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Molecular Genetic Variation in *Emmonsia crescens* and *Emmonsia parva*, Etiologic Agents of Adiaspiromycosis, and Their Phylogenetic Relationship to *Blastomyces dermatitidis* (*Ajellomyces dermatitidis*) and Other Systemic Fungal Pathogens

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***Emmonsia crescens*, an agent of adiaspiromycosis, *Blastomyces dermatitidis*, the agent of blastomycosis, and *Histoplasma capsulatum*, the agent of histoplasmosis, are known to form meiotic (sexual) stages in the ascomycete genus *Ajellomyces* (*Onygenaceae*, *Onygenales*), but no sexual stage is known for *E. parva*, the type species of the genus *Emmonsia*. To evaluate relationships among members of the putative *Ajellomyces* clade, large-subunit ribosomal and internal transcribed spacer region DNA sequences were determined from PCR-amplified DNA fragments. Sequences were analyzed phylogenetically to evaluate the genetic variation within the genus *Emmonsia* and evolutionary relationships to other taxa. *E. crescens* and *E. parva* are distinct species. *E. crescens* isolates are placed into two groups that correlate with their continents of origin. Considerable variation occurred among isolates previously classified as *E. parva*. Most isolates are placed into two closely related groups, but the remaining isolates, including some from human sources, are phylogenetically distinct and represent undescribed species. Strains of *B. dermatitidis* are a sister species of *E. parva*. *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* are ancestral to most *Emmonsia* isolates, and *P. brasiliensis*, which has no known teleomorph, falls within the *Ajellomyces* clade.**

Adiaspiromycosis is primarily a respiratory disease of rodents and many other small mammals but affects humans on occasion (8, 15, 24, 41). The causative agents of the mycosis are *Emmonsia parva* and *E. crescens*. Spores in tissue, called adiaspores, resemble the parasitic spherules of *Coccidioides immitis* but differ in that they lack internal spores (41). The finding of large, round or oval, thick-walled adiaspores in histopathological sections of tissue is the main criterion for a diagnosis of adiaspiromycosis (8, 15, 24, 41). Confirmation by culture of the organism from human tissues has rarely been achieved (12, 24, 41). *E. crescens* forms larger adiaspores and has a broader host range and geographic distribution than *E. parva*. Most human infections are attributed to *E. crescens*, but a recent report documented disseminated disease caused by *E. parva* in a patient with AIDS (12). Because *Emmonsia* species rarely affect humans and do not cause fulminant disease, as do the other dimorphic fungi, these fungal pathogens have received little attention.

The pathogenesis, history of discovery, and controversial taxonomy of the etiologic agents of adiaspiromycosis have been reviewed recently in detail (40, 41). Emmons and Ashburn (14) observed *C. immitis* and a second fungus forming spherule-like structures measuring up to 20 μm in diameter in the lungs of rodents trapped in Arizona. Initially named *Haplosporangium parvum* (31), the fungus was later transferred to the new genus *Emmonsia* as *E. parva* (5). A second fungus producing larger adiaspores but having similar morphology was discovered in rodents trapped in Alberta, Canada (9), and later named *E. crescens* (13). Ensuing studies have shown that the etiologic agents differ in maximum growth temperature, size of parasitic spores, host range, and geographic distribution, but no consensus has been reached on whether these differences are indicative of species or varieties.

Aspects of the saprobic and parasitic stages have led some workers to consider *Emmonsia* species to be close relatives of the dimorphic fungi (4, 9, 14, 30, 40), but this relationship has not been widely accepted because of the unusual nature of the parasitic form and the type of infection caused. This has led, even in 1998, to the treatment of *Emmonsia* species apart from the other dimorphic fungi and together with unusual organisms having unknown affinities (41). Similarly, the endosporulating spherule of *C. immitis* has been a dominant character contributing to disagreement over its phylogenetic affinity. Recent molecular (2, 33) and morphologic (43) studies have provided evidence that *C. immitis* is closely related to a species of *Malbranchea* having a sexual stage in the genus *Uncinocarpus* (*Onygenaceae*, *Onygenales*), as suggested by similarities in its alternate arthroconidia (7, 42) and development of spirally coiled hyphae (43).

Sigler (30) observed ascomatal hyphae and hyphal coils in an *E. parva*-like isolate cultured from the lung of an Australian wombat (27) and predicted that the sexual stage of *Emmonsia* species would belong in *Ajellomyces*. Molecular phylogenetic studies supported this suggestion and showed that *E. parva* and *Blastomyces dermatitidis* are united on a single branch, with *Histoplasma capsulatum* a close relative (2, 25). The latter species have sexual states in the genus *Ajellomyces*

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known as *Ajellomyces dermatitidis* and *A. capsulatus*, respectively (23, 28, 29). In 1996, Sigler (40) demonstrated the development of an *Ajellomyces* sexual state among crossed strains of *E. crescens*; however, only a few strains demonstrated mating competence (with low fertility). No strains of *E. parva* demonstrated compatibility, and in this sense they appear to be distinct species, but no corroborating evidence has been found. Also unresolved is the degree of distinction between species of the genus *Emmonsia* and *B. dermatitidis*. With the finding of *Ajellomyces* sexual stages (teleomorphs) for the agents of blastomycosis and adiaspiromycosis and their location on the same clade (2, 25), there are grounds for combining the species of *Emmonsia* and *Blastomyces* within a single anamorphic genus. This proposal has been rejected (40) because of nomenclatural issues concerning the genus *Blastomyces* that could result in the loss of this important name in common use.

Peterson and Kurtzman (36) examined ribosomal DNA (rDNA) sequence variability between sibling species of yeasts, and among the six regions tested, they showed that only variable domain D2 of large-subunit (lsu) nuclear rDNA (5′ end) is sufficiently variable to distinguish between those sibling species. Berres et al. (1) also found this lsu region useful for analysis of closely related species of auriculariaceous basidiomycetes. Kretzer et al. (21) examined variation among *Suillus* species by using the internal transcribed spacer (ITS) regions of rDNA.

These two regions of the rDNA repeat unit are suitable for examining species level variability and have been used here to examine the genetic variability of *Emmonsia* isolates and to assess evolutionary relationships among several related taxa. DNA sequences from the ITS1, ITS2, and 5.8S rDNA regions and domains D1 and D2 of lsu rDNA were determined for strains of *E. parva*, *E. crescens*, *B. dermatitidis*, *H. capsulatum*, *Paracoccidioides brasiliensis*, and out group species from the families *Onygenaceae* and *Arthrodermataceae* (ca. 1,200 bp, 43 strains). These data were analyzed phylogenetically to determine whether the species of *Emmonsia* are distinct from one another and to provide evidence concerning the separation of the anamorphic states of *Ajellomyces* species into different genera.

MATERIALS AND METHODS

Fungal cultures and growth. The fungal isolates used in this study (Table 1) are on deposit in the University of Alberta Microfungus Collection, Edmonton, Alberta, Canada. Isolates were revived from either freeze-dried or frozen (vapor phase of liquid nitrogen) samples and grown at 25°C on petri plates containing pablum cereal agar (19). All cultures were handled within a class II biological safety cabinet. After 14 to 21 days of growth, blocks (1 by 1 cm) of mycelium and agar from cultures of *Emmonsia* species were excised from the culture plates and transferred to sterile snap-cap polypropylene tubes (12 by 75 mm; Fisher Scientific, Nepean, Ontario, Canada). The mycelial blocks were freeze-dried by using an Edwards Moduylo freeze-dryer (Edwards High Vacuum, Burlington, Ontario, Canada). *B. dermatitidis*, *H. capsulatum*, and *P. brasiliensis* isolates were treated differently because of their hazard level. Instead of being freeze-dried, the blocks of agar and mycelium were placed in tubes containing a 1% thimerosal [ethyl(2-mercaptobenzoato-5)mercury sodium salt] solution for 24 h to kill the fungi.

DNA isolation. Freeze-dried blocks of agar and mycelium (100 mg) were placed in 1.5-ml microcentrifuge tubes and ground to a fine powder by using a 200-μl capacity pipettor tip. The fungal material was rehydrated with 500 μl of DNA extraction buffer (50 mM Tris, 10 mM EDTA, 1% sarcosyl, pH 8.0) with gentle agitation for 10 min. An equal volume of 1:1 chloroform-phenol was added to each tube, and an emulsion was formed by shaking. The emulsion was mixed for 20 min, and then the aqueous and organic phases were separated by centrifugation in a microcentrifuge for 5 min at full speed (14,000 × *g*). The aqueous phase was pipetted into a clean microcentrifuge tube, and 0.1 volume of 3 M sodium acetate (pH 6.0) and 1.3 volumes of ethanol were added. The tube was sealed, and the contents were mixed by inverting the tube several times. Precipitated nucleic acids were pelleted by centrifugation at full speed in a microcentrifuge for 1 min. Ethanol was decanted, and the pellet was dried by inverting the tube over absorbent paper for 5 min. Nucleic acids were dissolved in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 250 μl of a saturated NaI solution and 10 μl of glass milk (Gene Clean; Bio 101, Inc., La Jolla, Calif.) were added to the tube. The tube was inverted periodically, and DNA was adsorbed to the glass milk for 20 min. The glass milk was pelleted and rinsed in accordance with the manufacturer's instructions, and the genomic DNA was eluted into 50 μl of 1/10-strength TE. DNA was stored at −20°C until used.

For the thimerosal-treated samples, excess thimerosal solution was decanted and agar blocks were placed in 1.5-ml microcentrifuge tubes. Glass beads (0.3 g; 0.5 mm in diameter) were added to each tube along with 0.5 ml of DNA extraction buffer. Cell walls were broken by vortexing the tube for 45 to 60 s at full speed on a vortex mixer. DNA extraction and purification procedures were the same as for the freeze-dried samples. To determine whether killing the fungi affected the quality of the DNA, the same protocol was applied to thimerosol-treated mycelium of 10 *Aspergillus* strains for which sequences have already been published. The DNA was amplified and sequenced, and the sequences were found to be the same.

Gene amplification and sequencing. Genomic DNA (1 μl), 10 μl of 10× buffer (45), 1 μl of 50 μM primer ITS1 (45), 1 μl of 50 μM primer D2R (34), 0.5 μl (2.5 U) of *Taq* polymerase, 10 μl of a mixture containing each of the four deoxynucleoside triphosphates in a solution at a 1 mM concentration, and 76.5 μl of sterile deionized water (Milli-Q system) were placed in a reaction tube and overlaid with about 200 μl of mineral oil. The ca. 1,200-nucleotide fragment including ITS1, ITS2, 5.8S rDNA, and part of the 25S rDNA was amplified by 30 temperature cycles (96°C for 30 s, 53°C for 30 s, and 72°C for 150 s) followed by 10 min at 72°C. The amplified fragment was purified by using Gene Clean (Bio 101, Inc.) and eluted into 1/10-strength TE. The purified fragment was stored at −20°C until used in sequencing.

Sequencing was performed by using primers ITS1, ITS2, ITS3, ITS4, D1, D1R D2, and D2R and Applied Biosystems DyeDeoxy sequencing kits. The sequencing reaction mixture contained 1.5 pmol of primer, 200 to 400 ng of the purified DNA fragment, and sequencing reagents, in accordance with the manufacturer's instructions. DNA sequences were read on an ABI 373 automated DNA sequencer. Alignment of overlapping and reverse complement strands to form a consensus sequence for each isolate was accomplished with an ASCII text editor. Alignment of sequences from different isolates was performed visually by using an ASCII text editor. Phylogenetic relationships of isolates were determined by using PAUP 3.1.1 (44) on a Macintosh IIcx computer. Some tests were performed by using programs from the PHYLIP package (17).

RESULTS

The suitability of combining the ITS1, ITS2, and 5.8S-plus-lsu rDNA regions into a combined data set was assessed by analyzing each region separately and comparing the trees. The search algorithm was heuristic (PAUP 3.1.1), and the reliability of each branch was estimated from bootstrap values (1,000 replicates). The resulting trees revealed no contradictory branching patterns. Because mutation rates differ in each region of the rDNA repeat unit, and because the sequence length of each region is different, trees are not expected to reveal exactly the same branching pattern. The data were combined for further analysis.

Alignment of the sequences was accomplished by visual inspection of the bases, with the addition of dashes to account for length differences. Sequences from most *Emmonsia* strains could be aligned easily, but two strains, UAMH 2304 and UAMH 141, previously determined as *E. parva*, and the outgroup species were quite different from the *Emmonsia* isolates and not all of their ITS1 and ITS2 regions could be satisfactorily aligned with the other strains. Unalignable regions were excised from the data set. The 5.8S-and-lsu rDNA could easily be aligned for all of the studied species.

The data set with unalignable regions excised had 1,207 nucleotide positions, of which 703 were constant characters, 225 were variable but parsimony-uninformative characters, and 279 were parsimony-informative characters. Four equally parsimonious trees were found by heuristic searching (PAUP 3.1.1; random input order). The total length of each tree was 991 steps, the consistency index was 0.734, the homoplasy index was 0.266, the retention index was 0.788, and the rescaled consistency index was 0.578 for each tree. Tree topographies and branch lengths were the same for a data set that included all isolates, compared with a data set that excluded additional isolates having identical sequences. Bootstrap values were de-

TABLE 1. Origins and nucleotide sequence accession numbers of isolates used in this study

Species and strain no.	Host and/or source	GenBank nucleotide sequence accession no.
Emmonsia parva (Emmons & Asburn) Ciferri & Montemartini		
Genotypic group 1		
UAMH[a] 125	Isolated in Arizona by C. W. Emmons (no. 2370)	AF038331
UAMH 130	Isolated in Arizona by C. W. Emmons, 1946	AF038333
UAMH 434	Isolated from soil (?) (but see Carmichael [2]), Italy, by R. Ciferri	AF038332
UAMH 134	Isolated from *Neotama micropus*, Texas, by C. W. Emmons (no. 2372), 1949	AF038326
Genotypic group 2		
UAMH 139	Isolated from weasel, Ravelli County, Mont., by W. L. Jellison (no.27274)	AF038328
UAMH 4489	Isolated from bird's nest, Lost River Canyon, Alberta, by R. Currah, 1981	AF038327
UAMH 4770	Isolated from coyote dung, Edmonton, Alberta, by R. Currah, 1983	AF038325
UAMH 6312	Isolated from soil, Edmonton, Alberta, by J. Newton, 1988	AF038330
UAMH 7045	Isolated from bronchial washings, Winnipeg, Manitoba, by R. Summerbell (no. FR 0091), 1991	AF038329
Emmonsia crescens Emmons & Jellison		
Genotypic group 1		
UAMH 126	Isolated from mouse lung, Lethbridge, Alberta, by E. S. Dowding (no. 10), 1946 (=CBS[b] 191.55; ATCC[c] 10784)	AF038319
UAMH 127	Isolated from mouse lung, Peace River, Alberta, by E. S. Dowding (no. 190), 1946 (=CBS 475.77; ATCC 10785)	AF038344
UAMH 128	Isolated from mouse lung, Red Deer, Alberta, by E. S. Dowding (no. F29), 1946	AF038345
UAMH 129	Isolated from lung of *Peromyscus maniculatus borealis*, Athabasca, Alberta, by E. S. Dowding (no. 184), 1947	AF038343
UAMH 132	Isolated from lung of *Peromyscus maniculatus nebrascensis*, Lethbridge, Alberta, by E. S. Dowding (no. 19?)	AF038351
UAMH 136	Isolated from skunk, Lake County, Mont., by W. L. Jellison (no. 27128)	AF038341
UAMH 137	Isolated from muskrat, Lake County, Mont., by W. L. Jellison (no. 27242)	AF038342
UAMH 140	Isolated from lung of *Peromyscus maniculatus*, Alberta, by E. S. Dowding (no. 32), 1950	AF038350
UAMH 1067	Isolated from lung of wild field mouse, Edmonton, Alberta, by J. W. Carmichael, 1961	AF038346
UAMH 1140	Isolated from lung of wild field mouse, Bittern Lake, Alberta, by J. W. Carmichael, 1961	AF038347
UAMH 4076	Isolated from greenhouse soil, Edmonton, Alberta, by J. Weijer (no. V6), 1976	AF038348
UAMH 4077	Isolated from moldy straw bales in a mushroom house by J. Weijer (no. 2), 1975	AF038349
Genotypic group 2		
UAMH 349	Isolated from lung of mole (*Talpa europaea*), Exeter, United Kingdom, by P. K. Austwick (no. V.711), 1954	AF038336
UAMH 393	Isolated from lung of *Cleithrionomys* sp., Korea, by W. L. Jellison (no. 2405), 1953	AF038339
UAMH 394	Isolated from lung of *Cleithrionomys* sp., Korea, by W. L. Jellison (no. 2412), 1953	AF038340
UAMH 395	Isolated from lung of *Apodemus* sp., Korea, by W. L. Jellison (no. 2409), 1953	AF038338
UAMH 3008	Ex type strain, isolated from rodent (*Arvicola terrestris*) lung in Norway by Emmons and Jellison (=ATCC 13704; CBS 177.60)	AF038334
UAMH 7268	Isolated from lung of common brush-tail possum (*Trichosurus vulpecula*), New Zealand by A. Woodgyer, 1992	AF038337
UAMH 7365	Isolated from lung of common brush-tail possum (*Trichosurus vulpecula*), New Zealand, by A. Woodgyer (no. MY 92.307), 1992	AF038335
Other *Emmonsia* sp. isolates[d]		
UAMH 141	Isolated from barnyard soil, Boone County, Mo., by R. W. Menges (no. 3; Phillips strain soil 4), 1951 (28)	AF038321
UAMH 2304	Isolated from barnyard soil, Phillips barn, Kansas, by C. W. Emmons (no. 5117) (=CBS 273.77)	AF038320
UAMH 7172	Isolated from skin lesions of HIV[e]-positive patient, Saskatchewan, by H. Congly (no. 215M-92), 1992	AF038322
UAMH 7425	Isolated from granulomatous lesions on lip, hands and soft palate of otherwise healthy patient, Israel, by I. Polachek (Kemna no. 407-93), 1993	AF038323
UAMH 7426	Isolated from granulomatous lesions on lip, hands, and soft palate of otherwise healthy patient, Israel, by I. Polachek (Kemna no. 408-93), 1993	AF038324
Ajellomyces dermatitidis McDonough & Lewis		
UAMH 3538	Ex type of *Ajellomyces dermatitidis* (+ mating type); isolated from human infection (=ATCC 18187; CBS 673.68)	AF038355
UAMH 3604	Isolated from E. S. McDonough (no. 49A), 1968	AF038356
UAMH 5438	Isolated from dog lung, Alberta, 1986	AF038358
Ajellomyces capsulatus (Kwon-Chung) McGinnis & Katz		
UAMH 3536	Centers for Disease Control and Prevention, Atlanta, Ga. (CDC B-1392) (− mating type)	AF038354
UAMH 7141	Clinical isolate, Alberta	
Paracoccidioides brasiliensis Almedia & Lacaz		
UAMH 8037	Isolated from lung biopsy material, human, Alberta	AF038360
Auxarthron californiense Orr & Kuehn UAMH 1889	Isolated from pack rat dung, San Diego, Calif. *Aphanoascus fulvescens* (Cooke) Apinis	AF038352
Aphanoascus fulvescens (Cooke) Apinis		
UAMH 5117	Isolated from human toe cleft, New Zealand, 1985 *Trichophyton mentagrophytes* (Robin) Blanchard	AF038357
Trichophyton mentagrophytes (Robin) Blanchard		
UAMH 6256	Isolated from human, Edmonton, Alberta	AF038359

[a] University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada.
[b] Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
[c] American Type Culture Collection, Rockville, Md.
[d] Strains identified previously as *E. parva* (40).
[e] HIV, human immunodeficiency virus.

termined by using PAUP 3.1.1 (heuristic search, 1,000 replicates), and those values are placed on one of the four equally parsimonious trees (Fig. 1). The four trees differed in the lengths of the branch leading to *P. brasiliensis* and in the placement of *B. dermatitidis* strains as a sister group to one or both of the *E. parva* branches. The tree (Fig. 1) shows that all isolates of *Emmonsia* spp., *A. capsulatus* (*H. capsulatum*), *P. brasiliensis*, and *A. dermatitidis* (*B. dermatitidis*) are members of a single phylogenetic clade.

The rDNA and ITS nucleotide sequences of *E. crescens* strains fall into one strongly supported clade (99% bootstrap value) with two closely related phylogenetic subgroups. The Eurasian group (Fig. 1, 89% bootstrap value) includes the ex type strain of *E. crescens*, UAMH 3008, and six other strains, UAMH 349, UAMH 393, UAMH 394, UAMH 395, UAMH 7268, and UAMH 7365. The DNA sequences of these strains were either identical to that of the ex type isolate or varied from it at no more than three nucleotide positions. These strains were isolated from animal lungs in Northern Europe, Asia, or New Zealand (Table 1). Hosts for *E. crescens* include small rodents that have panboreal distributions in Europe and Asia (38). The inclusion of strains from New Zealand in this clade probably reflects long-distance transport of infected animals mediated by human commerce, since all New Zealand mammals are species which have been introduced to that country, mostly from Northern Europe.

The North American *E. crescens* group (91% bootstrap value) includes UAMH 126, UAMH 127, UAMH 128, UAMH 129, UAMH 132, UAMH 136, UAMH 137, UAMH 140, UAMH 1067, UAMH 1140, UAMH 4076, and UAMH 4077. Seven of the strains display identical nucleotide sequences, and the other isolates vary from each other at four or fewer nucleotide positions. All strains were isolated from animal hosts or from inanimate substrates in western North America. The Eurasian and North American groups differ at 6 to 10 nucleotide positions, depending on the isolates being compared. Strains cited by Sigler (40) as sexually compatible and producing the *Ajellomyces* state have zero to eight nucleotide differences (Table 2) and are drawn from both phylogenetic and geographic groups of the species (Fig. 1).

Isolates previously identified as *E. parva* (Table 1) (40) are phylogenetically more diverse. Most isolates are placed into two strongly supported groups closely related to *B. dermatitidis*, while five other isolates are not part of either clade (Fig. 1). One set of related *E. parva* strains includes UAMH 139, UAMH 4489, UAMH 4770, UAMH 6312, and UAMH 7045, and these strains form a monophyletic group (76% bootstrap value). These strains were isolated from animate or inanimate substrates in Montana, Alberta, and Manitoba. A closely related second group (UAMH 134, UAMH 125, UAMH 434, and UAMH 130) was obtained from rodents in Arizona and Texas and from soil in Italy (Table 1) and appears to be monophyletic (87% bootstrap value). Each of the *E. parva* groups displays 0 to 13 ITS nucleotide substitutions and 0 or 1 lsu nucleotide substitution among its strains, with 0 to 24 ITS and 0 to 3 lsu differences between groups.

Three isolates from human sources are genetically distinct. UAMH 7425 and UAMH 7426, previously reported as atypical *E. parva* strains (Table 1) (40), were isolated from an otherwise healthy male in Israel with oral skin lesions. The strains have identical nucleotide sequences but differ from other *E. parva* isolates, including the other human isolate, UAMH 7172, at 30 to 59 ITS and 5 to 8 lsu nucleotide positions. These two strains were ancestral to *E. parva* and *B. dermatitidis* in all of the most parsimonious trees. Isolate UAMH 7172 was isolated as a presumptive contaminant from skin lesions on a human immunodeficiency virus-positive human (Saskatchewan) and differs from other *E. parva* isolates at 59 to 65 ITS and 8 to 9 lsu nucleotide positions. In Fig. 1, it branches closer to *P. brasiliensis* than to *Emmonsia* species isolates, but the low bootstrap value makes this placement equivocal. Its sequence differs from that of *P. brasiliensis* at 89 ITS positions and 11 lsu rDNA positions.

Isolates UAMH 141 and UAMH 2304, isolated from barnyard soils in Kansas and Missouri, are genetically vastly different from other *E. parva* isolates, and this distinction had strong support in bootstrap analysis. The isolates have identical sequences and differ from all other *Emmonsia* strains at 100 to 115 nucleotide positions in the ITS regions and 22 to 28 nucleotide positions in the lsu region (Fig. 1).

The *B. dermatitidis* isolates (strains UAMH 3538 and UAMH 3604 are identical, UAMH 5438 has a single nucleotide difference) form a strongly supported branch (100% bootstrap value) and are a sister group to the *E. parva* groups (Fig. 1). *P. brasiliensis* and *H. capsulatum* have a most recent ancestor in common with all of the *Emmonsia* isolates except UAMH 141 and UAMH 2304 (Fig. 1), but the lack of strong bootstrap support on the branches leading to these species means that their placement in the tree is equivocal. A significant genetic difference exists between the clinical *H. capsulatum* strain and the ex type mating strain of *Ajellomyces capsulatus* (11 ITS1 differences, 15 ITS2 differences, and 3 D2 differences). Such differences are suggestive of sibling species (35).

DISCUSSION

The genus *Ajellomyces* contains three heterothallic species, *A. dermatitidis*, *A. capsulatus*, and *A. crescens*, the anamorphs (asexual stages) of which are placed in different genera, *Blastomyces*, *Histoplasma*, and *Emmonsia*. Historically, the genera have been distinguished by differences in conidial features and the morphologies of structures produced in tissue (i.e., budding yeast cells or thick-walled, nonbudding adiaspores). Mating studies are of value in proving relatedness but are problematic within the group because of low fertility, poor reproducibility, and risks of working with the cultures. A molecular approach was used here to determine whether nonmating isolates of the genus *Emmonsia* are members of the *Ajellomyces* clade and to further resolve relationships between them and members of the genera *Blastomyces* and *Histoplasma*. *E. crescens* and *E. parva* were found to be distinct species, but some isolates formerly determined as *E. parva* were found to be genetically distinct. Although microscopic morphology varies little among *Emmonsia* isolates, colonial variation has long been recognized (3, 9, 13). The colonial subgroups observed by Sigler (40) (roman numerals in Fig. 1) are similar, but not identical, to the phylogenetic groupings of strains.

The two phylogenetic groups of *E. crescens* correlate with the continents from which the isolates were obtained. Strains from North America form one clade; strains isolated from hosts having a Eurasian panboreal distribution form a second clade. The inclusion of strains from New Zealand in this clade is enigmatic since, to our knowledge, *E. crescens* has not been found in soil or native animals. Two isolates were found in a species of marsupial (18) that was introduced from Australia, where *E. parva*, but not *E. crescens*, has been reported (6, 27). Johnstone et al. (18) mentioned that *E. crescens* may also have been the cause of adiaspiromycosis in a ferret. No mammal is indigenous to New Zealand, and many have been introduced from northern Europe, where *E. crescens* is well known. Interanimal transmission of adiaspiromycosis is unknown, but adi-

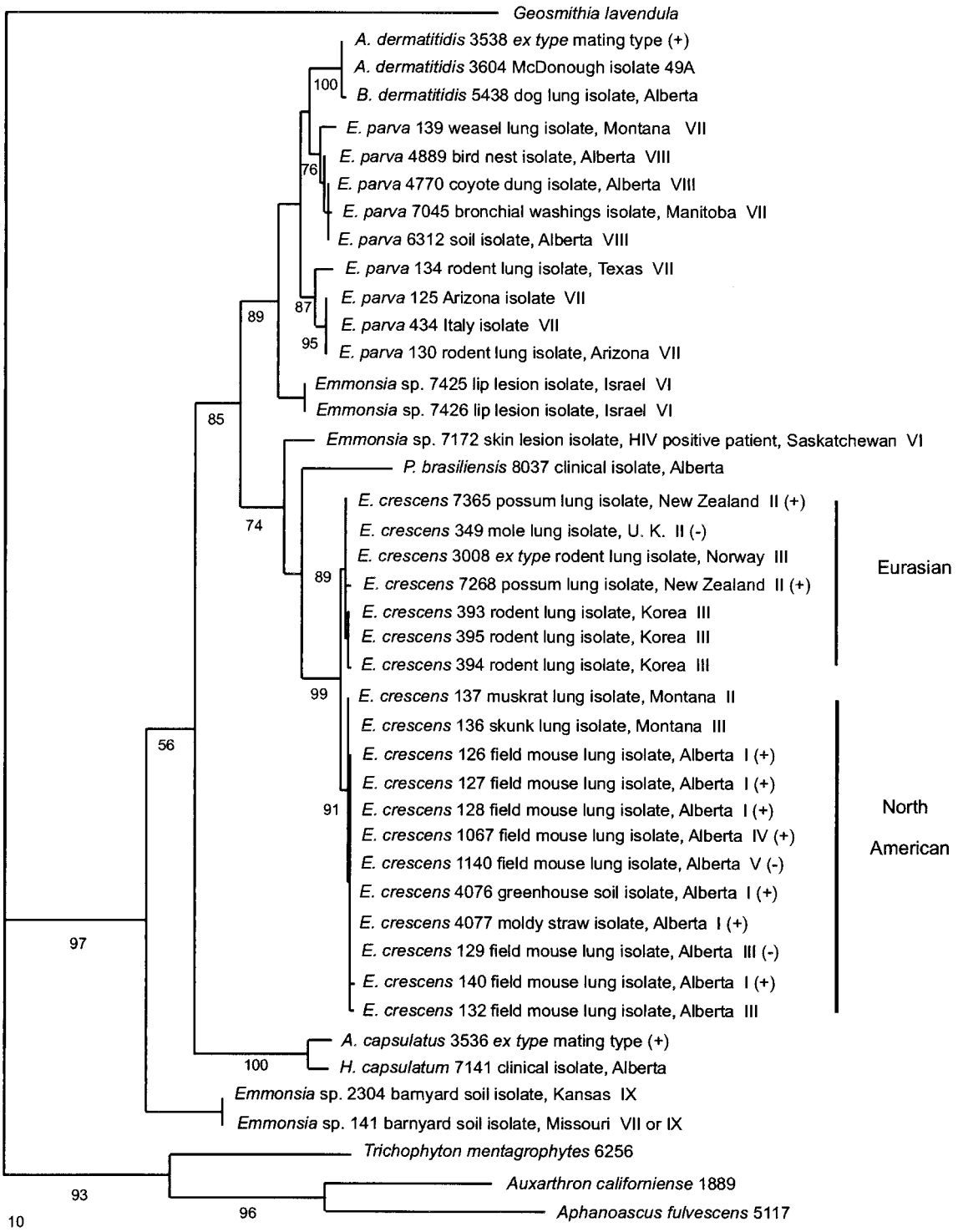

FIG. 1. Phylogram representing one of the four equally parsimonious trees found in a heuristic search (PAUP 3.1.1) of the ITS1, ITS2, and 5.8S-plus-lsu rDNA sequence data. The aligned data set included 1,207 nucleotide positions for each of 43 strains. The sequence of *Geosmithia lavendula* was obtained from GenBank (AF033385) and was used to root the tree since this species has been phylogenetically placed among the perithecial ascomycetes (32). Unalignable segments of ITS1 and ITS2 were excised from the alignment before analysis. The number of steps between nodes is proportional to branch length, and a scale bar for 10 steps appears at the bottom. The number below each internode is the bootstrap value (1,000 replicates) for that node. Isolation data, colonial subgroups designated by roman numerals and based on growth rates and colonial features on potato dextrose agar after 28 days at 22°C (data from reference 40) and mating type (plus or minus) (40), when known, are listed to the right of the isolate numbers. *B. dermatitidis* isolates are a sister group to *E. parva* strains. *E. parva* strains form two phylogenetic groups closely related to *B. dermatitidis*. Three isolates from humans, previously identified as *E. parva*, are on distinct lineages and represent undescribed species. Isolates of *E. crescens* form a clade with two subgroupings. Strains in each subgroup were isolated from locales in Eurasia or North America. They may represent geographic variants or incipient species. Low bootstrap values on the basal branches leading to *H. capsulatum* and *P. brasiliensis* make the placement of these species in the tree equivocal. Two *Emmonsia* strains, UAMH 141 and UAMH 2304, previously identified as *E. parva*, branch basally in the tree and represent an undescribed species. HIV, human immunodeficiency virus.

TABLE 2. *E. crescens* strains producing sequence ascospores in mating and numbers of nucleotide differences between paired strains

Mating-negative UAMH strain crossed	No. of nucleotide differences from mating-positive strain:								
	126	127	128	140	1067	4076	4077	7268	7365
129	0	0	0	1	0	0	0	8	7
349	7	7	7	8	7	7	7	1	0
1140	0	0[a]	0[a]	1[a]	0[a]	0[a]	0[a]	8	7

[a] Mating ability not tested.

aspores can survive passage through the digestive tracts of mice, birds, and carnivores (11, 41).

Sigler's mating data (40) help to place a taxonomic interpretation on the nucleotide substitution data. Isolates of *E. crescens* from a single geographic region displayed zero to two substitutions in the ITS regions and zero or one nucleotide substitution in the lsu regions. Comparisons of strains from the different geographic areas reveal five to eight ITS and one or two lsu region differences. The amount of DNA sequence divergence between the clades is similar to the amount of DNA difference observed in sexually isolated species of filamentous fungi and yeasts (26, 34–37). However, Sigler (40) found that isolates from different continents are sexually compatible, which indicates incomplete isolation. Additional mating experiments that assess viability in the F_1 and F_2 generations could resolve the degree of genetic isolation and provide good evidence of whether these geographically and phylogenetically defined clades represent sibling species or geographic variants (22).

Nine of the 14 isolates originally determined as *E. parva* were placed into two closely related groups and demonstrated phylogenetic diversity similar to that in *E. crescens*. One group of five closely related strains, including three authentic isolates examined by Emmons, was isolated in the northern short-grass prairie regions of North America, while another group of four related strains contains isolates from the desert southwest of the United States or from Italy (Fig. 1). The molecular data correlate, to some extent, with colonial subgroups observed by Sigler (40), who found that group VIII isolates UAMH 4489, UAMH 4770, and UAMH 6312 grew faster than group VII isolates UAMH 125, UAMH 130, UAMH 134, and UAMH 434. Carmichael (3) also recorded phenotypic variation and placed UAMH 139 in a position intermediate between subgroupings. These *E. parva* groups are closely related to *B. dermatitidis*. Guého et al. (16) reported similar findings of close relationship between authentic *E. parva* and *B. dermatitidis* isolates. There is no known sexual state in *E. parva*, but the phylogenetic diversity from one group to the other and to *B. dermatitidis* strains suggests diverging groups that may be varieties or sibling species.

Although literature reports suggest that *E. crescens* is the main cause of human adiaspiromycosis, primarily based on the larger size of the adiaspores observed in tissue (8, 11, 15, 24, 41), no isolate of *E. crescens* from a human source was available for this study, nor could we obtain for comparison the *E. parva* isolate described as causing disseminated infection in a patient with AIDS (12). Three isolates from human skin lesions, originally identified as *E. parva*, are shown here to be phylogenetically distinct and not closely related to any environmental or animal isolates of *E. parva*. UAMH 7172 was ancestral to *E. crescens* isolates and appears to represent an undescribed species. However, it requires comparison with an *Emmonsia*-like isolate that was recently isolated from an Italian patient also with AIDS and described as forming yeast cells in vivo (10). Guého et al. (16) showed their isolate to be phylogenetically related to *E. crescens*, similar to the placement of UAMH 7172. Two other slowly growing atypical isolates (40), UAMH 7425 and UAMH 7426, represent an undescribed species, according to the phylogenetic analysis. Both isolates came from oral skin lesions on a single male patient in Israel (Table 1) whose clinical and histopathological findings were suggestive of blastomycosis (20), but these isolates demonstrated morphology more consistent with that of an *Emmonsia* species, grew restrictedly at 37°C, failed to convert to a yeast phase in vitro, and demonstrated a negative exoantigen test with *B. dermatitidis* antiserum. The only other human isolate, UAMH 7045 (from bronchial washings in Manitoba), was included in a group of North American environmental isolates of *E. parva* (Fig. 1). It is notable that all of the human isolates examined in this study fall outside of the *E. crescens* clade, with pathogenicity known only for the Israeli isolates.

In the parsimony tree (Fig. 1), *B. dermatitidis* has a most recent ancestor in common with *E. parva* but is distinct from that species. Similar findings of phylogenetic relatedness between single isolates of *B. dermatitidis* and *E. parva* have been reported in prior studies (2, 25), but a direct comparison cannot be made because some of the isolates examined have been reidentified. Bowman and Taylor (2) examined UAMH 1067, and Leclerc et al. (25) used CBS 191.55 (=UAMH 126), both listed as *E. parva*, but both of these isolates have now been determined to be *E. crescens* (Table 1). Our study (Fig. 1) placed *B. dermatitidis* strains and *E. parva* isolates in the same clade, with high bootstrap support (89%), and *E. crescens* on a distinct branch of the tree. The study of Guého et al. (16) showed results similar to ours, but again two of their *E. parva* isolates (CBS 191.55 = UAMH 126 and CBS 475.77 = UAMH 127) have been reidentified as *E. crescens* (Table 1).

The distant phylogenetic position of isolates UAMH 141 and UAMH 2304, both from barnyard soils in the central United States, is surprising since they are basal to all other members of the clade. The isolates have identical sequences but differ from all other *Emmonsia* strains at 100 to 115 nucleotide positions in the ITS regions and 22 to 28 nucleotide positions in the lsu regions. These data prompted a reexamination of morphological data not reported in Sigler's previous study (40). UAMH 141 was slower growing on potato dextrose agar (40-mm diameter after 28 days at 22°C) than members of colonial subgroup VII, including UAMH 125, UAMH 130, UAMH 134, and UAMH 434 (average diameter, 53 mm), differed in colonial features, and produced larger adiaspores than other strains of *E. parva*. Carmichael (3) suggested that the species identification of this strain was in doubt. The colonial features of UAMH 2304 appeared to be intermediate between those of group VII and strain UAMH 141. Additionally, it produced slightly larger conidia and differed from all others in expressing acidity on bromcresol purple-milk solids-glucose agar (19). There is a possibility that these strains have a common origin. UAMH 141 is referred to as "Philips strain" in the study by Menges and Habermann (31). Although isolated in Missouri, the strain was used for skin testing of animals in Kansas. UAMH 2304 was received as "soil, Phillips barn, Kansas."

The other two systemic human pathogens, *H. capsulatum* and *P. brasiliensis*, cannot be placed in the tree with any statistical certainty, except to say that they belong in the same clade with the *Emmonsia* species and *B. dermatitidis*. Because *P. brasiliensis* is in a clade that contains *Ajellomyces* sexual states, it is likely that if a teleomorph is found for *P. brasiliensis*, it will be an *Ajellomyces* species. The data of Guého et al. (16)

also show that *Blastomyces*, *Histoplasma*, *Paracoccidioides*, and *Emmonsia* are part of a strongly supported clade but that the relationships of these four groups are unknown. Our findings are also similar to theirs in placing members of the *Ajellomyces* clade distant from out group species belonging to the families *Onygenaceae* (*Auxarthron californiense* and *Aphanoascus fulvescens*) and *Arthrodermataceae* (*Trichophyton mentagrophytes*). The muriculate ascospores of all *Ajellomyces* species appear to set them apart and are known elsewhere in the order *Onygenales* only in the genus *Polytolypa* (40).

Because the three species with *Ajellomyces* teleomorphic states occur in the same clade, as shown in this study (Fig. 1) and other studies (16), there are grounds for placing all of the anamorphic states in the same genus. Arguments supporting this approach have been made previously (4), but the genera have been retained because (i) phenotypic differences can be recognized, (ii) the names are in widespread use, and (iii) emphasis has been placed historically on the differences in their in vivo forms. In other genera, where different anamorphic states occur among species from the same teleomorphic genus (e.g., *Talaromyces* with anamorphs in *Geosmithia* and *Penicillium* [39]), identification of strains is aided by recognition of the phenotypic differences displayed by the anamorphs. The same rationale could be applied to the retention of separate anamorphic names for these members of the *Ajellomyces* clade. However, with the results of this study, which shows that isolates of *E. parva* are closer to *B. dermatitidis* than to *E. crescens*, and with the discovery that human-associated *Emmonsia*-like isolates have a propensity to form yeast cells in tissue (this study, 10, 20), there appears to be little basis for maintaining *Blastomyces* and *Emmonsia* as separate genera. *Blastomyces* is the older and best-known name, and its retention is important for the maintenance of a stable nomenclature. Normally, it would be a simple matter to transfer *Emmonsia* species, as has recently been suggested (16). However, an impediment to this proposal comes from the fact that the genus *Blastomyces* Gilchrist and Stokes is invalid under the International Code of Botanic Nomenclature and requires conservation first. A proposal for conservation is being initiated.

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