EUKARYOTIC CELL, Oct. 2005, p. 1629–1638 Vol. 4, No. 10
1535-9778/05/$08.00+0 doi:10.1128/EC.4.10.1629–1638.2005

Comparative Gene Genealogies Indicate that Two Clonal Lineages of *Cryptococcus gattii* in British Columbia Resemble Strains from Other Geographical Areas

Sarah E. Kidd,[1,2]* Hong Guo,[3] Karen H. Bartlett,[1] Jianping Xu,[3] and James W. Kronstad[2]

School of Occupational and Environmental Hygiene, University of British Columbia, 2206 East Mall, Vancouver, British Columbia, Canada V6T 1Z3[1]; The Michael Smith Laboratories, University of British Columbia, 2185 East Mall, Vancouver, British Columbia, Canada V6T 1Z4[2]; and Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1[3]

Received 10 May 2005/Accepted 1 August 2005

***Cryptococcus gattii* has recently emerged as a pathogen of humans and animals in the temperate climate of Vancouver Island, British Columbia (B.C.). The majority (~95%) of the isolates from the island belong to the VGII molecular type, and the remainder belong to the VGI molecular type. The goals of this study were to compare patterns of molecular variation among *C. gattii* isolates from B.C. with those from different areas of the world and to investigate the population structure using a comparative gene genealogy approach. Our results indicate that the *C. gattii* population in B.C. comprises at least two divergent lineages, corresponding to previously identified VGI and VGII molecular types. The genealogical analysis of strains suggested a predominantly clonal population structure among B.C. isolates, while there was evidence for sexual recombination between different molecular types on a global scale. We found no geographic pattern of strain relationships, and nucleotide sequence comparisons revealed that genotypes among isolates from B.C. were also present among isolates from other areas of the world, indicating extensive strain dispersal. The nucleotide sequence diversity among isolates from B.C. was similar to that among isolates from other areas of the world.**

The basidiomycete fungus *Cryptococcus gattii* is a primary pathogen of humans and animals. *C. gattii*, previously recognized as *Cryptococcus bacillisporus* and *Cryptococcus neoformans* var. *gattii*, has traditionally been associated with tropical and subtropical climates (20) and with the infection of immunocompetent hosts (30, 32). The latter feature distinguishes *C. gattii* from the related pathogens *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, which are opportunistic pathogens of immunocompromised hosts, including AIDS patients (6). An unprecedented emergence of *C. gattii* infection in humans and many animal species has occurred on Vancouver Island in British Columbia (B.C.), Canada, over the past 6 years (15, 33; M. Fyfe et al., unpublished), and there is no evidence of *C. gattii* infection in B.C. prior to 1999 (S. E. Kidd, unpublished results). This emergence is striking not only because of the temperate climate of this region but because the reported infection rate is currently the highest in the world, e.g., 37 times greater than that reported in Australia, where *C. gattii* is considered endemic (5, 15). Environmental sampling revealed that *C. gattii* has colonized trees and soil on Vancouver Island and that the fungus can readily be detected in air samples (15; K. H. Bartlett, L. MacDougall, S. Mak, C. Duncan, S. Kidd, and M. Fyfe, Abstr. 16th Biometeorol. Aerobiol. Meet. 2004, abstr. 5.5, 2004 [http://ams.confex.com/ams/pdfpapers/80027.pdf]). For this study, we were interested in the patterns of molecular variation among *C. gattii* isolates from B.C. as well as in their potential origins and mode of reproduction in nature.

Traditionally, cryptococcal isolates have been classified into five groups, i.e., A, B, C, D, and AD, by serotyping of the capsular polysaccharide (8, 37). More recently, PCR fingerprinting, restriction fragment length polymorphism (RFLP) analysis, and amplified fragment length polymorphism (AFLP) analysis have been used extensively in genotyping studies of the *C. neoformans* species complex that includes *C. gattii*. It has been demonstrated that eight major molecular types exist within the species complex, initially defined according to distinct PCR fingerprinting and randomly amplified polymorphic DNA profiles (26, 27, 29, 31) and supported by a number of different molecular typing techniques (17). *C. neoformans* var. *grubii* (serotype A) isolates correspond to molecular types VNI and VNII; *C. neoformans* var. *neoformans* (serotype D) corresponds to molecular type VNIV; the serotype AD hybrid corresponds to molecular type VNIII; and *C. gattii* (serotypes B and C) corresponds to four molecular types, namely, VGI, VGII, VGIII, and VGIV. Many genetic subtypes exist within each molecular type, representing different strains of the organism. In this context, a pilot study using isolates from clinical and environmental sources on Vancouver Island (collected between 1999 and 2002) revealed that approximately 95% of the isolates belong to the VGII molecular type (15). PCR fingerprinting and AFLP analysis also revealed two VGII subtypes among the Vancouver Island isolates. These were designated VGIIa/AFLP6A and VGIIb/AFLP6B, with approximately 90% of VGII isolates belonging to the VGIIa/AFLP6A subtype (15). In terms of the potential origins of the VGII strains in B.C., we note that a single *C. gattii* strain, NIH444 (also known as CBS6956 and ATCC 32609), isolated from a

* Corresponding author. Mailing address: Michael Smith Laboratories, University of British Columbia, 301-2185 East Mall, Vancouver, B.C. V6T 1Z4, Canada. Phone: (604) 822-2217. Fax: (604) 822-2114. E-mail: sakidd@interchange.ubc.ca.

human in Seattle, Wash., circa 1971, has also been typed as VGII/AFLP6 (1, 15). Washington State is geographically proximal to Vancouver Island and the B.C. mainland and shares a similar climate.

Besides the VGII isolates, one or two VGI isolates have been isolated each year since 2001 from clinical sources in B.C. (approximately 5% of typed cases) (15; S. E. Kidd, unpublished results), but no analogous environmental isolates of this molecular type were found. Many of the cases were associated with a travel history, leaving unclear the validity of VGI as an endemic molecular type for B.C. However, two VGI strains were recently (2004 and 2005) isolated from environmental sources in B.C. The first isolate was included in this study and came from an arbutus tree on Saltspring Island, located in the Strait of Georgia between Vancouver Island and the B.C. lower mainland (S. E. Kidd and K. H. Bartlett, unpublished results). The second isolate was obtained from a different tree on Saltspring Island following the completion of the analyses in this study. This finding indicates that a colonized source of the VGI molecular type may exist in B.C. and suggests that a deeper analysis of the population structure of *C. gattii* in B.C. is needed.

C. gattii is capable of both sexual and asexual (clonal) reproduction, and sexual recombination could potentially be occurring within the population on Vancouver Island, even though all isolates examined to date from B.C. have been of the alpha mating type (*MAT*α) (12, 15). Such a biased distribution of mating type alleles has also been observed in populations of *C. gattii* in other parts of the world (19, 25, 40). The skewed mating type distribution suggests that the B.C. population of *C. gattii* might be predominantly clonal. However, conclusive evidence is lacking at this time.

The genealogy of a given gene may be used to approximate the evolutionary history of an organism. However, comparative analyses of multiple gene genealogies can provide insight into the mode of reproduction that facilitates evolutionary change within a defined group of organisms, hence providing information about recombination, clonality, speciation, hybridization, and dispersal. Given a group of clonally reproducing organisms, the genealogies of multiple unrelated genes are expected to be the same since evolutionary changes arise mainly through random genetic drift. But given a group of organisms where sexual recombination has occurred to some degree, the genealogies of different genes may be expected to differ because of meiotic reassortment.

The relationships between isolates belonging to the same and different molecular types and subtypes from Vancouver Island are unclear, and additional studies are needed to examine aspects of clonality, dispersion, recombination, and hybridization for this population. To begin to explore these relationships, a comparative gene genealogy approach was used to assess the population structure of *C. gattii* isolates from B.C. The concordance of phylogenetic patterns (i.e., monophyly) and specific traits of the isolates, such as mating type, molecular type, host type, and geographic origin, were examined to further characterize the *C. gattii* lineages. Our other goals were to assess DNA sequence variation among B.C. isolates in the context of isolates from other areas of the world and to investigate potential epidemiological links between isolates.

MATERIALS AND METHODS

Yeast isolates. Twenty-four *Cryptococcus gattii* isolates from human ($n = 9$), animal ($n = 9$), and environmental ($n = 6$) sources on Vancouver Island and from other parts of B.C. and Canada were selected for analysis; these strains were isolated between 2001 and 2004. Nineteen *C. gattii* isolates collected in other parts of the world were used for DNA sequence comparisons. Serotyping of isolates was performed using the CryptoCheck slide agglutination test (Iatron, Tokyo, Japan). Detailed information for each of the isolates is provided in Table 1.

DNA manipulations. High-molecular-weight genomic DNA was isolated using previously described techniques (27, 39). The molecular types of all isolates were determined by *URA5*-RFLP analysis according to a previously described method (26). Some of the isolates in this study were included in a previous study, in which PCR fingerprinting and AFLP analysis revealed subtypes within the VGII molecular type (15).

Fragments of four unrelated genes were studied, namely, *LAC* (encodes diphenol oxidase/laccase) (36), *URA5* (encodes orotidine monophosphate pyrophosphorylase) (4), *FTR1* (encodes a high-affinity iron permease) (21), and *CAP1* (encodes a capsule-associated protein), located at the mating locus (11). To verify that these genes were physically unlinked, the positions of these loci in the serotype B genome were determined (www.bcgsc.ca). *LAC* and *URA5* both lie on chromosome 7 separated by 688 kb and the centromere, such that these loci are expected to undergo independent reassortment; *FTR1* lies on chromosome 3; and *CAP1* lies within the mating locus on chromosome 9. Primers designed to amplify and sequence the PCR products of each of the gene fragments were as follows (5′-3′): for *LAC* (565-bp product) (36), GGCGATACT ATTATCGTA (forward) and TTCTGGAGTGGCTAGAGC (reverse); for *URA5* (744-bp product) (4), ACGCCTGCCTGTTACTTAA (forward) and GG ACATGATGATTGGAGT (reverse); for *FTR1* (865-bp product), GTTCTCG GTCACCATCTTC (forward) and TCTCAGGCTCGCCATCTTC (reverse); and for *CAP1* (815-bp product), CGCCATAGAGAGAGGATGAC (forward) and CCGCCTTACCTTCACAGTCG (reverse). PCR products were gel purified using a Qiaquick gel purification kit (QIAGEN, Mississauga, Ontario, Canada) or Wizard spin columns (Promega, Madison, Wis.). Sequences for these genes from WM276 (VGI) were obtained from the genome assembly from the B.C. Genome Sciences Centre (www.bcgsc.ca). Orthologous sequences were obtained from genome assemblies of H99 (serotype A) (Duke Center for Genome Technology, NC [http://cneo.genetics.duke.edu/], and Broad Institute, MA [http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/]) and B-3501 (serotype D) (Stanford Genome Technology Center, CA [http://altoid.stanford.edu/sgtc/group/C.neoformans/index.html]) (24) for use as outgroups in the analyses.

The accuracy of the nucleotide sequences was assessed by a BLASTn search of the A1M R265 genome assembly that recently became available for this isolate (http://www.broad.mit.edu), using the nucleotide sequences obtained for all four loci for this strain.

The mating type was determined by using BLAST 2 sequences (34) to align the *CAP1* sequence from each isolate in our study to the recently available sequences of the *C. gattii* VGI *MAT*α (GenBank accession no. AY710430.1) and *MAT***a** loci (AY710429.1) (11), which share 87% nucleotide identity at the *CAP1* gene. Classification of the mating type in this way was consistent with previously determined mating types for some of the isolates used in this study (16). For those strains in which the *CAP1* sequence matched that of the *MAT***a** locus, classical mating tests were used for confirmation by previously described methods (15).

Data analyses. Phylogenetic analyses of the four individual gene fragments as well as the combined data (2,717 nucleotides) were performed using PAUP* v 4.0b10 software (D. L. Swofford, Sinauer Associates, Massachusetts), where transitions and transversions were weighted equally. The most parsimonious trees were obtained by a heuristic search based upon 500 random sequence additions. Statistical support for each clade was assessed using bootstrap analysis with 500 replicate samples of phylogenetically informative characters. Sequences from serotype A and D strains were used to root each tree. The sequence divergence between and within isolates grouped according to molecular types and geographical regions was estimated using mean pairwise Kimura 2 parameter distances (18).

The congruence of gene genealogies was assessed using the partition homogeneity test as well as pairwise comparisons of the tree topologies for the four genes (10). These tests were applied to the following six subsets of isolates: (i) all isolates used in the study ($n = 45$), (ii) *MAT*α isolates only ($n = 40$), (iii) VGI isolates only ($n = 10$), (iv) VGII isolates only ($n = 29$), (v) VGII *MAT*α isolates only ($n = 25$), and (vi) B.C. isolates only ($n = 22$).

TABLE 1. Cryptococcal isolates used in this study and details of their isolation, molecular types, and designated sequence subtypes

Isolate no.[g]	Serotype	Molecular type	Mating type	Source[f]	Year isolated	Geographical origin[f]	Sequence subtype			
							URA5	*LAC*	*FTR1*	*CAP1*
Cryptococcus gattii isolates from British Columbia										
A2M R314[A]	B	VGI	*MAT*α	Human, sputum	2002	Duncan, V.I. (travel history)	1	1	1	1
A2M R299[A]	B	VGI	*MAT*α	Human, CSF	2002	Lantzville, V.I. (travel history)	1	1	1	1
A4M R64[Aa]	B	VGI	*MAT*α	Human	2004	Vancouver (travel history)	1	1	1	1
KB7892[Ba]	B	VGI	*MAT*α	Arbutus tree	2004	Saltspring Island, B.C. (environs of parrot isolate KB7091)	3	2	2	1
A1M R794[A]	B	VGI	*MAT*α[c]	Human, CSF	2001	Vancouver, B.C. (travel history unknown)	4	2	2	1
A1M F2863[C]	B	VGI	*MAT*α[b]	Dall's porpoise	2002	Washed up on shore of southern V.I. (assumed travel history)	3	1	3	1
A1M R265[A]	B	VGII a[d]	*MAT*α[b]	Human, BAL	2001	Duncan, V.I.	5	3	4	2
A1M F3016[C]	B	VGII a[d]	*MAT*α[b]	Dall's porpoise	2002	Washed up on shore of a gulf island, B.C. (assumed travel history)	5	3	4	2
MAC-9[B]	B	VGII a[d]	*MAT*α[b]	Cedar	2001	Cathedral Grove, V.I.	5	3	4	2
AIM R272[A]	B	VGII b[d]	*MAT*α[c]	Human, BAL	2001	Ladysmith, V.I.	7	3	4	3
KB2045[Ba]	B	VGII a[d]	*MAT*α	Air sample	2002	Langley, B.C. (environs of tapir isolate KB1079)	5	3	4	2
RB28[B]	B	VGII b[d]	*MAT*α[b]	Tree stump	2002	Parksville, V.I.	7	3	4	3
RB67[B]	B	VGII b[d]	*MAT*α[c]	Douglas fir	2002	Parksville, V.I.	7	3	4	3
KB152A-6[B]	B	VGII b[d]	*MAT*α[c]	Air sample	2002	Parksville, V.I.	7	3	4	3
A2M R282[A]	B	VGII	*MAT*α	Human, sputum	2002	Victoria, V.I.	5	3	4	2
KB7091[Da]	B	VGII	*MAT*α	Companion parrot	2003	Saltspring Island (travel history)	5	3	4	3
A3M R535[A]	B	VGII a[d]	*MAT*α	Human, chest abscess	2003	Delta, B.C.	5	3	4	2
A3M R673[A]	B	VGII	*MAT*α	Human, BAL	2003	Sidney, V.I. (travel history)	5	3	4	2
KB5746[D]	B	VGII	*MAT*α	Horse	2003	Mill Bay, V.I.	7	3	4	3
KB7092[Da]	B	VGII	*MAT*α	Llama	2003	Chilliwack, B.C.	5	3	4	2
KB4672[D]	B	VGII	*MAT*α	Companion cat	2003	Nanaimo, V.I.	5	3	4	2
KB1079[Da]	B	VGIII	*MAT*α	Captive tapir	2002	Langley, B.C. (probably acquired infection in United States)	8	5	6	6
Cryptococcus gattii isolates from other parts of the world										
A3M R29[Aa]	B	VGI	*MAT*α	Koala	2002	Toronto Zoo, ON, Canada (previously from San Diego Zoo)	1	1	1	1
A2M R554[Aa] = UAMH9837	B	VGI	*MAT*α	Captive bottlenose dolphin	2000	San Diego, CA (no recent travel history)	2	1	1	1
WM179[Ia]	B	VGI	*MAT*α	Human, CSF	1993	Sydney, NSW, Australia	1	1	1	1
WM276[a,e]	B	VGI	*MAT*α	*E. tereticornis*	1993	Mt. Annan, NSW, Australia	1	1	1	1
KB10455[Da]	B	VGII	*MAT*α	Companion cat	2004	Edmonton, AB, Canada (travel history to V.I.)	5	3	4	2
NIH444[Fa] = ATCC 32609	B	VGII	*MAT*α	Human, sputum	ca. 1971	Seattle, WA	5	3	4	2
KB9944[Ba]	B	VGII	*MAT*α	Unidentified tree species	2004	CA (environs of parrot isolate KB7091)	5	3	4	2
CBS 7750[Ea]	B	VGII	*MAT*α	*E. camaldulensis*	1990	San Francisco, CA	5	3	4	2
LA55[Ha] = FOC417	B	VGII	*MAT***a**	Human, CSF	1995	NE region of Piaui, Brazil	5	3	4	5
LA57[Ha] = FOC506	B	VGII	*MAT*α	Human, CSF	1995	NE region of Piaui, Brazil	5	3	4	4
LA61[Ha] = FOC557	B	VGII	*MAT*α	Human, CSF	1997	NE region of Piaui, Brazil	7	3	4	4
LA499[Ga] = HOO58 I-106	B	VGII	*MAT***a**	Human, CSF	1990	Norte de Santander, Colombia	6	3	4	5
LA567[Ga] = HOO58 I-638	B	VGII	*MAT***a**	Human, CSF	1997	Caqueta, Colombia	6	3	4	5
LA584[Ga] = HOO58 I-675	B	VGII	*MAT***a**	Human, CSF	1998	Bolivar, Colombia	6	3	4	5
MC-S-115[Ia]	B	VGII	*MAT*α	Human, CSF	1993	Thailand	7	3	4	3
WM178[Ia]	B	VGII	*MAT*α	Human, lung	1991	Sydney, NSW, Australia	5	4	5	3
RAM005[Ia]	B	VGII	*MAT*α	*E. tetrodonta*	1999	Arnhemland, NT, Australia	7	3	4	3
WM1008[Ia]	B	VGII	*MAT*α	Insect frass	2000	Mt. Druitt, NSW, Australia	7	3	4	3
WM161[Ia]	B	VGIII	*MAT*α	*E. camaldulensis*	1992	San Diego, CA	8	5	6	6
NIH191[Fa]	C	VGIII	*MAT***a**[b]	Human, CSF	1978	CA	9	6	7	7
WM779[Ia]	C	VGIV	*MAT*α	Cheetah	1994	Johannesburg, South Africa	10	7	8	8
Outgroups										
H99[a,e]	A	VNI	*MAT*α[b]	Human	1978	New York, NY				
B-3501A[a,e]	D	VNIV	*MAT*α[b]	Laboratory cross	NA	NA				

Continued on following page

TABLE 1—*Continued*

[a] Isolates not known to be connected to Vancouver Island.
[b] Mating type of isolate was previously determined by laboratory crosses (15).
[c] Isolate was previously found to be infertile (15).
[d] Molecular subtype was determined from M13 PCR fingerprinting and/or AFLP analysis (15; S. E. Kidd, unpublished data).
[e] *URA5*, *LAC*, *FTR1,* and *CAP1* sequences were obtained from the following genome assemblies: H99, Duke Center for Genome Technology, NC, and Broad Institute, MA; B-3501A, Stanford Genome Technology Center, CA; WM276, British Columbia Genome Sciences Centre, Vancouver, B.C., Canada.
[f] V.I., Vancouver Island; B.C., British Columbia; NA, not applicable; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage.
[g] Isolates were donated by the following groups or individuals: [A], British Columbia Centre for Disease Control, Vancouver, B.C., Canada; [B], Karen Bartlett, School of Occupational and Environmental Hygiene, University of British Columbia, Vancouver, B.C., Canada; [C], Stephen Raverty, Animal Health Centre, Abbotsford, B.C., Canada; [D], Sally Lester, Central Laboratory for Veterinarians, Langley, B.C., Canada; [E], Teun Boekhout, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; [F], June Kwon-Chung, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.; [G], Elizabeth Castañeda, Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, Colombia; [H], Ricardo Pereira Igreja, Doencas Infecciosas e Parasitarias, Hospital Universittario Clementino Fraga Filho, Rio de Janeiro, Brazil; and, [I], Wieland Meyer, Molecular Mycology Laboratory, Westmead Hospital, Westmead, NSW, Australia.

To test whether there were significant phylogenetic patterns based upon mating types, molecular types (as determined by *URA5*-RFLP analysis), isolation host types, and geographic origins, we used the topology-dependent permutation tail probability test (T-PTP) (9). This test compares the lengths of maximum parsimony (MP) trees with and without the monophyletic constraint defined by each of the described traits. If the constrained trees are significantly longer than the MP tree without any constraint, the results would suggest a lack of a phylogenetic pattern based upon the specific traits. For each constraint analysis, the statistical significance was derived from permutation of the combined sequence data under the assumption of nonmonophyly to generate a null distribution of tree lengths. Statistical support for nonmonophyly is achieved when >95% of all permuted data sets have tree lengths shorter than the MP tree generated with the constraint of monophyly (9). The T-PTP test was implemented in PAUP* v. 4.0b10. One thousand permuted data sets were generated and analyzed for each of the constraint tests. The serotype A and D outgroup isolates were not included in these analyses.

Nucleotide sequence accession numbers. All sequences obtained in this study were submitted to GenBank under the following accession numbers: *LAC* sequences, AY973072 to AY973113; *URA5* sequences, AY973114 to AY973155; *FTR1* sequences, AY972002 to AY972043; *CAP1* sequences, AY971960 to AY972001.

RESULTS AND DISCUSSION

Nucleotide sequence diversity and lineages within *C. gattii*. To begin an analysis of *C. gattii* lineages, we examined the genealogy of each of the four genes using maximum parsimony and found that the genes possessed various levels of divergence (Fig. 1). *URA5* revealed the greatest diversity, with 10 alleles observed among the 43 *C. gattii* isolates in this study. There were eight *FTR1* alleles and seven *LAC* alleles. *CAP1* also revealed eight alleles, but this gene contained mating type-specific sequence variation due to its location at the mating locus (11); six *CAP1* alleles were observed among *C. gattii* *MAT*α isolates. These allele distributions were compiled to generate a multilocus sequence type (MLST) profile for each of the isolates in the study. The combined sequence data for the four gene fragments resulted in 2,717 aligned nucleotide sites. Of these, 614 sites were variable, including 388 that were phylogenetically informative. Figure 2 shows one of the 12 maximum parsimony trees generated from the combined sequence data that closely resembles the strict consensus tree.

The constraint of isolates belonging to the same mating type as a monophyletic group (for *MAT*α, $n = 38$; for *MAT***a**, $n = 5$) revealed little change in the tree length compared to the tree without constraint ($P = 0.999$), indicating significant support for isolates of each mating type representing two monophyletic groups. At the *CAP1* gene, sequence variation due to the mating type (104 and 110 of 672 sites within VGIII and VGII molecular types, respectively) was greater than that due to molecular type divergence (12 and 27 of 672 sites among *MAT***a** and *MAT*α isolates, respectively). This mating type-specific sequence variation at one locus outweighed variation due to the molecular type at all four loci in the combined sequence data set, with the creation of an artificial clade corresponding to *MAT***a** isolates (Fig. 2). These data support an ancient divergence of the cryptococcal mating locus (11) that predates the divergence of molecular types. Since the nucleotide sequences of genes at the bipolar cryptococcal mating locus are known to be mating type specific (11, 28, 34), all further analyses were performed with exclusion of the *MAT***a** isolates (for VGII, $n = 4$; for VGIII, $n = 1$) to avoid distortion of the data.

The *MAT*α isolates were constrained in monophyletic groups according to their molecular type (for VGI, $n = 10$; for VGII, $n = 25$; for VGIII, $n = 2$; for VGIV, $n = 1$), with little change in tree length compared to that without constraint ($P = 0.999$). This indicates significant support for isolates of different molecular types forming monophyletic groups representing potentially ancient lineages within the species, which is consistent with the findings of previous studies (14, 15). In addition, this reinforces the usefulness of molecular typing techniques for approximating sequence divergence and evolutionary distance between strains of *C. gattii*.

With the exception of the *CAP1* gene, most of the sequence loci that we investigated revealed greater diversity among the 10 VGI isolates used in this study than that observed among the 25 VGII *MAT*α isolates. The mean pairwise Kimura 2 parameter distance (Table 2) within VGI was significantly greater than that observed within the *MAT*α VGII isolates ($P = 0.000$), and the mean divergence between VGI and VGII *MAT*α isolates was >10 times greater than that within either the VGI or VGII isolates ($P = 0.000$). Other comparisons between and within molecular types showed a similar pattern of greater divergence between the molecular types than within them. A previous study also demonstrated greater nucleotide sequence diversity within VGI than within the other molecular types, using the internal transcribed spacer regions of the rRNA gene (14). Such a significant divergence between molecular types suggests the existence of several phylogenetic species within *C. gattii.*

Other T-PTP tests of geography- and host type-based phylogenetic patterns indicated significantly longer trees in the presence of the monophyletic constraints than those in the absence of such constraints. These results are consistent with the hypotheses of extensive strain dispersal among geographic

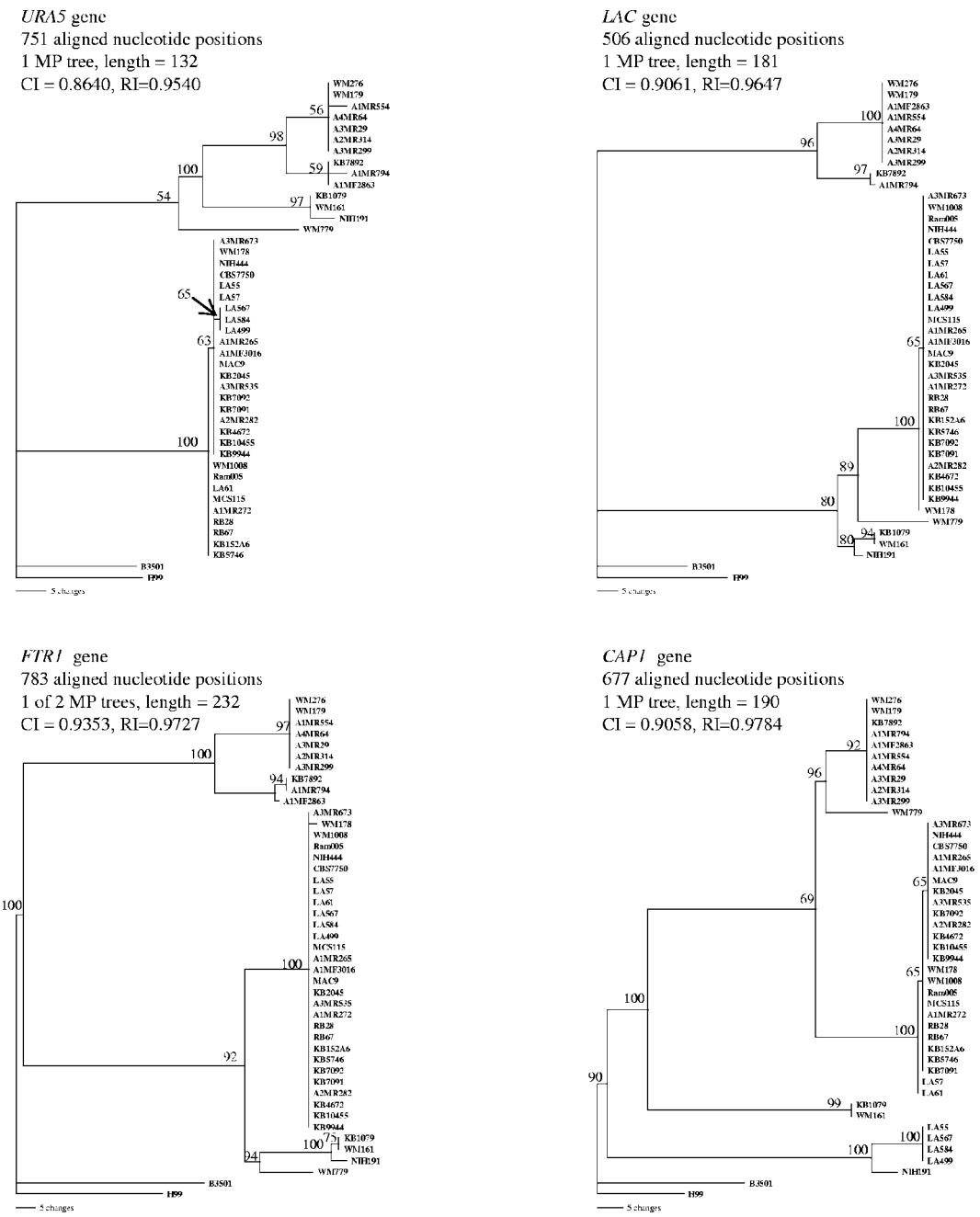

FIG. 1. Maximum parsimony trees for 43 isolates of *Cryptococcus gattii* from each of four gene regions sequenced. MP, maximum parsimony; CI, consistency index; RI, retention index. Values above branches indicate bootstrap support of >50% from 500 replicates.

areas and the lack of host specificity among the analyzed strains. Specifically, of the strains from each of the three host types (for humans, $n = 15$; for animals, $n = 11$; for the environment, $n = 12$), none formed a statistically robust monophyletic group ($P = 0.000$). Although the sample size is small, this indicates that the host type cannot be used to predict evolutionary groups among isolates and is consistent with previous studies that found clinical and environmental isolates to be genotypically indistinguishable (7, 23, 31).

Epidemiological links between B.C. isolates and those from other parts of the world. One of the goals of this study was to investigate the possibility of an epidemiological link between the VGII isolates from Vancouver Island and the NIH444 strain, isolated in Seattle circa 1971, and recently found to belong to the VGII molecular type (1, 15). The MLST profile for NIH444 was identical to those of many isolates from Vancouver Island (A1M R265, A1M R282, A3M R673, MAC-9, and KB4672), other parts of B.C. (A1M F3016, A3M R535, KB2045, KB7091, and KB7092), and other parts of North America (KB10455, CBS7750, and KB9944), some of which were previously subtyped as VGIIa/AFLP6A (15). It is also possible that NIH444 could share sequence identity with iso-

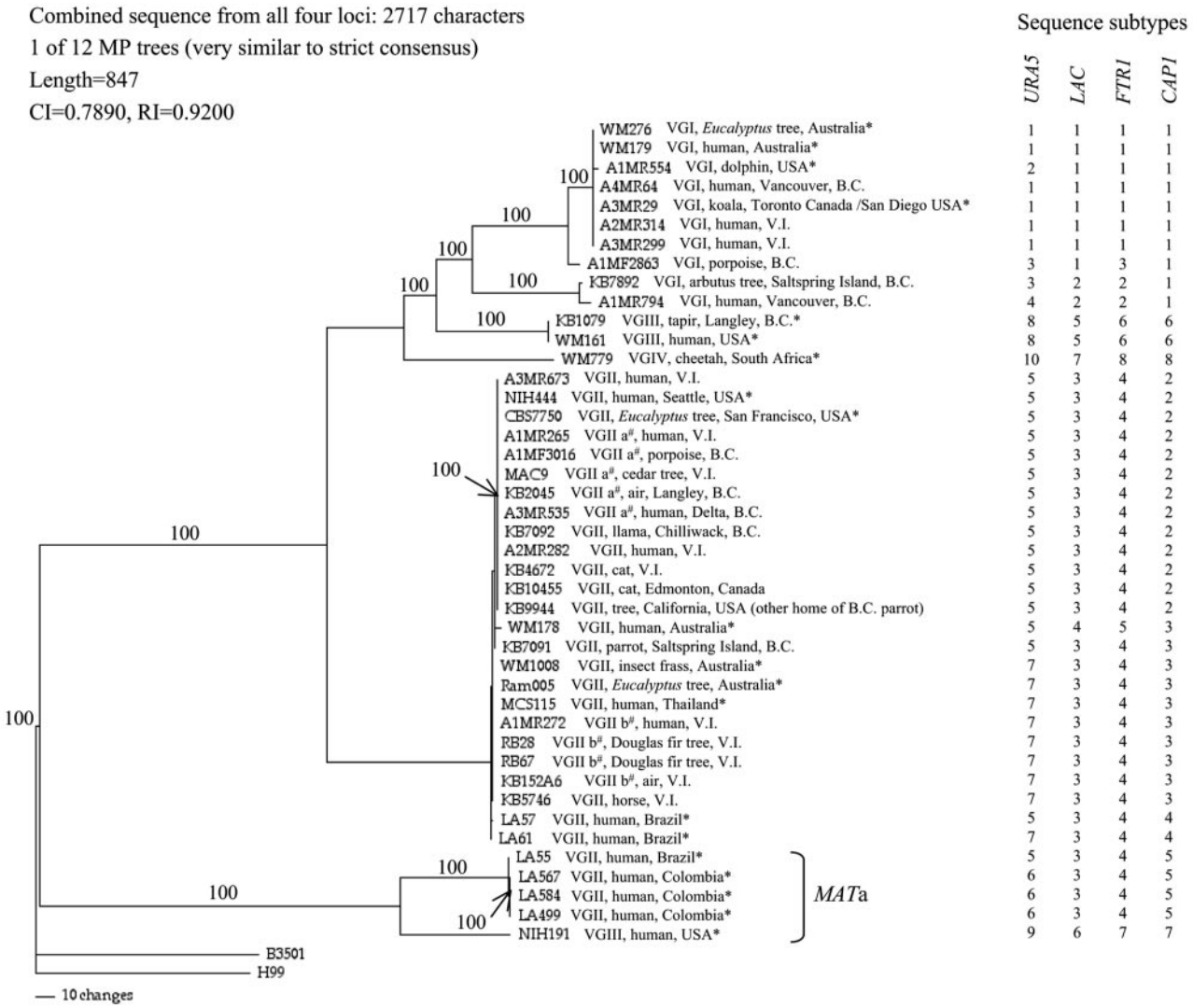

FIG. 2. One of 12 maximum parsimony trees from the combined DNA sequences of the *LAC*, *URA5*, *FTR1*, and *CAP1* genes. Numbers (100) above branches denote strict consensus branches. V.I., Vancouver Island; B.C., British Columbia. *, isolates not known to be related to the emergence of *C. gattii* in B.C.; #, previously determined VGII subtypes (15; S. E. Kidd, unpublished data). An artificial clade of *MAT***a** strains was observed, arising due to the mating type-specific sequences of the *CAP1* gene that outweighed the sequence variation due to molecular type divergence at all other loci.

lates from areas other than North America which were not included in this study. A recent study of the mini-intein sequence from the cryptococcal *PRP8* gene (2) revealed that NIH444 differed from seven Vancouver Island environmental VGII isolates at 1 of 510 nucleotide sites. We independently sequenced the *PRP8* mini-intein sequence for this strain and found that it was identical across all 510 nucleotides to the isolates from Vancouver Island. Therefore, it is possible that NIH444 may be related to isolates from B.C., but knowledge of the travel history of the patient from which NIH444 was isolated is lacking. On the other hand, the NIH444 *URA5* and *CAP1* sequences described in this study differ from those of the VGIIb isolates from Vancouver Island (RB28, RB67, KB152A-6, A1M R272, and KB5746). Rather, these VGIIb isolates share identical MLST profiles with VGII environmental isolates from Australia (Ram005 and WM1008) and a clinical isolate from Thailand (MC-S-115; travel history unknown). Thus, isolates from B.C. share similar or identical genotypes with isolates from several different areas of the world. However, there is insufficient evidence from these studies to conclude that *C. gattii* strains were introduced to B.C. from sources from any specific part of the world. Indeed, it is not implausible that strains from B.C. may have dispersed to other parts of North America or the world, thus accounting for the shared genotypes.

Based on their MLST profiles, four of the five VGI sequence variants observed in this study are associated with B.C. isolates, and three B.C. VGI isolates represent unique strains in this study. One of these unique strains, KB7892 (from an arbutus tree on Saltspring Island), represents the only VGI environmental strain isolated in B.C. at the time of these analyses. Therefore, there appears to be no epidemiological link be-

tween the B.C. VGI environmental isolate and any of the clinical VGI isolates from B.C. This is supported by preliminary studies of a second environmental VGI isolate, recently obtained from a different tree on Saltspring Island, which indicate that it is genotypically identical to KB7892 (S. E. Kidd, unpublished data). Clinical VGI isolates from B.C. (A2M R314, A2M R299, and A4M R64) possessed identical MLST profiles to those of an isolate from a captive koala (A3M R29) residing at Toronto Zoo, Ontario, Canada (and at San Diego Zoo, CA, 6 months prior to cryptococcal isolation) and environmental isolates from Australia (WM276 and WM179). As mentioned above, many of the clinical VGI cases from B.C. are associated with a known travel history (wild porpoises were assumed to have a travel history since their geographical ranges were not known), and it is possible that some or all of the VGI infections were acquired outside of B.C., particularly since there is limited evidence at this time for a truly colonized source of VGI in the B.C. environment. Further investigation is under way to determine whether the environmental VGI isolates from Saltspring Island represent colonization or a transient presence. Overall, these data indicate that the genetic variation among VGI strains isolated from sources in B.C. is roughly equivalent to that among VGI isolates from other parts of the world, although this conclusion is in the context of the limited number of isolates available for use in this study and the possibility that the VGI infections were acquired outside of B.C.

Population structure of *C. gattii* in B.C. The congruence of gene genealogies was tested to assess the population structure among the *C. gattii* isolates in this study. When all 45 isolates in the study were considered and when only the *MAT*α isolates ($n = 40$) were considered, the gene genealogies were found to be highly incongruent ($P = 0.000$). However, when the isolates from B.C. were considered alone, the four gene genealogies were highly congruent ($P = 0.450$), and when only the VGII isolates were considered, the four genes had highly congruent genealogies ($P = 0.734$), with increased congruence observed when the four VGII *MAT***a** isolates were excluded from this analysis ($P = 0.810$). When VGI isolates were considered alone, the four genes had marginally congruent genealogies ($P = 0.053$) which, in the absence of providing evidence for recombination, was interpreted as an indication of clonality. These data suggest that sexual recombination has occurred between *C. gattii* isolates of different molecular types on a global scale but that the *C. gattii* population in B.C. has a predominantly clonal mode of reproduction in nature. Furthermore, it appears that there is a clonal propagation of isolates within each of the VGI and VGII molecular types, both among isolates from B.C. and among the global isolates used in this study. This is consistent with the hypothesis presented in a previous study, in which all fertile *C. gattii* isolates tested from B.C. belonged to the alpha mating type and an association was observed between mating incompetence and the VGIIb/AFLP6B molecular subtype (15). In contrast, a recent study of *C. gattii* VGII isolates from Sydney and the Northern Territory of Australia found evidence for recombination among the isolates from each geographical area and genetic connectivity between the two populations (3). Studies of the population structure among environmental *C. gattii* VGI isolates from Australia and Papua New Guinea found no evi-

TABLE 2. Mean pairwise Kimura 2 parameter distances among *C. gattii* isolates within and between molecular types (or predetermined VGII subtypes [15])[a]

Molecular type	Kimura 2 parameter distance (mean ± SD)									
	*MAT*α isolates only ($n = 40$)						*MAT***a** isolates only ($n = 5$)		Subtyped isolates only ($n = 9$)	
	VGI ($n = 10$)	VGII ($n = 23$)	VGIII ($n = 2$)	VGIV ($n = 1$)	VNI ($n = 1$)	VNIV ($n = 1$)	VGII ($n = 4$)	VGIII ($n = 1$)	VGII a ($n = 5$)	VGII b ($n = 4$)
VGI	**0.00328 ± 0.00322**	0.04895 ± 0.00438	0.03847 ± 0.00070	0.04874 ± 0.00070	0.13075 ± 0.00063	0.13560 ± 0.00079				
VGII		**0.00052 ± 0.00046**	0.04766 ± 0.00048	0.04888 ± 0.00043	0.13888 ± 0.00053	0.13998 ± 0.00025	**0.00019 ± 0.00021**	0.04514 ± 0.00020		
VGIII			**0.00000**	0.04625 ± 0.00000	0.13045 ± 0.00000	0.13712 ± 0.00000				
VGIV					0.13742	0.13703				
VNI						0.08896				
VNIV										
VGII a									**0.00000**	0.00075 ± 0.00000
VGII b										**0.00000**

[a] Bold type indicates a mean genetic distance among isolates of a single molecular type or subtype.

TABLE 3. Mean pairwise Kimura 2 parameter distances among *C. gattii* isolates within and between different geographical regions

Region	Kimura 2 parameter distance (mean ± SD)								
	Vancouver Island (n = 12)	B.C., Excluding Vancouver Island (n = 10)	B.C., Including Vancouver Island (n = 22)	Rest of North America (n = 8)	South America (n = 6)	Australia (n = 5)	Thailand (n = 1)	South Africa (n = 1)
Vancouver Island	**0.01518 ± 0.02258**	0.02570 ± 0.02412	NA[b]	0.02589 ± 0.02813	0.02175 ± 0.02258	0.02175 ± 0.02433	0.00844 ± 0.01883	0.04883 ± 0.00039
B.C., excluding Vancouver Island		**0.03288 ± 0.02216**	NA[b]	0.03132 ± 0.02619	0.04551 ± 0.02551	0.02721 ± 0.02350	0.02471 ± 0.02535	0.04853 ± 0.00103
B.C., including Vancouver Island			**0.02383 ± 0.02403**	0.02840 ± 0.02732	0.03809 ± 0.02481	0.02423 ± 0.02400	0.01583 ± 0.02302	0.04870 ± 0.00074
Rest of North America				**0.03911 ± 0.02839**	0.04395 ± 0.02456	0.03320 ± 0.02709	0.02791 ± 0.03050	0.05207 ± 0.00915
South America					**0.01967 ± 0.01884**	0.04162 ± 0.02566	0.02504 ± 0.01897	0.06490 ± 0.01292
Australia						**0.02998 ± 0.02517**	0.01987 ± 0.02654	0.04874 ± 0.00033
Thailand								0.048430
South Africa								

[a] Bold type indicates a mean genetic distance among isolates from within a defined geographical area.
[b] NA, not applicable.

dence of sexual recombination, and all isolates were infertile (3, 13).

Studies of fertility among *C. gattii* isolates from Vancouver Island (obtained between 2001 and 2002) observed that all fertile isolates from clinical and environmental sources were of the alpha mating type (12, 15). Among the models presented in one of these studies (12), it was suggested that mating and meiotic recombination in a fertile clade may have given rise to a strain with increased virulence, resulting in the comparatively high rate of infection reported for Vancouver Island. A previously reported disparity in the fertility of VGIIa and VGIIb isolates (15) may provide a useful basis upon which to investigate this model further. While we observed no evidence for recombination specifically relating to the VGII isolates or the group of VGI and VGII isolates from B.C., the relationship between the VGIIa and VGIIb genotypes was unclear from our data set and should be further investigated. These VGII variants appear to be clonally propagated at present, but this does not preclude the possibility of a more ancient recombination. Other models invoking an increased ability of Vancouver Island strains to undergo haploid fruiting or a contribution of fertility to the formation of infectious propagules remain to be investigated. The recent demonstration that *C. neoformans* cells of the same (alpha) mating type can undergo sexual reproduction may be relevant in this context (22). Specifically, the fertile *MAT*α cells of the VGII molecular type found on Vancouver Island may be particularly well adapted for alpha-alpha sexual development leading to spore formation; this type of interaction may not be revealed by our methods if the strains were closely related. In general, it is clear that more detailed experiments are needed to understand the fertility properties of the Vancouver Island isolates in the context of their ecological niche and their virulence. The availability of sequenced genomes for strains of the VGI and VGII molecular types (see Materials and Methods) will facilitate such experiments.

Geographical relatedness of *C. gattii* isolates. To examine the phylogeographic patterns of the strains in this study, *MAT*α isolates were constrained within monophyletic groups according to their geographical origins (for Vancouver Island, $n = 12$; for other parts of B.C., $n = 10$; for other parts of North America, $n = 7$; for South America, $n = 2$; for Australia, $n = 5$; for Africa, $n = 1$; and for Asia, $n = 1$). These constraints led to significant increases in tree length ($P = 0.000$). To assess whether there was any bias introduced as a result of arbitrary limits of the geographical regions, we varied the geographical boundaries into which isolates were constrained, e.g., Canada ($n = 24$), the United States ($n = 5$), Brazil ($n = 2$), South Africa ($n = 1$), Australia ($n = 5$), and Thailand ($n = 1$) ($P = 0.000$), or North America ($n = 29$), South America ($n = 2$), Australia ($n = 5$), Africa ($n = 1$), and Asia ($n = 1$) ($P = 0.000$). Therefore, among the isolates used in this study, there was no correlation between sequence divergence among isolates and their geographic distance. Varying the limits of geographical regions that were constrained as monophyletic groups made no difference to the probability of constrained data, and all analyses yielded trees with significantly greater lengths than that of the tree with no geographic constraints. The mean Kimura 2 parameter (Table 3) distances between isolates from different geographical regions indicated that there is no significant difference in the level of sequence diversity among isolates from

Vancouver Island (or from all of B.C.) compared to that among isolates from other regions examined in this study. In addition, the global genetic diversity of the isolates used in this study is not subdivided according to geographical borders. Under the assumption that the dispersal of strains increases the heterogeneity of a given population at a greater rate than the accumulation of mutations among a clonal lineage (random genetic drift), these data indicate a significant migration of *C. gattii* strains between different regions of the world and are consistent with the findings of a previous study (38). However, since we cannot be certain that a given isolate is truly representative of the geographical region from which it was isolated (particularly for clinical isolates, which may have been acquired outside of the host's residential area) and since each geographic population appears to be as genetically diverse as the others, it was not possible to unambiguously infer the center(s) of origin for individual strains or populations. Indeed, the extent of cryptococcal strain migration throughout the world seems to be such that searching for specific sources of particular strains may be irrelevant.

Given that the B.C. *C. gattii* population appears to be clonal, there are a number of possible explanations for the recent emergence of *C. gattii* infection on Vancouver Island. These possibilities include (i) *C. gattii* on Vancouver Island represents an ancient population, where heterogeneity has arisen through random genetic drift, strain dispersal, or recombination that was not detectable within our data set; (ii) the Vancouver Island *C. gattii* population was recently established by introductions of both VGI and VGII strains from one or more parts of the world, which could have occurred independently or at the same time; and (iii) the Vancouver Island *C. gattii* population was recently established by the introduction, colonization, and rapid expansion of a VGII strain from another part of the world, where genetic drift, strain dispersal, or undetected recombination has effected at least two distinct strains within the VGII molecular type, and the presence of VGI isolates is due to strain dispersal.

Even though the emergence of *C. gattii* infection in B.C. is recent, no geographical area represented by isolates in this study revealed any more genetic diversity than other areas (Table 3), and the hypothesis of an ancient *C. gattii* population in B.C. cannot be rejected. Our data indicate that the B.C. *C. gattii* population possesses as much genetic diversity as those from other geographical areas and shares many identical or similar genotypes. Therefore, either the ancient population or the recent introduction hypothesis can explain the observed emergence of *C. gattii* in B.C. However, the evidence for clonal propagation of the isolates from B.C. investigated here suggests that the *C. gattii* isolates from B.C. are unlikely to represent an ancient population.

An ongoing environmental sampling study of *C. gattii* has amassed strong evidence for the colonization and propagation of VGII isolates on Vancouver Island (K. H. Bartlett and S. E. Kidd, unpublished results). However, despite the isolation of several VGI strains from clinical cases, there is little evidence at this time for permanent colonization of VGI in the B.C. environment, aside from two isolates from Saltspring Island. A comparison of MLST profiles revealed no similarity of the VGI environmental strain included in this study to any of the clinical strains. In the absence of a confirmed environmental reservoir for the VGI infections, we suggest that some or all of these cases may have been acquired through travel to other parts of the world; indeed, many of the patients had a travel history within the 12 months prior to diagnosis. Continued investigation of B.C. environmental isolates may make clear whether VGI is transient or has a colonized source in B.C.

Summary. The unprecedented emergence of *Cryptococcus gattii* as a saprophyte and infectious agent in the temperate climate of B.C. since 1999 (15, 33; Bartlett et al., Abstr. 16th Biometeorol. Aerobiol. Meet. 2004, abstr. 5.5, 2004 [http://ams.confex.com/ams/pdfpapers/80027.pdf]), together with evidence for extensive strain dispersion, indicates a dynamic distribution of strains throughout the world. However, despite their unforeseen colonization in a temperate region, the *C. gattii* isolates from Vancouver Island and B.C. do not appear to be exceptional in terms of their phylogeny compared to those from other areas of the world. These isolates are clonal, possess a level of nucleotide sequence diversity that is equivalent to that of the global population, and share identical MLST profiles with many isolates collected in other parts of the world.

ACKNOWLEDGMENTS

We thank the following groups and individuals for kindly providing cryptococcal isolates: British Columbia Centre for Disease Control, Vancouver, B.C., Canada; Sally Lester of the Central Laboratory for Veterinarians, Langley, B.C., Canada; Stephen Raverty of the Animal Health Centre, Abbotsford, B.C., Canada; Teun Boekhout of the Centraalbureau voor Schimmelcutures, Utrecht, The Netherlands; June Kwon-Chung of the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.; Elizabeth Castañeda of the Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, Colombia; Ricardo Pereira Igreja of the Doencas Infecciosas e Parasitarias, Hospital Universittario Clementino Fraga Filho, Rio de Janeiro, Brazil; and Wieland Meyer of the Molecular Mycology Laboratory, Westmead Hospital, Westmead, NSW, Australia. In addition, we thank Mary Berbee, Department of Botany, University of British Columbia, for her critical review of the manuscript.

This work was funded in part by grants from the Canadian Institutes of Health Research (to J.W.K.) and the National Sciences and Engineering Research Council of Canada (to J.X.). S.E.K. and K.H.B. are supported by the Michael Smith Foundation for Health Research. J.W.K. is a Burroughs Wellcome Fund Scholar in Molecular Pathogenic Mycology. DNA sequencing was performed by the Nucleic Acids and Protein Service (NAPS) Unit at the University of British Columbia and by the Mobix Laboratory at McMaster University.

REFERENCES

1. **Boekhout, T., B. Theelen, M. Diaz, J. W. Fell, W. C. J. Hop, E. C. A. Abeln, F. Dromer, and W. Meyer.** 2001. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology **147:**891–907.
2. **Butler, M. I., and R. T. Poulter.** 2005. The PRP8 inteins in *Cryptococcus* are a source of phylogenetic and epidemiological information. Fungal Genet. Biol. **42:**452–463.
3. **Campbell, L. T., B. J. Currie, M. Krockenberger, R. Malik, W. Meyer, J. Heitman, and D. A. Carter.** 2005. Clonality and recombination in genetically differentiated subgroups of *Cryptococcus gattii*. Eukaryot. Cell **4:**1403–1409.
4. **Casadevall, A., L. F. Freundlich, L. Marsh, and M. D. Scharff.** 1992. Extensive allelic variation in *Cryptococcus neoformans*. J. Clin. Microbiol. **30:**1080–1084.
5. **Chen, S., T. Sorrell, G. Nimmo, B. Speed, B. Currie, D. Ellis, D. Marriott, T. Pfeiffer, D. Parr, and K. Byth.** 2000. Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Clin. Infect. Dis. **31:**499–508.
6. **Chuck, S. L., and M. A. Sande.** 1989. Infections with *Cryptococcus neoformans* in the acquired immune deficiency syndrome. N. Engl. J. Med. **321:**794–799.
7. **Delgado, A. C., H. Taguchi, Y. Mikami, M. Myiajy, M. C. Villares, and M. L. Moretti.** 2005. Human cryptococcosis: relationship of environmental and

clinical strains of *Cryptococcus neoformans var. neoformans* from urban and rural areas. Mycopathologia **159:**7–11.
8. **Evans, E. E.** 1950. The antigenic composition of *Cryptococcus neoformans*. I. A serological classification by means of the capsular and agglutination reactions. J. Immunol. **64:**423–440.
9. **Faith, D. L.** 1991. Cladistic permutation tests for monophyly and nonmonophyly. Syst. Zool. **40:**366–375.
10. **Farris, J. S., M. Kallersjo, A. G. Kluge, and C. Bult.** 1995. Testing significance of incongruence. Cladistics **10:**315–319.
11. **Fraser, J. A., S. Diezmann, R. L. Subaran, A. Allen, K. B. Lengeler, F. S. Dietrich, and J. Heitman.** 2004. Convergent evolution of chromosomal sex-determining regions in the animal and fungal kingdoms. PLoS Biol. **2:**2243–2255.
12. **Fraser, J. A., R. L. Subaran, C. B. Nichols, and J. Heitman.** 2003. Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. Eukaryot. Cell **2:**1036–1045.
13. **Halliday, C. L., and D. A. Carter.** 2003. Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. J. Clin. Microbiol. **41:**703–711.
14. **Katsu, M., S. Kidd, A. Ando, M. Moretti-Branchini, Y. Mikami, K. Nishimura, and W. Meyer.** 2004. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* complex. FEMS Yeast Res. **4:**377–388.
15. **Kidd, S. E., F. Hagen, R. L. Tscharke, M. Huynh, K. H. Bartlett, M. Fyfe, L. MacDougall, T. Boekhout, K. J. Kwon-Chung, and W. Meyer.** 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. USA **101:**17258–17263.
16. **Kidd, S. E., T. C. Sorrell, and W. Meyer.** 2003. Isolation of two molecular types of *Cryptococcus neoformans var. gattii* from insect frass. Med. Mycol. **41:**171–176.
17. **Kidd, S. E.** 2003. Ph.D. thesis. University of Sydney, Sydney, Australia.
18. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
19. **Kwon-Chung, K. J., and J. E. Bennett.** 1978. Distribution of α and a mating types of *Cryptococcus neoformans* among natural and clinical isolates. Am. J. Epidemiol. **108:**337–340.
20. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiological differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
21. **Lian, T., M. I. Simmer, C. A. D'Souza, B. R. Steen, S. D. Zuyderduyn, S. J. M. Jones, M. A. Marra, and J. W. Kronstad.** 2005. Iron-regulated transcription and capsule formation in the fungal pathogen *Cryptococcus neoformans*. Mol. Microbiol. **55:**1452–1472.
22. **Lin, X., C. M. Hull, and J. Heitman.** 2005. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. Nature **434:**1017–1021.
23. **Litvintseva, A. P., L. Kestenbaum, R. Vilgalys, and T. G. Mitchell.** 2005. Comparative analysis of environmental and clinical populations of *Cryptococcus neoformans*. J. Clin. Microbiol. **43:**556–564.
24. **Loftus, B. J., E. Fung, P. Roncaglia, D. Rowley, P. Amedeo, D. Bruno, J. Vamathevan, M. Miranda, I. J. Anderson, J. A. Fraser, J. E. Allen, I. E. Bosdet, M. R. Brent, R. Chiu, T. L. Doering, M. J. Donlin, C. A. D'Souza, D. S. Fox, V. Grinberg, J. Fu, M. Fukushima, B. J. Haas, J. C. Huang, G. Janbon, S. J. Jones, H. L. Koo, M. I. Krzywinski, K. J. Kwon-Chung, K. B. Lengeler, R. Maiti, M. A. Marra, R. E. Marra, C. A. Mathewson, T. G. Mitchell, M. Pertea, F. R. Riggs, S. L. Salzberg, J. E. Schein, A. Shvartsbeyn, H. Shin, M. Shumway, C. A. Specht, B. B. Suh, A. Tenney, T. R. Utterback, B. L. Wickes, J. R. Wortman, N. H. Wye, J. W. Kronstad, J. K. Lodge, J. Heitman, R. W. Davis, C. M. Fraser, and R. W. Hyman.** 2005. The genome and transcriptome of *Cryptococcus neoformans*, a basidiomycetous fungal pathogen of humans. Science **307:**1321–1324.
25. **Madrenys, N., C. De Vroey, C. Raes-Wuytack, and J. M. Torres-Rodriguez.** 1993. Identification of the perfect state of *Cryptococcus neoformans* from 195 clinical isolates including 84 from AIDS patients. Mycopathologia **123:**65–68.
26. **Meyer, W., A. Castañeda, S. Jackson, M. Huynh, E. Castañeda, and the Ibero-American Cryptococcal Study Group.** 2003. Molecular typing of Ibero-American *Cryptococcus neoformans* isolates. Emerg. Infect. Dis. **9:**189–195.
27. **Meyer, W., K. Marszewska, M. Amirmostofian, R. P. Igreja, C. Hardtke, K. Methling, M. A. Viviani, A. Chindamporn, S. Sukroongreung, M. A. John, D. H. Ellis, and T. C. Sorrell.** 1999. Molecular typing of global isolates of *Cryptococcus neoformans var. neoformans* by PCR-fingerprinting and RAPD—a pilot study to standardize techniques on which to base a detailed epidemiological survey. Electrophoresis **20:**1790–1799.
28. **Moore, T. D., and J. C. Edman.** 1993. The alpha-mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. Mol. Cell. Biol. **13:**1962–1970.
29. **Ruma, P., S. C. A. Chen, T. C. Sorrell, and A. G. Brownlee.** 1996. Characterization of *Cryptococcus neoformans* by random DNA amplification. Lett. Appl. Microbiol. **23:**312–316.
30. **Sorrell, T. C.** 2001. *Cryptococcus neoformans* variety *gattii*. Med. Mycol. **39:**155–168.
31. **Sorrell, T. C., S. C. A. Chen, P. Ruma, W. Meyer, T. J. Pfeiffer, D. H. Ellis, and A. G. Brownlee.** 1996. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. J. Clin. Microbiol. **34:**1253–1260.
32. **Speed, B., and D. Dunt.** 1995. Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*. Clin. Infect. Dis. **21:**28–34.
33. **Stephen, C., S. Lester, W. Black, M. Fyfe, and S. Raverty.** 2002. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. Can. Vet. J. **43:**792–794.
34. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174:**247–250. (Erratum, 177:187–188.)
35. Reference deleted.
36. **Williamson, P. R.** 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J. Bacteriol. **176:**656–664.
37. **Wilson, D. E., J. E. Bennett, and J. W. Bailey.** 1968. Serological grouping of *Cryptococcus neoformans*. Proc. Soc. Exp. Biol. Med. **127:**820–823.
38. **Xu, J., R. Vilgalys, and T. G. Mitchell.** 2000. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. Mol. Ecol. **9:**1471–1481.
39. **Xu, J., A. R. Ramos, R. Vilgalys, and T. G. Mitchell.** 2000. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. J. Clin. Microbiol. **38:**1214–1220.
40. **Yan, Z., X. Li, and J. Xu.** 2002. Geographic distributions of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. J. Clin. Microbiol. **40:**965–972.