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Epidemiological investigation for grouped cases of *Trichosporon asahii* using whole genome and IGS1 sequencing

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Summary

Background: Trichosporonosis is a rare invasive infection in humans mainly due to *Trichosporon asahii*, and especially recovered from patients having haematological malignancy. Since 2012, IGS1 region sequencing is used as a genotyping method to distinguish isolates, with high frequency of one haplotype worldwide and a geographic specificity for some haplotypes.

Objectives: We compared the IGS1 genotyping method and whole genome sequencing (WGS) to study the relationship between clinical isolates involved in two grouped cases in France.

Methods: IGS1 sequencing and antifungal susceptibility testing were performed for 54 clinical isolates. Clinical data for 28 isolates included in surveillance programs were analysed. Whole genome was sequenced for 32 clinical isolates and the type strain.

Results: All isolates were intrinsically resistant to flucytosine, while voriconazole had the most potent in vitro activity. The majority of the isolates was recovered from patients with haematological malignancies (42.86%), with a high proportion of children (<15 yrs-old, 32.14%) and a high mortality rate at three months (46.15%). Based on the WGS analysis, isolates exhibiting IGS1 haplotype 1, 3 and 7 belonged to different clades. Five isolates recovered during the first grouped cases had the same IGS1 haplotype and shared 99% of SNPs similarity. For the second grouped cases, four isolates had 98.7% of SNPs similarity while the isolate recovered 4 years earlier was totally unlinked.

Conclusions: We confirmed the usefulness of IGS1 sequencing for grouped cases infection of *T. asahii*. We underlined its limitation for the study of population structure and the utility of WGS analysis for the study of epidemiologically unrelated isolates.

KEYWORDS

emerging yeast, epidemiology of trichosporonosis, IGS sequencing, *Trichosporon asahii*, whole genome sequencing

1 | INTRODUCTION

Trichosporon asahii is a basidiomycetous yeast-like species producing arthroconidia. It has a worldwide distribution in the environment, especially in the soil, pigeon droppings and houses (<http://www.mycobank.org>).¹⁻³ This species is able to grow up to 40°C and to produce biofilms providing potential virulence factors for humans.⁴ Previous classification placed the species in the clade *Ovooides*² with other pathogenic species such as *Trichosporon inkin* and *Trichosporon ovooides*. In 2015, a new classification of the *Tremellomycetes* class divided *Trichosporon* species into five genera in the new family *Trichosporonaceae*: *Trichosporon*, *Apiotrichum*, *Cutaneotrichosporon*, *Effuseotrichosporon*, *Haglerozyma*. *Trichosporon asahii* and other species affiliated to the clade *Ovooides* were maintained in the *Trichosporon* genus.⁵

Trichosporon asahii is considered to be part of the normal biota of the human skin⁶ and gastrointestinal tract.⁷ This species is rarely involved in white piedra but is frequently recovered from patients with summer-type hypersensitivity pneumonitis (SHP), an allergy developed after repeated inhalation of arthroconidia.⁸ It is also isolated from immunocompromised patients and is the major *Trichosporon* species responsible for life-threatening deep-seated infection called trichosporonosis,⁹⁻¹³ mainly in patients with haematological malignancies.¹⁴⁻¹⁶ Fungaemia due to *Trichosporon* sp. are frequently associated with pre-exposure to caspofungin.^{14,17} *Trichosporon asahii* is described in sporadic cases and also during local outbreaks of invasive infection.^{14,18} Infections can have exogenous (skin inoculation, inhalation) or endogenous (gut translocation) origins.

Since 2002, sequencing of the IGS1 region (intergenic spacer localised in the rDNA gene) is used as a genotyping method to differentiate *T. asahii* isolates.^{19,20} Up to now, 12 IGS1 haplotypes have been identified and a correlation between haplotype and geographical origin of the isolates has been described. Indeed, haplotype 1 is ubiquitous but predominant in Japan, China, South America, Turkey and Thailand, while haplotype 3 is more frequent in USA, Thailand and also in houses of SHP patients in Japan.^{12,20} Haplotype 5 is also frequent in USA and haplotype 7 in Thailand.^{20,21} Other haplotypes are described in Spain, Japan and Brazil.^{20,21} In 2012, Yang *et al* sequenced the whole genome of two strains of *T. asahii* (an environmental isolate, CBS8904, recovered from maize cobs, in 1998, Illinois, USA²² and a clinical isolate, CBS2479, the type strain, recovered in Japan in 1929 from a psoriasis lesion).²³

In 2014, two French hospitals notified to the NRCMA, grouped cases of colonisation and/or invasive infections due to *T. asahii* over a short period of time. At that time, the clinicians wondered if a unique strain was involved in each of these grouped cases. Given that the majority of the isolates recovered worldwide is of IGS1-haplotype 1, we considered that IGS1 sequencing was not sufficient to confirm link between isolates. We decided to sequence the whole genome of these isolates and others from our collection to study their genetic diversity and the relationship between isolates involved in these grouped cases.

2 | MATERIAL AND METHODS

2.1 | Isolates

Since 2003, 54 clinical isolates were sent to the National Reference Center for Invasive Mycoses & Antifungals (NRCMA, Institut Pasteur, Paris, France) in the context of the active surveillance of yeast fungaemia in the Paris area (YEASTS program, n = 17) or that of the nationwide Surveillance Network of Invasive Fungal Infections (RESSIF, n = 11). Other isolates (n = 26) were sent only for expertise (confirmation of species identification and/or determination of antifungal susceptibility). Clinical data (age, gender, underlying disease, prior exposure to antifungal agents, antifungal treatment and outcome at 90 days) when available were collected. The type strain of *T. asahii* CBS2479 was included in the analysis.

2.2 | Species identification

Purity was first checked using chromogenic medium (BBL™ CHROMagar™ Candida; BD Company, USA). After DNA extraction using NucleoMag plant kit extraction (Macherey-Nagel, Germany) in a KingFisher™ Flex system (Thermo Fisher Scientific), D1D2, ITS1/ITS2 and IGS1 regions were amplified and sequenced using pan-fungal primers (NL1/NL4,²⁴ V9D/LS266,^{25,26} 26SF/5SR,²⁰ respectively). Sequences were compared with sequences of type strains of *Trichosporon* species to confirm species identification. Haplotype of IGS1 was determined after comparison with the 12 haplotypes of *T. asahii* available in the literature.¹⁹ Sequences of IGS1 region were aligned with MUSCLE using Mega v. 6,²⁷ and maximum-likelihood tree was constructed with matrix alignment.

2.3 | Antifungal susceptibility testing

Minimal inhibitory concentrations (MICs) were determined for 5 antifungal agents (flucytosine, fluconazole, posaconazole, voriconazole and amphotericin B; all purchased from Alsachim, a Shimadzu Group Company) according to the EUCAST standardised microdilution broth method²⁸ except for amphotericin B testing since it was diluted in AM3 medium. ATCC22019 and ATCC6258 reference strains were used as quality control strains. MIC50 and MIC90, corresponding to the MIC value inhibiting at least 50% and 90% of the isolates, were calculated for each antifungal agent.

2.4 | Whole Genome Sequencing (WGS)

Epidemiologically related (n = 11 involved in the two grouped cases) or unrelated (n = 20) clinical isolates were selected in the NRCMA collection for WGS based on anatomical, geographical and temporal origins as well as IGS1 haplotype. The selected isolates were recovered from 29 patients diagnosed in 18 different hospitals between 2003 and 2016 and

TABLE 1 Characteristics of the 54 clinical isolates and the type strain of *Trichosporon asahii*

Strain	Site of isolation	Date of isolation	Haplotype IGS1	Patient	H#	Continent of diagnosis	NGS	MIC (mg/L)				
								Fluco	Vori	Posa	AmB	Fluicy
CNRMA14.90	Lung	05/07/2013	1	10	1	Europe	Yes	2	0.125	0.06	2	8
CNRMA14.89	Blood	26/06/2013	1	10	1	Europe	Yes	8	0.125	0.25	≥8	16
CNRMA14.717	Urinary tract	23/12/2013	1	7	1	Europe	Yes	2	≤0.015	0.125	1	≥64
CNRMA14.718	Digestive tract	20/01/2014	1	7	1	Europe	Yes	2	0.03	0.06	1	16
CNRMA14.716	Blood	27/01/2014	1	7	1	Europe	Yes	2	0.03	0.03	2	≥64
CNRMA17.64	Blood	11/01/2016	1	28	1	Europe		0.25	≤0.015	0.03	≥8	32
CNRMA12.1406	Blood	16/12/2012	3	45	2	Europe	Yes	0.5	0.06	0.06	0.5	8
CNRMA14.394	Skin	01/05/2014	1	11	3	Europe	Yes	8	0.25	0.5	0.5	≥64
CNRMA15.287	Blood	14/04/2015	4	6	4	Europe	Yes	1	0.06	0.5	1	16
CNRMA9.1206	Blood	29/11/2009	1	9	5	Europe	Yes	32	0.25	0.125	2	≥64
CNRMA18.365	Blood	27/04/2018	1	24	6	Europe		2	0.06	0.25	1	32
CNRMA8.1157	Blood	18/09/2008	7	16	7	Europe	Yes	16	0.5	0.5	2	≥64
CNRMA8.907	Blood	20/07/2008	1	31	8	Europe		4	0.06	0.125	≥8	≥64
CNRMA8.972	Blood	25/07/2008	1	31	8	Europe		4	0.06	0.125	≥8	≥64
CNRMA14.483	Bone	11/07/2014	3	14	8	Europe	Yes	8	0.06	0.5	0.5	32
CNRMA14.841	Bone	07/10/2014	4	15	8	Europe	Yes	0.25	0.03	0.125	0.25	16
CNRMA14.672	Blood	01/10/2014	Unknown	29	9	Europe	Yes	8	0.125	1	≥8	≥64
CNRMA8.358	Blood	17/03/2008	3	34	10	Europe		8	0.125	0.125	1	≥64
CNRMA13.273	Blood	25/03/2013	3	49	11	Caribbean Island	Yes	4	0.06	0.25	1	8
CNRMA12.448	Blood	03/05/2012	1	32	12	South America	Yes	4	0.06	0.125	≥8	≥64
CNRMA14.167	lung	27/08/2013	4	38	12	South America	Yes	4	0.25	0.5	2	8
CNRMA14.166	Urinary tract	27/08/2013	7	13	12	South America	Yes	8	0.25	0.25	4	8
CNRMA14.165	Urinary tract	27/01/2014	1	3	12	South America	Yes	2	0.125	0.25	2	8
CNRMA14.164	CSF	31/01/2014	1	44	12	South America	Yes	8	0.25	0.25	≥8	≥64
CNRMA14.401	Urinary tract	25/04/2014	1	23	12	South America	Yes	4	0.125	0.5	≥8	≥64
CNRMA14.391	Urinary tract	17/05/2014	1	12	12	South America	Yes	4	0.125	0.25	4	≥64
CNRMA18.15	Blood	02/01/2018	3	1	12	South America		4	0.125	0.25	0.5	≥64
CNRMA17.531	Blood	06/02/2017	1	22	13	Europe		2	0.06	0.03	1	≥64
CNRMA8.412	Blood	18/03/2008	3	34	14	Europe		8	0.125	0.125	1	≥64
CNRMA16.785	Blood	27/11/2016	3	4	14	Europe		2	0.03	0.06	0.5	≥64
CNRMA15.564	Blood	16/08/2015	1	27	15	Europe		8	0.125	0.25	1	≥64

(Continues)

TABLE 1 (Continued)

Strain	Site of isolation	Date of isolation	Haplotype IGS1	Patient	H#	Continent of diagnosis	NGS	MIC (mg/L)				
								Fluco	Vori	Posa	Amb	
CNRMA6.842	Blood	02/08/2006	1	19	16	Europe		4	0.125	0.5	2	4
CNRMA12.1055	Blood	22/08/2012	3	43	17	Carribbean Island	Yes	2	0.06	0.5	2	8
CNRMA17.857	Blood	06/09/2013	3	20	17	Carribbean Island		2	0.125	0.25	2	≥64
CNRMA11.755	Lung	27/07/2011	1	36	18	Africa	Yes	0.25	≤0.015	0.125	0.25	1
CNRMA14.475	BONE	07/07/2014	4	46	18	Africa	Yes	2	0.03	0.125	2	32
CNRMA11.1137	BLOOD	25/11/2011	1	40	19	Europe		4	0.25	1	≥8	≥64
CNRMA7.368	BLOOD	20/03/2007	3	17	20	Europe		4	0.06	0.25	2	32
CNRMA15.424	BLOOD	13/03/2015	1	25	21	Europe	Yes	4	0.06	0.5	0.5	≥64
CNRMA16.422	BLOOD	08/06/2016	1	33	21	Europe	Yes	64	0.5	0.25	4	32
CNRMA4.1415	BLOOD	14/12/2004	1	8	22	Europe		16	0.25	1	1	≥64
CNRMA16.827	BLOOD	22/09/2016	3	48	22	Europe		4	0.125	0.25	2	≥64
CNRMA8.212	BLOOD	11/03/2007	4	30	23	Europe	Yes	16	0.125	0.5	2	8
CNRMA10.64	BLOOD	14/01/2010	1	47	24	Europe	Yes	32	0.25	0.25	≥8	16
CNRMA14.826	Skin	09/12/2014	1	2	24	Europe	Yes	1	≤0.015	0.125	0.25	≥64
CNRMA16.701	Ear	17/10/2016	1	5	24	Europe		4	0.06	0.06	4	8
CNRMA18.102	Blood	30/01/2018	4	37	24	Europe		1	0.06	0.06	0.5	32
CNRMA3.276	Blood	04/11/2003	5	42	25	Europe	Yes	2	0.03	0.25	4	32
CNRMA14.203	Blood	15/02/2014	4	41	25	Europe	Yes	4	0.25	0.25	0.25	16
CNRMA17.767	Blood	28/09/2017	4	18	25	Europe		4	0.06	0.5	1	≥64
CNRMA17.910	Blood	29/11/2017	4	21	25	Europe		0.5	≤0.015	0.125	2	≥64
CNRMA18.705	Blood	01/10/2018	4	39	25	Europe		0.5	≤0.015	0.06	1	8
CNRMA17.259	Urine	05/03/2017	1	26	26	Africa		2	≤0.015	0.06	0.25	16
CNRMA14.72	Blood	01/01/2013	3	35	27	Europe	Yes	2	0.5	0.5	2	16
CBS2479	Psoriasis lesion	1929	1	50		Asia	Yes	4	0.06	0.25	≥8	≥64

Shadows were used to indicate isolates from a same hospital.

Abbreviations: H, hospital; Fluco, fluconazole; Vori, voriconazole; Posa, posaconazole; Amb, amphotericinB; Flucy, Flucytosin; CSF, cerebrospinal fluid.

involved in deep and superficial infections (53% blood culture, 16% urine, 9% lung, 6% skin). They belong to the 5/12 known IGS1 haplotypes found in the NRCMA collection, and one was of a yet undescribed haplotype. Thus, the whole genome of 32 strains including CBS 2479, the type strain of *T. asahii*, was sequenced at the Institut Pasteur P2M platform, using an Illumina NextSeq 500 sequencer. Libraries were Nextera® DNA Library Preparation Kit and sequencing using a 2 × 150 nucleotide paired-end strategy. Library adaptors of paired-ends read were trimmed with Cutadapt version 1.18 (<https://doi.org/10.14806/ej.17.1.200>) and then mapped to the 78 scaffolds of the reference genome of CBS2479²³ (GenBank accession number ALBS00000000) using the Burrows-Wheeler Alignment tool, BWA version 0.7.13²⁹ with the BWA-mem algorithm. SAMtools version 1.9 and Picard tools version 1.94 (<http://broadinstitute.github.io/picard>) were then used to filter, sort, convert SAM files and assign read groups and mark duplicate reads. Single-nucleotide polymorphism (SNPs) and insertion/deletion (indels) positions were called using Genome Analysis Toolkit version 3.1-1³⁰ according to the GATK Best Practices. Variant filtration, QD <2.0, LowQD, ReadPosRankSum <-8.0, LowRankSum, FS >60.0, HightFS, MQRankSum <-12.5, MQRankSum, MQ <40.0, LowMQ, HaplotypeScore >13.0, HaploScore parameters were used to filter SNPs and indels. SNPs that varied between isolates were combined into single multiple alignments. Phylogenetic analyses were performed with IQTree version 1.6.12³¹ with the TVM+F+R2 substitution model, the perturbation strength 0.5 and 1000 bootstraps replicates. Number of transitions [Ts, interchanges of purines (A ↔ G) or of pyrimidines (C ↔ T)] and transversions [Tv, interchanges of purine for pyrimidine bases] were determined, and ratio of Ts to Tv was calculated for SNPs positions, using vcftools v. 0.1.13.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to micro-organisms.

4 | RESULTS

4.1 | Characteristics of the isolates

Clinical isolates (1-3/patient) were recovered between October 2003 and December 2018 from 49 patients hospitalised in 27 French hospitals, mainly in metropolitan France (Europe: 35 patients-40 isolates) but also overseas territories (Africa: 3 patients, 3 isolates; South America: 8 patients, 8 isolates; Caribbean islands: 3 patients, 3 isolates). The majority of the isolates were recovered from blood culture (68.5%, 37/54) (Table 1). Detailed information was available for the 28 patients enrolled in the epidemiological surveys (Table 2 and Table S1). The majority were male (64.29%, 18/28) with a median age of 35 years (range 1month- to 74-year-old) and a high proportion of children <15-year-old (32.14%, 9/28). Haematological malignancies were diagnosed in 12/28 (42.86%). Half of the patients was treated with voriconazole alone (50%, 14/28). Global mortality was 46.2% at three months after

TABLE 2 Clinical characteristics of the 28 patients identified in the YEASTS and RESSIF surveys between 2003 and 2018

Characteristic of the patients	No./N ^a	%
Male gender	18	64.29
Mean age in years ± SD	35 ± 26.47	
Age		
<15 years	9	32.14
[15-44 years]	7	25
[45-64 years]	9	32.14
[65-79 years]	3	10.71
>80 years	0	0
Underlying disease		
Haematological malignancies	12	42.86
Lymphoma	4/12	33.33
Acute leukaemia	6/12	50
Others	2/12	16.67
Solid tumours	1	3.57
Other disease	7	25
Surgery within 30 days	6	21.43
Solid organ transplantation	1	3.57
Pre-exposure to antifungal drugs	11	39.29
Pre-exposure to echinocandins	7	25
Initial treatment (first 48 h)		
None	2	7.14
Voriconazole alone	14	50
Caspofungin alone	4	14.29
Lip-AmB alone	4	14.29
Combination	1	3.57
Lip-AmB + Caspo		
Other azoles	3	10.71
Death rate at 3 months	12/26	46.15

Abbreviation: Lip-AmB, liposomal Amphotericin B.

^aN = 28 except when value is indicated.

diagnosis (12/25). The grouped cases involved 2 patients (5 isolates) in hospital #1 and 6 patients (6 isolates) in hospital # 12 (Table 1), with a mixture of superficial and deep-seated infections.

MIC50 and MIC90 were high for flucytosine (32 mg/L and ≥64 mg/L, respectively), fluconazole (4 mg/L and 16 mg/L) and amphotericin B (2 mg/L and ≥8 mg/L) and lower for posaconazole (0.25 mg/L and 0.5 mg/L) and voriconazole (0.06 mg/L and 0.25 mg/L) (Table 1).

4.2 | IGS1 sequencing

Six different IGS1 haplotypes were identified among the 54 isolates studied (Figure 1): 28 isolates were haplotype 1 (51.9%), 12 haplotype 3 (22.2%), 10 haplotype 4 (18.5%), 2 haplotype 7 (3.7%), 1 haplotype 5 (1.9%) and one isolate had an unknown haplotype (CNRMA14.672, GenBank Accession no. MN936175). When looking at the geographic

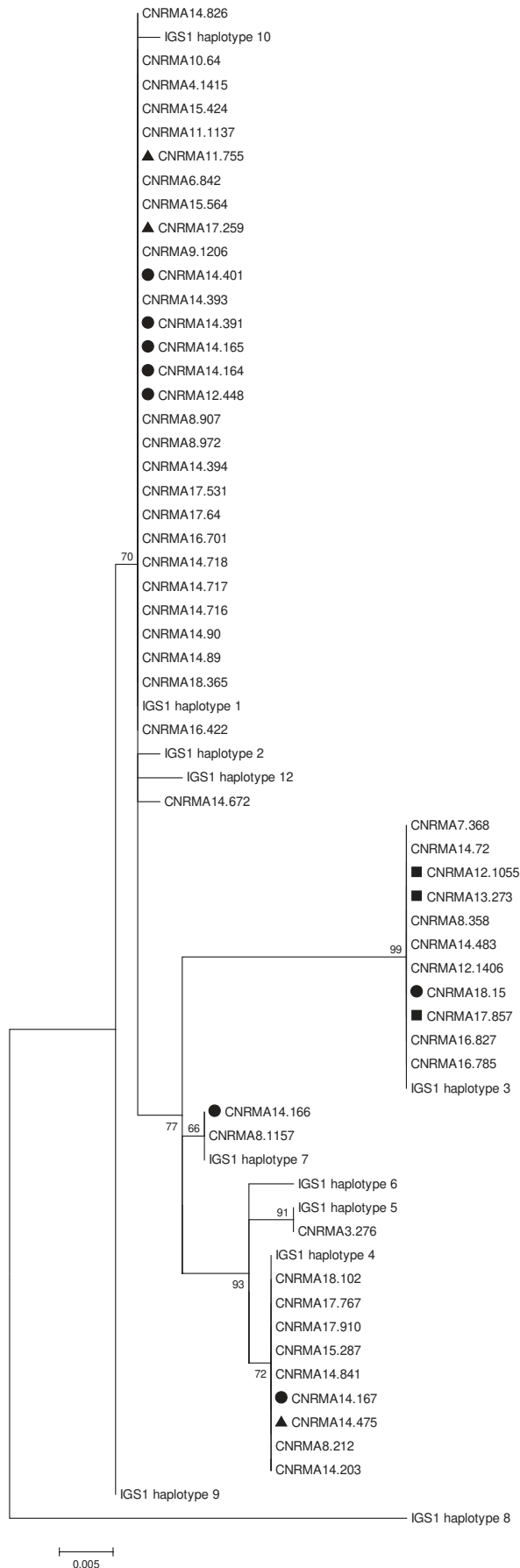


FIGURE 1 Maximum-likelihood tree constructed using matrix alignment (MUSCLE) with MEGA v. 6 for the IGS1 sequence of the 54 clinical isolates, and the 12 haplotypes of IGS1 deposited in public database. Bootstrap values are indicated. Black dots correspond to isolates recovered in South America, Black squares to Caribbean Islands and black triangles to Africa

distribution, the 6 haplotypes were identified among the 40 European isolates with a majority of haplotype 1 (21/40), 3 and 4 (8 isolates each). Haplotype 1 was also predominant in the South American (71%, 5/7) and African (66%, 2/3) isolates studied, while in the Caribbean islands, the 3 isolates were haplotype 3. Minor haplotypes were haplotype 3 in 1/7 South American isolates, haplotype 4 in 1/3 African isolates, haplotype 5 in 1/40 European isolates, and haplotype 7 in 1/40 European isolates and 1/7 South American isolates. The isolates from the same patient shared the same haplotype.

Concerning the two grouped cases (Table 1), the 5 isolates recovered from the two patients in hospital #1 exhibited haplotype 1. In hospital #12, two isolates recovered on the same day from the tracheal aspiration/BAL of patient #38 and the urinary tract of patient #13 were haplotype 4 and haplotype 7, respectively. Five months later, 4 isolates recovered from 4 different patients were all haplotype 1. Of note, an isolate recovered more than a year earlier (pt#32) was also haplotype 1, whereas another isolate responsible for fungaemia (pt#1) 4 years later was haplotype 3.

4.3 | Whole genome sequencing data

To better assess genetic relatedness, WGS was performed on selected isolates including all the grouped cases isolates. The percentage of mapping for isolates with reference genome of CBS 2479 varied between 93% and 99%, confirming the good species identification for all (Table S2). The percentage of GC varied between 55% and 59% for the whole genome. The number of indels was 1,156 for the genome of CBS 2479 re-sequenced in our laboratory and varied between 57,451 and 67,919 for the 32 clinical isolates. The number of SNPs positions was 2,039 in the ‘re-sequenced’ genome of CBS 2479 and varied between 148,294 and 175,236 for the clinical isolates. The Ts/Tv ratio was approximately equal to 2.

A total of 154,051 SNP positions were concatenated, and a multiple alignment was constructed for the 32 clinical isolates using the genome of CBS 2479 as reference. Then, a maximum-likelihood tree with 1,000 bootstraps was constructed using this SNP alignment (Figure 2). The tree included three major clades. The isolates belonging to IGS1 haplotype 4 (n = 6) were found grouped in one clade, while the majority (15/18) of isolates belonging to haplotype 1 composed a second clade. A third clade contained isolates belonging to haplotype 3 (n = 5), haplotype 1 (n = 2), haplotype 7 (n = 2) and the new haplotype. When looking at haplotype 1 isolates, three (CBS2479, CNRMA10.64 and CNRMA12.448) were unlinked while inside the clade containing the majority of them, subclades were observed with isolates sharing more than 98% of SNPs similarity.

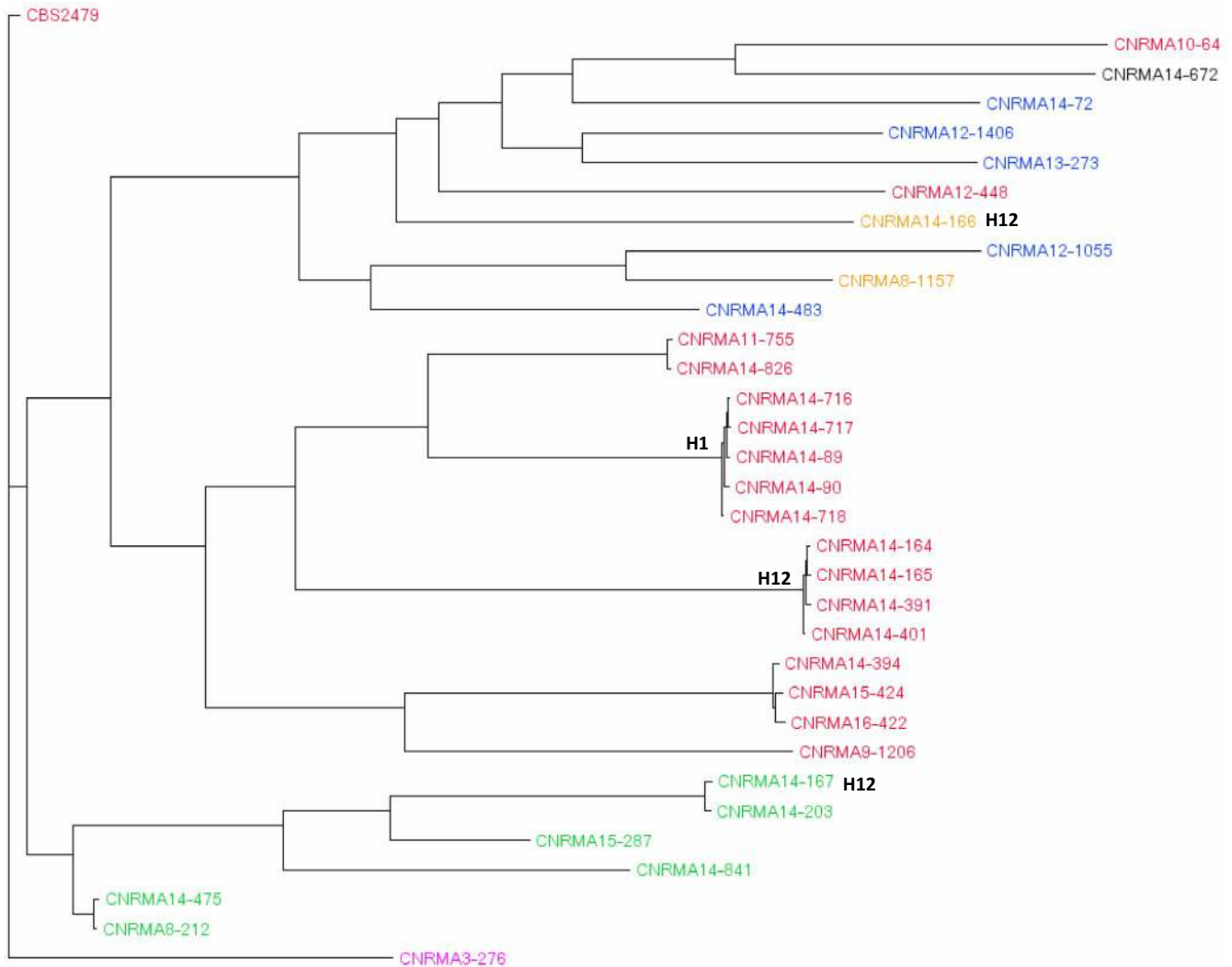


FIGURE 2 Maximum-likelihood tree constructed using matrix alignment, with IQTree, for the whole genome sequences of 32 clinical isolates and the type strain CBS2479 of *Trichosporon asahii*. The isolates previously identified as belonging to a given IGS1 haplotype are shown in red (haplotype 1), blue (haplotype 3), green (haplotype 4), orange (haplotype 7), pink (haplotype 5) and black (new haplotype)

We then analysed the haplotype 1 isolates involved in the grouped cases. For hospital #1, the five isolates recovered from two patients were grouped in a subclade with more than 99% of SNPs similarity. For hospital#12, four isolates were grouped in another subclade with 98.7% of SNPs similarity, while the two other isolates were unlinked with less than 37% of SNPs similarity. The two subclades shared 60% of SNPs similarity.

A maximum-likelihood tree was constructed by using indels information, and similar topology was obtained when compared to the maximum-likelihood tree constructed based on SNPs matrix alignment (data not shown).

5 | DISCUSSION

We took advantage of our assignments as National Reference Center for Invasive Mycoses & Antifungals to assess the usefulness of IGS1 genotyping for the study of the genetic relatedness between isolates

of *T. asahii* recovered during two grouped cases, using our collection of epidemiologically unrelated isolates.

Among the 54 clinical isolates of *T. asahii* analysed here, 28 were received in the context of active epidemiological surveillance programs (YEASTS and RESSIF programs). In a previous study,¹⁴ 19 isolates of *Trichosporon* species (*T. asahii*, *T. inkin*, *T. dermatis*, *T. japonicum*, *T. loubieri*, *T. mucoides*), grouped in the '*Trichosporon* sp.' group, represented 5.9% of the bloodstream infections between October 2002 and December 2014 in the Paris area. These rare species were significantly associated with haematological malignancies and pre-exposure to caspofungin, and the mortality rate was similar to that of patients infected with *C. albicans*. In the present study concerning only *T. asahii*, similar results were obtained with a high percentage (42.86%, 12/28) of patients with haematological malignancies and especially with acute leukaemia (50%, 6/12), followed by patients with recent surgery (21.43%, 6/28). We also found a high proportion of patient under 15 years (32.14%, 9/28) and a high mortality rate at three months (46.15%, 12/26). As reported in the literature,^{12,14,32-34}

all *T. asahii* isolates were intrinsically resistant to flucytosine and less susceptible to fluconazole and amphotericin B while voriconazole had the most potent in vitro activity with MIC₅₀ and MIC₉₀ of 0.06 and 0.25 mg/L, respectively. Voriconazole is the treatment usually recommended for deep-seated trichosporonosis.³⁴

All isolates were genotyped using IGS1 sequencing. The majority (51.9%, 28/54) belonged to IGS1 haplotype 1, which is also the most frequent haplotype found worldwide in clinical isolates. Five other haplotypes were found (haplotypes 3, 4, 5, 7 and one unknown). Interestingly, the 3 isolates recovered in 2 hospitals localised in the Caribbean islands were haplotype 3 which is also the most frequent haplotype in the USA. There was no other evidence of an association between geographic origin and haplotype among the isolates studied here.

The IGS1 sequencing was initially used to investigate grouped cases. In hospital#1, the multiple isolates recovered from two patients belonged to haplotype 1 suggesting a potential common source of infection that was not investigated. In hospital#12, a total of 8 patients were infected with *T. asahii* over 5 years. Two patients (#13 and 38) sampled the same day were infected by isolates exhibiting two different haplotypes (haplotypes 4 and 7), as did the last patient diagnosed 4 years later with haplotype 3 infection. This suggested different sources of infection that could include endogenous ones. The four remaining patients diagnosed over 4 months were infected with haplotype 1 with the hypothesis that a common source of infection could be responsible for these grouped cases. However, given that the majority of the isolates shared haplotype 1 and that it is also the most frequent haplotype worldwide, we were not confident to assert a potential link between isolates. We thus decided to sequence whole genome and selected 32 isolates representative of our collection in terms of the diversity of anatomical, temporal and geographic origins, as well as IGS1 diversity. The genome of the type strain CBS2479 was used as reference and was also re-sequenced.

The GC content can be of interest for the study of the orphan proteins,³⁵ mutation rate, spectrum of mutations.³⁶ The GC content for the reference strain CBS 2479 was 59%, as previously reported and varied between 55% and 59% for the clinical isolates. These values confirm data of the literature showing that Basidiomycetous yeast have usually a GC content higher than Ascomycetous yeast: 48.2% for *Cryptococcus neoformans* var. *grubii*,³⁷ 62% for *Rhodotorula toruloides*³⁶ while *Candida glabrata* and *Saccharomyces cerevisiae* are AT-rich with GC content of 38.8% and 38.3%, respectively.³⁸ The number of SNPs positions for the clinical isolates varied between 148,294 and 174,873. Given the 24 MB size of the genome, the SNPs positions corresponded to 0.62% of the genome. The number of transitions and transversions was calculated with a Ts/Tv ratio of ~2 confirming data obtained by Long et al demonstrating a predominance of transitions relative to transversions for Basidiomycetes.³⁶

The concatenation of the 154,050 SNP positions was used for construction of matrix alignment and of a maximum-likelihood tree. This tree confirmed the results obtained with IGS1 sequencing for the isolates involved in the two grouped cases. Indeed, the five

isolates recovered in hospital#1 shared 99% of SNPs similarity, suggesting a common source of infection. No specific investigation was carried out, but the hypothesis of a transmission through a healthcare worker seemed more likely than inter-patient transmission (since the patients did not share time in the same ward). For the second grouped cases, the four isolates recovered over 4 months had 98.7% of SNPs similarity suggesting also a common source of infection while the isolate recovered 4 years earlier was totally unlinked. In addition, nine isolates were grouped by 2 or 3, with more than 97% of SNPs identical. In one instance, it concerned two haplotype 1 isolates recovered 16 months apart from the same patient (Pt#21) grouped with an isolate recovered from a patient hospitalised 13 months earlier 700 kms away. For the other six isolates, the two pairs of IGS1 haplotype 4 isolates and one pair of haplotype 1 isolates were also uncovered with no temporal or spatial link. Environmental investigations were missing here to identify exogenous source of infection during the grouped cases.

IGS1 sequencing was thus helpful to rule out a common source of infection in several cases like in hospital #8 with 3 different haplotypes for 3 different patients including two (pt#14 and 15) diagnosed a few months apart. Analysis of the SNPs showed that sharing the same IGS1 haplotype does not always mean genetic relatedness (hospital #12) and that isolates can be genetically linked without obvious spatial or temporal relatedness complicating the investigation of grouped cases based only on IGS1 sequencing.

In conclusion, our study suggests that IGS1 sequencing of *T. asahii* is a good tool to investigate grouped cases but it is not sufficient to study the genetic diversity of the species and potential link between isolates. Although the presence of a rare species in several patients in the same hospital over a short period of time suggests a common source of infection, our results show that this is not always the case especially for a species that is part of the normal biota of the skin and/or gastrointestinal tract.

CONFLICT OF INTEREST STATEMENT

Authors have no conflict of interest.

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AUTHOR CONTRIBUTION

Marie Desnos-Ollivier: Conceptualization (lead); Data curation (lead); Formal analysis (equal); Writing-original draft (lead); Writing-review & editing (equal). **Corinne Maufrais:** Data curation (equal); Investigation (equal); Methodology (equal); Software (equal); Writing-review & editing (equal). **Marc Pihet:** Investigation (equal); Writing-review & editing (equal). **Christine Aznar:** Investigation (equal); Writing-review & editing (equal). **Francoise Dromer:** Data curation (equal); Investigation (equal); Methodology (equal); Supervision (lead); Validation (equal); Writing-original draft (equal); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

The sequence of isolate CNRMA14.672 with an unknown IGS1 haplotype is deposited to GenBank (Accession no. MN936175).

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