The basidiomycete fungus *Cryptococcus gattii* is a primary pathogen of humans and animals. *C. gattii*, previously recognized as *Cryptococcus baccilisporus* 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has occurred on Vancouver Island in British Columbia (B.C.), Canada, over the past 6 years (15, 33; M. Fyfe et al., unpublished), and there is no evidence of *C. gattii* infection in B.C. prior to 1999 (S. E. Kidd, unpublished results).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 VGIa/AFLP6A and VGIb/AFLP6B, with approximately 90% of VGII isolates belonging to the VGIa/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
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 Salt Spring 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 Salt Spring 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 occur within the population on Vancouver Island, even though all isolates examined to date from B.C. have been of the alpha mating type (MATa) (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 VGI molecular type (15).

Fragments of four unrelated genes were studied, namely, LAC (encodes diaphen oxidase/lactase) (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 reassociation; FTR1 lies on chromosome some 3; and CAPII lies within the mating locus on chromosome 9.

Primers (Table 1) were designed to amplify and sequence the PCR products of each of the genes. The PCR conditions were as follows (5'-3') for LAC (565-bp product) (36), GGCGATATTATCGTATA (forward) and TCTCCTGTTGAGTTAGAACG (reverse); for URA5 (744-bp product) (4), AAGCTTTGCTTGTAACTTA (forward) and GCACATGATTAGTGG (reverse); for FTR1 (865-bp product), GGCGGCATTTACCTTA (reverse); and for CAP1 (815-bp product), CGCATAGAGAGGAGGATG (forward) and CGCGCTACCTTACACG (reverse). PCR products were gel purified using a Qiaprep 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.bgcsc.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/index.html] and B3501 (serotype D) (Stanford Genome Technology Center, CA [http://altdoit.stanford.edu/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 CAPII sequence from one isolate in our study to the recently available sequences of the C. gattii VGI MATa (GenBank accession no. AY710430.1) and MATa 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 MATa 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) MATa isolates only (n = 40), (iii) VGI isolates only (n = 10), (iv) VGI isolates only (n = 29), (v) VGI MATa 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. | Serotype | Molecular type | Mating type | Source | Year isolated | Geographical origin | Sequence subtype |
|-------------|----------|----------------|-------------|--------|---------------|---------------------|-----------------|
| **Cryptococcus gattii isolates from British Columbia** | | | | | | | |
| A2M R314<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human, sputum | 2002 | Duncan, V.I. (travel history) | 1 1 1 1 |
| A2M R299<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 2002 | Lantzville, V.I. (travel history) | 1 1 1 1 |
| A4M R64<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human | 2004 | Vancouver (travel history) | 1 1 1 1 |
| KB7892<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Arbutas tree | 2004 | Saltspoint Island, B.C. (environ of parrot isolate KB7091) | 3 2 2 1 |
| A1M R794<sup>a</sup> | B | VGI | MAT<sup>b</sup> | Human, CSF | 2001 | Vancouver, B.C. (travel history) | 4 2 2 1 |
| A1M F2863<sup>c</sup> | B | VGI | MAT<sup>b</sup> | Dall’s porpoise | 2002 | Washed up on shore of southern V.I. (assumed travel history) | 3 1 3 1 |
| A1M R265<sup>a</sup> | B | VGI | MAT<sup>b</sup> | Human, BAL | 2001 | Duncan, V.I. | 5 3 4 2 |
| A1M F3016<sup>c</sup> | B | VGI | MAT<sup>b</sup> | Dall’s porpoise | 2002 | Washed up on shore of a gulf island, B.C. (assumed travel history) | 5 3 4 2 |
| **MAC-9<sup>b</sup>** | B | VGI | MAT<sup>b</sup> | Cedar | 2001 | Cathedral Grove, V.I. | 5 3 4 2 |
| A1M R272<sup>a</sup> | B | VGI | MAT<sup>c</sup> | Human, BAL | 2001 | Ladsmith, V.I. | 7 3 4 3 |
| KB2045<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Air sample | 2002 | Langley, B.C. (environ of tapir isolate KB1079) | 5 3 4 2 |
| **RB26<sup>b</sup>** | B | VGI | MAT<sup>b</sup> | Tree stump | 2002 | Parksville, V.I. | 7 3 3 4 |
| **RB27<sup>b</sup>** | B | VGI | MAT<sup>b</sup> | Douglas fir | 2002 | Parksville, V.I. | 7 3 3 4 |
| KB152A-6<sup>b</sup> | B | VGI | MAT<sup>c</sup> | Air sample | 2002 | Parksville, V.I. | 7 3 3 4 |
| **A2M R282<sup>a</sup>** | B | VGI | MAT<sup>a</sup> | Human, sputum | 2002 | Victoria, V.I. | 5 3 4 2 |
| KB7091<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Companion | 2003 | Saltspoint Island (travel history) | 5 3 4 3 |
| A3M R535<sup>a</sup> | B | VGI | MAT<sup>c</sup> | Human, chest abscess | 2003 | Delta, B.C. | 5 3 4 2 |
| A3M R673<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human, BAL | 2003 | Sidney, V.I. (travel history) | 5 3 4 2 |
| KB7092<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Horse | 2003 | Mill Bay, V.I. | 7 3 4 3 |
| KB4672<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Llama | 2003 | Chilliwack, B.C. | 5 3 4 2 |
| KB1079<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Companion cat | 2003 | Nanaimo, V.I. | 5 3 4 2 |
| **NIHI444<sup>e</sup>**<sup>a</sup> | B | VGI | MAT<sup>a</sup> | 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**

| Isolate no. | Serotype | Molecular type | Mating type | Source | Year isolated | Geographical origin | Sequence subtype |
|-------------|----------|----------------|-------------|--------|---------------|---------------------|-----------------|
| A3M R29<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Koala | 2002 | Toronto Zoo, ON, Canada (previously from San Diego Zoo) | 1 1 1 1 |
| A2M R554<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Captive bottlenose dolphin | 2000 | San Diego, CA (no recent travel history) | 2 1 1 1 |
| WM179<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1993 | Sydney, NSW, Australia | 1 1 1 1 |
| WM276<sup>e</sup> | B | VGI | MAT<sup>a</sup> | E. tereticornis | 1993 | Mt. Annan, NSW, Australia | 1 1 1 1 |
| KB1045<sup>asu</sup> | B | VGI | MAT<sup>a</sup> | Companion cat | 2003 | Edmonton, AB, Canada (travel history to V.I.) | 5 3 4 2 |
| **NIHI444<sup>e</sup>**<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human, sputum ca. 1971 | 2004 | Seattle, WA | 5 3 4 2 |
| KB9944<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Unidentified tree species | 2004 | CA (environ of parrot isolate KB7091) | 5 3 4 2 |
| CBS 7750<sup>a</sup> | B | VGI | MAT<sup>a</sup> | E. camaldulensis | 1990 | San Francisco, CA | 5 3 4 2 |
| LA55<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1995 | NE region of Piaui, Brazil | 5 3 4 4 |
| LA57<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1995 | NE region of Piaui, Brazil | 5 3 4 4 |
| LA61<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1997 | NE region of Piaui, Brazil | 7 3 4 4 |
| LA499<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1997 | Norte de Santander, Colombia | 6 3 4 5 |
| HOO58 I-106 | B | VGI | MAT<sup>a</sup> | Human, CSF | 1997 | Caqueta, Colombia | 6 3 4 5 |
| HOO58 I-638 | B | VGI | MAT<sup>a</sup> | Human, CSF | 1998 | Bolivar, Colombia | 6 3 4 5 |
| HOO58 I-675 | B | VGI | MAT<sup>a</sup> | Human, CSF | 1998 | Bolivar, Colombia | 6 3 4 5 |
| MC-S-115<sup>a</sup> | B | VGI | MAT<sup>a</sup> | Human, CSF | 1993 | Thailand | 7 3 4 3 |
| WM179<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Human, lung | 1991 | Sydney, NSW, Australia | 5 4 5 3 |
| RAM005<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | E. tetrodonta | 1999 | Arnhemland, NT, Australia | 7 3 4 3 |
| WM108<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | Insect frass | 2000 | Mt. Druitt, NSW, Australia | 7 3 4 3 |
| WM161<sup>amu</sup> | B | VGI | MAT<sup>a</sup> | E. camaldulensis | 1992 | San Diego, CA | 8 5 6 6 |
| NIH191<sup>a</sup> | B | VGI | MAT<sup>b</sup> | Human, CSF | 1978 | CA | 9 6 7 7 |
| WM779<sup>s</sup> | B | VGI | MAT<sup>b</sup> | Cheetah | 1994 | Johannesberg, South Africa | 10 7 8 8 |

**Outgroups**

| Isolate no. | Serotype | Molecular type | Mating type | Source | Year isolated | Geographical origin | Sequence subtype |
|-------------|----------|----------------|-------------|--------|---------------|---------------------|-----------------|
| H99<sup>a</sup> | A | VNI | MAT<sup>a</sup> | Human | 1978 | New York, NY | NA |
| B-3501A<sup>a</sup> | D | VNI | MAT<sup>a</sup> | Laboratory cross | NA | NA |  

Continued on following page
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 scytanthé 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* MATα 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 MATα, n = 38; for MATα, 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 MATa and MATα 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 MATα 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 MATα isolates (for VGII, n = 4; for VGIII, n = 1) to avoid distortion of the data.

The MATα isolates were constrained in monophyletic groups according to their molecular type (for VGII, 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 MATα isolates. The mean pairwise Kimura 2 parameter distance (Table 2) within VGI was significantly greater than that observed within the MATα VGI isolates (P = 0.000), and the mean divergence between VGI and VGII MATα 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
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. 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.
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 VGII sequence variants observed in this study are associated with B.C. isolates, and three B.C. VGII isolates represent unique strains in this study. One of these unique strains, KB7892 (from an arbutus tree on Saltspring Island), represents the only VGII environmental strain isolated in B.C. at the time of these analyses. Therefore, there appears to be no epidemiological link be-
between 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 MATα 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 MATα 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 3. Mean pairwise Kimura 2 parameter distances among C. gattii isolates within and between different geographical regions

| Region               | Vancouver Island | B.C., excluding Vancouver Island | B.C., including Vancouver Island | Rest of North America | South America | Australia | Thailand | South Africa |
|----------------------|------------------|----------------------------------|----------------------------------|-----------------------|---------------|----------|----------|--------------|
| Kimura 2 parameter distance (mean ± SD) | 0.01518 ± 0.00258 | 0.02570 ± 0.02112 | 0.03380 ± 0.02216 | 0.02833 ± 0.01900 | 0.03031 ± 0.01889 | 0.03911 ± 0.02839 | 0.09197 ± 0.01884 | 0.02998 ± 0.02517 |

- Bold type indicates a mean genetic distance among isolates from within a defined geographical area.

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* 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* 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 VGII strains from clinical cases, there is little evidence at this time for permanent colonization of VGII in the B.C. environment, aside from two isolates from SaltSpring Island. A comparison of MLST profiles revealed no similarity of the VGII 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/pdpapers/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.

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