequently planted in gardens.

(Descriptions of distributions of conifers based on the study by HAYASHI (1960).)

Table 3-2. Records of *Armillaria* classed according to condition of host

Species	Category						
	a	a’	b	c	d	NR	total
A. mellea	3	7	0	0	2	2	14
A. cepistipes	0	2	1	0	9	2	14
A. ostoyae	1	3	0	1	5	12	22
A. sinapina	0	0	0	1	9	1	11
A. nabsnona	0	0	0	0	1	0	1
Nagasawa’s E	0	0	0	0	2	0	2
A. tabescens	0	1	0	0	0	0	1

(a) Isolates from mycelium or a fruit-body in/on a living tree showing no obvious predisposing factor for infection.
(a') Isolates from mycelium or a fruit-body in/on a tree that had been killed in the previous year.
(b) Isolates from mycelium or a fruit-body in/on a living tree stressed by some factor other than *Armillaria*.
(c) Isolates from mycelium or a fruit-body in/on decayed heart wood of a living tree showing no symptoms.
(d) Isolates from mycelium, rhizomorph, or a fruit-body on a tree or a stump that had been killed more than one year ago, on a wind-thrown tree or on wood debris.
NR: no record.

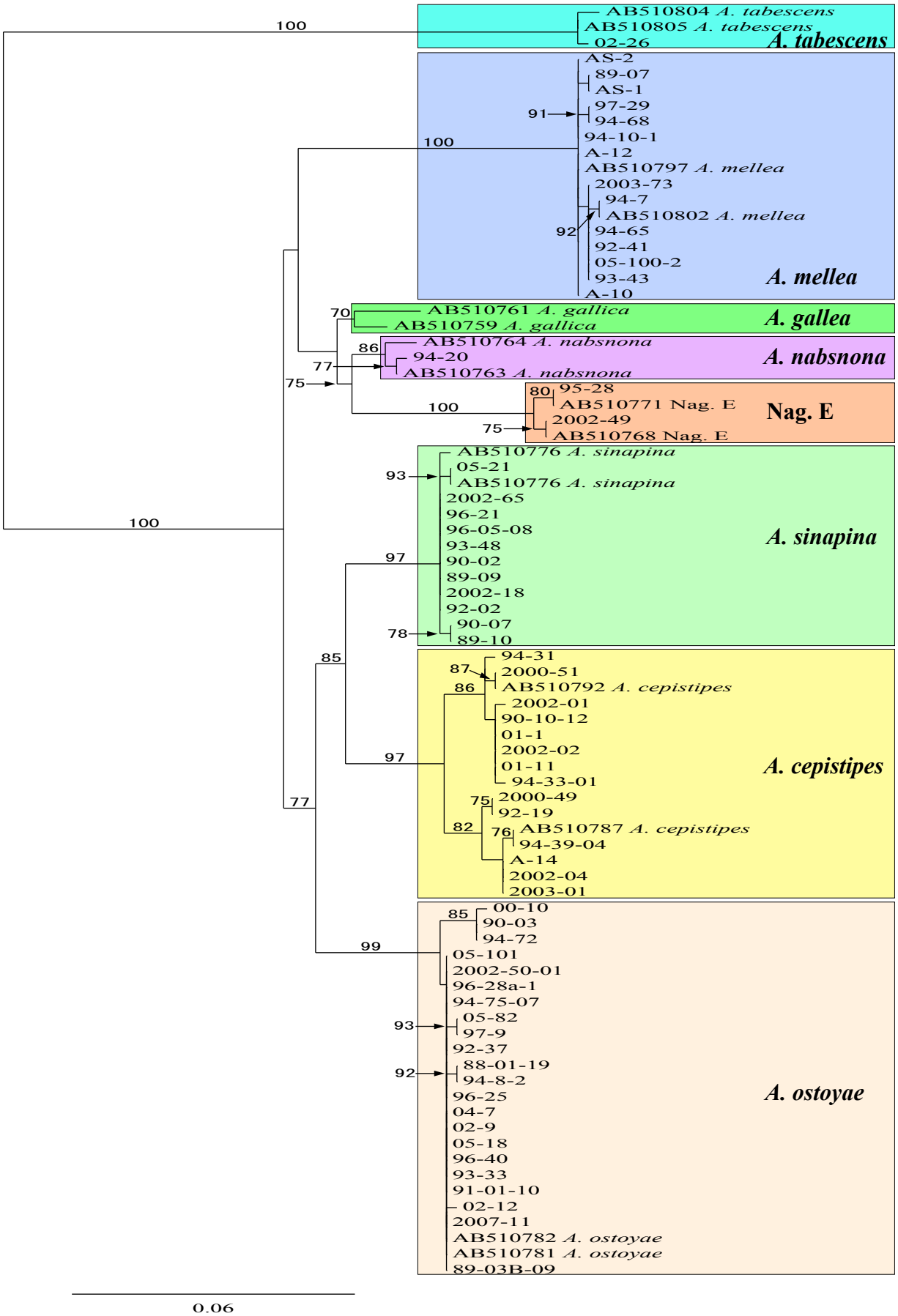


Figure 3-1. The maximum-likelihood tree generated with the EF-1α sequences of *Armillaria* isolates from conifers in Japan. GenBank accession numbers of reference isolates are indicated by numbers following the letters 'AB'. Nodes supported by reliability values (aLRT values) greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

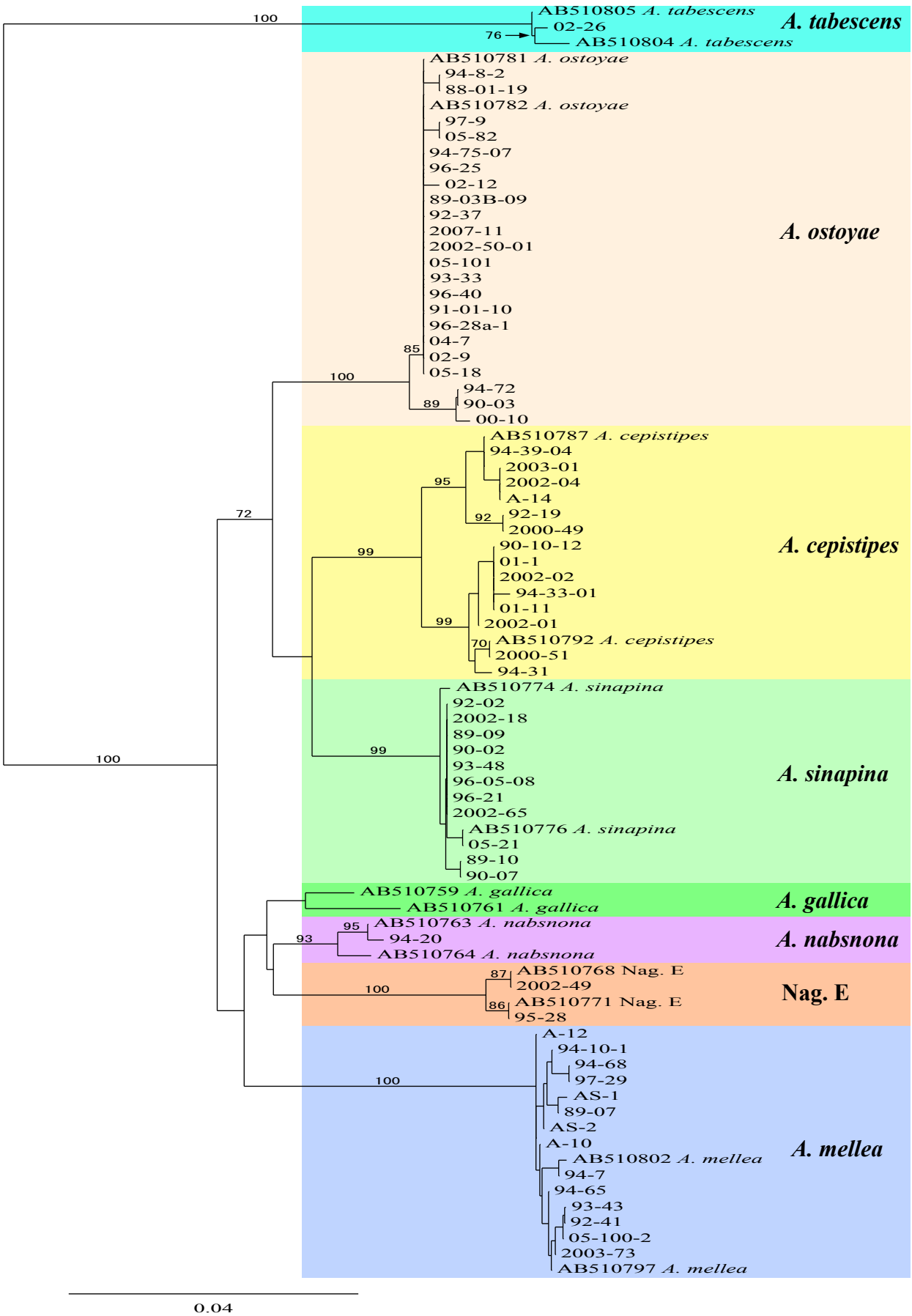


Figure 3-2. The neighbor-joining tree generated with the EF-1α sequences of *Armillaria* isolates from conifers in Japan. GenBank accession numbers of reference isolates are indicated by numbers following the letters 'AB'. Nodes supported by bootstrap values greater than 70% are indicated by numeric values above the tree branches. Branch lengths and the bar below the tree correspond to the distances measured in terms of the proportion of nucleotide substitutions between sequences.

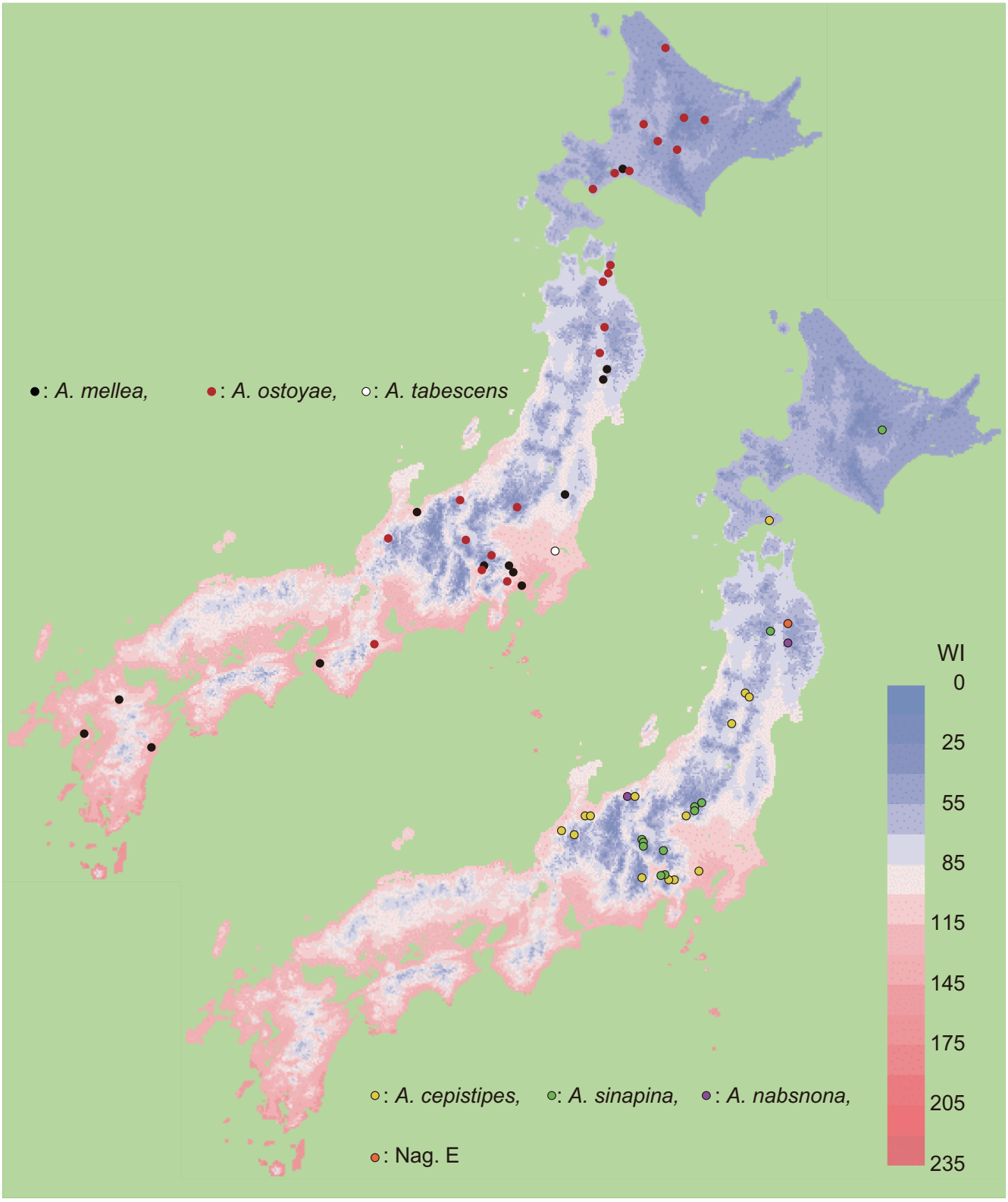


Figure 3-3. Sites from which *Armillaria* isolates were collected from conifers in Japan. WI: Kira's warmth index. WI = Σ(t - 5), where t is the mean monthly temperature exceeding 5°C. Upper map: collection sites of *A. mellea*, *A. ostoyae* and *A. tabescens*. Lower map: collection sites of *A. cepistipes*, *A. sinapina*, *A. nabsnona* and Nag. E. The maps of Japan were generated by a software in Japan Meteorological Agency (2002).

Figure 3-4. Fruit bodies of *Armillaria* species collected from conifers.
a: *Armillaria ostoyae* (94-75-07); b: *A. sinapina* (96-21); c: *A. cepistipes* (94-33-01); d: *A. mellea* (94-65).

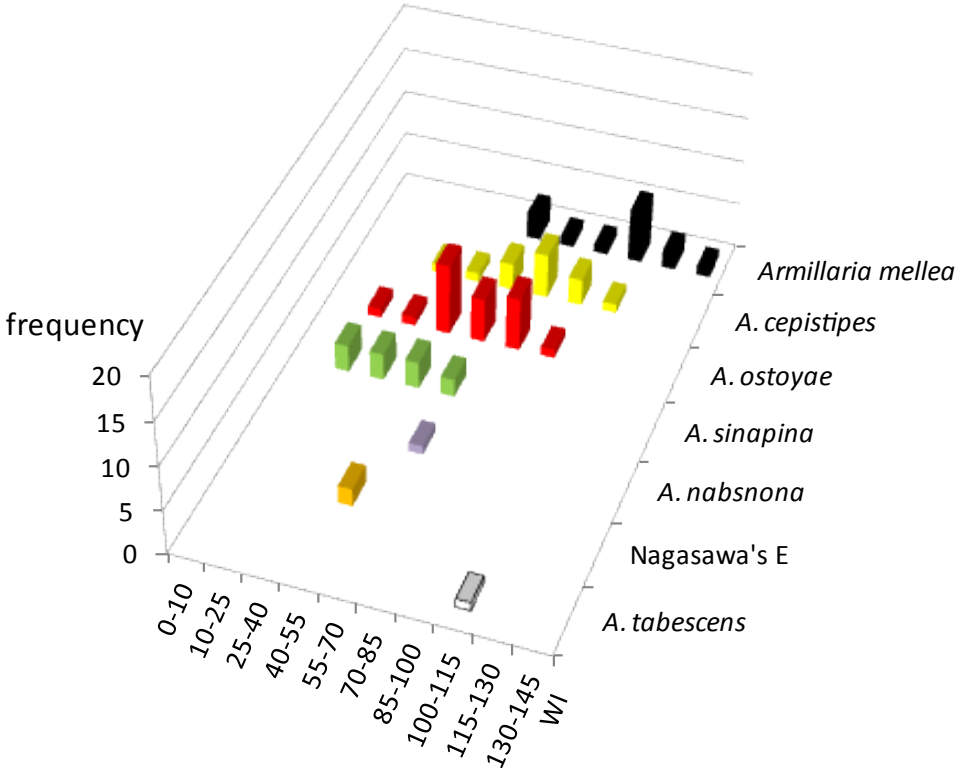


Figure 3-5. Kira's warmth index (WI) of the collection sites of *Armillaria* species from conifers.

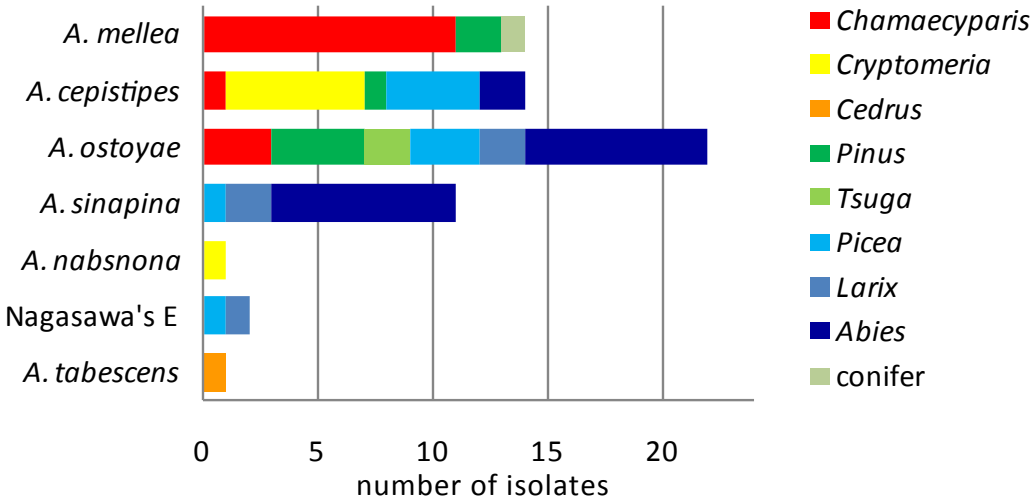


Figure 3-6. Hosts of *Armillaria* species collected from conifers

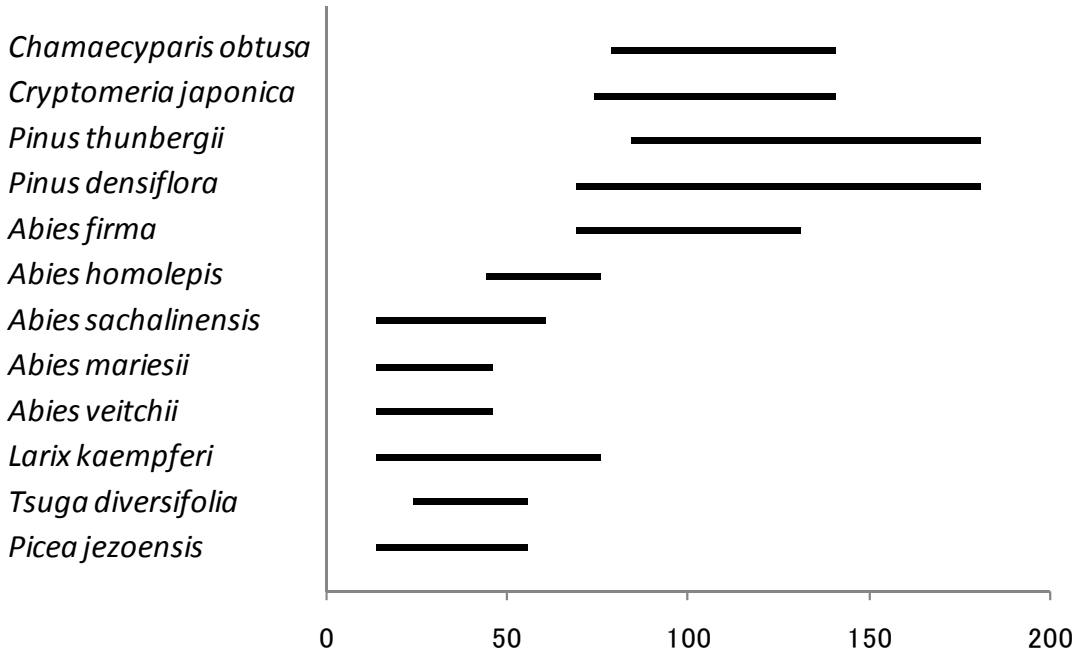


Figure 3-7. Kira's warmth index (WI) of distribution areas of Japanese conifers (Kira 1949 with modification).

4 Concluding Remarks

For effective control of Armillaria root disease, elucidation of ecological and pathological characteristics of each *Armillaria* species is required. When stands damaged by Armillaria root disease should be investigated in ecological and pathological viewpoints, a rapid and reliable technique to identify species, would be fundamentally important, of many fungal samples such as basidiocarps, rhizomorphs and mycelial mats. The present thesis aimed to find an excellent technique for rapid, reliable and easy identification of Japanese *Armillaria* species, and thereafter, to characterize interspecific differences in ecology and pathology among each species.

After Mayer (1942) proposed the biological species concept, dozens of alternative species concepts have been proposed (de Queiroz 2005). In fungal biology, species concepts have evolved from strictly morphological descriptions of the fruit body and the spore, through the embrace of the biological species concept, to a call for phylogeny-based species concepts (Harrington and Rizzo 1999). There are practical difficulties in the former two: morphological identification needs fruit bodies, whereas fruit bodies of most fungal species are obtained in only restricted season in the year or never; mating tests are time-consuming and not practical to identify biological species of homothallic or asexual fungi. Among these three, the last one may be the most practical in fungal ecology.

Species based on these three concepts are not necessarily consistent with each other. For instance, *A. mellea* in the northern hemisphere are morphologically conspecific but the allopatric counterparts are not fully compatible in mating tests. Another example is the case of the IGS sequence, which is successfully applied to identification of European *Armillaria* species, but cannot discriminate some isolates of North American *A. gallica* from *A. calvescens*. Consequently,

it is indispensable to develop a phylogeny-based technique (or techniques) whose result agrees with the results of other two methods as well as possible.

As phylogeny-based techniques for *Armillaria* species identification, regions in rDNA such as the ITS, IGS and 16S rRNA gene have usually been used. Recently, Maphosa et al (2006) applied EF-1α gene for molecular phylogenetic analysis of *Armillaria* species in the northern and southern hemisphere. Their result was largely consistent with the previous results obtained from the sequence analyses of the ITS and IGS of rDNA. Antonín et al (2009) utilized this technique for phylogenetic analysis of two closely related European species, *A. gallica* and *A. cepistipes*. The Czech *A. cepistipes* clade and *A. gallica* clade was clearly distinct and the method proved to be more appropriate to distinguish the two species than the method based on the sequences and RFLPs of rDNA.

In the present study, isolates of Japanese *Armillaria* species were examined by mating tests and phylogenetic analyses with three DNA regions, ITS, IGS and EF-1α. There was no critical conflict among these techniques in species identification. However, the results of EF-1α analysis showed higher resolution, and were more correlated with biological species than analyses with the ITS and IGS regions of rDNA. In conclusion, this thesis demonstrated that the identification technique based on EF-1α is a powerful tool for identification of Japanese *Armillaria* species. The present study also focused on the ecological and pathological descriptions written in sampling records of the Japanese *Armillaria* isolates. The results revealed that the Japanese *Armillaria* species showed obvious differences in their distribution in Japan, host preference and potential pathogenicity to conifers. Such kind of information on interspecific differences in ecology and pathology may be useful to predict and control Armillaria root disease in nature.

Comparing between *Armillaria* species in Japan and other countries, there seems to be ecological and pathological intraspecific differences between them. Seven of the eight Japanese wood-inhabiting *Armillaria* species have their counterpart species in the northern hemisphere outside Japan. Although the Japanese and the allopatric counterpart species are conspecific in terms of taxonomy and mating behavior, virtually they do not necessarily share the same ecological and pathological characteristics. Such kind of intraspecific differences in ecology and pathology should be taken into consideration for deeper understanding of Armillaria root disease in future.

Harrington and Rizzo (1999), seeking for practical species concept in fungal ecology, defined species simply as " ... the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters." They emphasized the importance of population-based approach for characterizing the diversity apparent at the population level, because the process of speciation is a population-level phenomenon (Harrington and Rizzo 1999). They also emphasized importance of ecological and physiological characters because ecological adaptations are key to the process of speciation, and members of a population that share such ecological adaptation should have certain phenotypic characters in common.

Their species concept by a population-based approach may lead to more detailed intraspecific criteria of ecological and pathological differentiation in *Armillaria* species. Development of genetic markers will enable population-level identification of field isolates, and genetic markers linked to ecological and pathological characteristics will be a great help to study fungal population dynamics, speciation and evolution related to forest epidemiology and ecology.

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要旨

ナラタケ属菌は汎世界的に分布し，樹木の根系腐朽病害である「ならたけ病」の病原である。日本ではこれまでに約 10 種のナラタケ属菌が報告されている。本論文では，ナラタケ属菌の分類の歴史を概括し，これまでの種の識別方法について，実用上の見地から検討を加えた。その知見にもとづき，日本産ナラタケ属菌の DNA 配列による識別を試み，日本の針葉樹に産するナラタケ属菌の生態を，採集データから明らかにした。

ナラタケ属の分類学的な位置づけに歴史的な問題があったことと，子実体の形態に基づいた種の識別に伴う困難から，1970 年代後半まで，ナラタケ属の種の分類と識別は混乱状態にあった。現在，ナラタケ属には約 40 種があるとされているが，かつて多くの樹病学者は，ならたけ病の病原である，子実体につばのあるナラタケ属の種を「ナラタケ」一種としていた。そのため，「ナラタケ」は汎世界的に分布し，子実体の形態の変異に富み，強い病原とされたり，あるいは腐生菌とされたりするなど幅広い生理・生態的性質を示し，針葉樹・広葉樹を含む広い範囲の宿主を持つ謎に満ちた種とされた。一方，分類学的には，属の性質を決定するタイプ種の選定に複数の説があったため，属の範囲自体にいくつかの解釈が併存するという混乱があった。その混乱を乗り超えてのちにも，子実体の形態の記載の困難が難問として残った。

1970 年代後半に，複数の子実体由来の単胞子分離菌株の交配試験の結果，ナラタケ属の子実体につばのあるものの中には数種の「生物学的種」が含まれることが発見された。「生物学的種」のそれぞれは固有の形態的特徴を備えていることが明らかになり，子実体につばのあるナラタケ属菌は生物学的種の区割りにもとづいて記載されるようになった。これに伴い，種毎の生理・生態が記録されるようになり，ナラタケの謎とされた多様な性質は多種の混同の結果であったことが明らかになりつつある。1980 年代には，分子生物学的手法による種の識別，および種の系統関係の解析が盛んに行われるようになった。その結果，交配試験による生物学的種の識別を行わずに，分子生物学による系統解析と形態の記述のみで種を記載する例が見られるようになった。

今日，ナラタケ属の種の識別法には，子実体の形態，テスター菌株との交配，分子生物学的手法の 3 通りがある。これらの方法のもたらす結果はおおむね一致するが，細部では必ずしも一致しない。例えば異なる生物学的種の子実体がほとんど同じ形態を示したり，よく種の識別に用いられる領域の DNA 配列が，異なる生物学的種の間で極めて違いが少なく種が識別できない例があるなどであり，各手法はそのことに留意して使用する必要がある。各手

法の実用上の問題は，子実体の形態には子実体の発生する期間が限られることが，交配試験にはテスター菌株の一揃いと時間と労力が必要で，特に組織分離菌株の識別の結果が不明瞭な場合があることが挙げられる。分子生物学的手法は，迅速で再現性が高く明瞭な結果が得られる手法である。さらに，分子生物学的手法は単胞子分離菌株・組織分離菌株の別なく明瞭な種の識別が可能なことから，菌糸膜・腐朽材片などからの組織分離菌株を採集して使用することの多い菌類生態の研究では，活用すべき手法と考えられる。

一方，生物学的種の発見以降，ナラタケ属各種の生理・生態についての知見が世界的に蓄積されつつある。日本と共通する種の多い欧米では，多くの種の分布・寄主選好・病原性等が，野外観察と接種試験の両面から調査されており，比較的好温性で暖温帯に分布する種から逆に北方林に分布する種，しばしば針葉樹の病原である種や広葉樹の病原である種，主に腐生菌である種などが知られている。日本では，主要造林樹種のすべてにならたけ病が記録されているにもかかわらず，種の識別に着目したならたけ病研究が非常に少ない。その原因の一つに，種の識別の困難が挙げられるだろう。本論文では，分子生物学的マーカーを用いた迅速かつ明瞭な種の識別を試み，野外で針葉樹から採集された菌株にその方法を適用し，林業上重要な樹種を含む針葉樹に発生する日本産ナラタケ属菌の生態の解明を目指した。

日本産ナラタケ属菌の種の識別の正確性を比較するため，交配試験で識別されたナラタケ属 8 種 (*Armillaria mellea*，*A. ostoyae*，*A. gallica*，*A. cepistipes*，*A. nabsnona*，*A. sinapina*，*A. tabescens*，生物学的種 Nagasawa's E) に属する 49 菌株を用い，ポリペプチド鎖翻訳伸長因子 1α (EF-1α) 遺伝子，リボゾーム RNA をコードする DNA クラスタ (rDNA クラスタ) の internal transcribed spacer (ITS) 領域，intergenic spacer (IGS) 領域の塩基配列を分析した。ITS と IGS の解析では 5 種が識別できたが，残りの 3 種である *A. gallica*, *A. cepistipes*, *A. sinapina* の識別はできなかった。IGS-1 領域の制限酵素断片多型ではほとんどの種を識別できたが，4 つの制限酵素を用いても，*A. cepistipes* と *A. sinapina* の一部の菌株の識別ができなかった。EF-1α 遺伝子の解析の結果は交配試験の結果とよく一致し，供試した 8 種を識別できた。結論として，今回用いた手法のうち，EF-1α 遺伝子の DNA 配列が最もナラタケ属の種の識別に適していることが明らかになった。一部の菌株の rDNA 配列が判読できなかったが，rDNA は重複遺伝子であり，ゲノム内ヘテロがあったためと考えられた。このようなケースで DNA 配列を判読するには，シーケンス前にクローニングを行うことが必要である。菌類の EF-1α 遺伝子は通常単一遺伝子とされる。

海外のナラタケ属菌の既報の EF-1α 遺伝子の情報を加えて系統樹を作成したところ，おおむね同種は同クレードまたは近接したクレードを形成した。しかし，一部の菌株は同種のま

とまりから離れた位置に配されたため，海外菌株を加えた解析には，より多くの菌株データを用いた検討が必要と考えられた。Nagasawa's E はいずれの系統樹でも独立したクレードを形成し，交配のみならず分子系統でも明確な種であることが示された。

日本の針葉樹に産するナラタケ属菌の生態を解明するため，採取時の記録に基づき，ナラタケ属各種の分布・寄主選好・潜在的な病原性を解析した。子実体組織・菌糸膜・根状菌糸束・腐朽材片・単胞子を分離源とし，日本の主要な造林樹種 6 種を含む 19 種の針葉樹から 65 菌株が採集された。これらの菌株とテスター菌株との交配および EF-1α 遺伝子の DNA 配列により，7 種のナラタケ属菌が識別された。組織分離菌株とテスター菌株との交配試験では不明瞭な結果があったが，EF-1α 遺伝子を利用した方法では全ての菌株が明瞭に識別された。これらの菌株のうち *A. mellea*，*A. ostoyae*，*A. cepistipes*，*A. sinapina* が高い頻度で検出され，日本の針葉樹に生ずる代表的ナラタケ属菌と考えられた。採集地の吉良の暖かさの指数から，各菌種の温度への指向性を調べたところ，*A. sinapina* は寒冷地に，*A. mellea* は温暖地に出現する傾向が表れた。*A. sinapina* は初めて北海道以南で記録され，関東・中部の亜高山のシラベ・オオシラベから多く分離された。*A. ostoyae* および *A. cepistipes* は多くの樹種を利用していたが，*A. mellea* は特にヒノキから頻繁に分離されることが明らかになった。*A. nabsnona*，*A. tabescens*，Nag. E の採集は稀であり，採集されなかった *A. gallica* を含め，これら 4 種は主に広葉樹を基質として利用すると考えられた。各菌種の潜在的な病原性を評価するため，菌株が分離された基質の状態を，ナラタケ属菌が形成層に寄生している生立木，ナラタケ属菌が形成層に寄生している枯死後 1 年以内の枯死木，ナラタケ属菌が形成層に寄生している枯死後 1 年以上の枯死木，ナラタケ属菌が心材腐朽を起こしている病徴のない生立木，ナラタケ属菌が腐朽を起こしている倒木・伐根等の 5 つにクラス分けしたところ，*A. mellea*，*A. ostoyae*，*A. cepistipes*，*A. tabescens* は生立木かつ形成層に寄生されている状態，および枯死後 1 年以内の枯死木かつ形成層に寄生されている状態の寄主から分離されていた。これらの種は生立木に感染して衰弱・枯死させる能力がある，もしくはその可能性が高く，日本の針葉樹の中程度～強い病原と考えられた。

針葉樹から分離された 7 種のうち 6 種は，ヨーロッパもしくは北アメリカに同種が存在している。これらの日本の種と，遠隔地に生育する同種は，かならずしも同じ生態的・病理学的性質を持つとは限らない。同種内の遠隔地個体群間の変異の研究は，ならたけ病のより深い理解につながる。個体群を識別する遺伝子マーカー，および生態的・病理学的性質に関連する遺伝子マーカーの開発は，菌の種分化や生態，疫学に大きく貢献すると考えられた。