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Molecular Phylogeny and Evolutionary Divergence of North American Biological Species of *Armillaria*

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Armillaria **is a genus of root infecting basidiomycetes, which includes nine North American biological species. Anonymous nucleotide sequences obtained from four different primer pairs were combined to produce a data set which was analyzed phylogenetically. The data indicated that randomly chosen sequences from the genome were capable of resolving the phylogenetic history of species of *Armillaria* and provided strong support for intraspecies clustering. NABS III and VII formed a significant monophyletic clade, with III being derived from the more broadly distributed NABS VII. Sequences of isolates of NABS V showed a high degree of variation. This variation may be an indication of recent sympatric speciation, with NABS IX and X diverging from a genetically diverse NABS V. NABS I formed a monophyletic clade despite the variation in geographic distance among the isolates. The position of NABS II as ancestral to NABS I was discussed. However, literature evidence favored divergence of NABS II from NABS I, while this study illustrated genetic similarity of NABS II with NABS VI. NABS VI was the most divergent of the North American species and represented the outgroup. A molecular clock of NABS *Armillaria* was proposed.**

Key Words: **NABS *Armillaria*; molecular phylogeny; anonymous sequences; combined data sets; molecular clock; evolutionary divergence.**

INTRODUCTION

The genus *Armillaria* (Fr.:Fr.) Staude is a group of root infecting basidiomycetes composed of several reproductively isolated groups, or biological species. Species are distributed worldwide and vary in their pathogenicity. Some species are necrotrophic pathogens, while others are saprotrophic (Gregory *et al.,* 1991) or form mycotrophic associations with achlorophyllous plants, such as the orchid *Galeola septentrionalis* Reichb. f. (Terashita and Chuman, 1987).

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The genus *Armillaria* consists of a wide range of annulate and exannulate morphological species. Until recently the annulate species were considered a single species, *Armillaria mellea* (Vahl: Fr.) Kummer. Hintikka (1973) showed that *Armillaria* has a bifactorial, sexual incompatibility system. This system is present in all North American species of *Armillaria* that have been investigated (Ullrich and Anderson, 1978). The *Armillaria mellea* group has been separated into nine North American biological species (NABS), five European, and five Australasian species (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979; Kile and Watling, 1983). Isolates of different species of *Armillaria* produce incompatible matings due to intersterility barriers (Anderson and Ullrich, 1979; Anderson *et al.*, 1980; Anderson, 1986; Korhonen, 1978). Most biological species of *Armillaria* can also be distinguished by characteristics of fruit bodies or rhizomorphs (Marxmüller, 1982; Kile and Watling, 1983; Bérubé and Dessureault, 1988, 1989; Volk *et al.*, 1996).

Species of *Armillaria* have also been shown to be distinct with respect to mitochondrial and nuclear DNA (Jahnke *et al.*, 1987). Restriction maps of ribosomal DNA (rDNA) identified related clusters of *Armillaria* species (Anderson *et al.*, 1989; Smith and Anderson, 1989). Anderson and Stasovski (1992) compared sequences of the intergenic region (IGR) of rDNA to assess the phylogeny of *Armillaria.* Isozyme analysis (Morrison, 1982; Morrison *et al.*, 1985a) and immunological techniques (Lung-Escarment *et al.*, 1985) were successful in differentiating among some groups within the genus *Armillaria.* Characteristics of basidiome morphology have also been used to differentiate among closely related species (Bérubé and Dessureault, 1988, 1989).

Separate or combined analysis of phylogenetic data has become a popular approach in recent literature and its efficacy has been debated (Hillis, 1987, 1995; Kluge, 1989; Omland, 1994; de Queiroz *et al.*, 1995; Lutzoni and Vilgalys, 1995). Quicke (1993) suggested that an increase in the size of the data set would produce a tree closer to the true phylogenetic tree. However, Miya-

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moto and Fitch (1995) believed that separate analyses on individual data sets should be performed to provide insight into different evolutionary histories.

There were two objectives to this study. First, to determine the effectiveness of combining data sets of four different conserved anonymous fragments to estimate a phylogeny. Second, to infer phylogenetic relationships among NABS of *Armillaria* and to place NABS *Armillaria* into an historical framework.

MATERIALS AND METHODS

Material Examined

North American biological species of *Armillaria* are indicated by Roman numerals (Anderson and Ullrich, 1979). Fungal material was obtained from various sources (Table 1).

DNA Extraction

Crude genomic DNA was extracted from approximately 200 mg lyophilized fungal tissue using a 2× CTAB procedure modified from Zolan and Pukkila (1986). DNA was extracted with chloroform:isoamyl alcohol, precipitated with isopropanol, and resuspended in TE-8 buffer.

Polymerase Chain Reaction

PCR amplification components included 0.1 µM each oligonucleotide primer (Table 2), 2 mM each of dATP, dCTP, dGTP, and dTTP, 2.0 mM $MgCl_{12}$, 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 0.1% Triton X-100, and 0.4 units *Taq* DNA polymerase (Promega). Two microliters of 1:10 dilution of approximately 10 ng/µl genomic DNA was added for each 20-µl amplification for regular PCR. Five microliters of 1:10 genomic DNA (10 ng/µl) was added to each 100-µl PCR reaction in preparation for sequencing. One drop of light white mineral oil was placed in each tube to prevent evaporation. Amplification occurred on a Perkin–Elmer Cetus 480 Thermal cycler. PCR cycle conditions varied depending on the nature of the primers used. All PCR cycles began with 1 min preheat at 94°C. Twenty nucleotide (nt) primers

TABLE 1

Strains of *Armillaria* Showing Name of Collector, Host Species on Which It was Collected, and Location of Collection

Sample	Collector	Host species	Location
I DMR 20	T. Harrington	*Abies balsamea*	Wildcat Mountain, NH
I BOW PK	J. Bérubé	Undetermined wood debris	Bowring Park, St. John's, NF
I JB 08	J. Bérubé	*Acer saccharum*	Oka, Ottawa River Valley, QC
I JB 09	J. Bérubé	*Betula populifolia*	Sainte-Therese, north Montreal, QC
I JB 13	J. Bérubé	*Betula papyrifera*	Chicoutimi, Lake St-Jean, QC
I GG 12D	J. Bérubé	*Pinus banksiana*	Chicoutimi, Lake St-Jean, QC
I NOF 1076	Y. Hiratsuka	*Pinus contorta*	Hinton, Alberta
I NOF 830	F. J. Emond	*Pinus contorta*	Cow Lake, Alberta
II JB 38	J. Bérubé	*Acer saccharum*	Duchesnay, near Quebec City, QC
II JB 39	J. Bérubé	*Acer saccharum*	Duchesnay, near Quebec City, QC
II 160-8	J. Anderson	Undetermined	Smuggler's Notch, VT
II JB 85	J. Bérubé	*Acer saccharum*	Bromont, Eastern Township, QC
III JB 56	J. Bérubé	*Acer saccharum*	St.-Ange, Beauce, QC
III JB 61	J. Bérubé	Unknown	Donnaconna, near Quebec City, QC
V 83 62 1	C. G. Shaw III	Unknown	New York
V 83 91 1	C. G. Shaw III	Unknown	Petersburg, Alaska
V 48-3	J. Anderson	Unknown	Ithaca, NY
V JB 75	J. Bérubé	*Acer saccharum*	St-Odilon, Beauce, QC
V JB 66	J. Bérubé	*Acer saccharum*	St-Odilon, Beauce, QC
V Bow Pk	J. Bérubé	American Mountain ash	Bowring Park, Newfoundland
V JB 07	J. Bérubé	*Acer saccharum*	Bromont, Eastern Townships, QC
V JB 72	J. Bérubé	*Acer saccharum*	St.-Odilon, Beauce, QC
V JB 19C	J. Bérubé	*Pinus strobus*	St.-Jean, Vianney, Lake St.-Jean, QC
VI PD 37	J. Bérubé	*Quercus alba*	Oka, Ottawa River Valley, QC
VI KJS-6	T. Harrington	*Acer rubrum*	New Market, NH
VI GB 898	D. Bills	Unknown/hardwoods	Augusta Co, VA
VI 97-1	J. Anderson	*Acer rubrum*	Provincetown, MA
VII 90-10	J. Anderson	*Fraxinus americana*	Burlington, VT
VII HHB 11912	H. H. Burdsall	Dead *Ulmus*	Madison, WI
IX TJV 179-1	T. Volk	*Picea sitchensis*	Jefferson Co., WA
IX TJV 200	T. Volk	*Acer* sp.	Olympic National Park, Jefferson Co., WA
IX TJV 188-4	T. Volk	*Acer macrophyllum*	Olympic National Park, Jefferson Co., WA
IX 121-2	J. Anderson	*Acer macrophyllum*	Vancouver, BC
IX 139-1	J. Anderson	Soil surface	Moscow, Idaho
X SP812015	D. Morrison	Conifer stump	South of Nelson, BC

TABLE 2

Primer Sequences Used to Obtain Monomorphic Fragments in Eight NABS *Armillaria*

Primer	Primer sequence (5′–3′)
V250a	CGA ACT GAT CGT CGT CGA
V250b	GTT TCG AAC GCG AAT ATG CTC
III180a	ACC ACA TCC TTG TCG CCG AG
III180b	GTG GTT GAT GAG ATT GTT CG
III520-1	CAT GGT CGC TAC TTA CTC TGA TAA CGG
III520-2	GAG TTG ACG TAG ACT AC
83	GGG CTC GTG G
66	GAG GGC GTG A

were subjected to 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 45° to 50°C (depending on the primer used) for 1 min, and extension at 72°C for 1 min with a 50-s ramp time. Amplification with the 10-nt primers consisted of 42 cycles at the same temperatures and times, except annealing occurred at 38°C. Two different 10-nt primers were used in each amplification reaction to allow for sequencing of the fragments. This method was described by Burt *et al.* (1994) as sequencing with arbitrary primer pairs (SWAPP). A set of 200 10-nt primers was examined and 42 pairs of these were screened before selecting the 4 pairs in this study. Twenty-nucleotide primers were developed from 3 pairs of 10-nt primers, and the resulting monomorphic fragments were used for the phylogeny. The 10-nt primers were obtained from The Regional DNA Synthesis Lab, Calgary, Alberta. Twenty-nucleotide primers were obtained from Laboratoire d'Analyse et de Synthèse d'Acides Nucléiques, Université Laval, Québec. Primer sequences are provided in Table 2. Amplified fragments that were similar in size and present in all species of NABS *Armillaria* were selected for sequence analysis.

Purification of PCR Product

PCR product was visualized on 1% NuSieve (FMC) plus 2% agarose (Sigma) gel in 0.5× TBE buffer by staining with ethidium bromide and fluoresced with ultraviolet light. Agarose plugs were removed from bands and DNA was resuspended in 100 µl sterile, distilled water and heated to 70°C for 30 min. DNA was purified with Magic PCR Preps DNA Purification system (Promega) and reamplified using the same 10-nt primers as in PCR. Three 100-µl amplifications (30 ng/µl DNA) were pooled for each sample and again purified with the Magic PCR purification kit.

Sequencing

Purified DNA was quantified on a TKO 100 Fluorometer (Hoefer Scientific Instruments) and subjected to electrophoresis on agarose gels as described under *Purification of PCR Product*. DNA was sequenced on an Automated 373A DNA Sequencer (ABI) using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (ABI). Cycle sequencing reactions involved 25 or 30 temperature cycles (depending on the primers used) of denaturation at 98°C for 1 s, annealing at 45° or 50°C (depending on the primer used) for 15 s, and extension at 60°C for 4 min.

Molecular Clock

A distance matrix from aligned sequences for the combined data set was produced using SeqEd, version 1.0.3 (ABI, 1990), and manually corrected for missing data. Divergence times were calculated using the methods described in Berbee and Taylor (1993), with a minor modification. Substitution rates for groups of lineages were not normalized as they were in Berbee and Taylor (1993). Hence, divergence times represent noncorrected distances.

Calculation of substitution rates and calibration point. Rates of substitution vary among lineages of *Armillaria* (Figs. 1 and 2). Since two isolates of NABS VI were used as outgroup, and their distances were similar to one another, calculation of all rates was determined using VIPD37. The distance from VIPD37 to present was determined by calculating the midpoint between lowest and highest dissimilar values in the distance matrix. The midpoint calculated as 14.60% was very similar to the average of 14.33%. In contrast to Berbee and Taylor (1993), substitutions for lineages were not normalized, and rates were based on distance. Since the use of genetic distance assumes equal amounts of homoplasy across taxa, Mindell and Thacker (1996) discouraged the use of genetic distance in relative rate tests. However, topologies between the neighbor joining distance (unpublished data) and the parsimony-based tree (Fig. 2) were very similar in this study. Rate variation between isolates, indicated by the staggering end points of the terminal branches (Fig. 2), would also affect molecular clock estimates of divergence times.

Divergence times of basal branches were calculated using two of the highest and two of the lowest dissimilarity values to obtain an average to represent the divergence. Divergences occurring near terminal branches were calculated as an average of all possible rates for each divergence.

Berbee and Taylor (1993) provided molecular evidence that the Basidiomycetes emerged 200 to 300 million years ago (MYA) at the beginning of the Mesozoic and that mushrooms appeared about 130 MYA, coinciding with the radiation of the Angiosperms. The origin of Agaricales was also placed in the Mesozoic era by Pirozynski (1976). Divergence of mushroom genera, *Athelia* and *Spongipellis*, occurred approximately 120 MYA (Berbee and Taylor, 1993), and the divergence of *Coprinus* was even more recent (Bruns *et al.*, 1992).

The oldest fossil gilled mushroom was found in the mid Cretaceous (90–94 MYA). A more recent finding was dated 25–30 MYA (Hibbett *et al.*, 1995), revised

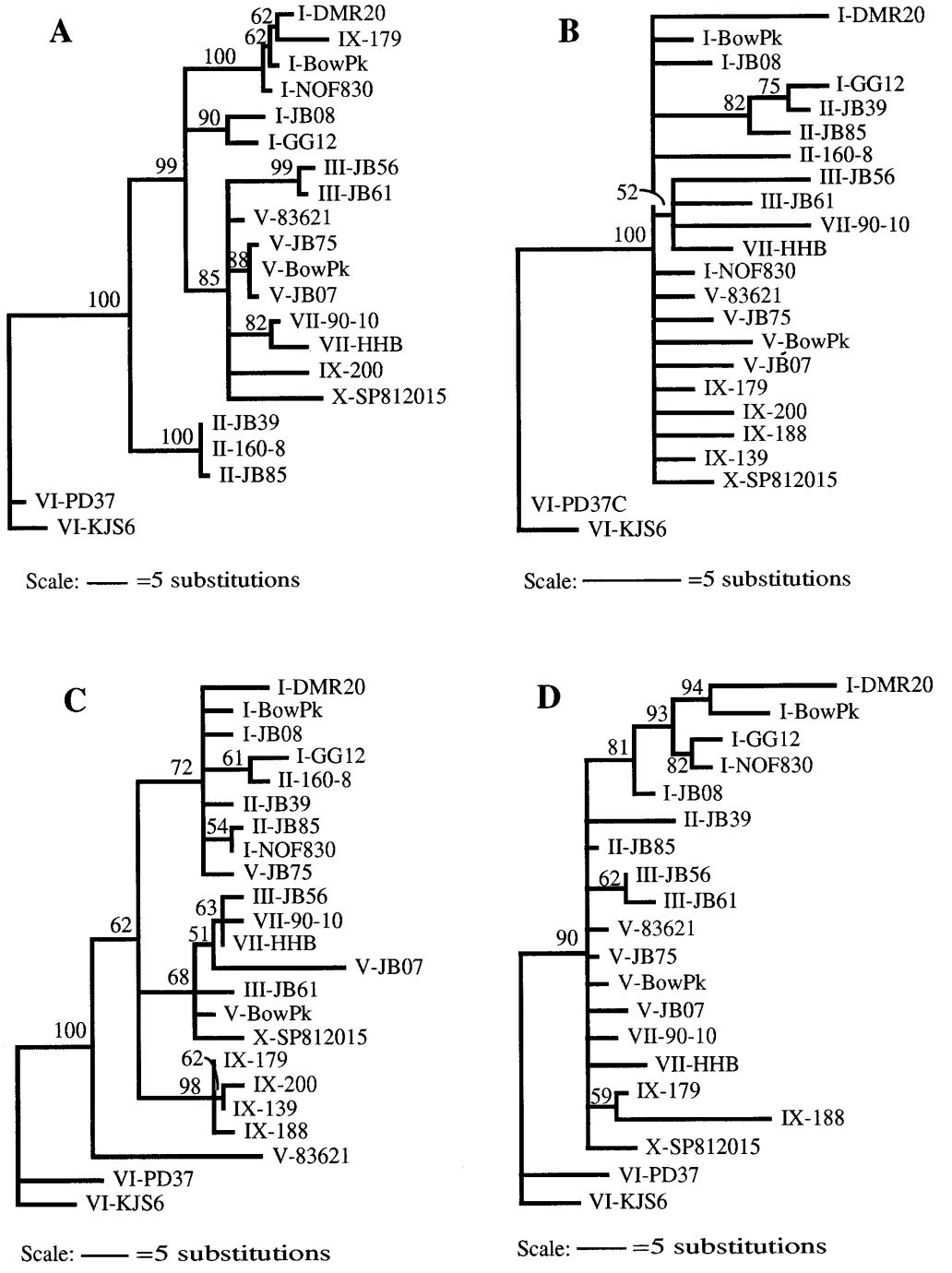

FIG. 1. Comparison of topologies obtained by maximum parsimony from anonymous nucleotide sequences amplified by (A) V250 for 224 bp, (B) III180 for 177 bp, (C) III520 for 122 bp, and (D) 83/66 for 150 bp. Primer V250 showed the highest degree of resolution of the four trees, and III180 showed the least resolution. Two isolates of NABS VI were user-specified outgroups. Isolate names correspond to those in Table 3. Numbers indicate bootstrap proportions from 100 replications, and horizontal branch length is proportional to distance as shown by scale bars (number of substitutions). There were 9, 25, 13, and 25 equally parsimonious trees produced by V250, III180, III520, and 83/66, respectively.

from an earlier estimate of 40 MYA (Poinar and Singer, 1990). This mushroom, *Coprinites dominicana*, was considered a member of the Agaricales and had many similarities with the present-day genus *Coprinus* (Poinar and Singer, 1990). Although accelerated morphological divergence has been observed from mushroom genera (Bruns *et al.*, 1989), and rare recessive alleles can produce extensive morphological change in the basidiocarp (Hibbett *et al.*, 1994), Hibbett *et al.* (1995) suggested that extant morphologies such as gilled

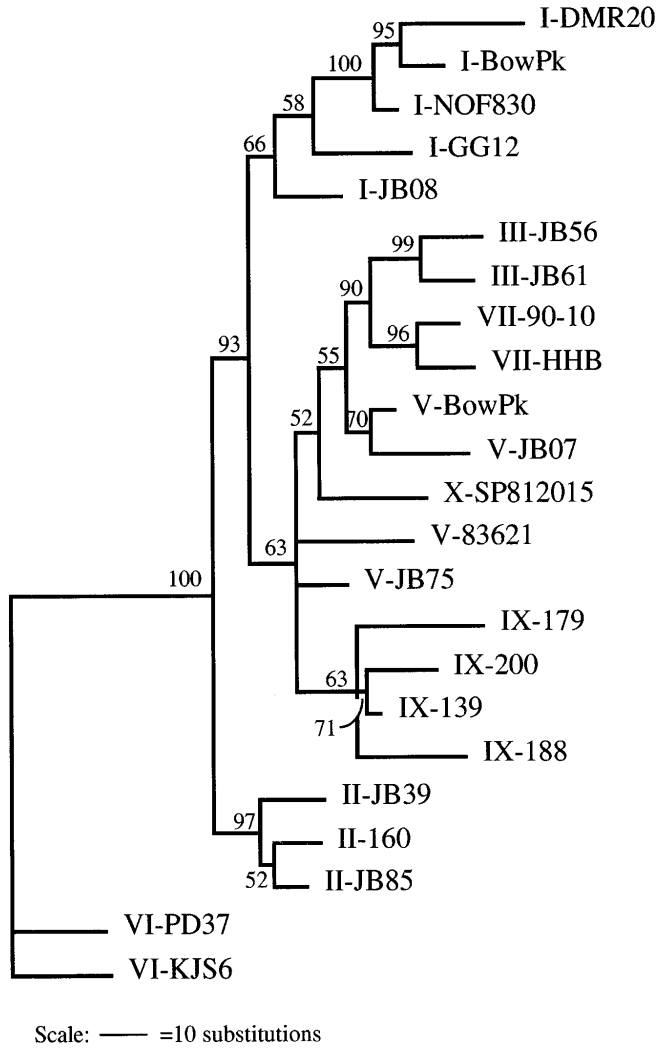

FIG. 2. The single most parsimonious tree obtained by maximum parsimony from combined anonymous nucleotide sequences. Four primer sets were used to amplify four different data sets using 23 taxa of eight NABS *Armillaria*. Two isolates of NABS VI were user-specified outgroup. Isolate names correspond to those in Table 3. Numbers indicate bootstrap proportions from 100 replications, and horizontal branch length is proportional to distance (number substitutions), as shown by scale bars.

mushrooms may be of ancient origin. However, the difficulties of relating extinct fossil mushrooms to extant genera suggest that present-day genera may be fairly recent. Assuming that NABS VI is as old as *Coprinites*, the calibration point in this study will be 30 MYA for NABS VI, with 14.60% substitution in that time. NABS VI may be younger than 30 MYA, but this calibration point was used as an estimate based on limited available fossil evidence.

Analysis

Crude sequence alignments were performed on a Macintosh computer using SeqEd, version 1.0.3 (ABI, 1990) followed by manual adjustment using the same program. The phylogeny was produced with PAUP, version 3.1.1 (Swofford, 1993), using NABS VI as the outgroup. Preliminary analysis using *A. tabescens* as outgroup always placed NABS VI basal to the rest of NABS *Armillaria* (unpublished data). The principle of maximum parsimony was used to infer five phylogenies from each of the four individual data sets and a combined data set containing the four individual sets. Robustness of the data was determined using 100 bootstrap replications (Felsenstein, 1985) on each data set, keeping minimal trees only, and producing a single 50% majority rule consensus tree for each of the five data sets. Character-state optimization was performed using delayed transformation. However, accelerated transformation produced the same topology.

Optimal trees were found using heuristic search methods, and random stepwise addition was used to obtain starting trees. By initiating branch swapping from different starting trees, the probability of landing on more than one island was increased. This decreased the possibility of limiting all optimal trees to a single island. Random addition sequences were also used to evaluate the effectiveness of the heuristic search. Optimal tree numbers were most stable using 10, 50, 100, and 300 replications for V250, III520, III180, and 83/66 data sets, respectively, and 10 replications for the combined data set. The nearest neighbor interchange branch swapping algorithm was used since it decreased computer time to a reasonable length for analyses. Due to technical limitations a constraint was placed on analysis of the data sets produced by III180, III520, and 83/66. No more than 90,000 trees were saved during the nearest neighbor interchange branch swapping algorithm with a length $\geq$20 steps, rather than saving all optimal trees.

A parsimony-based incongruence length difference (ILD; Farris *et al.*, 1995) test was used to examine congruency of the data sets. It was performed using Arnie in Random Cladistics 4.0 (Siddall, 1996) for 1000 randomizations. ILD is defined as the difference between the sum of the number of steps of individual analyses and the combined analysis (Farris *et al.*, 1995). ILD was calculated first for the original maximum parsimony trees and then for a series of randomized trees. The ILD from the original trees was then compared to the distribution of ILDs from randomized trees, and a *P* value obtained. The ILD test was applied to the four data partitions simultaneously.

Networks were produced manually by comparing all substitutions to a consensus sequence. The consensus sequence was the sequence containing the least number of substitutions in all sequences combined. The type and number of substitutions were then scored for each sample.

Sequences were deposited in GenBank with Accession numbers corresponding to samples in Table 3; AFO13777 to AFO13797 (for V250), AFO14514 to

TABLE 3

***Armillaria* Isolates Amplified by the Primer Sets Indicated at the Top of Each Column**

V250	III180	III520	83/66
I DMR 20	I DMR 20	I DMR 20	I NOF 1076
I Bow Pk	I Bow Pk	I Bow Pk	I Bow Pk
I JB 08	I JB 08	I JB 09	I JB 09
I GG 12D	I GG 12D	I GG 12A	I JB 13
II JB 39	II JB 38	I JB 38	II JB 38
II 160-8	II 160-8	II 160-8	Missing
II JB 85A	II JB 85A	II JB 85B	II JB 39
III JB 56	III JB 56	III JB 56	III JB 56
III JB 61D	III JB 61D	III JB 61D	III JB 61D
I NOF 830	I NF 830	I NOF 830	I NOF 830
V 83621	V 48-3	V 48-3	V 48-3
V JB 75B	V JB 75B	V JB 75B	V JB 66
V Bow Pk	V Bow Pk	V JB 72	V Bow Pk
V JB 07	V JB 07	V JB 19	V 82911
VI PD 37C	VI PD 37C	VI PD 37C	VI GB 898
VI KJS-6	VI KJS-6	VI KJS-6	VI 97-1
VII 90-10	VII 90-10	VII 90-10	VII 90-10
VII HHB 11912	VII HHB 11912	VII HHB 11912	VII HHB 11912
IX TJV 179-1	IX TJV 179-1	IX TJV 179-1	IX TJV 179-1
IX TJV 200-9	IX TJV 200-9	IX TJV 200-5	Missing
Missing	IX TJV 188-4	IX 121-2	IX TJV 188-4
Missing	IX 139-1	IX 139-1	IX TJV 188-4
X SP812015	X SP812015	X SP812015	X SP812015

Note. Isolate names correspond to those in Table 1. This table illustrates the layout of the combined data set in the NEXUS file for PAUP. Isolate names in the first column (V250) were used to label all branch termini in the topologies.

AFO14533 (for 83/66), AFO14534 to AFO14556 (for III180), and AFO14557 to AFO14579 (for III520). Sequence alignment is available at the web site: http://vaughan.fac.unbc.ca/egma_lab/protdata/index.html.

RESULTS

Results from electrophoresis showed an agarose gel with a single band for each sample, resulting in a single row of bands across the gel for each primer pair. These bands were approximately the same size and present in all samples for each primer set, leading to four different data sets corresponding to each primer pair, with fragments of four different sizes. Primer set V250 produced a nucleotide sequence 224 base pairs (bp) long, III180 produced a sequence 177 bp long, III520 produced a sequence 122 bp long, and 83/66 produced a sequence 150 bp long for each of the isolates in Table 3.

Comparison of each of the sequences with those in GenBank showed very little similarity with known sequences. Start and stop codons were randomly scattered throughout the sequences.

Analysis of Individual Data Sets

Each of the four individual data sets resolved different groups of species (Fig. 1). The V250 tree resolved NABS II, III, and VII supported by bootstrap values of 100, 99, and 82%, respectively. Primer III180 weakly supported a monophyly containing NABS III and VII (Fig. 1B). Primer III520 resolved NABS IX with strong bootstrap support of 98%, and 83/66 resolved NABS I, III, and IX with bootstrap support of 81, 62, and 59%, respectively (Figs. 1C and 1D). The individual trees collectively resolved all groups except NABS V. Consequently, it was expected that the tree produced from the combined data set resolved all NABS but split isolates of NABS V into two groups (Fig. 2). There were 9, 25, 13, and 25 equally parsimonious trees for data sets V250, III180, III520, and 83/66 respectively.

The numbers and types of characters present in each of the four sequence data sets yielded insight into the relative contribution of each type of character to the resolution of each tree (Table 4). The number of informative characters in the V250 fragment was high and the number of constant characters was relatively low (Table 4). Analysis of this V250 fragment produced a phylogenetic tree yielding the most resolution, as indicated by topology of the tree (Fig. 1A) and the network (Fig. 3A). However, the network produced a monophyly for NABS III and VII (Fig. 3A), whereas they were paraphyletic in the tree (Fig. 1A). Analysis of the data set containing the second longest fragment (III180) produced the tree yielding the least information of the four fragments (Fig. 1B). Fragment III180 had the largest number of constant characters (Table 4), indicating that the fragment was more conserved than the others, and the least number of informative characters, resulting in unresolved intraspecific clustering (Fig. 1B), a lower consistency index, and a higher homoplasy index (Table 4). Although maximum parsimony produced an unresolved tree with a weakly supported monophyly for NABS III and VII (Fig. 1B), the network for III180 formed clusters of related groups sharing substitutions including resolution for NABS III and VII, as well as the group of NABS I and II, and three isolates of NABS IX (Fig. 3B). The remaining two data sets, from III520 and 83/66, contained smaller number of total characters, and the number of informative characters was intermediate between V250 and III180. However, the CI was higher and the HI was lower than those of either V250 or III180. The information gained from the branching pattern of phylogenetic trees of III520 and 83/66 was intermediate. The network for III520 was in agreement with the phylogenetic tree, except that one isolate of NABS V was placed in the NABS I and II cluster in the tree (Fig. 1C). The network for 83/66 produced varied results. It resolved NABS VII and the clade NABS III and VII, but dissolved NABS IX. The high degree of variation within this sequence was evident in the low bootstrap values for the monophylies (Fig. 1D).

Phylogenetic Signal and Congruency

The data sets were tested for phylogenetic signal by analysis of frequency distributions of 10,000 random

TABLE 4

Comparison of Goodness of Fit Statistics for Most Parsimonious Heuristic Trees, Frequency Distribution of 10,000 Randomly Sampled Tree Lengths, and Character Information Content for Each of the Four Individual Data Sets, V250, III180, III520, and 83/66, as well as the Combined Data Set

	Heuristic search			Random search			Character information content			
Primer set	CI	HI	Tree length	Mean tree length	SD	*g*1	Total	Constant	Uninform	Inform
V250	0.734	0.266	139	286 [224–310][a] (263)[b]	11.7	−0.98	224	35	118	66
III180	0.704	0.296	71	110 [92–118] (104)	3.1	−0.71	177	128	18	28
III520	0.752	0.248	125	232 [186–254] (214)	9.0	−0.52	122	24	48	48
83/66	0.786	0.214	112	161 [133–171] (152)	4.7	−1.06	150	19	87	42
Combined	0.665	0.335	501	781 [646–832] (744)	20.9	−0.68	673	206	271	184

Note. The heuristic search statistics show consistency index (CI), homoplasy index (HI), and the most parsimonious tree length (number of substitutions). The random search produced a mean tree length (number substitutions) with standard deviation (SD) and a *g*1 statistic for skewness. Character information content shows total number of characters, number of constant characters, number of uninformative characters, and number of phylogenetically informative characters for each data set.

[a] Square brackets enclose the range of random tree lengths (number substitutions) for each data set.

[b] Parentheses enclose the tree length above which 95% of all random tree lengths were located.

trees. Frequency distributions were all skewed to the left as shown by negative *g*1 values (Table 4) (Sokal and Rholf, 1981), and length of each most parsimonious tree of all five data sets was at least 2 standard deviations shorter than 95% of the random trees (Table 4).

The ILD test produced a *P* value of 0.001, indicating that the ILD of the real trees was less than 99% of that of the randomized ILDs. Although this suggested that incongruency was significant, combining the data was an option.

Phylogeny of Armillaria

The combined data set produced phylogenetic signal sufficient to resolve the phylogeny of most NABS *Armillaria*. A single most parsimonious tree separated the eight species into three clades; NABS I, NABS II, and all other NABS *Armillaria* (Fig. 2). Three isolates of NABS I were separated by large geographical distances but were similar, with strong bootstrap support of 100%. Variability within NABS I was indicated by the weak bootstrap support of 66% for the entire clade of five isolates. NABS II formed a strong monophyletic group, supported by a 97% bootstrap value, and was placed ancestral to NABS I (Fig. 2). NABS III, VII, V, IX, and X formed the third clade in the phylogenetic tree (Fig. 2). NABS III and VII formed a monophyletic clade with strong bootstrap support (90%). NABS IX formed another monophyletic group but bootstrap support was weak (63%). The resolution of isolates of NABS V was poor and indicated polymorphism within the sequences examined. NABS X was weakly associated with NABS III, VII, and part of V. The combined data set contained missing sequences and ambiguity at some character positions (see Table 3).

Although minimum-length networks from each of the four separate data sets were very similar to the trees produced by maximum parsimony (Figs. 2 and 3), there were minor differences in the 83/66 data set, and there was a higher degree of resolution in the III180 network (Fig. 3B) than in the corresponding parsimony tree (Fig. 1B).

Neighbor joining analysis of the combined data set suggested a phylogeny that was consistent with phylogenetic relationships produced by maximum parsimony (unpublished data).

Molecular Clock

NABS VI was placed at 30 MYA based on fossil evidence from other Agaricales (Hibbett *et al.*, 1995). The phylogeny was superimposed on the molecular clock (Fig. 4) based on a calibration point of 30 MYA.

DISCUSSION

Sequences did not match any entries from GenBank (Fig. 5). The most probable identification of each of the fragments was that of an unused protein coding sequence such as a pseudogene, since start and stop

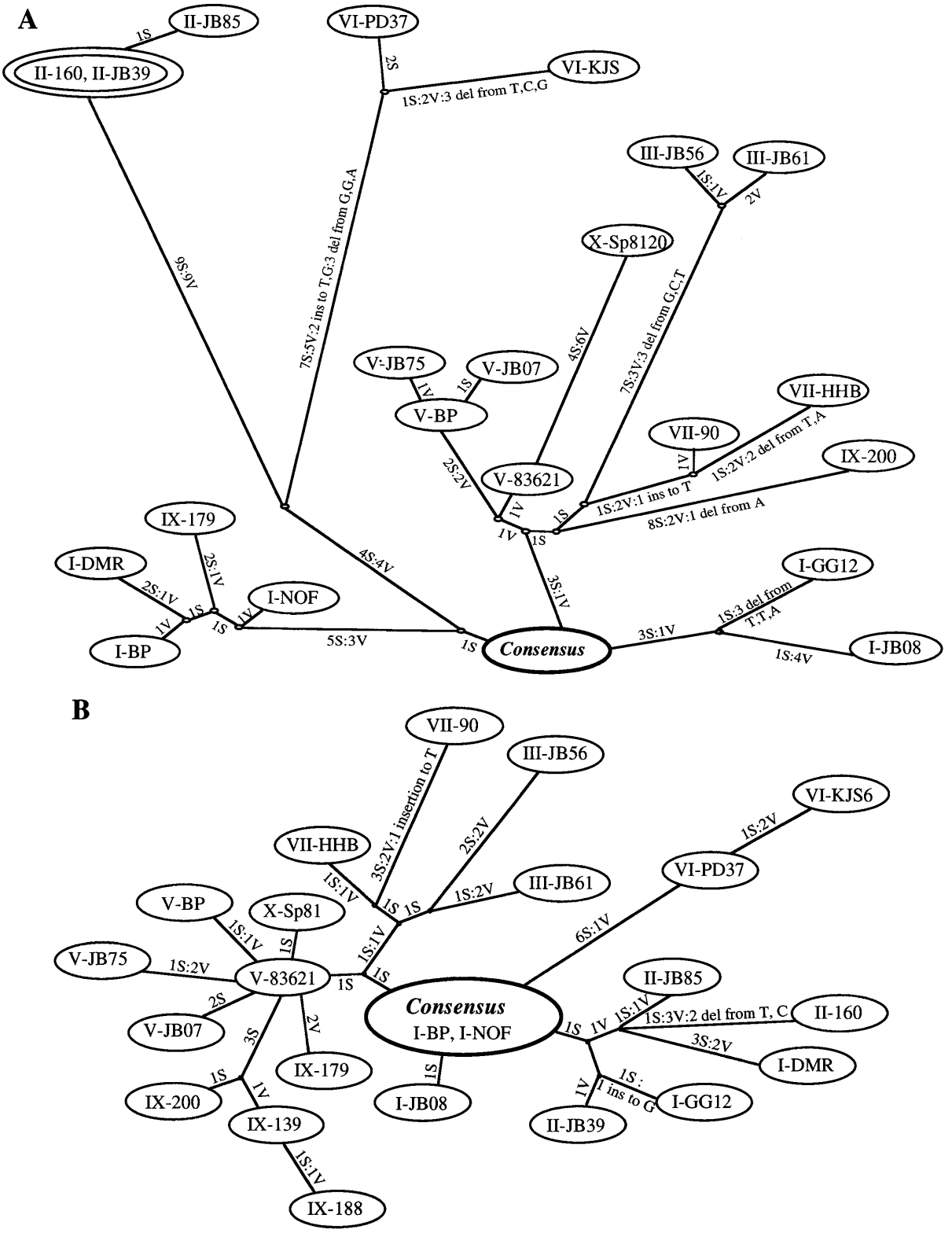

FIG. 3. Minimum-length network diagrams based on anonymous nucleotide sequences amplified by (A) V250, (B) III180, (C) III520, and (D) 83/66 comparing variation to a consensus sequence. Substitutions were determined manually to illustrate all substitution changes among taxa. Length of branch is proportional to number of substitutions. Transition:transversion ratios are indicated on each branch; S, transition; V, transversion; del, deletion; and ins, insertion. Taxa epithets correspond to those in Fig. 1.

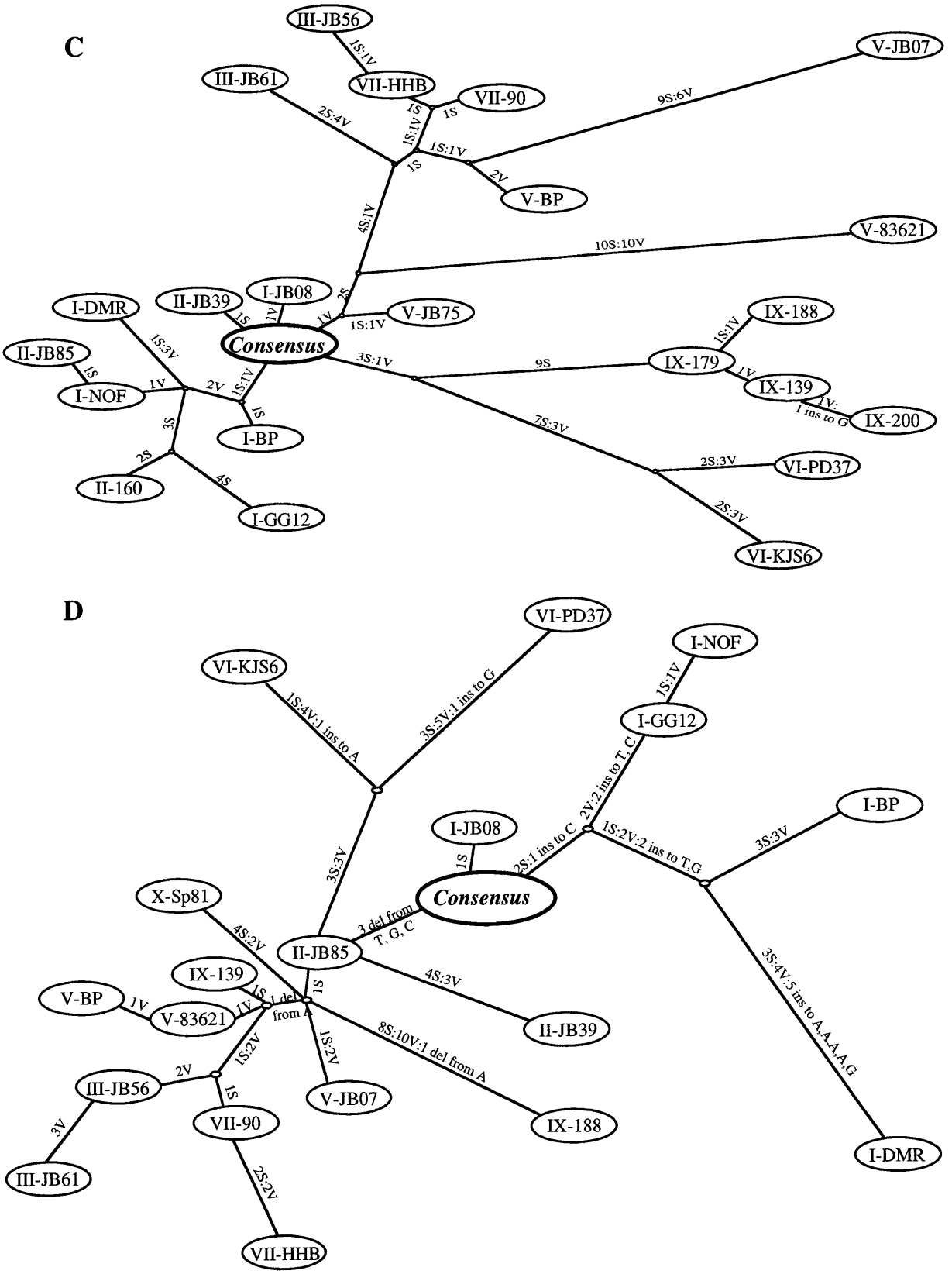

FIG. 3—*Continued*

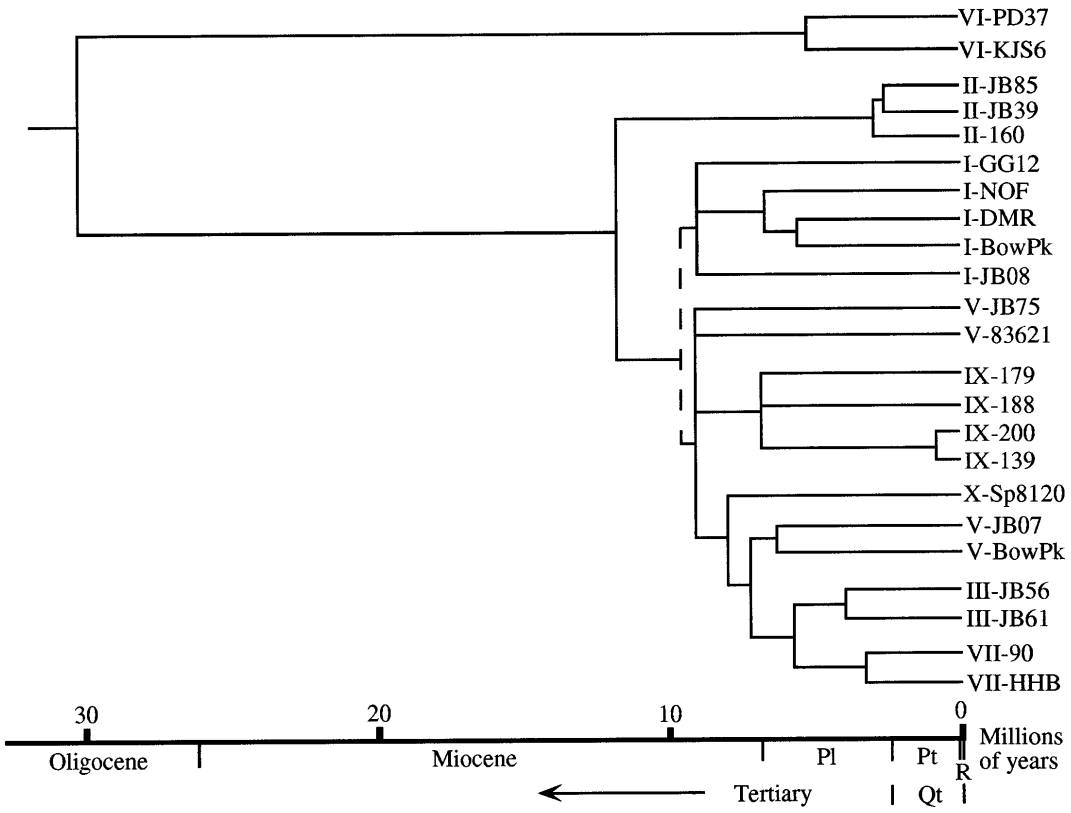

FIG. 4. Divergence times of NABS *Armillaria* superimposed on phylogeny (Fig. 2) showing branch lengths proportional to the percentage of nucleotide substitutions (see text for calculations). Dashed line indicates variation in calculations. Pl is Pliocene, Pt is Pleistocene, R is recent, and Qt is Quarternary. Numbers refer to millions of years ago.

codons were present throughout the sequences. All transition/transversion (S/V) ratios were greater than 1 except in the 83/66 fragment (see Fig. 3). Transitions are more common than transversions in protein coding genes (Mason, 1991; Brown *et al.*, 1982). Since the fragments in this study were randomly chosen and were of unknown function and location, they may be part of pseudogenes with lower S/V ratios than mtDNA, yet evolving fast enough to accumulate more transitions than transversions (except 83/66). The 83/66 fragment may contain more noncoding DNA undergoing less evolutionary constraint, or transitions have become saturated, giving the appearance that there were more transversions occurring. More transitions were apparent in the shared substitutions in the networks, and transversions became more common near taxa differentiation (Fig. 3), reflecting the importance of transversions in determining changes in amino acids responsible for taxa differentiation.

Phylogenetic Signal and Congruency

Each of the fragments amplified by the four primer sets was monomorphic in size and present in most isolates of all NABS *Armillaria*, indicating that the regions amplified were well conserved and therefore good candidates for a phylogenetic study. Frequency distributions of tree lengths, homoplasy, and consistency indices were examined to test that the data contained more phylogenetic signal than would be expected purely by chance (Table 4). Frequency distributions of tree lengths of each of the individual trees as well as the combined tree were all skewed to the left, as indicated by the negative $g1$ values (Sokal and Rohlf, 1981) (Table 4), indicating the presence of phylogenetic signal in the data set (Hillis and Huelsenbeck, 1992). The lengths of the heuristic trees were located at least 2 standard deviations below 95% in the frequency distributions (Table 4), indicating that the tree length obtained by maximum parsimony was significantly shorter than a tree produced with random data ($\alpha = 0.05$). Although the function and location of the fragments within the genome were unknown, the left-skewed frequency distributions of tree lengths and the location of the tree length obtained by maximum parsimony outside the 95% confidence intervals supports the use of these fragments in a phylogenetic study.

The CI of the combined data set was lower than that any of the four separate sets (0.665), yet the combined set produced the most highly resolved tree. A high ILD (61 steps) would suggest that homoplasy had increased when the data sets were combined (Farris *et al.*, 1995),

accounting for the decrease in CI in the combined tree. Archie (1989) critically evaluated the CI and reported that the CI was insensitive in data sets where the number of trees outnumber the characters and when the absolute levels of homoplasy increase. This may have occurred in the three data sets which had a limitation placed on the number of trees saved in the branch swapping algorithm (III180, III520, and 83/66).

A parsimony-based incongruence test, ILD (Farris *et al.*, 1995), was applied to the individual data sets in this study. Cunningham (1997) showed that the ILD test was the best of three tests able to distinguish between degree of incongruence between genes. Interpretation of the *P* value ($P = 0.001$) in this study would suggest that analysis of separate phylogenetic trees be discussed, as well as the combined tree. Individual trees in this study resolved specific groups, leaving others unresolved. For example, III180 resolved NABS III and VII only, leaving all others unresolved (Fig. 1B), whereas III520 resolved NABS IX only, leaving all others partially or unresolved (Fig. 1C). Farris *et al.* (1995) suggested that ILD is large when groups that are well supported by one tree conflict with groups well supported by another tree. Discrepancy among groups in this study was not a result of incongruent fully resolved trees, but rather because different components of each data set were resolving different groups, leaving a lack of information available to form the remainder of the tree.

Congruence among independent data sets and their combination provides strong evidence for correct estimation of a phylogenetic hypothesis (Hillis, 1987). Kluge (1989) argued for "total evidence" in a phylogeny. He contended that it was not necessary for individual results to be consistent with the combined result, since the explanatory power of the data in the combined analysis would then be maximized. Insights into the evolution of separate data sets (Miyamoto and Fitch, 1995) in this study were discussed with reference to analysis of individual fragments, but the individual fragments provided insufficient resolution to justify using them alone. The combined analysis of these data sets emphasizes the value of Kluge's (1989) total evidence concept to maximize the explanatory power of the data, as well as Quicke's (1993) argument that a combination of more than one data set would increase the amount of data available for phylogenetic resolution. In this study results obtained from the combined data set were in agreement with a previous phylogenetic hypothesis (Anderson and Stasovski, 1992) and morphological groups (Bérubé and Dessureault, 1989).

Consequently, if different data sets were sampled from the same individuals, yet the data sets by themselves incompletely resolved a phylogenetic history, it would seem appropriate to combine them in the same way that longer nucleotide sequences or a larger number of morphological characters would improve resolution. Since each of the four fragments were chosen from random segments of the DNA, with the only criterion that they be conserved within the genus, then the final result is that the length of DNA sequence has been increased to a size sufficient to resolve a phylogenetic history. Cunningham (1997) showed that whenever a *P* value from the ILD test was greater than 0.01, the combination of the data sets either improved or did not reduce phylogenetic accuracy. However, if the *P* value was less than 0.001, the combined data set would not improve phylogenetic accuracy. In this study the *P* value was exactly 0.001, and combination of the data sets significantly improved phylogenetic resolution (Fig. 2).

Phylogeny of Armillaria

Both parsimony and distance analyses produced similar results resolving relationships among NABS *Armillaria*. Most of the sequence variation was between rather than within species. Initial observations of the combined phylogenetic tree of NABS *Armillaria* (Fig. 2) indicated that one group of species, NABS III, VII, V, IX, and X, were more closely related to one another than to NABS I, II, and VI. This observation was also supported by similarity data (unpublished data).

NABS VI is a temperate species, with a wide distribution found in both Northern and Southern hemispheres, and is parasitic on hardwood hosts. It has been considered the most divergent of the North American species (Anderson and Stasovski, 1992) based on morphological features (Bérubé and Dessureault, 1988), the lack of clamp connections on the basidia (Korhonen, 1978), and having a larger rDNA repeat, placing it in an rDNA class of its own (Anderson *et al.*, 1989), and mtDNA digests (Anderson and Smith, 1988). Isolates of NABS VI grouped together in the networks with a larger number of common base substitutions than other isolates (Fig. 3).

NABS III and VII. The close phylogenetic relationship hypothesized by Anderson and Stasovski (1992) and Smith and Anderson (1989) between NABS III and VII was supported by this study. NABS III and VII formed a monophyletic cluster with a marginally significant bootstrap proportion of 90% (Fig. 2). NABS III and VII were both found in the same rDNA class (Anderson *et al.*, 1989). They show similarity in cluster analyses of RFLPs of mtDNA (Smith and Anderson, 1989) and in fruit body morphology (Bérubé and Dessureault, 1989) and are considered closely related species based on IGR sequences of rDNA (Anderson and Stasovski, 1992). They are also found as weak pathogens or saprophytes on hardwood hosts. NABS III and VII are both distributed in eastern North America, but the European counterpart of VII, *A. lutea*, is distributed widely in Europe and far east Asia. Consequently, NABS VII may

have given rise to the more narrowly distributed NABS III.

Anderson *et al.* (1989) placed the European species, *A. cepistipes*, in the same rDNA class as III and VII. *A. cepistipes* exhibited lower interfertility with NABS V (Bérubé *et al.*, 1996) and is partially interfertile with NABS X. It is not surprising in this study that isolates of NABS V and NABS X formed a paraphyletic group with the clade containing NABS III and VII (Fig. 2), implying similarity between the monophyletic clade, NABS III and VII, and the more variable isolates, NABS V, IX, and X. This is consistent with IGR sequences in which V, IX, and X form a close common ancestry with III, VII, and *A. cespistipes* (Anderson and Stasovski, 1992).

NABS V, IX, and X. In this study four isolates of NABS IX formed a monophyletic clade weakly supported with a 63% bootstrap value (Fig. 2). Missing data in 3 of 16 fragments may explain the low bootstrap value in this study (Table 3). NABS IX may have diverged from the same common ancestor that gave rise to NABS V and X. This is consistent with phylogenetic analysis of IGR sequences of rDNA of *Armillaria* placing NABS IX more distantly related to V and X (Anderson and Stasovski, 1992). Anderson *et al.* (1989) showed that NABS V, IX, and X formed the same rDNA class. Further evidence supporting their relatedness was reported for similarities in DNA reassociation values in which Miller *et al.* (1994) were unable to resolve relationships among III, VII, V, IX, and X. However, Harrington and Wingfield (1995) were able to distinguish NABS IX from other NABS *Armillaria* using RFLP–PCR. NABS V is distributed across temperate North America and Japan and is found on hardwoods. Both NABS IX and X are small populations found on the west coast of North America but IX generally colonizes hardwoods (Volk *et al.*, 1996) and X colonizes conifers (Anderson *et al.*, 1980; Morrison *et al.*, 1985b). NABS IX and X may be derived from the more widely distributed NABS V, a hypothesis consistent with shared morphological features of NABS V and IX such as absence of scales, small black hairs present on the surface of the pileus, and a more orange coloration than other species (Bérubé and Dessureault, 1988; Volk *et al.*, 1996).

Two isolates of NABS V, VBowPk and VJB07, were collected a large distance (more than 2000 km) apart in Newfoundland and Quebec, respectively. The phyloge-

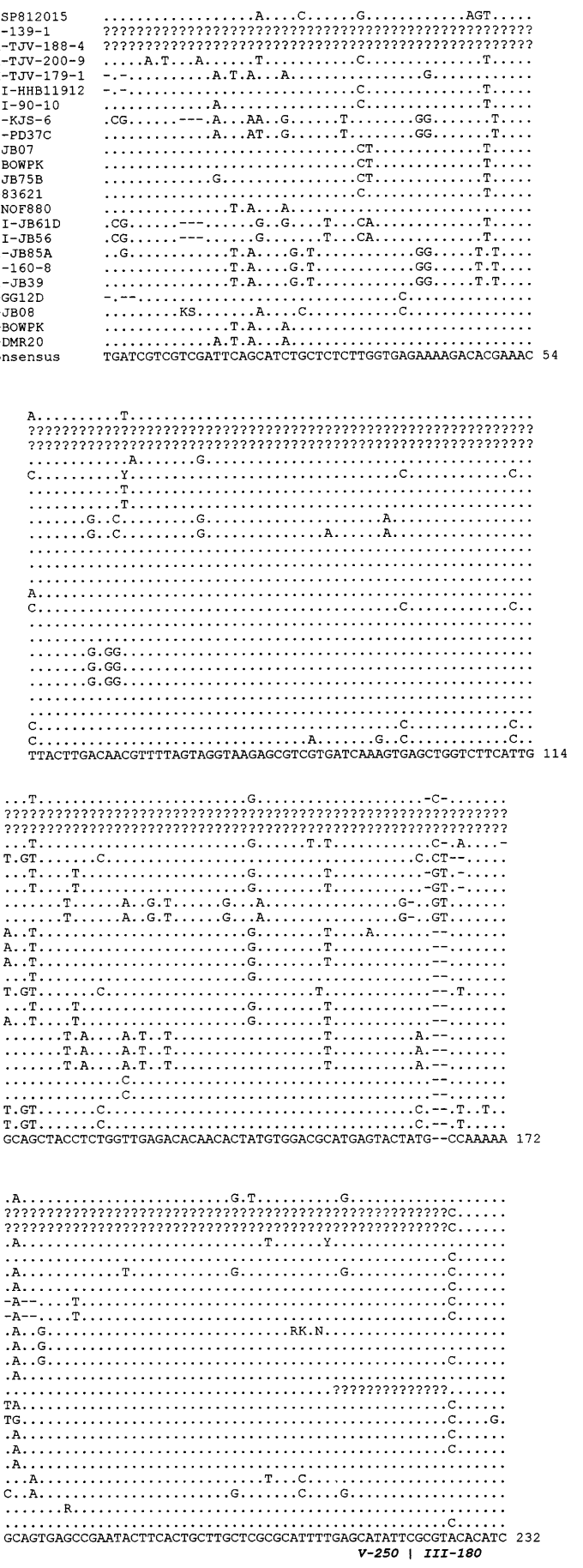

FIG. 5. Sequence alignment of combined data set amplified from four primer sets, V250, III180, III520, and 83/66. *Armillaria* isolate and species is indicated at left of sequence, distance in base pairs is indicated at bottom right of consensus sequence, beginning and ending of each fragment is indicated below consensus sequence as primers, dots represent matches with consensus sequence, dashes represent deletions, mismatches are represented by bases, and question marks indicate missing data.

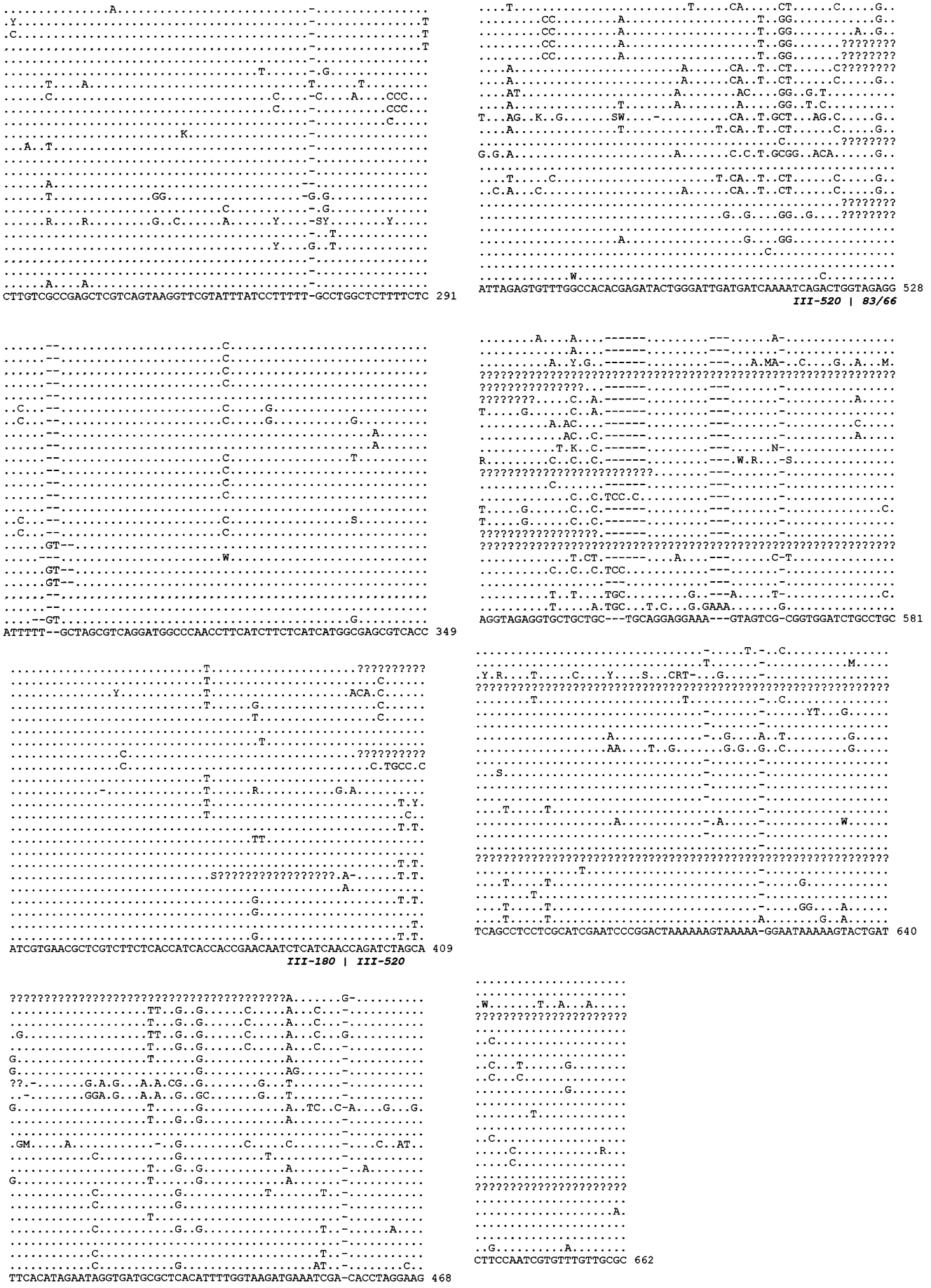

FIG. 5—*Continued*

netic positions of the other two isolates of NABS V, V83621 and VJB75, collected from New York and Quebec, respectively, were unresolved. Geographic distance does not appear to be proportional to sequence polymorphisms in NABS V. The paraphyly of isolates of NABS V indicated a high degree of polymorphism within the sequences relative to the other species (Fig. 2). Isolates of NABS V also seem to be variable with respect to pathogenicity (Mallett, 1990). This variation may be an indication of recent sympatric speciation of NABS V, IX, and X which was discussed by Anderson *et al.* (1989) and Miller *et al.* (1994) as occurring between NABS I and II. The phylogenetic position of NABS X was unclear since only a single isolate with weak amplification and ambiguous sequence was used in this study.

NABS I and II. Despite the large geographical separation of the isolates IDMR20, IBowPk, and INOF830, they were phylogenetically related, with a significant bootstrap value of 100% (Fig. 2). Addition of two more isolates, IJB08 and IGG12, reduced bootstrap support to 66% for the clade containing NABS I. Sequence variation among isolates of NABS I in this study coincided with a mixture of soft- and hardwood species for the isolates (Tables 1 and 3), variation in degree of pathogenicity (Rishbeth, 1982; Guillaumin *et al.,* 1983), and variation in fruit body morphology (Bérubé and Dessureault, 1988).

This study found less variation in DNA of the narrowly distributed NABS II (bootstrap support = 66%) than that of the widely distributed NABS I (bootstrap support = 67%). However, the phylogenetic analysis placed NABS II ancestral to NABS I and all other NABS (Fig. 2). This contradiction may be explained in several ways. The placement of the NABS II clade may have been an artifact resulting from inherent characteristics of the samples collected and the collection sites (Fig. 2). The high degree of similarity in DNA (Miller *et al.,* 1994) and fruit body morphology (Bérubé and Dessureault, 1989) between NABS I and II, and the presence of intersterility barriers producing separate biological species (Bérubé and Dessureault, 1989), indicates that divergence has occurred. However, the direction of this divergence is questionable. NABS II, being limited in distribution, may have diverged from the widely distributed NABS I, as supported by networks of fragments III180 and III520 (Fig. 3). This type of sympatric speciation was suggested by Anderson *et al.* (1989) and supported by Anderson and Stasovski (1992).

Sequences in this study may reflect similarity between NABS II and VI or differences between NABS II and I. A similarity between NABS II and VI is that they both colonize hardwoods, whereas NABS I mainly colonizes softwoods. Differences between NABS II and I involve virulence and rhizomorph morphology (Korhonen, 1978, 1980). NABS I is highly virulent and has dichotomously branched rhizomorphs. NABS II is saprotrophic and has monopodial branched rhizomorphs. The similarity between NABS II and VI may be considered a symplesiomorphy because the shared feature is ancestral, being present in NABS VI and again in NABS II, but absent (or more variable) in NABS I. This may be explained by either of two hypotheses. NABS II may have diverged from NABS VI, and later NABS I appeared, causing NABS II to recede toward the Great Lakes region. In this case the symplesiomorphy was retaind in NABS II, but lost in NABS I. Alternatively, NABS I may have diverged from NABS VI, and later NABS II arose in the Great Lakes region. The symplesiomorphy was lost in NABS I, but the ancestral sequences were retained in the genome and became functional again in NABS II.

The possibility of paralogous genes presenting difficulties in phylogenies has been discussed (Springer *et al.,* 1995; Montchamp-Moreau *et al.,* 1993; Voytas *et al.,* 1992). However, there was no evidence of multiple copies of the anonymous sequences used in this study. Since the anonymous sequences were obtained by nonspecific RAPD primers under low-stringency PCR conditions, amplification of duplicate copies of a sequence might have been expected. However, a single band was amplified in all cases, and ambiguous sequencing positions were not consistently found. Assuming that single-copy sequences indicate orthology, anonymous sequences in this study may indeed reflect a finer level of resolution between NABS I and II and hence the divergence of NABS I from II. Expanding on this concept, we cannot rule out the possibility that NABS I diverged from NABS II, rapidly colonizing the Northern Hemisphere and causing the distribution of NABS II to recede toward the Great Lakes area.

Molecular Clock

The nucleotide substitution rate of 48.7% per 100 MYA for anonymous gene sequences in *Armillaria* in this study was higher than the 1% per 100 MYA used for 18S ribosomal gene sequences (Berbee and Taylor, 1993). Since sequences used in this study were conserved only within the genus *Armillaria,* and rRNA genes are found in all fungi, then a faster rate of evolution would be consistent with the degree of conservation of the genes.

Coevolution of fungus and host. Angiosperms, appearing 140 MYA, were thought to have evolved from gymnosperm ancestors, which appeared about 300 MYA (Stewart and Rothwell, 1993). Meeuse (1975) and Hughs (1977) suggested that angiosperms evolved from a heterogeneous gymnosperm ancestry displaying a combination of characters, some found only in angiosperms, some only in gymnosperms, and some that fit neither group. The rapid evolution and diversification of the angiosperms in the Cretaceous (60–140 MYA) may account for the establishment of ancestral *Armillaria* species as pathogens of angiosperm hardwoods. If

this early *Armillaria* ancestor had been exposed to heterogenetic traits of conifers from the Carboniferous (190–369 MYA), an already established group related to the angiosperms, perhaps present-day *Armillaria* retained its variable genetic traits, allowing the pathogen compatibility with the genetic diversity of its host. The variation in soft- and hardwood host pathogenicity displayed by the extant genus *Armillaria* may be reflected in exposure of its ancestor to host heterogeneity. Basidiomycetes that radiated in the Mesozoic are considered the most aggressive wood rotters in present environments (Robinson, 1990). *Armillaria* can subsist as a saprotroph on decaying wood debris in the soil until a suitable host becomes available. The ancestor of NABS VI may have been one of those wood rotters, with extant species maintaining both saprotrophic and pathogenic modes of life.

Major climatic changes occurred about 50 to 60 MYA (Hopkins *et al.*, 1971). Radiation of the more modern type of angiosperms occurred, while diversity of the gymnosperm flora decreased (Stewart and Rothwell, 1993). Major continental uplifts and climatic cooling occurred during the Miocene (7–26 MYA), which was characterized by more mesophytic vegetation in which broadleaf evergreens were limited to lower lattitudes, and present-day forest associations became established (Stewart and Rothwell, 1993). If the ancestor of NABS VI contained genetic diversity suitable for colonization of both soft- and hardwoods, then diversification of the ancestor would occur as the angiosperms radiated 50 MYA. The angiosperm success persisted for 30 million years during the Tertiary before climatic changes altered the flora again. Dramatic climatic fluctuations occurred during the Tertiary; however, it was characterized mainly by tropical forest (Wolfe, 1971). The proposed establishment of two lineages of *Armillaria* during these changes is supported by the present worldwide distribution of NABS VI and the radiation of eight other more recent NABS *Armillaria* throughout the Northern Hemisphere (Fig. 4). Phylogenetic study of *Pleurotus* demonstrated that early evolving species are presently broadly distributed, whereas more recently evolved species are restricted in their geographic distributions (Vilgalys and Sun, 1994). Perhaps the widely distributed NABS VI continued to colonize the successful hardwood hosts, and the other less widely distributed ancestral group colonized both soft- and hardwood hosts.

Vegetation was adapted to drier, cooler environments at the Miocene/Pliocene boundary (Heusser and King, 1988), which would include softwood conifers of the present-day boreal forest. Since conifers began to invade the habitat already occupied by *Armillaria* in the Miocene, a coniferous niche was opened which required adaptations of the pathogen to a different host in a cooler, drier environment. The second lineage of *Armillaria* diverged into two major groups as these environmental changes occurred (Fig. 4). One group became highly pathogenic on conifer hosts forming NABS I, and the other group formed five different species, neither entirely pathogenic nor saprotrophic, and colonizing hardwood and softwood hosts (NABS III, V, VII, IX, and X).

Isolates of NABS I were successful in the Pliocene and presently colonize the Northern Hemisphere. Although two data sets in this study supported the similarity between NABS I and II (Figs. 1C, 3B, and 3C), it also demonstrated synplesiomorphies present between NABS II and VI (Figs. 1A, 3A, and 3D). These synplesiomorphies affected the combined phylogenetic tree placing NABS II basal to all NABS *Armillaria* including NABS I, with significant bootstrap support of 93% (Fig. 2). This in turn affected the placement of NABS II in the molecular clock, making it appear ancestral to NABS I. However, NABS I and II have been shown to be very similar based on morphological characters (Bérubé and Dessureault, 1989) and molecular characters (Anderson *et al.*, 1989; Anderson and Stasovski, 1992; Miller *et al.*, 1994), with the exception of host preference and base substitutions from anonymous sequences in this study (Figs. 3A and 3D). NABS I is presently distributed throughout the Northern Hemisphere, while NABS II occupies a limited area east of the Great Lakes region. Literature evidence is in favor of NABS II being sympatrically derived from NABS I, but this study suggests that NABS II is ancestral to NABS I.

As the Laurentide ice sheet receded from the Great Lakes region about 13 thousand years ago (KYA), the area was first invaded by conifers such as spruce and fir (Davis and Jacobson, 1985), and later hardwoods such as sugar maple and chestnuts followed (Davis, 1981). Other species present in this area today include beech, yellow birch, basswood, ash, and oak, making these forests highly diversified. If literature evidence predominates then perhaps the phylogenetic species of *Armillaria* had gone through a bottleneck, from the more primitive character of colonizing a hardwood host in NABS VI to a more recent character of colonizing a softwood host in NABS I and reverting back to colonizing a hardwood again in NABS II. The opening of the hardwood niche by the retreat of the Laurentide ice sheet provided a habitat in which these primitive recessive characters, retained from the ancestral genotype, could resurface in a sympatric speciation event, creating NABS II, which presently colonizes hardwoods (Table 1). The base substitutions in the sequences in this study reflect independent characters common to NABS II and VI.

The divergence producing NABS I, specializing as pathogens of softwood hosts, also produced a second lineage, an ancestral form which was not quite as specialized, colonizing both soft- and hardwoods and sometimes producing disease, but mostly obtaining

nutrients saprotrophically (NABS III, V, VII, IX, and X). All members from this second lineage, as well as NABS II, produce monopodial branched rhizomorphs which are typical of less virulent *Armillaria*. NABS I retained an ancestral character from NABS VI in that both produce dichotomously branching rhizomorphs, which are typical of more virulent forms. The ancestor to NABS III and VII diverged about 6 MYA (Fig. 4) and was probably more similar to the more widely distributed NABS VII than the more narrowly distributed NABS III. The remaining nonspecialists include NABS V, IX, and X. The high degree of variation in NABS V in this study has also been shown by Harrington and Wingfield (1995). The consistency in variation may have been overlooked and explained as being an unresolved group, whereas the variation may be evidence that speciation is occurring in this group. If species accumulate variation due to limited gene flow between widely separated populations, they may slowly edge toward speciation. The diversity present in NABS V may be an indication that the group designated NABS V is actually more than one species. The wide distribution of NABS V throughout Asia and North America may represent the ancestral state which produced local outgrowths forming the more narrowly distributed monophyletic NABS IX and X.

CONCLUSIONS

The phylogenetic relationship among NABS of *Armillaria*, inferred from the combined data set, is more or less consistent with that proposed by Anderson and Stasovski (1992). A molecular clock was superimposed on this phylogenetic tree and placed NABS *Armillaria* in an historical framework with its host species. In order to assimilate evidence in this study, which placed NABS II ancestral to NABS I, with that of current literature, NABS II was discussed in terms of a phylogenetic species rather than a biological species. It was hypothesized that NABS II had gone through a bottleneck from colonization of a hardwood host (as NABS VI) to colonizing a softwood host (as NABS I) and reverting back to a hardwood host again (as NABS II).

Lineages containing large geographic distances among collection sites of isolates of NABS I and small distances among those of NABS II produced significant bootstrap values. Similar results occurred with NABS V. Therefore, geographic distance appeared to have no correlation with sequence polymorphisms in biological species of *Armillaria*. Anderson *et al.* (1989) reported a similar conclusion for rDNA classes of *Armillaria*.

Combining data sets from four conserved fragments is an effective way to estimate a phylogeny. DNA regions examined in this study represented regions conserved within species and were variable among species, containing a level of variation suitable for a phylogenetic study. The use of SWAPP PCR is a promising technique for locating regions of DNA suitable for phylogenetic studies. SWAPP regions tend to be short, randomly positioned, and numerous, which would increase the sample size and improve the "total evidence" (Kluge, 1989) of the phylogenetic history of a group of organisms.

The use of goodness of fit statistics such as those used in this study would confirm the presence of phylogenetic signal for anonymous sequences. However, an apparent incongruency of the data sets may result from lack of resolution of trees due to an insufficient number of informative sites in short sequences, rather than disagreement among trees. Combination of more than one fragment incorporates a larger part of the whole organism into a molecular phylogeny and can be valuable for identifying missing pieces of a phylogenetic history. This phylogeny represented a gene tree of NABS *Armillaria* comparing anonymous gene characters with biological species. The genes represented a small aspect of the relationship between species used to assemble an evolutionary history of *Armillaria*. Similarly, the consistency of the disjunct group of NABS V in previous and present studies has been considered problematic. However, the variability of NABS V may act as a clue for resolving the evolutionary history of the group. Nevertheless, this study illustrated the effect that different characters can have on the topology of a phylogenetic tree and that more study is required to resolve the evolutionary history of closely related species of *Armillaria*.

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