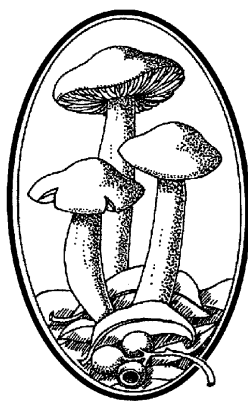


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MOLECULAR EPIDEMIOLOGY OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *CRYPTOCOCCUS NEOFORMANS* FROM TAMIL NADU, INDIA

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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.

S.E. Kidd *et al.* (2002). Molecular epidemiology of clinical and environmental isolates of *Cryptococcus neoformans* from Tamil Nadu, India. *Australasian Mycologist* 20 (3): 105–114.

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. blakeyi* [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 triisopropyl-naphthalene 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 μ L was added to the frozen, ground cells. The tubes were incubated for 2 min. at 55°C, shaken occasionally; 500 μ L 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 μ L 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 μ L 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 μ L 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 <i>C. neoformans</i> var. <i>neoformans</i> 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
<i>Cryptococcus neoformans</i> var. <i>neoformans</i> 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' GAGGGTGGCGTTCT 3') and the microsatellite specific sequence (GACA)₄. The amplification reactions were performed in 50 μ L volumes, containing 25 ng DNA, with 1x PCR buffer (1 mM Tris-HCl, pH 8.3, 5 mM KCl, 0.15 mM MgCl₂) (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 μ L 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 μ L 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 analysis

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 MgCl₂ (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 (NH₄)₂SO₄, 750 mM Tris-HCl, pH 8.8, 0.1% Tween, 15 mM MgCl₂) (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 H₂O, 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 H₂O) 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*.

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FURTHER HYGROPHORACEAE OF VICTORIA

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Abstract

Further information on the macrocharacters of *Hygrocybe conica* var. *conicoides* is provided together with an extension of its known geographical range. The European taxon *Hygrocybe persistens* var. *persistens* is recorded for the first time for Australia from a Victorian coastal community.

Young, A.M. (2002). Further Hygrophoraceae of Victoria. *Australasian Mycologist* 20 (3): 115–117.

Introduction

Several recent collections of Hygrophoraceae have added to the number of known Victorian taxa and increased the extent of previously known distributions. Both species noted here are from coastal communities based on sand. Material was examined in accordance with Young (1999). If no collection number is designated by the collector, this is so shown by the abbreviation 's.n.'. Where holotypes for established European taxa do not exist the species concepts of Boertmann (1995) are used. Material not seen by the author is indicated by 'n.v.'. Victorian material considered in this paper is deposited with the National Herbarium of Victoria (MEL).

Taxonomy

Hygrocybe conica* var. *conicoides (P.D. Orton) Boertman, *Fungi of Northern Europe* 1: 162 (1995)
Hygrophorus conicoides P.D. Orton, *Trans. Brit. Mycol. Soc.* 43: 262 (1960); *Hygrocybe conicoides* (P.D. Orton) P.D. Orton & Watling, *Notes Roy. Bot. Garden Edinburgh* 29: 131 (1969). Type: England. Somerset. Bossington, 31.x.1957, P.D. Orton (holotype K, n.v.).

Illustration: Boertmann (1995), p. 163; Young (2000), p. 15.

Pileus 20–30 mm diam., orange but rapidly blackening with maturity or bruising and often completely black when fully expanded, conical expanding to broad-conical or near applanate, smooth, margins often lobed or ragged. *Lamellae* ascending, orange, blackening when bruised. *Stipe* 40–70 _ 5–7 mm, pallid orange and blackening above ground with maturity or when bruised, cylindrical, hollow, splitting longitudinally, often twisted.

Basidiospores 10.5–14.5 _ 5.5–7.5 µm, mean 12.2 _ 6.0 µm, Q: 1.7–2.3, mean Q: 2.03, very long-ellipsoidal to cylindrical, hyaline, smooth, often showing constrictions or indented on one side. Remainder of characters in accordance with *Hygrocybe conica* var. *conica* (Schaeff. : Fr.) P. Kumm.

Habitat: gregarious amongst exotic grasses in sandy soil.

Material examined: Victoria. Pt Lonsdale, 9.vii.2000, K. Ralston (*Ralston 2069*) (MEL 2082595).

Remarks: *Hygrocybe conica* var. *conicoides* has very long-ellipsoidal to cylindrical basidiospores with an average Q >2.0. These basidiospores readily distinguish it from *H. conica* var. *conica* which has ellipsoidal basidiospores measuring 9–11 _ 6–7.5 µm with a mean Q <2.0. This collection has provided the first good information about the macrocharacters for the taxon as it occurs in Australia and it confirms the occurrence in coastal sands as noted in Young (2000). A second collection made by Kathleen Ralston (*Ralston 2070*, MEL 2082596) from the Pt Lonsdale area salt marshes (outer edges) was also thought to be var. *conicoides* but examination of its basidiospores has shown that it is intermediate between var. *conica* and var. *conicoides*. The basidiospores have the correct range of values for var. *conicoides* as they measure 11.5–14.0 _ 5.5–7.5 µm, mean 12.3 _ 7.0 µm; however, their Q values (1.4–2.0, mean Q: 1.75) are less than 2.0 and the basidiospores are ellipsoidal rather than very long-cylindrical. Such intermediates are also known in European collections (Boertmann 1995) and it suggests that the two accepted variants of *Hygrocybe conica* might actually be extremes of a single large range.

Figure 1. *Hygrocybe persistens* var. *persistens*. A. Longitudinal diagram, B. basidiospores, C. basidia. Habit sketch bar = 10 mm; basidiospores and basidia bars = 10 µm.

Hygrocybe persistens (Britzelm.) Singer, *Rev. Mycol. (Paris)* 5: 8 (1940)

Hygrophorus conicus var. *persistens* Britzelm. in *Ber. Naturwiss. Vereins Schwaben Augsburg* 30: 200 (1890).

Type: none designated.

Illustration: Boertmann (1995), p. 155, top photograph from English sand dunes; Fuhrer (1985), p. 47 as *Hygrocybe* sp.

Pileus 30–65 mm diam., yellow-orange to dark orange with age, conical becoming expanded-conical, smooth, slightly viscid or sticky, margin even or a little lobed. *Lamellae* ascending-adnexed, orange, margins even and concolorous or a little paler. *Stipe* 40–90 _ 8–12 mm, white below the sand but yellow to bright yellow above the sand, cylindrical, smooth, dry. Fig. 1.

Basidiospores 11.5–16.5 _ 5.5–8 µm, mean 13.8 _ 6.4 µm, Q: 1.8–2.6, mean Q: 2.16, long-ellipsoidal to cylindrical often with a constriction or one side depressed, smooth, hyaline, occasionally with large, transparent inclusions. *Basidia* 43–62 _ 9–14.5 µm, mean 51.1 _ 10.7 µm, Q: 4.2–5.4, mean Q: 4.77, 4-spored, clamped. *Cystidia* absent. *Hymenophoral trama* regular and consisting of tubular, aseptate, hyaline, thin-walled elements 2000–3000 _ 8–14.5 µm, tapered at their ends and sometimes protruding into the lamellae margins to form pseudo-cheilocystidia. *Pileipellis* an ixocutis of repent, thin-walled, hyaline, septate hyphae 3–18 µm diameter, clamps present, surface hyphae usually with tapered ends and some internal pigmentation at the apex. *Stiptipellis* a cutis of repent, thin-walled, hyaline, septate hyphae 2–8 µm diam., clamps present.

Habitat: gregarious amongst calcareous, moving sand.

Material examined: Victoria. Pt Lonsdale, vii.2000, K. Ralston (Ralston 2075) (MEL 2082597).

Remarks: *Hygrocybe persistens* var. *persistens* is very widespread in Europe, and Boertmann (1995) reports that in Denmark it is found in small groups in grasslands on calcareous soils, fixed dunes, lawns, etc. The Australian material is more closely related to the English subvariety which is found on sand dunes and which has bright orange lamellae rather than the light yellow lamellae of the European material. Macroscopically, the English subvariety is indistinguishable from the Australian material. The basidiospores of the English subvariety measure (11–) 12–14.5 (–15.5) _ (5–) 5.5–6.5 µm, Q: 2.0–2.7, mean Q: 2.3, which compares very well with the Australian material. *Hygrocybe persistens* var. *persistens* is easily distinguished from *Hygrocybe persistens* var. *konradii* as the latter has very broadly ellipsoidal to globose basidiospores measuring (9.5–) 10–13 (–14) _ 7–9.5 (–10.5) µm (Young 2000).

With the confirmation of *Hygrocybe persistens* var. *konradii* (R. Haller Aar) Boertm. from Sydney where it appears in ‘buffalo grass’ lawns (Young 2000), it seems that the European *Hygrocybe persistens* with its complex of varieties has been introduced to Australia and is now well established in selected communities which agree almost perfectly with the known European habitats.

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PHYLLACHORA CLADII-GLOMERATI AND P. SCHOENICOLA: NEW AUSTRALIAN RECORDS FROM SEDGES

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Abstract

Two new records of *Phyllachora* species on sedges (Cyperaceae) in Australia are recorded and described. *Phyllachora cladii-glomerati* which occurs on *Baumea* spp. was known previously only from New Zealand, and *P. schoenicola* on *Schoenus apogon* was recorded only from the Philippines. Each is widely distributed in Australia.

Pearce, C.A. & Hyde, K.D. (2002). *Phyllachora cladii-glomerati* and *P. schoenicola*: new Australian records from sedges. *Australasian Mycologist* 20 (3): 118–122.

Plate 1. *Phyllachora cladii-glomerati*. **a** Leaf spots, **b** Vertical section through ascoma illustrating central ostiole, peridium, and adjacent host tissue, **c** Vertical section through ostiolar canal illustrating periphyses, **d** Vertical section through peridium, **e, f** Asci, **g–j** Ascospores. **Scale bars:** **a** = 1 mm, **b** = 50 µm, **c–j** = 10 µm.

Plate 2. *Phyllachora schoenicola*. **a** Leaf spots, **b** Vertical section through ascomata illustrating central ostiole, peridium, adjacent ascomata and host tissue, **c** Vertical section through ostiolar canal, **d** Vertical section through peridium, **e–g** Asci, **h–k** Ascospores. **Scale bars:** **a** = 0.5 mm, **b** = 50 μm , **c–k** = 10 μm .

Introduction

During a recent survey of phyllachoraceous fungi in Australia for the Australian Biological Resources Study (Pearce & Hyde 2001, Pearce, Reddell & Hyde 1999, 2000, 2001), two previously unrecorded species of *Phyllachora* on sedges were discovered. Both were found while examining dried specimens of Cyperaceae in plant herbaria. Comparison of the foliicolous tar spots with type specimens confirmed their identity as *Phyllachora cladi-glomerati* and *P. schoenicola*. *Phyllachora cladi-glomerati* has previously been recorded only on *Baumea rubiginosa* in New Zealand. *Phyllachora schoenicola* has previously been recorded only on *Schoenus apogon* in the Philippines. Both taxa are described in this paper and illustrated with photomicrographs.

Material and methods

The holotypes of *P. cladi-glomerati* and *P. schoenicola* were borrowed from herbaria PDD and S and compared with specimens found on sedges in herbarium collections at AD, BRI, BRI (MBA), MELU and PERTH. Key characters of the fungi including morphology and size of colonies, ascomata, asci, and ascospores were examined (Cannon 1991, Parbery 1967, Parbery and Langdon 1963, 1964). Specimens were prepared for light microscopy using the methods of Cannon (1996). This involved dissection of leaf spots in a drop of water. Following rehydration, squash mounts were prepared in water, lactophenol cotton blue, and Melzer's reagent. Sections were cut

using a freezing microtome. Unless otherwise stated, all photographs and measurements were made from material mounted in water.

***Phyllachora cladii-glomerati* Hansf., *Proceedings of the Linnean Society of New South Wales* 82: 221 (1957)**

Colonies: containing several perithecia beneath a common clypeus on culms, clypei 1–4.5 _ 0.5–2.5 mm, black, shiny, ellipsoidal, elongate, parallel with leaf veins, sometimes slightly raised and flattened, ostioles minute and indistinct, occasionally surrounded by a narrow halo of reddish brown discoloured host tissue, up to 1 mm wide.

Anamorph: not known.

Teleomorph: *Ascomata* immersed in the host parenchyma tissue, 136–420 µm diam., 294–600 µm high, usually ellipsoidal, occasionally globose, with a cylindrical to wide conical ostiolar canal, lined with fine hyaline periphyses. Upper peridium clypeate, consisting of deeply melanised, dark brown to black host epidermal cells and occasionally cuticle, often infiltrating the host parenchyma adjacent to the ostiolar canal, generally amorphous, but sometimes resembling *textura intricata*, up to 130 µm thick and extending laterally up to 1 mm from the ostiole, usually not involving host vascular bundles. Peridium also strongly melanised, comprising multiple layers of brown to brown-black, elongate, thin-walled, flattened cells, sometimes resembling *textura intricata*, c. 40–90 µm thick, merging inwardly with a hymenium consisting of several layers of hyaline, thin-walled, flattened cells, 5–10 µm thick. Peridium laterally merges with a narrow region of compressed host cells. *Paraphyses* numerous, filiform, as long as asci, 2.5–4 µm wide, tapering to rounded apices, thin-walled, hyaline, not constricted at the septa. *Asci* 114–194 _ 9–16 µm, 2–4–6-spored, rarely 8-spored, narrow-cylindrical, short-pedicellate, thin-walled, unitunicate, apex truncate with an opaque, cup-shaped, ring-like, apical apparatus, 4–5 µm wide, 2.5 µm thick, non-reactive in Melzer's reagent. *Ascospores* arranged uniseriately, often oblique, rarely overlapping, 17–26 _ 5–10 µm, oblong, poles rounded, occasionally slightly ovoid or slightly inaequilateral, aseptate, hyaline, thick-walled, enclosed in a clear mucilaginous sheath up to 13 µm thick. Plate 1.

Known host: *Baumea rubiginosa* (Spreng.) Boeck., *B. teretifolia* (R. Br.) Palla.

Known distribution: Australia, New Zealand.

Material examined: Australia. W.A.: south side of Toodyay Rd, 9 km from Great Northern Highway (just north of Northam), on culms of *B. rubiginosa*, 30 Nov. 1975, *A.M. George* (14) PERTH 02091267; Walpole-Nornalup National Park, Monastery Road, 1.4 km from junction with Gully road, on culms of *B. rubiginosa*, 2 Dec. 1992, *J.R. Wheeler* 3666 and *S.T. Patrick* PERTH 03823393; Yeaganup Lake, 34°32'S, 115°52'E., on culms of *B. rubiginosa*, 13 May 1991, *C.J. Robinson* (620) PERTH 03555593. Qld: Cape York Peninsula, 2.4 km north of Harmer Creek on track from Spencer's Lease to Heathlands, Mapping Site SBN 13, 11°57'S, 142°54'E, on living culms of *B. teretifolia*, 12 Oct. 1991, *J.R. Clarkson* (JRC9144) and *V.J. Neldner* BRI MBA. New Zealand. Silverdale, North Auckland, on *Cladium glomeratum* (= *Baumea rubiginosa*), Oct. 1950, *J.M. Dingley* PDD 17245 (holotype).

Notes: *Phyllachora cladii-glomerati* was originally described from New Zealand by Hansford (1957). The Australian collections differ from the holotype, in having 2, 4, or rarely 6 to 8-spored asci. The asci of Australian collections are also variable in size, and often slightly longer than those of the type, 114–194 _ 9–16 µm versus 120–140 _ 10–11 µm respectively. The size of the ascospores in the Australian collections are also more variable (17–26 _ 5–10 µm versus 22–26 _ 7.5–9.5 µm for the type) and in this study have been found with a hyaline, mucilaginous sheath up to 13 µm thick.

Phyllachora cladii-glomerati most closely resembles *P. epicladii* (Cooke & Masee) Arx, described on *Cladium* from Port Phillip, Victoria. Although we have been unable to locate the holotype, or any other collections of *P. epicladii*, Arx (1957) originally reports the asci as being 8-spored, and the ascospores forming a slimy yellow spore mass at the ostiole. *Phyllachora cladii-glomerati* rarely has 8-spored asci, and the gelatinous ascomatal contents are generally hyaline.

***Phyllachora schoenicola* Syd., *Annales Mycologici* 11: 265 (1913)**

Colonies: amphigenous, containing several perithecia beneath a common clypeus on culms, clypei 0.4–3 _ 0.1–0.8 mm, black, shiny, roughly ellipsoidal, elongate, parallel with leaf veins, sometimes coalescing to form irregular lines, apex slightly to moderately raised and flattened, ostioles minute.

Anamorph: not known.

Andromorph: not known.

Teleomorph: *Ascomata* immersed in the parenchyma, occupying 1/2 to 3/4 leaf thickness, developing between vascular bundles, sometimes slightly distorted by them, often forming close to adjacent ascomata, up to five in an amphigenous group with ostioles opening to different leaf surfaces, 129–250 µm diam., 130–195 µm high, globose to oblate-sphaeroidal, with a central, or sometimes off-centre, wide conical ostiolar canal, lined with fine, hyaline periphyses. Upper and often lower peridium clypeate, consisting of deeply melanised, brown-

black, amorphous host epidermis and adjacent parenchyma, sometimes incorporating the host cuticle, usually not infiltrating the host vascular tissue, up to 52 µm thick. Lateral peridium of variable thickness depending on number of ascomata involved. Single ascomata often thin-walled, lateral peridium 8–13 µm thick, consisting of multiple layers of thin-walled, flattened, light brown cells, sometimes resembling *textura intricata*, becoming hyaline on the inner hymenial surface. Groups of ascomata have thicker walls, similarly composed, but more deeply melanised, brown-black, 10–25 µm thick. The lateral peridium merges outwardly with a narrow region of discoloured yellow-brown, compressed host cells. *Paraphyses* numerous, filiform, slightly longer than asci, up to 4 µm diam., tapering to rounded apices, not constricted at septa, no branching observed. *Asci* 60–104 _ 8.5–13 µm, 8-spored, cylindrical to cylindric-clavate, tapering to a rounded apex, short-pedicellate, unitunicate, thin-walled, no apical structure visible. *Ascospores* arranged obliquely uniseriate, sometimes biseriate and overlapping, 15.5–23 _ 3–6.5 µm, fusiform with attenuated poles, sometimes ovoid, slightly inaequilateral, hyaline, guttulate, aseptate. Plate 2.

Known host: *Schoenus apogon* Roem. & Schult.

Known distribution: Australia, Philippines.

Material examined: Australia. S.A.: Region 11 southern Lofty, Horsnell Gully, 34°56'S, 138°43'E, on *S. apogon*, 12 Nov. 1977, *Tineka Kempen* AD 9775012. Qld: Girraween National Park, 29°2'S, 152°2'E, on foliage of *S. apogon*, 11 Nov. 1974, *W. McDonald (690)* BRI 187960. N.S.W.: Gibraltar Range National Park, on leaves and stems of *S. apogon*, Apr. 1973, *C. Bell (no 573)* BRI 160018. Vic.: Grampians, roadside Epacris Falls, on *S. apogon*, 13 Nov. 1959, *D.E. Symon (293)* AD 98673880. Vic.: Warburton, 37°4'S, 145°4'E, on *S. apogon*, 19 Jan. 1935, *S.J. Blake (7216)* BRI 206883. Tas.: Hobart, 45°53'S, 147°19'E, on leaves and stems of *S. apogon*, 17 Feb. 1943, *W.M. Curtis* BRI 206882.

Philippines. Mt. Banahao, on living stems and leaves of *S. apogon*, 18 Feb. 1913, leg. *E.B. Copeland (C.F. Baker no.853)* S (holotype); collection site not given, on living foliage of *S. apogon*, date not given, *R.T. Patton* MELU 5833F.

Notes: *Phyllachora schoenicola* is the only *Phyllachora* species described from *Schoenus*, and was previously known only from the Philippines (Sydow & Sydow 1913). The Australian collections differ from the holotype, in having slightly shorter asci, 60–85 _ 8.5–13 µm versus 75–104 _ 10–13 µm respectively, and slightly shorter and narrower ascospores, 15.5–23 _ 3–6 µm versus 18–23 _ 5–6.5 µm respectively.

We recognise six *Phyllachora* species from sedges in Australia, including *P. anceps*, *P. cladii-glomerati*, *P. cyperi*, *P. epicladii*, *P. fimbriatilis* and *P. schoenicola* (Pearce 2000).

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OCCURRENCE OF TRITERPENOIDS AND POLYSACCHARIDES ON *GANODERMA TROPICUM* WITH *GANODERMA LUCIDUM* AS REFERENCE

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Abstract

A local strain of *Ganoderma tropicum* has been analyzed for the appearance of triterpenoids and polysaccharides, with *Ganoderma lucidum* as reference. These two compounds are reported to have medicinal value in *G. lucidum*. *Ganoderma tropicum* was isolated from a *Delonix regia* tree, an ornamental tree locally known as Flamboyant, in December 1999. The fungus is parasitic to the tree and is quite aggressive, killing the tree within one to five years. The fruiting bodies of both *G. tropicum* and *G. lucidum* were obtained by growing them in sawdust after three months' incubation. Extraction for triterpenoids was conducted in wash benzene and ethanol, while extraction for polysaccharides was done in hot water. Analysis for triterpenoids was conducted by using silica gel-thin layer chromatography (TLC) with eluent of chloroform:methanol (10:1) for the first extract and dichloromethane for the second extract. Detection of triterpenoids was done by spraying the plate with Carr-Price as well as Lieberman-Burchard reagents then observing under UV light (366 nm). The same principle, except for the eluent n-butanol : acetic acid : ether : water (9:6:3:1) and reagent of aniline ftalate, was used for detecting polysaccharides in the form of their monomers (glucose, galactose, xylose and rhamnose) under normal light. Both triterpenes and polysaccharides were detected on *G. tropicum* and *G. lucidum*, suggesting that the local strain of *G. tropicum* also possesses medicinal value.

Keywords: *Ganoderma tropicum*, *Ganoderma lucidum*, triterpenes, polysaccharides, medicinal mushroom.

Aryantha, I.N.P. *et al.* (2002). Occurrence of triterpenoids and polysaccharides on *Ganoderma tropicum* with *Ganoderma lucidum* as reference. *Australasian Mycologist* 20 (3): 123–129.

Introduction

Ganoderma mushrooms, particularly *G. lucidum*, have some medicinal properties including antitumor (Kishida *et al.* 1988, Zhang *et al.* 2000), antiviral (Eo *et al.* 2000), anti-inflammatory (Patocka 1999), lowering blood pressure (Morigiwa *et al.* 1986) and increased immune system (Kino *et al.* 1989, Tomoda *et al.* 1986, Yu *et al.* 1997). Other species such as *G. tsugae*, *G. chinense* and *G. applanatum* also possess medicinal properties. Extensive research has been done on *G. lucidum* and other species, but there is very little information available for *G. tropicum*. Jun & Shuqin (1987) reported that *G. tropicum* has been used in China for curing coronary heart disease and also inhibited sarcoma cells up to 70 per cent.

The two main substances in the fruiting body of *G. lucidum* which are considered to possess medicinal properties are polysaccharides and triterpenoids (Min *et al.* 2000). A polysaccharide isolated from spores of *G. lucidum* was found to be a complex glucan (Bao *et al.* 2001). Other reports stated that *G. lucidum* contains polysaccharides called GLA (galactan) with monomers of mainly galactose and others such as glucose, rhamnose and xylose and GLB (glucan) with monomers of mainly glucose and others such as rhamnose and xylose (Qing-Yao *et al.* 1994). Triterpenoids contained in *Ganoderma* mushrooms are classified as ganoderic acid (C30) and lusidenic acid (C27) (Mizuno 1984). Indonesia has mega-diversity of natural resources including its mycoflora, yet lacks information available on chemical composition of mushroom isolates.

The aim of this study was to investigate whether triterpenoids and polysaccharide are present in *G. tropicum* as they are in *G. lucidum*.

Materials and Methods

Isolate of *G. lucidum* and *G. tropicum*

Ganoderma lucidum was obtained from Fungi Perfecti, U.S.A. *Ganoderma tropicum* was isolated, by using tissue culture techniques, from a fresh fruiting body collected from a *Delonix regia* tree (Fig. 1) in Bandung during 1999. The flesh edge part of the mushroom was cut into small pieces with a scalpel aseptically, then cultured onto PDA before incubation at room temperature. Young hyphae growing on the plate were cut and transferred into fresh slant PDA as stock culture.

Fruiting body production

Cereal substratum consisting of corn cob, rice bran and red rice was inoculated with seven-day-old mycelium growing on a plate then incubated for 20 days at room temperature. Mycelium growing from this substratum (spawn) was inoculated into log substratum made mainly of sawdust and rice bran. Four weeks' incubation at room temperature was required to complete the colonization of the log before the log was exposed to fresh air in a humid condition (RH 85%). A total of three months was required to obtain mature fruiting bodies.

Analysis for Triterpenoids

Extraction. A fresh fruiting body was washed, then sliced into small pieces and dried in an oven at 50°C before being blended to granules. Twenty-five grams of dried granules were then extracted with 250 ml wash benzene in soxhlet for 24 hours. Another extraction was done by using 250 ml ethanol. Both extracts were concentrated by vacuum evaporation and heating evaporation at 70°C in a water bath before running the Thin Layer Chromatography.

Thin Layer Chromatography (TLC). Detection was conducted by spotting the extract with a micro pipette onto a silica gel plate (60 F 254) as a stationary phase and developing solvent of dichloromethane for the first extract and chloroform : methanol (10:1) for the second extract. After developing then drying the plate, spraying with Carr-Price reagent (20% antimon solution chloroform) at 100°C for 10 minutes and Lieberman Burchard (1 ml H₂SO₄ conc. 20 ml acetic anhydride and 50 ml CHCl₃) at 85–95° for 15 minutes were done before observing under UV light (366 nm).

Analysis for polysaccharides

Extraction. Ten (10) grams of granules of fruiting body were soaked in 100 ml of hot distilled water (90–95°C) over 48 hours. The extract was then hydrolyzed with H₂SO₄ 1M at 100°C over 8–16 hours then neutralized with Ba(OH)₂. Precipitation of BaSO₄ was then removed after centrifugation. Ultimately, the filtrate was concentrated by evaporating at 70°C on a water bath before running TLC.

Thin Layer Chromatography (TLC). The same principle as used for detecting triterpenoids was used for detecting polysaccharides, except for the developing solvent (n-butanol : acetic acid : ether : water 9:6:3:1) and reagent (aniline hydrogen ftalat) at 100°C for 10 minutes were applied before observing under UV light (366 nm).

Results and Discussion

Isolation of *G. tropicum*. Incubation over four days was required for hyphae to develop from tissue pieces before transferring into slant agar. No purification was required since the mycelium growing from tissue culture was found to be axenic. This can be achieved by careful handling when opening the fresh fruiting body before cutting the inner part into pieces. Opening should start from the centre towards the edge of the fruiting body. The knife should be used to just open the fruiting body slightly then continued by hands without touching the inner part of the fruiting body to avoid contamination. This way was proven to be mostly successful in tissue culture isolation.

Fruiting body production of *G. tropicum* and *G. lucidum*. Both *G. tropicum* and *G. lucidum* were grown on the same sawdust substratum to obtain fruiting bodies (Fig. 2). At first, the fruiting initials for both species appeared after 1.5–2 months, then maturation took place in 1–1.5 months. *G. lucidum* grew faster than *G. tropicum* both in mycelium running and fruiting initiation. As can be seen on Fig. 2, the fruiting body of *G. lucidum* is bigger than that of *G. tropicum* since the formula was initially developed for producing *G. lucidum* commercially. The fruiting body of *G. tropicum* produced from cultivation was different from the fruiting body obtained from the field (Figs 1 & 2). The environmental condition, which is different between field and indoor condition, can explain this phenomenon. In addition, the substratum used in producing fruiting bodies in 1,5 L log is also different from the natural substratum of a live tree. The size of fruiting bodies in the

field may reach 40 cm in diameter on a big tree. According to our observations, the more substratum available (as on the big tree) the bigger size of fruiting bodies produced.

Occurrence of triterpenoids. First extraction using wash benzene, a non-polar solvent, was able to extract non-polar substances. TLC pattern for non-polar triterpenoids without applying any reagent can be seen in Fig. 3. Without reacting agent Carr Price or Lieberman Burchard, only four spots for *G. lucidum* and five spots for *G. tropicum* were observed under UV light (Fig. 3). After applying Carr Price reagent, both *G. lucidum* and *G. tropicum* gave 11 spots (Table 1). However, when applying Lieberman Burchard to the TLC plate less spots were produced, eight spots for *G. lucidum* and 10 spots for *G. tropicum* (Table 2). This means that Carr Price reagent is better for detecting triterpenes diversity as also stated by Harborne (1996). Some of the spots have the same value of retention factor (Rf) and colour under UV light indicating the same substance (Tables 1 and 2). This phenomenon may explain that both species possess medicinal value which have been traditionally used for medicinal herbs in Asia. From Table 2 it can also be noticed that *G. tropicum* produces more spots than *G. lucidum*, suggesting that *G. tropicum* contains more types of triterpenoids.

Table 1. TLC spots of *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane after applying Carr Price reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	<i>G. lucidum</i>	<i>G. tropicum</i>
1	0.051 (White fluorescent)	0.051 (White fluorescent)
2	0.204 (White fluorescent)	0.204 (White fluorescent)
3	0.264 (Red fluorescent)	0.264 (Red fluorescent)
4	0.381 (White fluorescent)	0.381 (White fluorescent)
5	0.457 (White fluorescent)	0.457 (White fluorescent)
6	0.498 (White fluorescent)	0.498 (White fluorescent)
7	0.572 (White fluorescent)	0.558 (White fluorescent)
8	0.660 (White fluorescent)	0.660 (White fluorescent)
9	0.762 (White fluorescent)	0.742 (White fluorescent)
10	0.871 (White fluorescent)	0.863 (White fluorescent)
11	0.901 (White fluorescent)	0.888 (White fluorescent)

Table 2. TLC spots of *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane after applying Lieberman Burchard reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	<i>G. lucidum</i>	<i>G. tropicum</i>
1	0.204 (White fluorescent)	0.204 (White fluorescent)
2	0.264 (Red fluorescent)	0.264 (Red fluorescent)
3	0.457 (White fluorescent)	0.335 (White fluorescent)
4	0.498 (White fluorescent)	0.381 (White fluorescent)
5	0.559 (White fluorescent)	0.457 (White fluorescent)
6	0.711 (White fluorescent)	0.498 (White fluorescent)
7	0.863 (White fluorescent)	0.558 (White fluorescent)
8	0.888 (White fluorescent)	0.660 (White fluorescent)
9	-	0.863 (White fluorescent)
10	-	0.888 (White fluorescent)

Two spots are very strong *i.e.* Rf: 0,204 (white fluorescent) and Rf: 0,264 (red fluorescent) under UV (yellow and blue) under normal light after spraying with both reagents. These two spots indicate the presence of steroids, one group of triterpenes (Harborne 1996). This may explain how some people become awake and feel fresh even working until late after drinking crude water extract of *Ganoderma* fruiting body (personal experience).

Ethanol extraction is able to separate the polar compounds including glycosidic triterpenoid. Nishitoba *et al.* (1987) discovered that some triterpenoids of *G. lucidum* can be obtained from ethanol extraction. TLC plate after spraying with Carr Price reagents gave 11 spots for *G. lucidum* and 12 spots for *G. tropicum* (Table 3). However, Lieberman Burchard reagent gave less spots *i.e.* eight spots for *G. lucidum* and nine spots for *G. tropicum* (Table 4). Both reagents can react with triterpenoids producing various fluorescent colour ranging from blue, purple and red fluorescent under UV light 366 nm (Harborne 1996). All spots observed from TLC plate were white fluorescent, ranging from weakly to strongly fluorescent. Weak fluorescence indicates less quantity of the metabolites and strong fluorescence indicates more quantity. Again more spots are observed on the treatment with Carr Price reagent indicating that Carr Price is more sensitive than Lieberman Burchard to

react with triterpenoid metabolites. As was observed on previous extract, *G. tropicum* contains more types of metabolites than *G. lucidum*.

The metabolite production is also influenced by the way the culture is maintained (Garraway & Robert 1984). *Ganoderma lucidum* was first obtained from Fungi Perfecti in the early 1990s. For more than 10 years the isolate was subcultured routinely on synthetic medium. On the other hand, *G. tropicum* was isolated in 1999 from a fruiting body collected from nature, in other words, it is only two years since the first isolation. Theoretically, its genetic diversity is more than the isolate maintained for more than 10 years in the laboratory. This may explain why more types of metabolite were obtained from the *G. tropicum* extract than from the *G. lucidum* extract. Another explanation is that *G. tropicum* naturally (genetically) may produce more metabolites than *G. lucidum*.

Table 3. TLC spots of *G. lucidum* and *G. tropicum* (Ethanol extract) run on Chloroform:Methanol (10:1) after applying Carr Price reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	<i>G. lucidum</i>	<i>G. tropicum</i>
1	0,022 (White fluorescent)	0,058 (White fluorescent)
2	0,058 (White fluorescent)	0,134 (White fluorescent)
3	0,089 (White fluorescent)	0,301 (White fluorescent)
4	0,301 (White fluorescent)	0,334 (White fluorescent)
5	0,357 (White fluorescent)	0,368 (White fluorescent)
6	0,435 (White fluorescent)	0,446 (White fluorescent)
7	0,479 (White fluorescent)	0,479 (White fluorescent)
8	0,535 (White fluorescent)	0,580 (White fluorescent)
9	0,569 (White fluorescent)	0,624 (White fluorescent)
10	0,624 (White fluorescent)	0,680 (White fluorescent)
11	0,736 (White fluorescent)	0,769 (White fluorescent)
12	-	0,814 (White fluorescent)

Table 4. TLC spots of *G. lucidum* and *G. tropicum* (Ethanol extract) run with Chloroform:Methanol (10:1) after applying Lieberman Burchard reagent under UV light (366 nm)

No of spot	Rf value and colour under UV light (366nm)	
	<i>G. lucidum</i>	<i>G. tropicum</i>
1	0,203 (White fluorescent)	0,220 (White fluorescent)
2	0,305 (White fluorescent)	0,305 (White fluorescent)
3	0,339 (White fluorescent)	0,339 (White fluorescent)
4	0,424 (White fluorescent)	0,424 (White fluorescent)
5	0,636 (White fluorescent)	0,534 (White fluorescent)
6	0,721 (White fluorescent)	0,636 (White fluorescent)
7	0,778 (White fluorescent)	0,721 (White fluorescent)
8	0,825 (White fluorescent)	0,778 (White fluorescent)
9	-	0,835 (White fluorescent)

TLC is not a very precise technique for detecting chemical compounds of any natural products. Nevertheless, Carr Price and Lieberman Burchard are the most popular reagents for detecting triterpenes (Harborne 1996). The further steps of using HPLC technique for quantitative analysis or even GCMS and NMR for determining chemical structure of the compounds are required. With the last technique, Kleinwachter *et al.* (2001) were able to determine seven new triterpenoids metabolites (colossalactones 1-7) from *Ganoderma colossum*. Meanwhile, Su *et al.* (2001) divided *Ganoderma* mushroom into 18 groups based on triterpenoids patterns analyzed with HPLC technique. This grouping was also confirmed with morphological data and infertility testing by dimonokaryotic mating.

Overall, triterpenoids from *G. tropicum* and *G. lucidum* are quite similar, based on the Rf values and colour reaction with both Carr Price and Lieberman Burchard. *Ganoderma lucidum* has been extensively studied for active compounds, including triterpenoid metabolites which are mainly reported as ganoderic acid and lusidenic acid (Komoda *et al.* 1985, Nishitoba *et al.*1987). Nevertheless, overall TLC patterns for triterpenoids of both species are not exactly the same. This tells that *G. tropicum* is a different group from *G. lucidum* (Moncalvo *et al.* 1994).

Figure 1. *Ganoderma tropicum* fruiting body parasitizing a *Delonix regia* tree in nature.

Occurrence of polysaccharides based on their monomer forms. TLC results of polysaccharides analysis are presented in Table 5. It can be seen that there are four spots produced after running the extract on a TLC plate with spots 1 and 2 having the same Rf value. Based on the standard, these four spots are glucose, galactose, xylose and rhamnose. Spots 1 and 2 (Rf = 0.333) belong to glucose and galactose. This may happen owing to the similarity of the chemical structures of both sugars. Previous findings have reported that *G. lucidum* can produce GLA which consists of glucose, galactose, xylose and rhamnose. The same pattern of TLC spots was observed from *G. lucidum* and *G. tropicum*, suggesting both species produce the same polysaccharides. All colours of spots are not the same contrast, (intensity) indicating different quantity of each monomer.

Table 5. TLC pattern of monomer forms of polysaccharides of *G. lucidum* and *G. tropicum* (hot water extract) run on n-butanol : acetic acid : ether : water (9:6:3:1) and spraying with Aniline Ftalat

No of Spot	Rf value and colour after spraying with Aniline Ftalat	
	<i>G. lucidum</i>	<i>G. tropicum</i>
1	0,333 Glucose (Light brown)	0,333 Glucose (Light brown)
2	0,333 Galactose (Light brown)	0,333 Galactose (Light brown)
3	0,444 Xylose (Red)	0,444 Xylose (Red)
4	0,555 Rhamnose (Yellow)	0,555 Rhamnose (Yellow)

In this study, only the occurrence of four sugar monomers was observed. It is not conclusive as to whether the monomers belong to GLA or GLB since both polysaccharides were not separated before running TLC. Qing-Yao *et al.* (1994) determined the Mol ratio of GLA and GLB monomer forms based on their retention time of the peak and the areas of HPLC analysis. They found that GLA consists of glucose, galactose, xylose and rhamnose with Mol ratio of 3.2:2.7:1.8:1.0, whilst GLB consists of glucose, xylose and rhamnose with Mol ratio of 6.8:2.0:1.0.

Conclusion

From this preliminary examination of the fruiting bodies of *G. tropicum*, it can be concluded that *G. tropicum* contains triterpenoids and polysaccharides as contained in *G. lucidum*. TLC pattern for triterpenoids of both isolates is not exactly the same, indicating they are different groups of *Ganoderma*.

A.

B.

Figure 2. A. *Ganoderma tropicum* and B. *Ganoderma lucidum* fruiting bodies grown on sawdust based substratum.

Figure 3. TLC of triterpenoids pattern from *G. lucidum* and *G. tropicum* (wash benzene extract) run on dichloromethane observed with UV light 366 nm.

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A REPLY TO HEINO LEPP'S 'A TALE OF TWO CDS'

Dear Editor,

All authors learn to take both good and bad reviews of their published work as normal events; however, Heino Lepp's comparative review of the *Fungimap* and *101 Forest Fungi* CDs (*Australasian Mycologist* 20: 100–103) demands a reply. While parts of his criticism may be justified, two points should be brought to all readers' attention. Firstly, these two CDs target substantially different user groups, have completely different internal structures and very different aims. For these reasons they should never have been compared. Secondly, a comparison of the reviews shows that the review style of each CD is quite different. The *Fungimap* CD is shown to have faults but these are put to one side and the positive aspects are emphasised; the *101 Forest Fungi* CD has faults, but these are generally the only aspects that are emphasised and where good aspects are present, these are denigrated. Fair criticism is absolutely essential to the scientific process, but it should always be unbiased, constructive, cover both good and poor aspects of an author's work and demonstrate the reviewer's understanding of the intent of the author.

There is no doubt that *101 Forest Fungi* has some typographical errors and text mistakes but these can be dealt with in a later publication. Testing of the CD by the Lucid software centre has also shown that the reviewer is incorrect in his statements with respect to full pictures in the key and netsearch operation. The bibliography of the CD was chosen to reflect books readily available on public or school library shelves and the reviewer's comments are therefore irrelevant.

An aspect of the reviewer's comparison which must be addressed is the fact that the most important function of the CD was essentially skimmed over: the Lucid key itself. For the reviewer to devote just over one paragraph out of ten to the specific operation of the Lucid keying software is a considerable omission of fact. One could readily draw the inference that the reviewer concentrated on a subset of 'browser' aspects of the *101 Forest Fungi* CD which allowed comparison with the *Fungimap* CD, rather than examine and explore the unique aspects of the first published key to fungi written in the Lucid key software.

Despite the unfortunate inference that can be made from the reviewer's article, the CDs are not in competition and I consider that they should never have been comparatively reviewed. In essence, the *Fungimap* CD targets 'those involved in the Fungimap project'. The *101 Forest Fungi* CD targets the student population with absolutely no prior interest or knowledge of these organisms. Information from the publisher indicates that the CD is already fulfilling its aims and I am delighted that the love of fungi is being presented to the mycologists of the future.

Dr Tony Young
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ENVIRONMENT PROTECTION AND BIODIVERSITY CONSERVATION ACT 1999

The Commonwealth Government has amended the laws governing exchange of museum and herbarium specimens, export of native wildlife, the import of endangered species and import of live animals and plants. These new provisions will come into effect on 11 January 2002 and have been incorporated into the *Environment Protection and Biodiversity Conservation Act 1999*.

For further information about the new legislation see:

<http://www.ea.gov.au/biodiversity/trade-use/index.html>

The text of the Act can be viewed at: <http://scaleplus.law.gov.au/html/comact/11/6370/top.htm>

The explanatory memorandum can be viewed at:

<http://scaleplus.law.gov.au/html/ems/0/2001/0/0642460051.htm>

Information on obtaining permits for the export or import of specimens for research purposes is available on:

<http://www.ea.gov.au/biodiversity/trade-use/permits>

Contacts

If you have any queries relating to the facilitated exchange system please contact Assistant Director, Wildlife Science and Management, telephone 02 6274 2313 or by email at: tom.kaveney@ea.gov.au

NEW MEMBERS OF THE AUSTRALASIAN MYCOLOGICAL SOCIETY

Full members:

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7303

John Flanagan, Library, Royal Botanic Gardens, Kew,
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REFEREES FOR 2001

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