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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.

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

Palabras clave:
Cryptococcus gattii
Tipado por secuenciación de múltiples loci
Polimorfismo de tamaño de fragmentos amplificados
Muestreo medioambiental
Caesalpinia coriaria

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.

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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.

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′ 6.366″), 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 I'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]

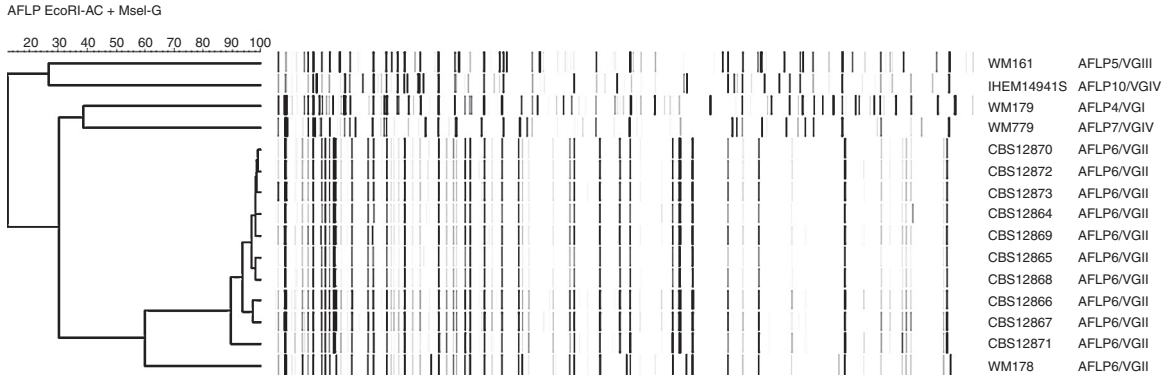

Fig. 1. Amplified fragment length polymorphism fingerprint dendrogram of ten *C. gattii* isolates from Bonaire compared to the reference strains WM179 (AFLP4/VGI), WM161 (AFLP5/VGIII), WM178 (AFLP6/VGII), WM779 (AFLP7/VGIV) and IHEM14941 (AFLP10/VGIV) representing all known AFLP genotypes within *C.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