



## Molecular Epidemiology of *C. neoformans* var *grubii* Clinical Isolates from Western India with Three Novel STs

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### ABSTRACT

**Introduction:** Cryptococcosis is a fungal infection predominantly caused by *Cryptococcus neoformans*. The prevalent genotype VNI of *C. neoformans* has a two-fold higher risk of fatality in cryptococcosis coinfecting with HIV patients.

**Objective:** We have isolated 49 *C. neoformans* from cryptococcosis patients during Apr 2010 to Jun 2015 from different samples. About 26 of these isolates were molecularly typed to identify the prevalent molecular types from Western Maharashtra, India. **Methods:** Out of 47 clinical cryptococcal isolates from suspected cryptococcosis 26 were further subjected to genotyping by Multilocus sequence typing (MLST) method using 8 different house-keeping loci. All 28 DNA sequences were deposited in Genbank under the following accession numbers MF580727 to MF580754.

**Results:** The phylogenetic tree was made to determine the relatedness between the isolates. About 26 *C. neoformans* var *grubii* isolates were divided into two genotypes VNI (%) and VNII (%). The 2 allele numbers for CAP59, SOD1, and URA5, 3 for LAC1 and PLB1 and 4 for GPD1 and IGS1 per gene loci were noted. Based on these allelic polymorphism genotypes were sub-typed into seven sequence types including three novel Sequence types (STs) from the Western part of India. **Conclusions:** Intra-species variation was observed among the isolates from this region of India. The molecular epidemiology from the current study suggests that there is a need to look for an environmental source for these novel STs.

**Keywords:** Molecular epidemiology, *C. neoformans*, Novel STs

**Abbreviations:** MLST: Multi Locus Sequence Typing; ST: Sequence Type

### INTRODUCTION

*Cryptococcus neoformans/gattii* species complex is responsible for a life-threatening infection in immune-compromised as well as in immune-competent individuals [1]. This species complex is divided into five serotypes as serotype A (*C. neoformans* var *grubii*), serotype B and C (*C. neoformans* var *gattii*), serotype D (*C. neoformans* var *neoformans*) and serotype AD (hybrid). Genotypically, it is divided into eight subgroups (genotypes) such as VNI to VNIV of *C. neoformans* and *C. gattii*. Among these genotypes, VNI is the predominant genotype in Southeast Asian countries [1,2]. The molecular epidemiology of the organism has been studied from north India extensively but not from another part of India. Hence this study was undertaken in Department of Microbiology, B.J. Government Medical College and Sassoon General Hospital, Pune, a district of Maharashtra, India to identify the prevalent molecular type as well as a genetic variation among the clinical isolates of *Cryptococcus neoformans* from this region. Multilocus sequence typing (MLST) method was selected for genotyping of *C. neoformans* isolates. The advantage of MLST over other molecular typing methods is that it not only identifies the genotype of *Cryptococcus neoformans/C. gattii* species complex but also discriminate between the same genotype into sequence types. It has more discriminatory power. This is possible due to use of highly polymorphic multiple genetic loci used in MLST technique. MLST gives the exact molecular global epidemiological status of particular *Cryptococcus* ST prevalence.

## PATIENTS AND METHODS

Cerebrospinal fluid and respiratory samples from patients of cryptococcal meningitis or pulmonary infection were followed up to culture *C. neoformans* over a 5 year period from April 2010 to June 2015. Around 47 *C. neoformans* clinical isolates were obtained. These isolates were confirmed to be *C. neoformans* by using conventional physiological and biochemical methods such as growth at 37°C, negative staining for capsule demonstration (ref), urease test (ref), phenoloxidase test, nitrate and inositol assimilation test and serovar typing by using L-canavanine-glycine-bromothymol blue medium [3-5]. Out of 47 isolates, 26 were further genotyped by ISHAM consensus multilocus sequence typing scheme at Center of Advanced Research in Medical Mycology, Department of Medical Microbiology, PGIMER, Chandigarh [6]. For this study, the 8 gene loci were selected as 6 housekeeping genes, virulence factor coding genes such as CAP59 (capsular polysaccharide), GPD1 (glyceraldehyde-3-phosphate dehydrogenase), LAC1 (laccase), PLB1 (phospholipase), SOD1CN (Cu, Zn superoxide dismutase of *C. neoformans*), SOD1 CG (Cu,Zn superoxide dismutase of *C. gattii*), URA5 (Orotidine monophosphate pyrophosphorylase) and one the ribosomal RNA intergenic spacer (IGS1) region.

All the isolates were obtained in pure form on SDA plate for DNA isolation by using Phenol: Chloroform: Isoamyl alcohol method. Conventional PCR was done using the above mentioned 7 genes using the cycling parameters as mentioned by Meyer, et al. [6].

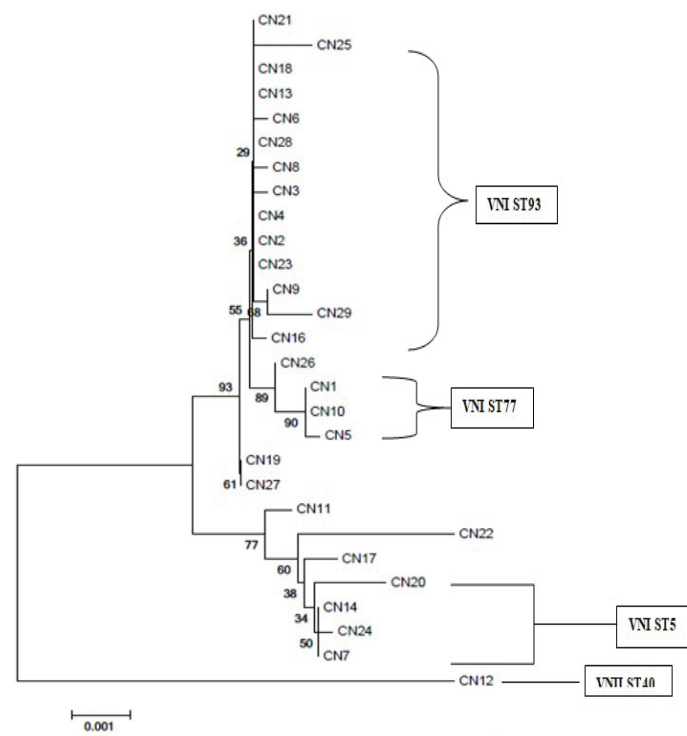
Each amplified fragment was sequenced using Big Dye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) according to manufacturer's guidelines. For each gene fragment, sequencing was carried out for both forward and reverse primers in separate tubes. Sequences were analyzed with an ABI 3130 Genetic Analyser (Applied Biosystems), capillary sequencer. The sequences were aligned for the alleles by using BioNumerics software (Applied Maths, Belgium) and the phylogenetic tree was made using the Neighbour-joining method. After alignment of the sequences for each strand, allelic and sequence type (ST) were assigned for every unique allelic profile. BLASTn (Basic Local Alignment Search Tool) search was carried out for all sequences obtained against NCBI NR (non-redundant) database to find homologous sequences. (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was constructed using MEGA6. The sequences were taken in fasta format and aligned using ClustalW. The best DNA/Protein model was selected using the maximum likelihood method. The model with the lowest BIC (Bayesian Information Criterion) scores was considered to describe the substitution pattern the best. The tree was constructed using the unrooted neighbor-joining method with a bootstrap value of 1000. The numbers at each branch indicate bootstrap values, based on 1,000 replicates. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

## RESULTS

Out of 26 clinical *C. neoformans* (serotype A and AD) isolates, 25 were from CSF and one from sputum sample from 25 patients having Cryptococcosis. The query sequence was aligned with all the sequences deposited in the NCBI database and had an alignment score of  $\geq 200$  which imply that all the sequences were from a single species, *Cryptococcus neoformans* var *grubii*, however, the strains or isolates varied. There were a total of 4257 positions in the final dataset. Maximum likelihood phylogenetic analysis showed these isolates were *C. neoformans* var *grubii* (serotype A) and the predominant genotype was VNI (96.1%, n=25). However a single (3.8%) isolates from CSF (HIV uninfected patient) was of VNI genotype, however, all other strains were from an HIV infected patients.

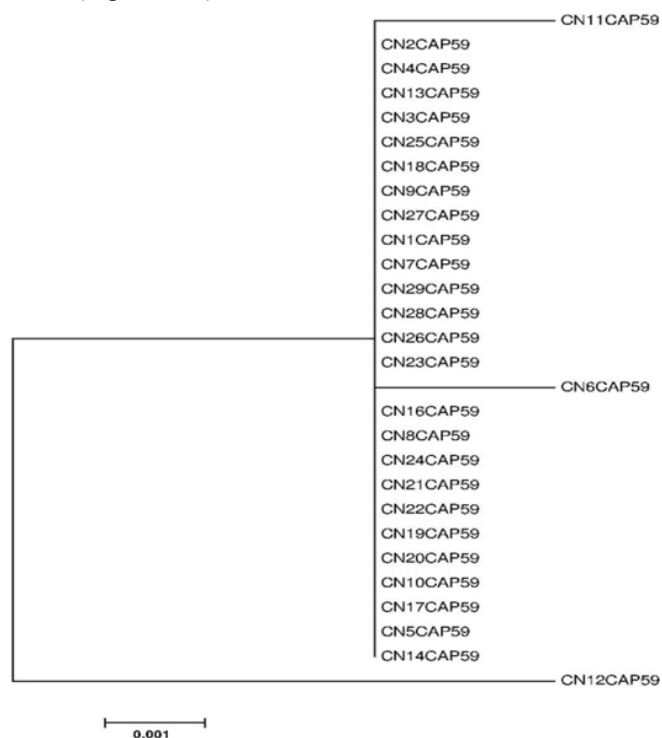
### Phylogenetic Analysis

When all the Indian isolates along with all the genes were aligned for phylogenetic analysis, the analysis showed that CN12 was considered as an out-group. It was different from rest of all the isolates. The tree generated showed two distinct clades, in which isolates CN7, CN11, CN14, CN17, CN20, CN22 and CN24 were clustered together in one clade. This showed that the isolates are closely related. In the other clade, the isolates CN1, CN5 and CN10 were closely related whereas isolates CN9 and CN29 were somewhat similar (Figure 1). All 28 DNA sequences were deposited in Genbank under the following accession numbers MF580727 to MF580754. The sequences of VNI molecular type were similar to the standard reference strain H99 and WM148. Whereas, CN12 (VNII molecular type) was similar to the standard reference strain WM626.



**Figure 1 Molecular phylogenetic analysis of all genes of all isolates**

The sequences of all the housekeeping genes and other isolates obtained from the database were submitted to MEGA6 software. The sequences were aligned and a phylogenetic tree was generated for the same (Figures 2-15). Intraspecies genetic diversity has been observed (Figures 2-8).



**Figure 2 Phylogenetic rectangular tree of CAP59 sequences of our isolates. It shows two clades were formed: all the isolates in one clade and CN12 in another clade as an out-group. CN12 belongs to VNII whereas all other grouped as VNI. CN6 and CN11 strains are different from the rest.**

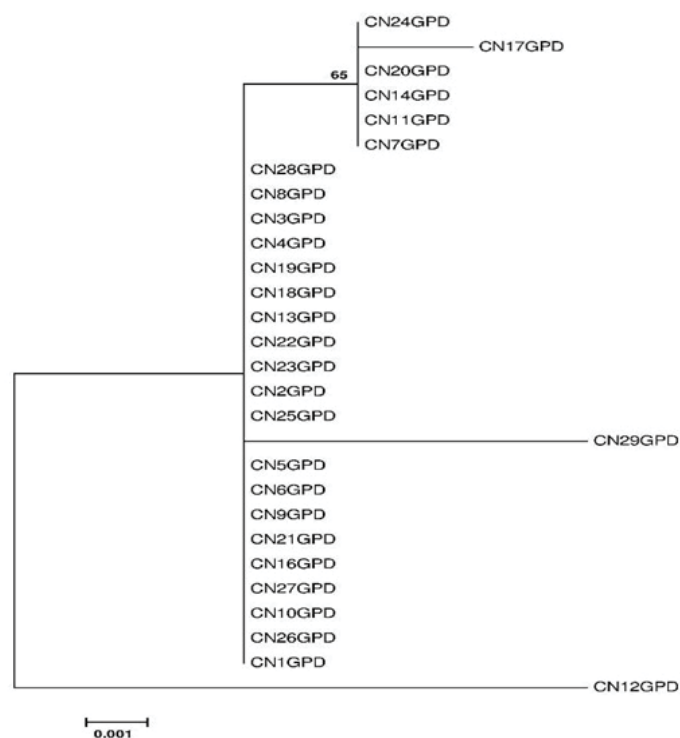


Figure 3 Phylogenetic rectangular tree of GPD sequences of our isolates. Three clades were obtained. The isolate CN12 was generated as an out-group. CN29 and CN17 were different. CN24, CN20, CN14, CN11, and CN7 belonged to one group.

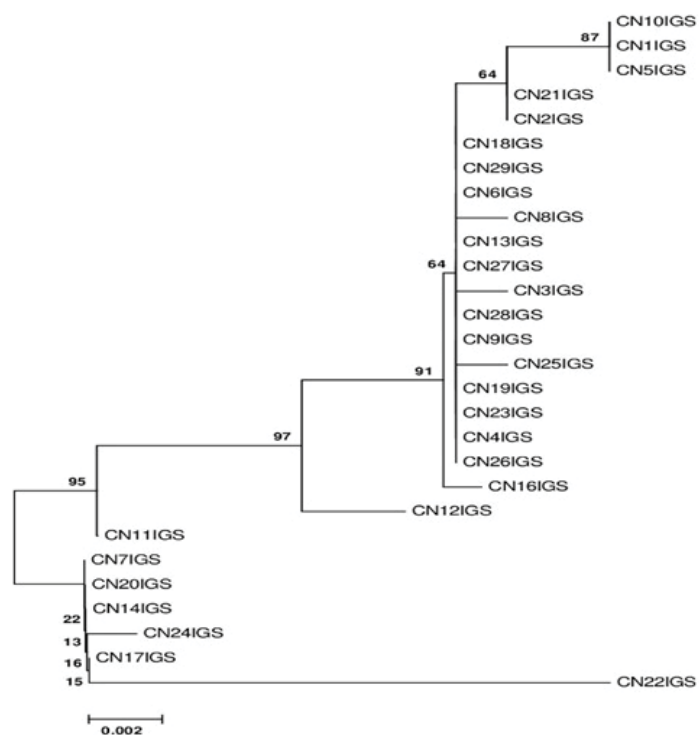


Figure 4 Phylogenetic rectangular tree of IGS sequences of our isolates. It shows two major clades were formed in this tree viz: 1) CN7, 20, 14, 24, 17 and rest in another clade. CN22 is different from these two clades. Similarly, CN24, which is a sputum sample, is different from the rest of clinical sources samples.

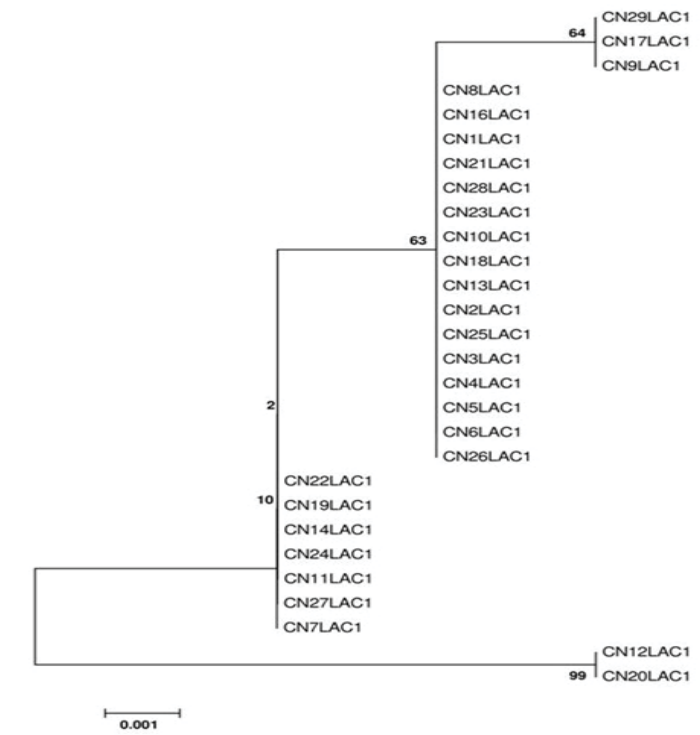


Figure 5 Phylogenetic rectangular tree of LAC1 sequences of our isolates. It shows three clades formed. CN12 and CN20 were different and considered as out-group in this tree. CN29, 17 and 9 different from rest and were included in another clade.

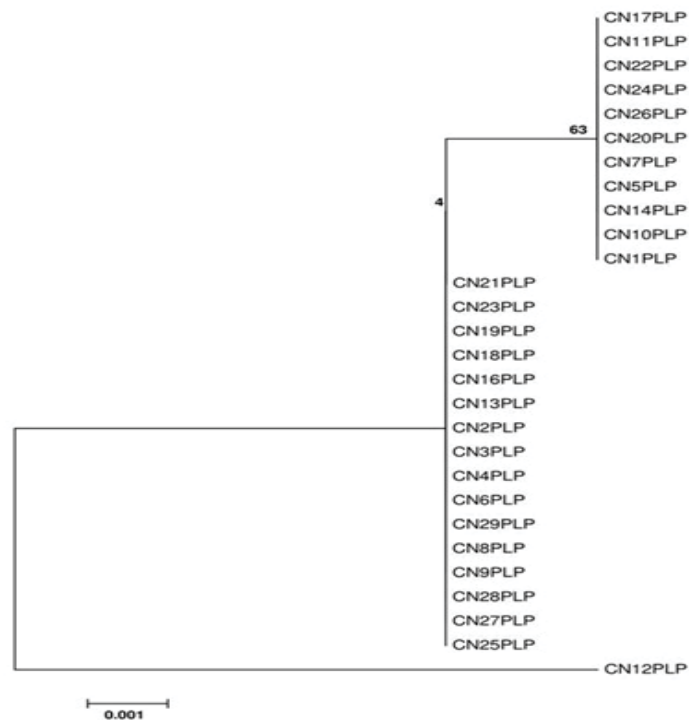


Figure 6 Phylogenetic rectangular tree of PLP1 sequences of our isolates. It shows two clades formed. CN12 showed as an out-group and CN24 is similar to CN26 which is a blood sample isolate.



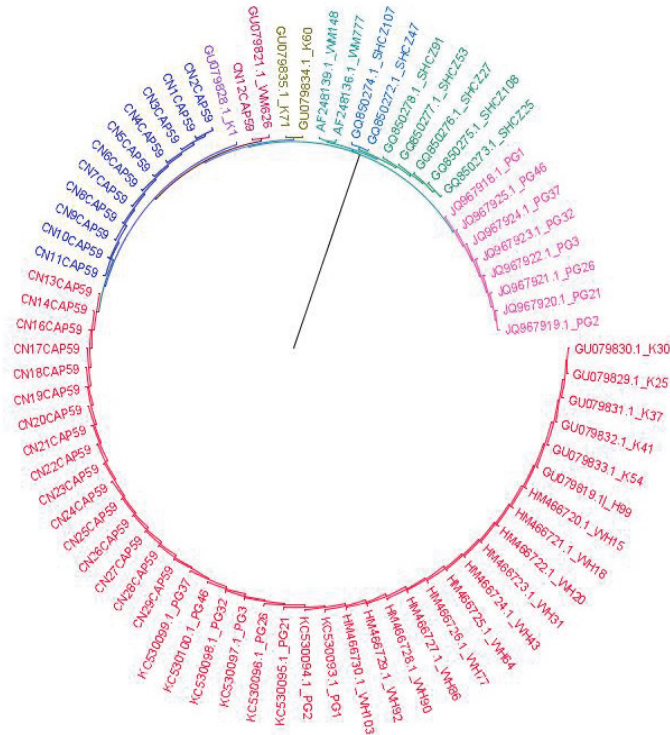
**Figure 7** Phylogenetic rectangular tree of SOD1 gene sequences of our isolates. It shows two clades, CN12 showed as an out-group. CN5 is different as compared to other strains.



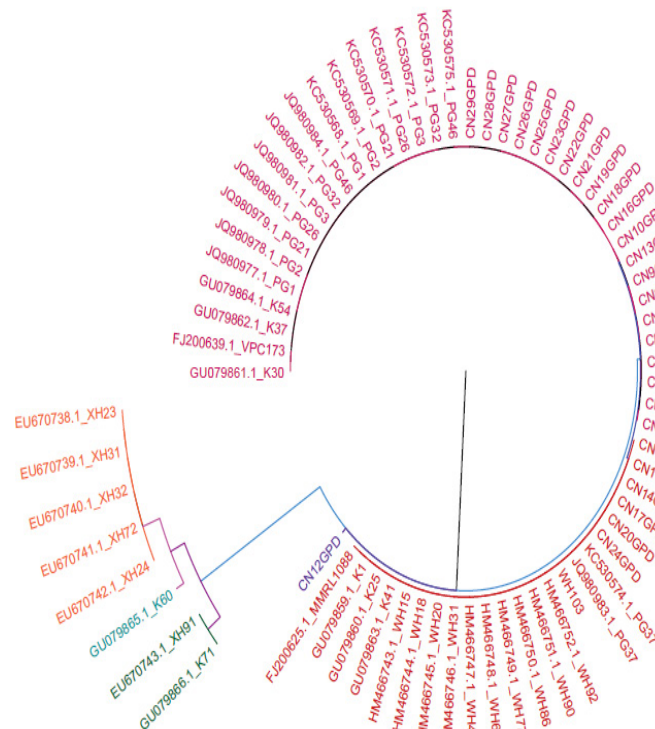
**Figure 8** Phylogenetic rectangular tree of URA5 sequences of our isolates: It shows two clades formed. CN12 showed as an out-group. All the other sequences were similar in one clade

The Indian isolate sequences were clustered in one group and were closer to the sequences from Indian strains (PG, Chandigarh)

and Republic of Korea (Kr). The CN12 sequence was closer to *Filobasidiella neoformans* strain Korean isolates (Figures 9-15).



**Figure 9** Radial phylogenetic tree of CAP59 with other Asian isolates. All sequences were in one clade. CN12 isolate sequence was closer to WM626 isolate while CN2 and few isolates were closer to the K1 strain of Korean clinical isolate (Choi YH, 2010)



**Figure 10** Radial phylogenetic tree of GPD1 with other Indian and Asian isolates. It shows two distinct clades. The Indian isolate sequences were clustered in one group and were closer to the sequences from Indian strains (PG, Chandigarh) and







formed. CN12 and CN20 strain sequences were also similar to the K1 strain sequences. LAC1 sequences of most of the isolates matched with PG strains from Chandigarh, India

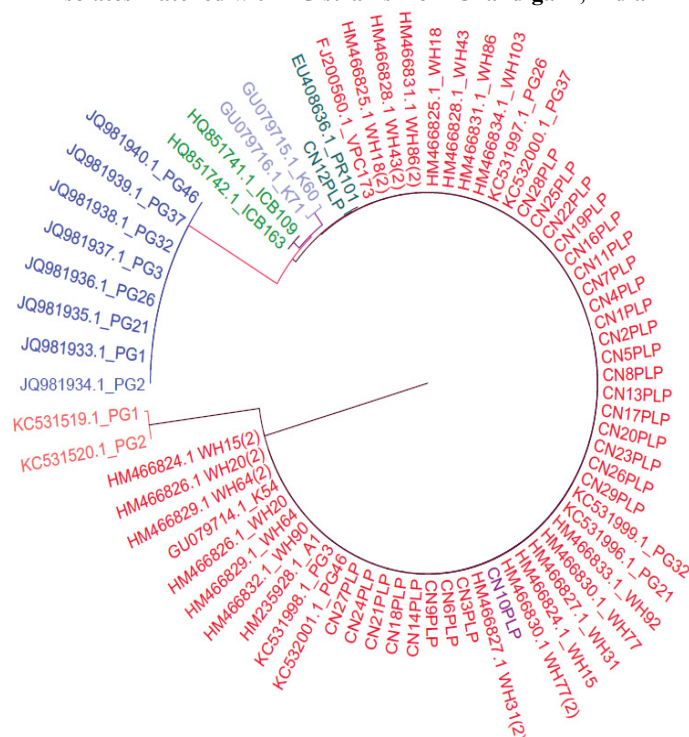


Figure 13 Radial phylogenetic tree of PLP1 gene sequences with other Indian and Asian isolates. It shows two clades were formed. CN10 matches with Chinese isolates WH strains. CN12 which out-group matches with Indian isolate PR101, Korean K60, and K71 strain's sequences

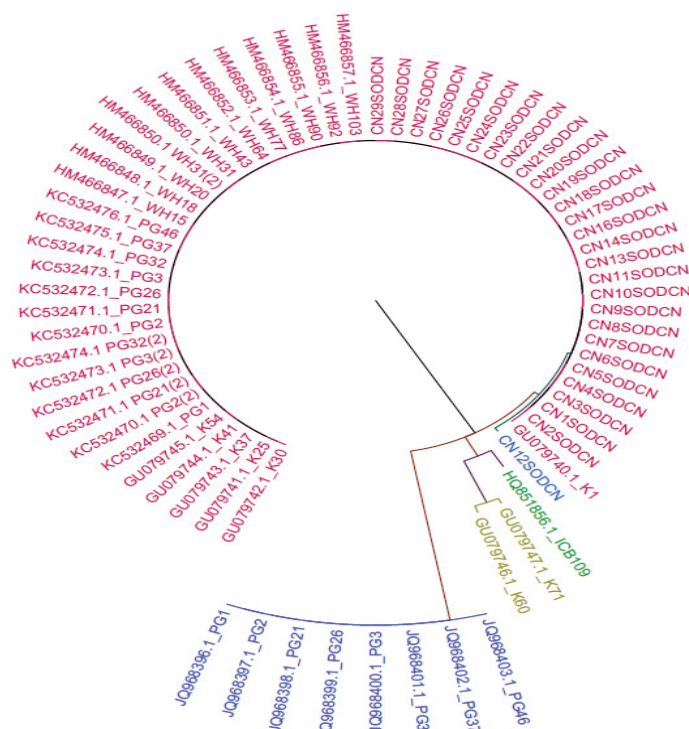
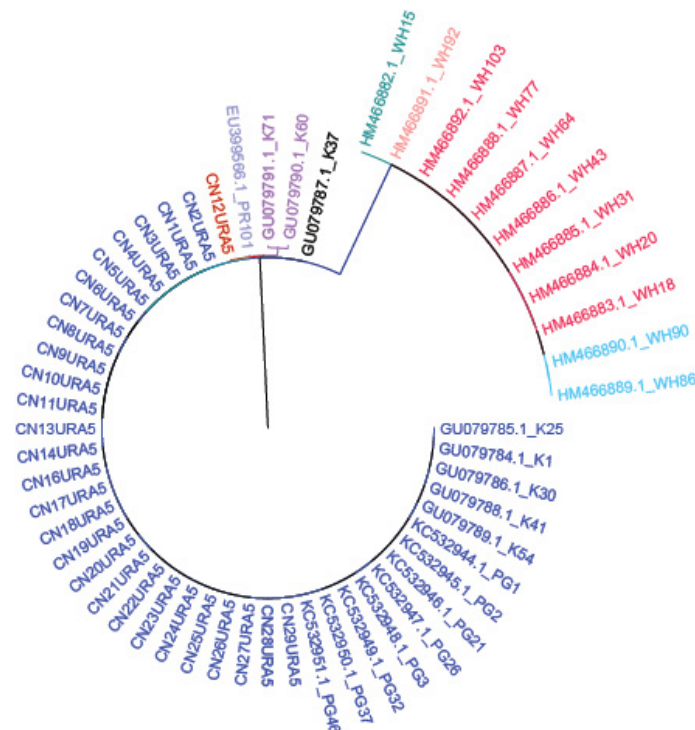


Figure 14 Radial phylogenetic tree of SOD1 gene sequences with other Indian and Asian isolates. It shows two clades,

most of the sequences are similar to Korean and Indian strain's sequences. CN12 sequence match with Chinese and Korean strains



**Figure 15** Radial phylogenetic tree of URA5 gene sequences with other Indian and Asian isolates. It shows two clades were formed. Most of the sequences of our isolates are similar to PG (PG1-3, 21, 26, 30, 32, 46) and K37 strain sequences. CN12 sequence matches with PR101 Indian strain and is also closer to K1 strain

Most of the *C. neoformans* isolate's sequence type clustered in one group belongs to sequence type (ST) 93 (53.8%) followed by ST5 (19.2%) and ST77 (11.5%) (Table 1). There were 2 (CAP59, SOD1, and URA5) to 4 (GPD1 and IGS1) alleles numbers per gene loci noted which gave 7 different sequence types.

**Table 1** Genotyping of *C. neoformans var grubii* isolates with sequence types (STs)

Molecular Type	ST	Source	Identification matching %	Total n=28 N (%)
VNI	93	CSF	99.8-100%	14 (53.8) %
VNI	5	CSF	99.79-100%	5 (19.2) %
	-	Sputum	99.88%	-
VNI	77	CSF	99.87-99.98%	3 (11.5) %
VNI	32	CSF	99.9%	1 (03.8) %
VNI	53	CSF	99.84%	1 (03.8) %
VNI	81	CSF	99.4%	1 (03.8) %
VNII	40	CSF	99.79%	1 (03.8) %

## DISCUSSION

The genotyping of *C. neoformans/C. gattii* species complex is useful for epidemiological purposes. Clonality would indicate a common source. The spread of the organism from country to country or continent to continent can also be appreciated. It is thus essential for the development of strategies will be helpful for its surveillance and prevention.

The current study for the first time provides the molecular epidemiology of *C. neoformans var grubii* strains from patients from Western Maharashtra, India. Very few reports are there of genotyping of *Cryptococcus neoformans* and *C. gattii* from India however extensive studies have been there from other Asian countries and rest of the world. All

the obtained sequences of isolates from the current study were similar to other Indian PG (Chandigarh) and Korean strains.

Predominant molecular type detected was VNI (96.1%), however, a single isolate from (3.8%) CSF was of VNII genotype from (Table 1). All these patients were HIV positive except one among VNI molecular type, sequences of isolates such as CN7, CN11, CN14, CN17, CN20, CN22, CN24, and CN29 were different from the rest of the VNI sequences (Figure 1). The intra-species genetic difference was seen when the phylogenetic rectangular tree was drawn of each gene (Figures 2-8).

The sequences of VNI molecular type are similar to the standard reference strain H99 and WM148. Whereas CN12 (VNII molecular type) was similar to the standard reference strain WM626.

Phylogenetic analysis reveals that most of the sequences shared a common relationship with the Asian strains shown particularly by the Korean strains of *C. neoformans* var *grubii* (K61 and K71) (Figures 9-15). MLST genotyping method has not been used previously from other of India. Chowdhary, et al., in 2012 and Duggal, et al., in 2014 from north India have reported all the clinical isolates of *C. neoformans* were VNI/AFLP1 molecular type. Chowdhary, et al., have also done molecular typing of *C. gattii* and all were VGI/AFLP4. These reports indicate homogeneity among the north Indian isolates [7,8].

An earlier study of molecular typing of *Cryptococcus* isolates from Delhi by Jain, et al., in 2005 found 89.5% were of VNI/AFLP1 molecular type, 1.7% VNIV/AFLP2 (*C. neoformans* var. *neoformans*, serotype D) and 8.8% VGII (*C. gattii*, serotype B, and C). No homogeneity has been observed in this study among their clinical isolates [9]. The prevalence of molecular types VNI and VNII of *C. neoformans* from clinical isolates were reported globally is as shown in Table 2.

**Table 2 Distribution of *C. neoformans* var *grubii* molecular type VNI and VNII in Asian vs. rest of the world**

Genotype	Asian Countries	%	Rest of the world:	%
VNI	Malaysia (Tay ST et al, 2006) [10]	84.6%	Australia, New Zealand Papua New Guinea(Cogliati M, 2013) [18]	27.0%
	China (Feng X et al, 2008) [11]	89.6%	Brazil (Trilles L et al, 2008) [19]	64.0%
	Japan (Mihara T et al, 2013) [12]	91.4%	South Africa ((Beale MA et al, 2015) [20]	76.0%
	Taiwan (Tseng HK et al, 2013) [13]	94.6%	Brazil (Martins LMS et al, 2011) [21]	86.5%
	Thailand (Kaucharoen S et al, 2013)[14]	94.6%	Italy (Cogliati M et al, 2013b) [22]	92.4%
	Thailand (Simwami SP et al, 2011)[15]	94.8%	-	-
	Present study, India (2016)	96.3%	-	-
	Korea (Park SH et al, 2015) [1]	97.1%	-	-
	Taiwan (Liaw SJ et al, 2010) [16]	98%	-	-
	Korea (Chau TT et al, 2010) [17]	100%	-	-
	India (Duggal S et al, 2014) [8]	100.0%	-	-
VNII	Taiwan (Liaw SJ et al, 2010) [16]	1.0%	Colombia (Cogliati M et al, 2013) [18]	1.6%
	India (Khayhan K et al, 2013) [2]	1.60%	Brazil (Martins LMS et al, 2011) [21]	4.3%
	Taiwan (Tseng HK et al, 2013) [13]	1.8%	Italy (Cogliati M et al, 2013b) [22]	5.6%
	Thailand (Simwami SP et al, 2011) [15]	2.0%	Australia, New Zealand Papua New Guinea(Cogliati M et al, 2013) [18]	6.9-7.0%
	Thailand (Kaucharoen S et al, 2013) [14]	2.1%	South Africa (Beale MA et al, 2015) [20]	20.0%
	India (Present Study, 2016)	3.6%	-	-
	Malaysia((Tay ST et al, 2006) [10]	5.1%	-	-
	Japan (Mihara T et al, 2013) [12]	8.6%	-	-

Other than Asian countries multiple genotypes of *C. neoformans* and *C. gattii* have been reported by researchers such as VNIIc, VNB, VNII, VNIII, VNIV, VGI, VGII, VGIIa, and VGIII (Table 3).

Table 3 Other molecular types reported globally (in %)

Variables	VNIc	VNIB	VNIII	VNIV	VGI	VGII	VGIIa	VGIII	VGIV
India (Jain N et al (2005) [9]	-	-	-	1.7%	-	8.8%	-	-	-
Thailand (Kaocharoen S et al , 2013) [14]	-	-	3.1%	-	0.3%	2.1%	-	-	-
Republic of Korea (Choi YH, et al 2010) [23]	89.7%	-	-	-	-	-	-	-	-
European countries (Cogliati M, 2013a) [18]	-	-	18.5%	18.3%	3.4%	0.3%	0.1%	0.1%	-
N.E Brazil (Martins LMS et al,2011) [21]	-	-	-	-	-	8.1%* and 80.8%**	-	-	-
American Continent (Trilles L et al, 2008) [19]	-	-	-	3%	3%	21%	-	4%	-
Sub Saharan Africa (Litvintseva AP, 2005) [24]	-	-	-	-	-	-	-	-	4%*
S. Africa (Beale MA et al, 2015) [20]	-	4%	-	-	-	-	-	-	-

\*From HIV infected cryptococcal meningitis; \*\*From non-HIV infected cryptococcal meningitis

An interesting finding was observed by Aminnejad, et al., among the 4 clinical strains that, a mixed genetic pattern of *C. neoformans* and *C. gattii* species. The 3 strains were VNI/VGII and one VNI/VGI. These were from Brazil (2 nos.), one each from Colombia and India [25].

Though VNI is a globally common molecular type responsible for cryptococcosis, it has been reported predominantly from most of the Asian countries as compared to other countries. It has a two-fold higher risk of fatality in cryptococcosis co-infected with HIV [7]. VNII molecular type has been reported less frequently from Asian countries (Table 2).

MLST profiling of this 26 VNI and one VNII was studied on MLST scheme of ISHAM. Seven sequence types (STs) in 3 major clusters were found (Figure 1).

We have identified 7 MLST STs among 26 *C. neoformans* var. *grubii* isolates from Western India based on the allele polymorphism. There were 2 allele types in CAP59, SOD1 and URA5, 3 in LAC1 and PLP1 and 4 in GPD1 and IGS1 alleles numbers per gene loci were noted which gives 7 different sequence types.

We found that the predominant sequence type was ST93 (53.6%) followed by ST5 (17.8%) (ST77) (Table 4). Similar predominant *C. neoformans* ST was reported previously by Khayhan, et al., in Indian and Indonesian isolates [2]. However, in the present study, 3 novel STs were detected which have not been detected previously in India. Also, VNII molecular type showed an unusual type of ST40.

- **ST5:** This is the first report in which high frequency of sequence type ST5 was observed as the second most predominant ST from India which is the South Asian country. Other countries from South Asia the molecular epidemiology have been not reported. It has been earlier reported by East Asian countries like China, Japan, Hong Kong and South Korea [2,26].
- **ST32:** About 3.8% reported in the present study. Previously it has been reported by Mihara, et al., from Japan in a clinical isolate from a patient of cryptococcal meningitis without HIV [12].
- **ST81:** In the present study, 3.8% were reported for cryptococcal meningitis isolates. It is an Asian origin strain first time reported by Simwami, et al., from Thailand and later by Cogliati, et al., from an Italian clinical isolate [15,22].

These 3 new STs detected in the present study could be due to the dispersal of these organisms in the environment by migratory birds.

Table 4 Predominant ST reported from various studies

Country	Predominant ST	%
Thailand (Khayhan K et al, 2013) [2]	ST4	41.9%
China (Khayhan K et al, 2013)(872) [2]	ST5	86%
Hong Kong (Khayhan K et al, 2013) [2]	ST5	85.7%
China (Fan X et al, 2016) [26]	ST5	89.2%
Korea((Park SH et al, 2015) [1]	ST5	96.0%
South Africa (Beale MA et al, 2015) [20]	ST69	14.0%
	ST93	14.0%

Indonesia (Khayhan K et al, 2013) [2]	ST93	35.0%
India (Khayhan K et al, 2013) [2]	ST93	47.5%
Present Study (2016)	ST93	53.8%

High genetic diversity among the cryptococcal isolates from south/south-east Asian countries has been reported in the literature [1,2,14]. In the present study, we have reported even in a small number of isolates (26 isolates), 7 STs including the three novel STs and a rare ST40. VNII/ST40 strain has earlier been reported by Khyahan, et al., from India (1.6%) and Thailand (3.5%) by Kaocharoen, et al., [2,14]. However, it has not been identified from other Asian countries. Beale, et al., have reported 13% ST40 strains from South Africa [20].

Sanchini, et al., have reported vast genetic diversity in *C. neoformans var neoformans* isolates (10 STs among 15 isolates) than in *C. neoformans var grubii* (16 STs in 78 isolates) from Germany [27].

From the above studies, it was found the worldwide predominant genotype is VNI. Its occurrence was comparatively less in other countries than in Asian countries where various genotypes of *C. neoformans* have been observed. MLST results of the current study are consistent with results reported from India and other Asian countries. The prevalent sequence type in the present study observed was ST93. This observation was similar to that reported from Chandigarh, India, and Indonesia. [2] Meyer, et al., suggested that gene loci coding for cryptococcal virulent factors (CAP59, LAC1, and PLP1 and IGS) were selected based on high allelic diversity, however, in the present study GPD1 and IGS1 showed high allelic diversity and thus this can be added for screening of isolates [6]. The intra-species genetic difference was also seen so this implies that there was clonal expansion among the VNI sequences.

MLST has been extensively used to analyze the evolutionary relatedness between isolates. The change in the molecular epidemiology of Indian isolates has been observed in the present study as mentioned that migratory birds may visit India based on the climate and may spread the strain from another part of the world in the local environment. An extensive study on an ecological survey to find out the reservoirs enable to understand the population structures in and around Pune located in Western Maharashtra, India.

### CONCLUSION

Nowadays increase in immunocompromised patients with chronic underlying diseases/disorders led to an increase in the risk of invasive fungal infections like cryptococcosis. The prevalent genotype was found VNI and in sequence types ST93 from this Western India region. The current study has enlightened on the clonal diversity of *C. neoformans var grubii* isolates by reporting three novels STs. Therefore, the molecular epidemiological study needs to be carried out in clinical as well as environmental isolates to find out the source of the organism.

### DECLARATIONS

#### Conflict of Interest

The content submitted to the journal is not submitted to the journal or similar content under my authorship is not submitted for consideration or published elsewhere.

#### Human and Animal Rights Policy

After approval from the B.J. Government Medical College and Sassoon General Hospital, Pune Institute ethical committee, the actual project started with an enrolment of patients. Informed consent was taken from the enrolled subjects.

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