

Note

Molecular characterization of *Cryptococcus gattii* genotype AFLP6/VGII isolated from woody debris of divi-divi (*Caesalpinia coriaria*), Bonaire, Dutch Caribbean



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ABSTRACT

Background: The basidiomycetous yeast *Cryptococcus gattii* is an emerging and primary pathogen. There is a lack of information about its environmental spread outside outbreak regions in Mediterranean Europe, North and South America. Environmental sampling for *C. gattii* and molecular characterization of the obtained isolates will provide an insight into the global spread of the various genotypes.

Methods: Woody debris of native divi-divi (*Caesalpinia coriaria*) trees were sampled across Bonaire, Dutch Caribbean. Colonies suspected for *Cryptococcus* species were subjected to standard mycology investigations and identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Isolates identified as *C. gattii* were subjected to amplified fragment length polymorphism genotyping, mating-type analysis and multi-locus sequence typing.

Results: Ten colonies of *C. gattii* were cultured from different trunk hollows of the same divi-divi tree. Molecular characterization showed that all isolates were genotype AFLP6/VGII and mating-type α . Multi-locus sequence typing revealed that all isolates were genetically indistinguishable from each other.

Conclusions: *C. gattii* is present in the environment of Bonaire, which suggests that this yeast is likely to be present in the environment of other Caribbean islands.

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Caracterización molecular del genotipo AFLP6/VGII *Cryptococcus gattii* aislado de restos de madera de divi-divi (*Caesalpinia coriaria*), Bonaire, Caribe Holandés

RESUMEN

Antecedentes: La levadura *Cryptococcus gattii* es un basidiomiceto emergente y patógeno primario. Existe poca información acerca de su dispersión en medio ambiente fuera de las regiones con brotes descritos por esta levadura en la Europa mediterránea, Norte y Sur de América. Los muestreos del medio ambiente para la búsqueda de *C. gattii* y la caracterización molecular de los aislamientos obtenidos puede proveer de una visión global sobre la dispersión de varios de sus genotipos.

Métodos: Se tomaron muestras de residuos de madera de árboles nativos divi-divi (*Caesalpinia coriaria*) en Bonaire, Caribe Holandés. Las colonias susceptibles de pertenecer a las especies de *Cryptococcus* se sometieron a un estudio micológico estándar e identificación por espectrometría de masas MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight). Los aislamientos identificados como *C. gattii* se sometieron a genotipado mediante AFLP (Amplified Fragment Length Polymorphism), obtención del tipo sexual y MLST (Multi-locus Sequence Typing).

Resultados: Se obtuvieron diez colonias de *C. gattii* en el cultivo de nuestras de diferentes agujeros de un mismo árbol divi-divi. La caracterización molecular mostró que todos los aislamientos eran genotipo AFLP6/VGII y tipo sexual α . El tipado mediante MLST reveló que todos los aislamientos eran genéticamente indistinguibles unos de otros.

Palabras clave:

Cryptococcus gattii

Tipado por secuenciación de múltiples loci

Polimorfismo de tamaño de fragmentos

amplificados

Muestreo medioambiental

Caesalpinia coriaria

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Conclusiones: *C. gattii* está presente en el medio ambiente de Bonaire, lo que sugiere que esta levadura podría estar presente en el ambiente de otras islas del Caribe.

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Cryptococcus gattii and its sibling *Cryptococcus neoformans* are the major basidiomycetous yeast pathogens within the genus *Cryptococcus* that comprises over one hundred recognized species.¹⁴ Annually, nearly an estimated one million individuals with HIV/AIDS develop cryptococcal meningitis with high estimated death rates of 6,25,000 subjects.¹⁹ *C. neoformans*, with its two varieties *grubii* and *neoformans*, has a global distribution and affects mainly immunocompromised individuals, such as those with HIV/AIDS or those under immunosuppressive therapy. Although *C. gattii* is mainly restricted to subtropical and tropical climate zones and is known to be a major source of cryptococcosis among immunocompetent subjects, it has also emerged as a significant pathogen in Canada and the Pacific Northwest of the USA.^{1,2,9,10,20}

Molecular techniques, such as amplified fragment length polymorphism (AFLP) fingerprinting, PCR-fingerprinting, restriction fragment length polymorphism fingerprinting and multi-locus sequence typing (MLST), have provided a detailed insight into the genetic diversity of the *C. neoformans/C. gattii* species complex.^{1,18} *Cryptococcus neoformans* var. *grubii* (serotype A) can be divided into the three genotypes: AFLP1/VNI, AFLP1A/VNII/VNB and AFLP1B; *C. neoformans* var. *neoformans* (serotype D) is represented by genotype AFLP2/VNIV, and the intervarietal *C. neoformans* hybrid (serotype AD) corresponds with genotype AFLP3/VNIII.^{1,18} *C. gattii* consists of five haploid genotypes, with genotypes AFLP4/VGI, AFLP6/VGII and AFLP10/VGIV representing the serotype B isolates, and genotypes AFLP5/VGIII and AFLP7/VGIV corresponding with isolates exhibiting the C serotype.⁹ Interspecies hybrids have been described and thus far have only been isolated from clinical sources.¹

During the past decade ongoing and expanding outbreaks of *C. gattii* occurred in temperate climate zones affecting previously healthy humans and animals.^{6,10,20} Besides the changing distribution pattern, it has been observed that certain *C. gattii* genotypes may be found more often in immunocompromised individuals than in immunocompetent subjects.⁹ Notably, environmental isolation of *C. gattii* was found to be a valuable tool to enhance the current knowledge of the geographical spread, genotypic diversity and the environmental niche of this pathogenic species.^{3,4,6,16,20}

Within the Caribbean, *C. gattii* has rarely been reported as clinical or veterinary infection.^{9–11,13} Despite large-scale environmental sampling of a plethora of environmental sources that includes many tree and cacti species^{9,10,12,15} the fungus has been, up until

now, only recovered from the environment of Puerto Rico. Here we describe the environmental isolation and molecular characterization of *C. gattii* isolates from decayed woody debris of a native divi-divi tree (*Caesalpinia coriaria*) in Bonaire, Dutch Caribbean.

Woody debris, collected from inside trunk hollows of living divi-divi trees in April 2013, were cultured on simplified niger seed agar as described.⁴ The sampled divi-divi trees were located in Lagun Goto (N12° 14' 3.1344", E-68° 22' 6.3666"), Rincon village (N12° 14' 24.1116", E-68° 19' 32.5662") and neighboring surroundings of Hato village (N12° 10' 11.8734", E-68° 17' 1.2366"). Plates were incubated at 28 °C and periodically observed for chocolate brown colonies of *C. gattii* and *C. neoformans* up to 7 days. Suspected colonies of *Cryptococcus* spp. were purified by dilution plating and identified by their morphological and biochemical profiles using VITEK2 and API 20C AUX (bioMérieux, Marcy l'Etoile, France). *Cryptococcus* spp. colonies were also inoculated on L-canavanine-glycine bromothymol blue medium; a blue-color change suggestive of *C. gattii* was observed.¹ Identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry was performed using the MALDI Biotyper, and spectra were compared to the reference spectra in the commercial database (Bruker Daltonics, Bremen, Germany).¹⁷

Ten colonies identified as *C. gattii* were obtained from decayed wood of different trunk hollows of the same divi-divi tree. The isolates have been deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre under accession numbers CBS12864–CBS12873.

All ten *C. gattii* isolates were subsequently subjected to molecular characterization as described previously.^{4,6,9} Analysis of the AFLP fingerprints, using UPGMA clustering and the Pearson correlation coefficient in BioNumerics version 6.6.8 (Applied Maths, Sint-Martens-Latem, Belgium) showed that all ten isolates clustered together with the reference strain representing genotype AFLP6/VGII (Fig. 1). Mating-type analysis by conventional PCR revealed that all ten isolates exhibit the *STE12*-allele with the α mating-type.⁹

In-depth genetic analysis was initiated by amplification and sequencing of the ten nuclear loci *CAP10*, *CAP59*, *GPD1*, *IGS1*, *LAC1*, *MPD1*, *PLB1*, *SOD1*, *TEF1* and *URA5*, as previously described.⁹ Multi-locus sequence typing links clinical isolates to their geographical origin and demonstrates their relatedness to the isolates obtained from environmental and veterinary sources.^{4,5,7,9,11–13}

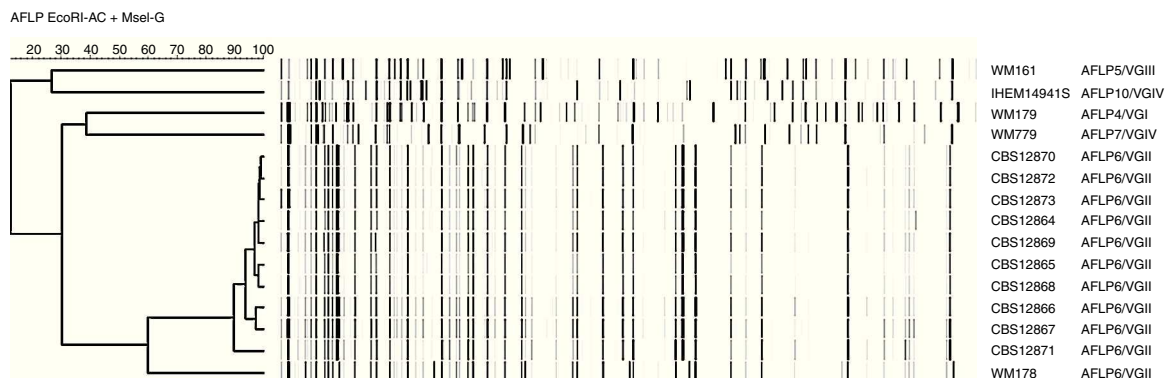


Fig. 1. Amplified fragment length polymorphism fingerprint dendrogram of ten *C. gattii* isolates from Bonaire compared to the reference strains WM179 (AFLP4/VGI), WM161 (AFLP5/VGIII), WM178 (AFLP6/VGII), WM779 (AFLP7/VGIV) and IHEM14941 (AFLP10/VGIV) representing all known AFLP genotypes within *C. gattii*.

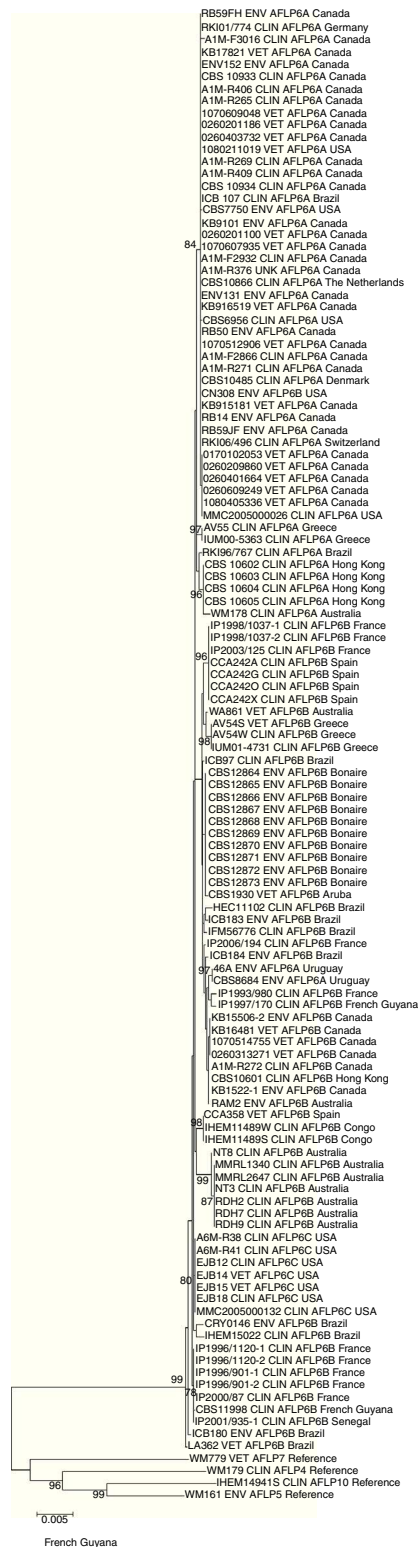


Fig. 2. Bootstrapped maximum likelihood phylogenetic analysis of the multi-locus sequence typing data from a global set of *C. gattii* genotype AFLP6/VGII isolates (data from 9) compared with those isolated from a divi-divi tree on Bonaire.

Sequences for each of the ten loci and for all ten *C. gattii* isolates revealed that they were genetically indistinguishable from each other. Genbank accession numbers for sequences identical to those obtained in the current study were DQ861598 (=CAP10), DQ096433 (=CAP59), DQ096382 (=GPD1), KF439871 (=IGS1), DQ096400 (=LAC1), DQ861595 (=MPD1), DQ198344

(=PLB1), HM207449 (=SOD1), DQ861593 (=TEF1) and HQ606091 (=URA5).

Phylogenetic analysis was performed as described before by using a bootstrapped maximum likelihood analysis (1000 bootstraps) in MEGA version 5.2 with the best fitting nucleotide substitution model being Hasegawa-Kishino-Yano plus gamma distributed with invariant sites and with *C. gattii* reference strains from the other genotypes as an outgroup.⁹ To compare the *C. gattii* isolates with those from other localities, the recently published MLST-dataset of *C. gattii* AFLP6/VGII isolates was included.⁹

As depicted in Fig. 2, all isolates from Bonaire clustered together with the genotype AFLP6/VGII isolate CBS1930 from Aruba. This isolate, obtained in July 1953, originated from clinical material of a child. This isolate was experimentally inoculated in a goat to see whether the atypical yeast isolate was the culprit of infection (original correspondence about this isolate in the CBS-KNAW archive). Other available isolates from Latin America were only distantly related, with Brazilian and French Guyana isolates as the closest siblings (Fig. 2).

Interestingly, isolate CBS1930 was found to have mating-type α while the isolates from Bonaire were all mating-type α . Genetically, the isolates from Aruba and Bonaire differed slightly from each other suggesting that there is a clonal population of *C. gattii* AFLP6/VGII on these Caribbean islands. However, due to the presence of different mating-types within this set of isolates the conclusion of one clonal population needs to be taken with caution. Another explanation may be that although isolates from Dutch Caribbean islands are genetically closely related they may form different clonal populations. This scenario is identical to the one observed in Mediterranean population of *C. gattii* AFLP4/VGI, where Italian isolates with the mating-type α and Spanish isolates with the mating-type α were genetically indistinguishable from each other using the same set of ten sequenced nuclear loci.⁹

Unfortunately, the only *C. gattii* AFLP6/VGII isolates available from the Caribbean, isolated from cacti on the island of Puerto Rico, could not be compared with our data since these isolates are not available to the research community.^{8,15}

To conclude, *C. gattii* AFLP6/VGII is present in the environment of the Dutch Caribbean and Puerto Rico, suggesting that *C. gattii* might be present in other Caribbean localities. Little is known about the epidemiology of *C. gattii* in Central America and the Caribbean; therefore environmental sampling at other Caribbean islands, as well as investigating the occurrence of this pathogenic yeast among clinical samples from this region, should be considered.

Conflict of interest

The authors declare that there are no competing interests.

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