MOLECULAR EPIDEMIOLOGY OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *CRYPTOCOCCUS NEOFORMANS* FROM TAMIL NADU, INDIA

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Abstract

The genetic relatedness of twelve clinical and five environmental isolates of *Cryptococcus neoformans* var. *neoformans* from the Indian state Tamil Nadu was analysed by PCR-fingerprinting and Randomly Amplified Polymorphic DNA (RAPD). Both typing methods separated the isolates into three major molecular types VNI, VNII and VNIII. The majority of the isolates belonged to molecular type VNI. Cluster analyses of the isolates using *GelCompar* II revealed a high degree of homogeneity (77%), with a similarity of 88% among the isolates of molecular type VNI. Ten of the 12 clinical isolates, from HIV positive hosts, were found to be of molecular type VNI.

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Introduction

Cryptococcus neoformans is an encapsulated, basidiomycetous yeast that is pathogenic to both humans and animals. While there is a significant incidence of cryptococcal infection in apparently immunocompetent persons, cryptococcosis predominantly affects those that are immunocompromised, as a consequence of organ or tissue transplantation, high dose steroid therapy, cancer or Human Immunodeficiency Virus (HIV) infection [1, 2]. Infection with *C. neoformans* typically occurs by inhalation, and initially affects the lungs. Dissemination to the central nervous system (CNS) results in life-threatening meningoencephalitis [1].

Currently there are two varieties of *C. neoformans* accepted: variety *neoformans* (serotypes A, D and A/D), and variety *gattii* (serotypes B and C). Recently it has been recommended that there is sufficient genetic distinction between serotypes A and D, that serotype A should be given a separate varietal status as *C. neoformans* var. *grubii* [3]. Under this system of classification it is unclear where the serotype A/D should be placed. The present epidemiological study and those done previously have the original classification of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*.

The two varieties of *C. neoformans* differ ecologically and epidemiologically. Variety *neoformans* has a worldwide distribution. It is most frequently isolated from pigeon droppings, but has also been isolated from soil, wood and trees [2, 4, 5]. In contrast, *C. neoformans* var. *gattii* is mostly limited to tropical and subtropical regions and has a known association with trees, notably eucalypts such as *Eucalyptus camaldulensis*, *E. tereticornis*, *E. rudis*, *E. gomphocephala* and *E. blakelyi* [6–9], Almond trees (*Terminalia catappa*) [10], Pottery trees (*Moquilea tometosa*) [11] and decaying wood. Variety *neoformans* predominantly affects immunocompromised persons while *C. neoformans* var. *gattii* nearly always infects apparently immunocompetent hosts [12, 13].

Previous studies of *C. neoformans* in India have shown that isolates of *C. neoformans* var. *gattii* obtained from several cities in the North Indian state of Punjab, were acquired from a variety of eucalypt species where either the tree or the seedlings from which the tree was grown, could be traced back to Australia [4]. Another study,

following up the infection of a patient from Vellore in the South Indian state of Tamil Nadu, found that 86 samples taken from *Eucalyptus* trees, leaves or debris, yielded no isolates of *C. neoformans* [14].

A variety of molecular techniques have been used to study the epidemiology of *C. neoformans*. These include electrophoretic karyotyping [13], Restriction Fragment Length Polymorphisms (RFLP) [16], DNA-fingerprinting [17, 18], Randomly Amplified Polymorphic DNA (RAPD) [20–24] and PCR-fingerprinting [21, 24–26]. These methods are reproducible, rapid and technically simple, making them powerful tools for large-scale epidemiological studies.

It has been demonstrated that PCR-fingerprinting and RAPD analysis discriminate between strains of *Cryptococcus neoformans*. In recent studies, eight major molecular types have been identified based upon the major bands present in typical profiles generated by PCR-fingerprinting or RAPD analysis [21]. For *C. neoformans* var. *neoformans* these molecular types correlate with the serotype: VNI and VNII correspond to serotype A, VNIII to serotype A/D, and VNIV to serotype D. For *C. neoformans* var *gattii* a correlation between molecular type and serotype has not been observed. Molecular types VGI, VGII, VGIII and VGIV contain both serotypes B and C. Subtypes within each molecular type may be determined by minor bands that are present or absent in each profile [21, 24].

Knowledge of the relationship between strains of an organism is useful in the investigation of its pathogenicity and evolution. Recent work on the molecular epidemiology of *C. neoformans* has demonstrated genetic variation between isolates. There appears to be a high degree of heterogeneity among isolates from the U.S.A. [21, 27]. In comparison, isolates from most other areas of the world, including Australia, show very little genetic diversity, possibly with some clonality. The reasons for the high genetic variation of isolates from the U.S.A. compared with those examined from other areas of the world are not understood.

The purpose of this study was to determine the genetic diversity within a group of *C. neoformans* var. *neoformans* isolates obtained from the Indian state Tamil Nadu, using PCR-fingerprinting and RAPD analysis.

Materials and Methods

Fungal isolates

Seventeen isolates from Tamil Nadu previously identified as *C. neoformans* with the VITEK YBC card (bioMérieux Vitek, Inc. Anglum, MO, U.S.A.) were provided by the Post Graduate Institute of Basic Medical Sciences, Department of Microbiology, University of Madras, Chennai (Madras), India. Twelve of the 17 isolates were obtained from the cerebrospinal fluid of immunocompromised patients (10 of 12 have been isolated from HIV positive patients), while five were isolated from pigeon droppings. A set of standard strains representing each of the major molecular types for *C. neoformans* var. *neoformans* was amplified in parallel. Table 1 lists all strains used in the study. Fig. 1 illustrates the geographic relationships of the isolates.

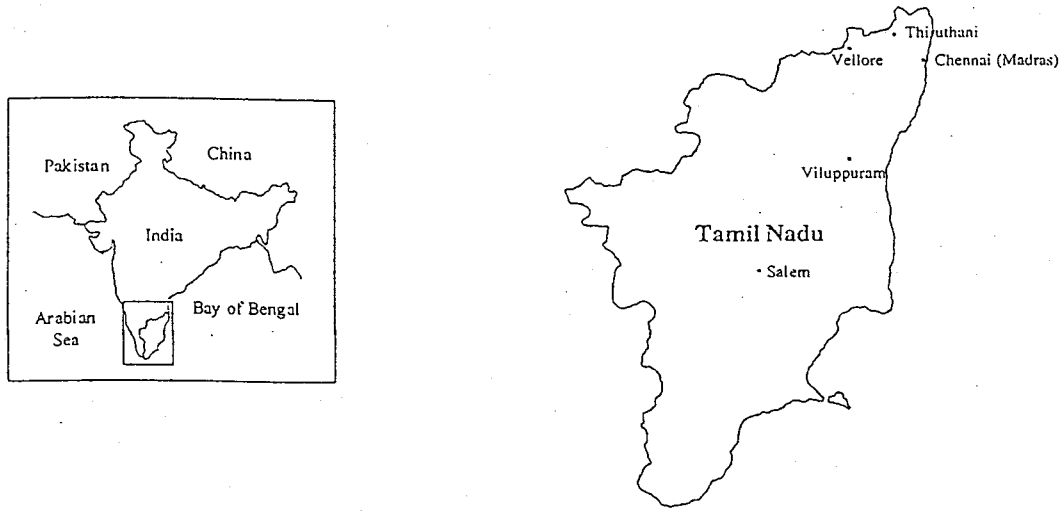

Figure 1. Map of the Indian state Tamil Nadu, showing the cities in which the *C. neoformans* strains were isolated.

Isolation of high molecular weight genomic DNA

High molecular weight genomic DNA was extracted from the isolates, using a method described previously [26]. Briefly, *C. neoformans* isolates were grown on Sabaroud's Dextrose Agar (SDA) at 37°C for two days. A loopful of cells from the culture was mixed with deionized water and centrifuged for 15 min. at 14,000 rpm. The supernatant was discarded and the tube containing the yeast cell pellet was frozen in liquid nitrogen. The frozen cells were ground using a miniature pestle. An extraction solution containing 100 mg triisopropylnapthalene sulfonic acid, 600 mg *para*-aminosalicylic acid, 10 mL deionized water, 2.5 mL extraction buffer (1 M Tris-HCl, 1.25 M NaCl, 0.25 M EDTA, pH 8.0), and 7.5 mL phenol saturated with Tris-EDTA, was preheated to 55°C and 700 µL was added to the frozen, ground cells. The tubes were incubated for 2 min. at 55°C, shaken occasionally; 500 µL of chloroform was added to each tube and then incubated for a further 2 min. at 55°C, again with occasional shaking. The tubes were centrifuged for 10 min. at 14,000 rpm and the aqueous phase was removed and transferred to a new tube. 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube, shaken for 2 min. at room temperature and centrifuged at 14,000 rpm for 10 min. The aqueous phase was transferred to a new tube. 500 µL of chloroform was added and again shaken and centrifuged as in the previous step. The aqueous phase was transferred to a clean tube. To precipitate the genomic DNA, 0.03 volumes of 3.0 M sodium acetate (pH 5.2) and 2.5 volumes of cold 96% ethanol were added to the tube, shaken gently and incubated at -20°C for at least one hour or overnight. The solution was centrifuged for 30 min. at 14,000 rpm to pellet the DNA. The DNA pellet was washed with 70% ethanol and centrifuged for 10 min. at 14,000 rpm and then air dried, resuspended in 200 µL deionised water at 4°C, then stored at -20°C.

Table 1. List of *Cryptococcus neoformans* var. *neoformans* strains used in the study

No.	Lab No.	Origin	Comments	MOLECULAR TYPE [SUBTYPE]
Standard *C. neoformans* var. *neoformans* strains				
1	WM148	Sydney, Australia	Clinical	VNI Standard
2	WM626	Melbourne, Australia	Clinical	VNII Standard
3	WM628	Melbourne, Australia	Clinical	VNIII Standard
4	WM629	Melbourne, Australia	Clinical	VNIV Standard
***Cryptococcus neoformans* var. *neoformans* from the Indian state Tamil Nadu**				
5	TBS 19	Viluppuram	Clinical, HIV+	VNI [d]
6	TBS 28	Chennai (Madras)	Clinical, HIV+	VNIII [a]
7	TBS 54	Chennai (Madras)	Clinical, HIV+	VNIII [b]
8	TN/ENV/1 = WM720	Chennai (Madras)	Environmental, pigeon droppings	VNI [a]
9	TN/ENV/2 = WM721	Chennai (Madras)	Environmental, pigeon droppings	VNI [a]
10	TN/ENV/3 = WM722	Chennai (Madras)	Environmental, pigeon droppings	VNI [a]
11	TN/ENV/4	Vellore	Environmental, pigeon droppings	VNI [c]
12	TN/ENV/7	Vellore	Environmental, pigeon droppings	VNI [c]
13	PR 2	Thiruthani	Clinical, HIV+	VNI [c]
14	PR 12	Chennai (Madras)	Clinical, HIV+	VNI [c]
15	PR 13	Chennai (Madras)	Clinical, HIV+	VNI [b]
16	PR 15	Chennai (Madras)	Clinical, HIV+	VNI [b]
17	PR 16	Chennai (Madras)	Clinical, HIV+	VNI [b]
18	PR 18	Chennai (Madras)	Clinical, HIV+	VNI [b]
19	PR 20	Chennai (Madras)	Clinical, Kidney transplantation	VNI [b]
20	PR 25	Chennai (Madras)	Clinical, Hodgkin's lymphoma	VNI [d]
21	PR 101	Salem	Clinical, HIV+	VNII [a]

Note: HIV+ denotes the patient tested positive for the Human Immunodeficiency Virus at the time of isolation; Standard strains supplied by Westmead Hospital/University of Sydney, Molecular Mycology Laboratory culture collection, Sydney, Australia; Tamil Nadu strains supplied by P. Balakrishnan, Department of Microbiology, Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai (Madras), India. Indicated [subtypes] are assigned for purposes of this study only.

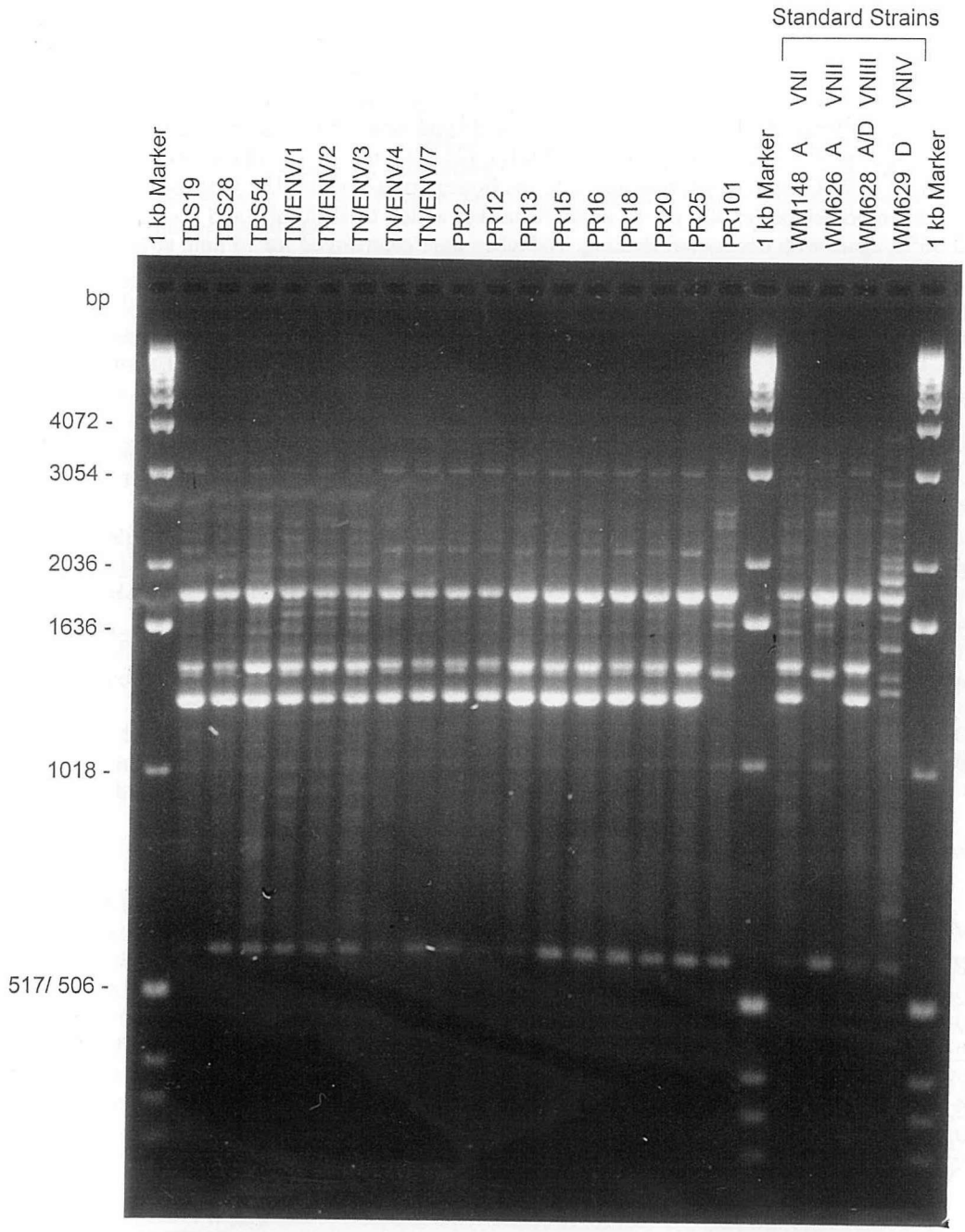

Figure 2. PCR-fingerprints of *C. neoformans* var. *neoformans* strains, using the primer M13. PCR products were separated on a 1.4% agarose gel.

PCR-fingerprinting

PCR-fingerprinting was performed according to a method described previously [21]. Briefly, two single primers were utilised for PCR-fingerprinting; the minisatellite specific core sequence of the wild-type phage M13 (5' GAGGGTGGCGGTTCT 3') and the microsatellite specific sequence $(GACA)_4$. The amplification reactions were performed in 50 µL volumes, containing 25 ng DNA, with 1x PCR buffer (1 mM Tris-HCl, pH 8.3, 5 mM KCl, 0.15 mM $MgCl_2$) (Applied Biosystems, Foster City, CA, U.S.A.) 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), 3 mM magnesium acetate, 30 ng primer and 2.5 U AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). Thirty-five PCR cycles were performed in a Perkin Elmer thermal cycler (model 480) with 20 s of denaturation at 94°C, 1 min. annealing at 50°C, 20 s of extension at 72°C. This was followed by a final extension of 6 min. at 72°C. 10 µL of PCR-products were separated by electrophoresis on a 1.4% agarose gel using 1 x Tris-borate-EDTA (TBE) buffer (10.8 g/L Tris-Base, 5.5 g/L Boric Acid, 4.0 mL/L 0.5 M EDTA pH 8.0). The gel was supplemented with

2 µL ethidium bromide (10 mg/mL) per 100 mL of gel. The gel electrophoresis was performed in a Gel-o-submarine system electrophoresis chamber (model JSB-120, Jordan Scientific, Bloomington, IN, U.S.A.), at 60 V to a length of 14 cm and visualised under ultraviolet light.

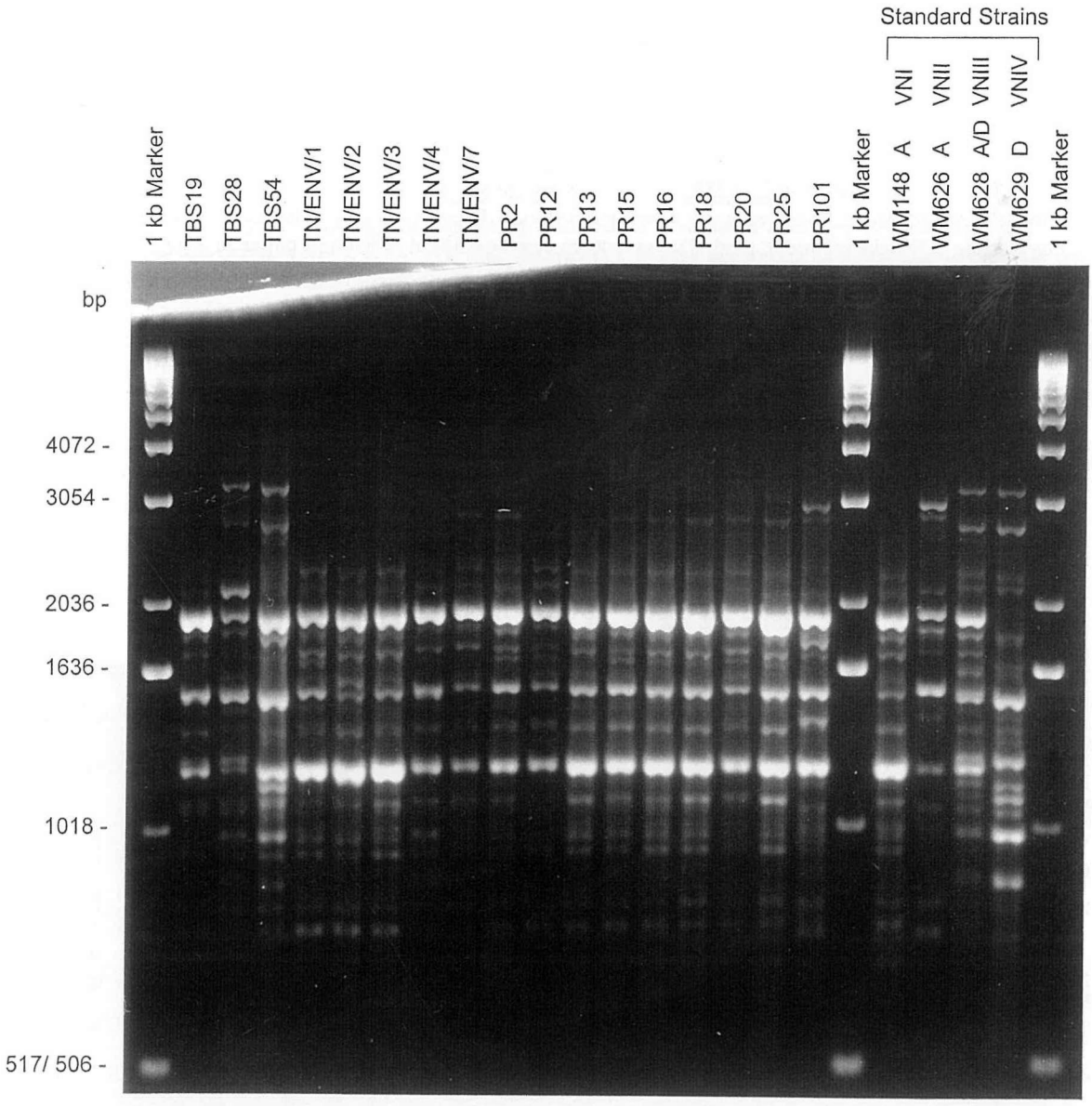

Figure 3. PCR-fingerprints of *C. neoformans* var. *neoformans* strains, using the primer $(GACA)_4$. PCR products were separated on a 1.4% agarose gel.

RAPD analysisfs

RAPD reactions were set up modified from a method previously described [20]. Briefly, the arbitrary primers 5SOR (5' ATGGGAATACGACGTGCTGTAG 3') and MYC1 (5' GAGGAAGGTGGGGAT-GACGT 3') were used as the primer pair 5SOR/MYC1. The amplification reactions were performed in 25 µL volumes, containing 10 ng DNA, 6 mM $MgCl_2$ (Advanced Biotechnologies, Surrey, U.K.), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), 5 pmol each of the primers and 1 U *Taq* DNA polymerase (Advanced Biotechnology, Surrey, U.K.) in buffer (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, pH 8.8, 0.1% Tween, 15 mM $MgCl_2$) (Advanced Biotechnology, Surrey, U.K.). The PCR reactions were performed in an MJ Research PTC-100-60 thermal cycler (MJ Research, Waltham, MA, U.S.A.). The initial denaturation was for 3 min. at 93°C with 10 cycles of annealing under low stringency with 1 min. of denaturation at 93°C, 1 min. annealing at 35°C and 1 min. extension at 72°C. This was followed by 20 cycles of annealing at high stringency with 1 min. of denaturation at 93°C, 1 min. annealing at 55°C and 1 min. extension at 72°C. RAPD fragments were separated on a 20 mL 10% polyacrylamide gel (2 mL 10 x TBE, 13 mL H_2O, 5 mL 40% (w/v) acrylamide/Bis (29:1) (BIO-RAD, La Jolla, CA, U.S.A.), 140 µL 10% ammonium persulfate

and 20 µL 6.6 M TEMED (BIO-RAD)) using 1 x TBE as the running buffer. 5 µL of PCR product was combined with 2 µL of 6x blue/orange loading dye (Promega, Madison, WI, U.S.A.) and loaded onto the gel. The electrophoresis was executed in a Joey Gel Casting System (model JGC-2, Owl Scientific, Woburn, MA, U.S.A.) at 200 V until the blue dye front was 3 cm from the bottom of the chamber. The gels were silver stained using a modification of the method of Bassam and Caetano-Anollés [28]. The gels were fixed by shaking with 10% glacial acetic acid solution for 10 min., then rinsed three times in water for 2 min. each time. The staining solution (15 mL 1% silver nitrate, 225 µL 49% (w/w) formaldehyde and 135 mL H_2O) was prepared. Gels were stained during constant shaking for 30 min. at room temperature and then twice rinsed briefly with water. The gels were developed with developing solution, containing: 3% sodium carbonate (prepared in advance and chilled to 4°C), 180 µL of a 0.2–0.28% sodium thiosulphate stock solution and 450 µL 49% (w/w) formaldehyde. The developer was replaced when the solution became cloudy. The developing process was stopped by adding 10% glacial acetic acid. The gels were dried on 3MM Whatman paper in a gel dryer (model 583, BIO-RAD, U.S.A.) at 80°C for 30 min. and then laminated.

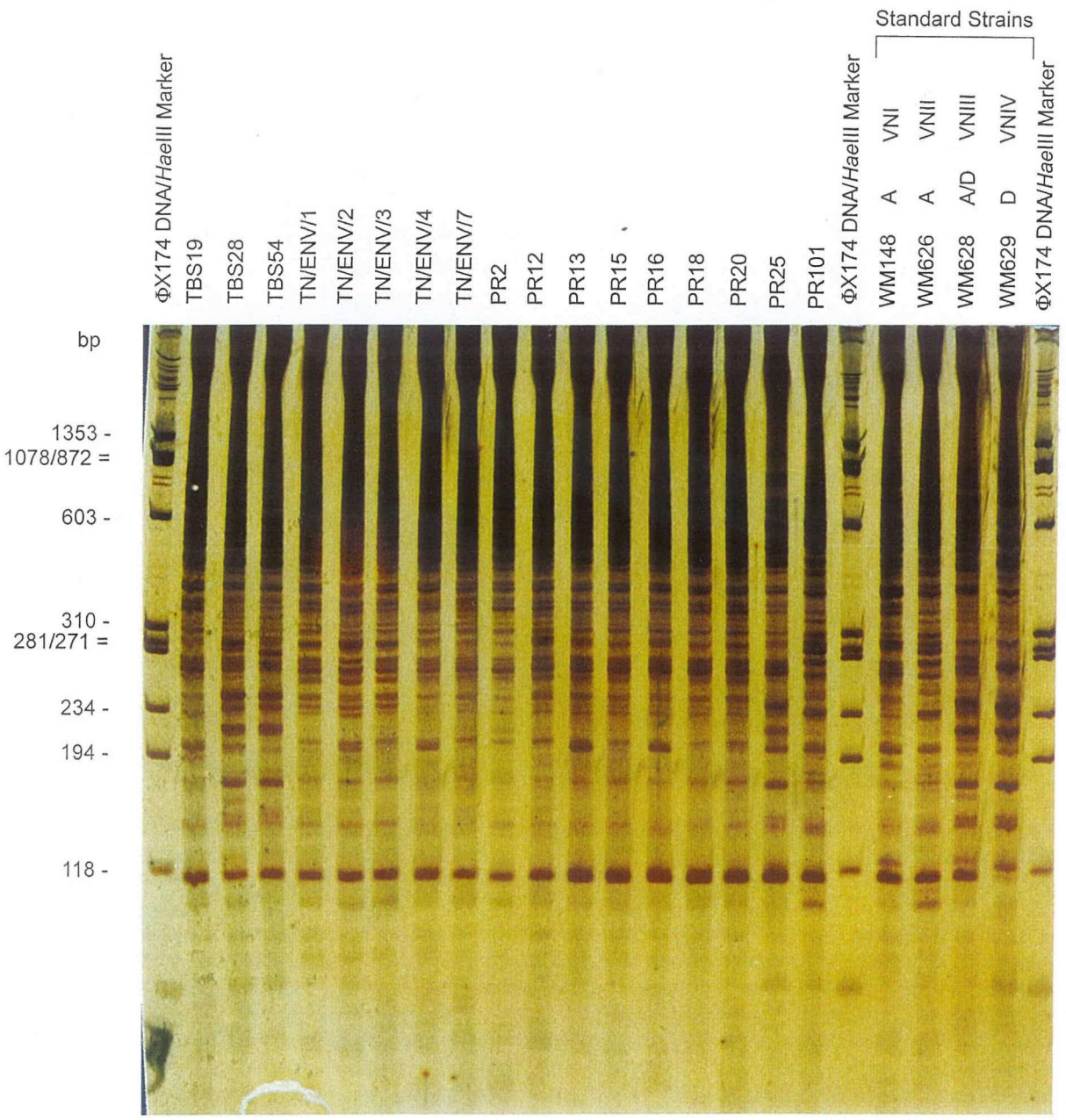

Figure 4: RAPD patterns of *C. neoformans* var. *neoformans* strains, using the primer pair 5SOR/MYC1. PCR products were separated on a 10% polyacrylamide gel.

Analysis of Genetic Relatedness

Initially, individual fingerprints for the Tamil Nadu isolates were analysed visually by comparison to the standard strains, which were amplified in parallel, to determine the major molecular type for each strain. For the PCR-fingerprints, all visible bands were included in the analysis regardless of the band intensity. In the case of the RAPD analysis, all visible bands between 100–600 bp were included, regardless of their intensity.

The *GelCompar* II, version 1.01 software (Applied Maths, Kortrijk, Belgium) was used to determine the genetic relatedness of the strains. The DNA bands for each fingerprint pattern were defined manually with a band position tolerance of 0.8%; this was the minimum position tolerance within which the molecular size markers were recognised as 100% identical. Similarity coefficients were calculated using the dice algorithm, and cluster analyses performed by the unweighted pair group method for arithmetic averages (UPGMA). Fingerprint data obtained using the primers M13, $(GACA)_4$ and 5SOR/MYC1 were combined as a composite data set corrected for internal weights, to conduct cluster analyses based upon the combined fingerprint data.

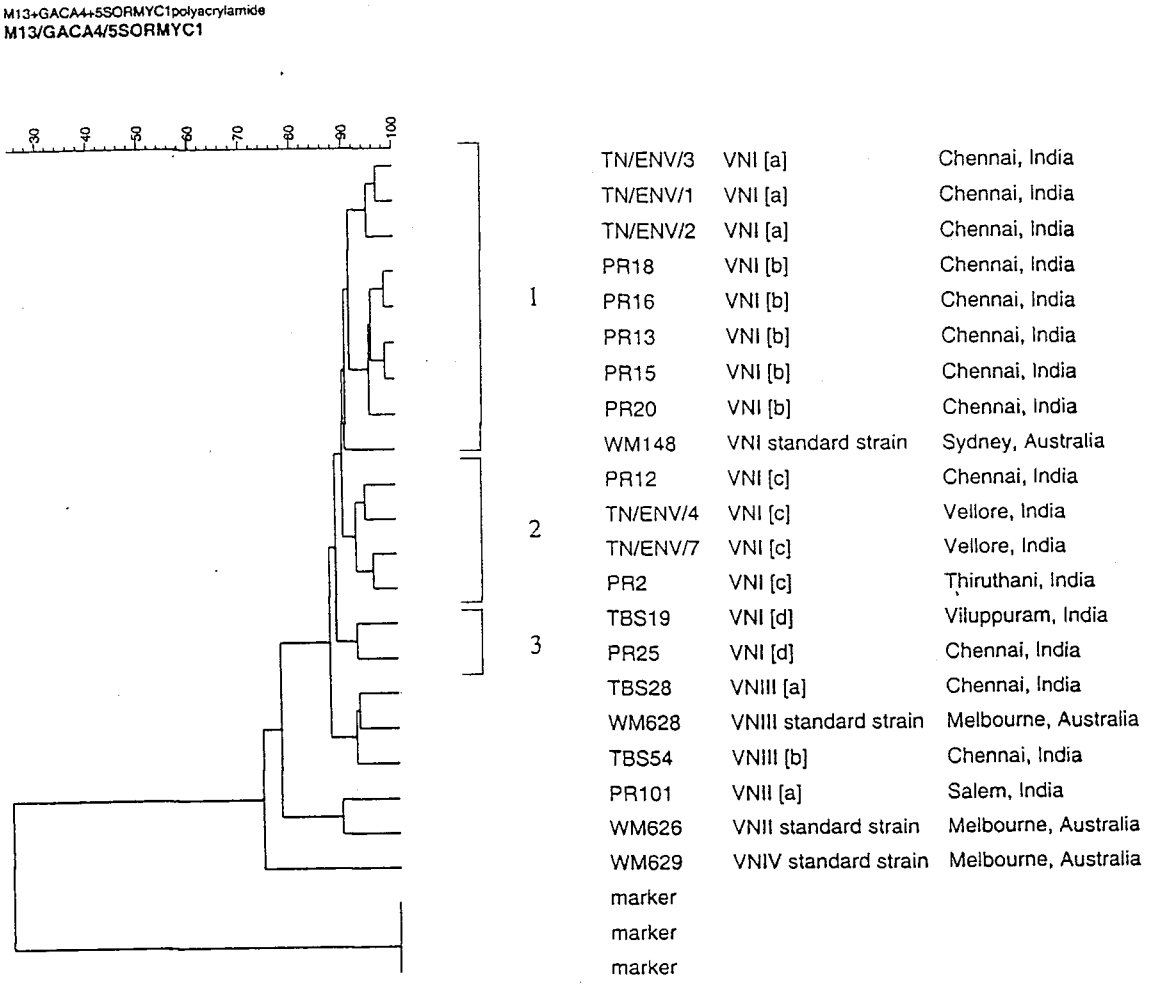

Figure 5. Combined cluster analysis of *C. neoformans* var. *neoformans* isolates using the M13, $(GACA)_4$ and 5SOR/MYC1 fingerprint data. Similarity coefficients were calculated by the Dice algorithm using a band position tolerance of 0.8%. The dendrogram was constructed using UPGMA. Clusters of isolates within molecular type VNI are labelled 1–3 based upon a cut-off point of 90% similarity.

Results

Twelve clinical and five environmental isolates of *C. neoformans* var. *neoformans* were investigated by PCR-fingerprinting and RAPD. The M13 and $(GACA)_4$ PCR-fingerprints of these isolates are shown in Figs 2 and 3, while the 5SOR/MYC1 RAPD profiles are shown in Fig. 4. Three molecular types were identified; VNI was the

predominant molecular type, comprising 14 of the 17 strains (82.3%) while there was one strain of molecular type VNII (5.9%) and two of VNIII (11.7%). Strains of each molecular type were examined visually to determine the number of subtypes within each, as a broad indicator of genetic diversity. Within molecular type VNI there were four subtypes, and there were two subtypes within VNIII. Ten of the 12 clinical isolates belonged to molecular type VNI, while the remaining two clinical isolates were found to be VNIII.

Cluster analyses of these isolates grouped strains of the same molecular type together. The overall similarity of the Indian isolates was 77%, based upon the combined fingerprint data set (see Fig. 5). The similarity among the isolates of VNI was 88%. There were three distinct clusters of isolates within the molecular type VNI, based upon a cut-off point of 90% similarity. Clusters were labelled 1–3 with cluster 1 comprising isolates TN/ENV/1, TN/ENV/3, TN/ENV/2, PR18, PR16, PR13, PR15, PR20 and the Australian standard strain WM148; cluster 2 contained the isolates PR12, TN/ENV/4, TN/ENV/7, and PR2; and cluster 3, TBS19 and PR25. All Indian strains in cluster 1 were isolated from patients who lived in Chennai (Madras) and from environmental sources in Chennai (Madras), while strains in clusters 2 and 3 were isolated from patients living in Thiruthani, Chennai (Madras), Viluppuram and from environmental sources in Vellore.

Discussion

The purpose of this study was to ascertain the genetic relatedness of a group of *C. neoformans* var. *neoformans* isolates from the Indian state, Tamil Nadu. It was not surprising that 10 out of the 12 clinical isolates were found to be molecular type VNI, given that these strains were isolated from HIV positive patients. Previous studies have noted that immunocompromised patients are invariably infected with strains of variety *neoformans*, particularly those of serotype A [12, 29].

Cluster analyses of the fingerprint data obtained for individual primers showed distinctions between the molecular types. Some variation was observed in the relationships between isolates when the data obtained with each primer were analysed individually (data not shown). The variation in the relationships calculated between strains using different primers can be attributed to differences in primer sensitivities, caused by disparities in the frequency of primer annealing sites throughout the genome and evolutionary rates of polymorphic loci. In order to overcome the bias introduced by the individual primers, fingerprint data sets obtained from all primers were combined to produce a more accurate representation of the clustering patterns of these isolates.

Cluster analyses of the combined fingerprint data showed distinctions between the varieties and the major molecular types, demonstrating that there are significant genetic differences between these groups. Although there appears to be a high degree of genetic homogeneity within these isolates, three distinct clusters of isolates within the molecular type VNI were observed. Cluster 1 contained isolates from patients living in Chennai (Madras) and environmental isolates from the same area, while clusters 2 and 3 were comprised of environmental isolates from Vellore (150 km from Chennai), and from patients living in Chennai, Thiruthani (120 km from Chennai), and Viluppuram (200 km from Chennai). While there are insufficient strains in this study to draw definitive conclusions, these findings suggest that genetic differences between the isolates are related to geographical location and that concordance of patterns from environmental and clinical isolates in a given location support earlier evidence of an epidemiological link [24].

The overall genetic diversity of globally obtained *C. neoformans* var. *neoformans* strains was estimated to be 65% (Kidd *et al.*, unpublished). This is comparable to the 77% similarity found among the Indian isolates in this study. A small group of Indian isolates of molecular type VNI in a previous study, showed a similarity of 93% (Kidd *et al.*, unpublished), which is consistent with 88% similarity observed for the VNI isolates in this study.

Combined analysis of the typing patterns generated by PCR-fingerprinting and RAPD provided initial insights into the genetic relationships between *C. neoformans* var. *neoformans* isolates from India, even if the isolates used in this study are not completely representative of the Indian isolates in general. In previous studies it has been shown that both varieties of *C. neoformans* are present in India, including serotypes A, B and AD. Serotype B has been isolated from environmental sources mainly in the northern states of India [4, 14]. Our group has earlier noted the existence of isolates of molecular type VGIV (serotype B) from Himachal Pradesh and Punjab, in northern India.

Conclusions

The present study analysed the genetic relatedness of 12 clinical and five environmental isolates of *C. neoformans* var. *grubii/neoformans* from the Indian state Tamil Nadu using two independent molecular typing techniques, PCR-fingerprinting and RAPD analysis. The isolates were grouped into three major molecular types VNI, VNII and VNIII expanding the currently limited knowledge of the epidemiology of this important human fungal pathogen in India. Continued sampling of isolates from all areas of the world is required in order that a better understanding may be gained of the epidemiological and evolutionary relationships of *C. neoformans.*

Acknowledgments

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