



# Sexuality and mating types of *Ganoderma philippii*, *Ganoderma mastoporum* and *Ganoderma australe*, three basidiomycete fungi with contrasting ecological roles in south-east Asian pulpwood plantations

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## Abstract

Species of *Ganoderma*, particularly *G. philippii*, *G. australe* and *G. mastoporum*, are commonly found in Indonesian *Acacia mangium* plantations. *Ganoderma philippii* is a root rot pathogen while the other two species are secondary root invaders and wood rotters. Management of *G. philippii* can be supported by knowledge of its gene flow, genetic diversity and population dynamics. This investigation was undertaken to determine the sexuality and mating systems of *G. philippii* and co-occurring *Ganoderma* species, observing the somatic interactions between monokaryotic and dikaryotic mycelia and noting any incompatibility mechanisms. In all three species monokaryons were self-sterile. By examining the contact-zone hyphae, it was determined that in all three species, full sexually compatible matings occurred in 26–33% of the crossings. Two mating type loci were identified, as is the case for a wide range of Basidiomycetes. Dikaryons generated from monokaryotic isolates showed morphological changes as cultures aged. The results of this study indicate that outcrossing is favoured in all three species, *G. australe*, *G. philippii* and *G. mastoporum*, therefore promoting adaptation to new hosts and environments.

**Keywords** *Ganoderma australe* · *Ganoderma philippii* · *Ganoderma mastoporum* · Fungal mating systems

## Introduction

*Ganoderma philippii* (Bres. & Henn. ex Sacc.) has been identified as the dominant pathogen causing red root-rot disease in *Acacia mangium* Willd. (Coetzee et al. 2011; Mohammed et al. 2012; Yuskianti et al. 2014), a key industrial species for pulpwood production in Indonesia (Arisman and Hardyanto 2006). Red root rot is considered an economically damaging disease (Eyles et al. 2008; Potter et al. 2006). *Ganoderma mastoporum* (Lév.) Pat. and *Ganoderma australe* (Fr.) Pat. have also been associated with *A. mangium* in Sumatra (Glen et al. 2009); the former has been isolated from

roots (Yuskianti et al. 2014) but in the absence of pathogenicity tests, it is unclear whether this species can act as a primary pathogen or is merely a secondary coloniser of damaged roots. We reject the synonymy proposed by Wang et al. 2014 for *G. mastoporum* and *G. orbiforme* as even their own data shows high DNA sequence variation between *G. boninense/orbiforme* and *G. mastoporum/G. cupreum* and the oil palm pathogen does not, to our knowledge, occur on hardwoods. It is, however, possible that *G. cupreum* is synonymous with *G. mastoporum* (Glen et al. 2009). *Ganoderma australe* represents a diverse species complex that is widespread across both the northern and southern hemispheres (Moncalvo and Buchanan 2008); it is known as a decay agent of dead wood but not a root pathogen.

Management and prevention of basidiomycete stem and root rots in forest systems is generally achieved by integrating breeding programs for the development of resistant host cultivars and high risk site avoidance with targeted applications of silvicultural, chemical and biological control treatments (Cleary et al. 2013; Eyles

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et al. 2008; Laflamme 2010; Möykkynen and Pukkala 2011; Susanto et al. 2005). Although growers of *A. mangium* can learn from accumulated knowledge and experience of basidiomycete root-rot diseases in other forest systems, direct transference of practices known to reduce inoculum potential in temperate (Woodward et al. 1998) and tropical (Eyles et al. 2008; Nandris et al. 1987) tree crops have met with little success, e.g. planting techniques, root excision and isolation trenching, stump removal and thinning (Ariffin et al. 2000; Pratt 1998), fungicide drenching, stump urea (Pratt 2001) and *Trichoderma* antagonism (Sundram et al. 2008; Susanto et al. 2005). The poor level of control in *A. mangium* plantations is almost certainly due to the non-specificity of the treatments tested, and exacerbated by poor understanding of the pathosystems involved. Economic considerations also severely limit the feasibility of many control strategies (Pratt 1998). For example, the practice of individually removing diseased trees and the underlying soil in high-value crops such as oil palm and rubber may not effectively target the primary mode of spread in pathogens associated with *A. mangium*. Regardless, these intensive approaches to controlling root disease are not economically viable to low-value pulpwood forestry applications (Mohammed et al. 2014).

Disease management may be enhanced by a thorough understanding of the pathogen, and knowledge of its biology and aetiology (Chee 1990; Cooper et al. 2011; Sariah 2003). For example, elucidating the genetic structures of *Ganoderma boninense* Pat. populations in oil palm revealed high levels of genetic diversity within and between plantations, strongly implicating basidiospore dispersal in disease progression (Miller 1995; Miller et al. 1999; Pilotti 2005; Pilotti et al. 2003). Basidiospores are now considered to be the primary means of disease spread, by direct infection of cut fronds and indirect root infection via colonized debris. New infections from mycelial contacts do occur, but far less frequently than was previously thought (Rees et al. 2011; Sanderson 2005). Whilst disease control by the development of resistant material and methods to reduce inoculum at replanting continue to be pursued, management strategies now also involve routine removal of basidiocarps, particularly in first rotation plantings with low levels of infection and few basidiocarps (Hunt and Pilotti 2004).

A knowledge of the sexuality, gene flow, genetic diversity and population dynamics of *Ganoderma* is prerequisite to determining the primary mode of root rot disease infection and spread in *A. mangium* plantations, as has been achieved for *G. boninense* (Sanderson 2005; Sanderson et al. 2000). Studies in *Ganoderma lucidum*

(Tiratana and Chaiprasert 1991), *Ganoderma tsugae* (Adaskaveg and Gilbertson 1986), *Ganoderma collosum*, *Ganoderma microsporium*, *Ganoderma fornicatum* (Hseu and Wang 1996) and *G. boninense* (Pilotti et al. 2002) have revealed that these species possess a tetra-polar mating system with alleles for heterothallism at two loci. Although Rajchenberg (2011) reports this finding as a strong characteristic feature of basidiomycetes, it should not be assumed that all species of the same genus have the same genetics of sexuality. The sexuality of *G. philippii*, *G. mastoporum*, and *G. australe* are unknown. Lim (1977) conducted studies on basidiospore germination of *Ganoderma philippii* (syn. *G. pseudoferreum*) but could only germinate the basidiospores following passage through the digestive system of an insect and did not publish any mating studies. Until recently, germination of *G. philippii* has remained problematic (Page et al. 2017) and this may have prevented such studies in this species.

Genomic sequence analyses have elucidated the genetic architecture of basidiomycete mating systems (James et al. 2013) but determination of mating type using molecular markers is not as reliable as using pairing tests (Skrede et al. 2013). To date, this level of genomic information is not available for the three *Ganoderma* species in this study.

This investigation was undertaken as the first step in a study of the population genetics of *G. philippii* on *A. mangium* in Sumatra, Indonesia. Its aim was to determine the sexuality and mating systems of *G. philippii*, *G. mastoporum*, and *G. australe*. In addition, population genetic studies, as planned for *G. philippii*, will be enhanced by demonstrating independent segregation of microsatellite markers in a set of monokaryotic offspring from a single, heterozygous parent. Knowledge of the genetics, key mode and loci of infection and the propensity to accumulate aggressiveness genes will provide aetiological and ecological insights to programs currently screening for host resistance and biological control agents.

## Materials and methods

### Spore collection

Spores were collected opportunistically and non-destructively from basidiocarps at the following locations:

- Langgam, Lat. 0.13°N, Long 101.6 °E: two *G. philippii* basidiocarps (from separate stumps, approximately 1 km apart in a stand of *E. pellita*); BO22947, BO22948. Spore

collections were made from a third sporocarp in this area in April 2014 but the sporocarp was not collected.

- Logas, Lat. 0.29°S, Long 101.27 °E: one *G. mastoporium* basidiocarp (growing in a mature-age stand of *A. mangium*); BO22949.
- Baserah, Lat. 0.21°S, Long. 101.48°E: two *G. australe* basidiocarps (from two mature-age *A. mangium* wildling trees, approximately 750 m apart in a mature-age stand); BO22945, BO22946.

Following overnight collection of basidiospores onto filter paper (Page et al. 2017) spores were air-dried for 15 min before storage at room temperature in sealed, opaque containers. The basidiocarps were removed from the tree, placed in paper bags, dried for two weeks and then vacuum sealed for storage.

Basidiospore suspensions were incubated in antibiotic solution for 4 h (Page et al. 2017), except for the *G. philippii* basidiospores that were collected in April 2014, which were incubated in antibiotic solution for 24 h. Spore density was calculated using an Optik-Labor Neubauer Improved 0.0025 mm<sup>2</sup> haemocytometer and aliquots diluted to  $3 \times 10^4$  spores ml<sup>-1</sup> (Page et al. 2017).

### Spore germination and single spore isolation

Petri dishes were prepared using optimal media for germination in 50 mm Petri dishes (Page et al. 2017). Spores of *G. philippii* were pipetted onto 1% malt agar plus 2% ethanol (20 g agar, 10 g malt extract in 980 mL dH<sub>2</sub>O, 20 ml technical grade 98% ethanol added post-sterilisation prior to pouring). *G. australe* and *G. mastoporium* were pipetted onto rice dextrose agar (20 g agar, 5 g dextrose, 106 g rice flour in 1 L dH<sub>2</sub>O). For each species, 250 µl spore suspension was spread over the agar surface. Plates were sealed with parafilm and incubated in the dark at 27 °C (*G. philippii* and *G. mastoporium*), or 22 °C (*G. australe*) (Page et al. 2017).

Each plate was assessed for germination every 24 h under 50× phase contrast magnification using a Zeiss Axioskop. Single and suitably spaced germinated spores were cut from the agar surface by using a sterile hypodermic needle and placed, one to a plate, onto 3% potato dextrose agar (47 g Difco PDA in 1 L dH<sub>2</sub>O). Plates were sealed and re-incubated in the dark at the above temperatures. After 6–7 days, the cultures had reached a size of approximately 1.5–2.0 cm diameter. Confirmation of monokaryon isolation was achieved by removing a small portion of mycelium and inspecting for clamp connections under 100× phase contrast magnification: isolates lacking clamp connections were subcultured separately onto PDA,

placed again in dark incubation and allowed to grow to 7 cm diameter.

### Species confirmation

*Ganoderma philippii* and *G. mastoporium* basidiocarps and isolates were confirmed by species specific PCR (Yuskianti et al. 2014) and *G. australe* basidiocarps and isolates by PCR and rDNA ITS sequencing (Glen et al. 2014).

DNA was extracted from sporocarps and fresh mycelium from single spore isolates. Sporocarp material, approximately 20 mg dry weight, was ground by hand with a plastic pestle under liquid nitrogen. Mycelial samples were ground with a plastic pestle and a Kontes motorised pellet mixer in a 1.5-ml microcentrifuge tube. A total of 250 µl extraction buffer (Raeder and Broda 1985) was added and the tubes incubated at 65 °C for one hour. Tubes were centrifuged at 14,000 rpm for 15 min and the supernatant removed. DNA was extracted and purified according to Yuskianti et al. (2014). DNA was eluted in 20 µl of TE buffer and an aliquot diluted 1/10 in TE for PCR.

### Pairing technique and scoring

To study sexual incompatibility, pairings were made as per Adaskaveg and Gilbertson (1986) and Pilotti et al. (2002) with three replicates per pairing. Mycelial plugs of 5 mm<sup>2</sup> were cut from the growing edge of each isolate and placed approximately 1 cm apart in a petri dish containing 18 ml of 3% PDA (47 g Difco), and incubated in the dark at 25 °C. Plates were examined at 10 days, and then every 7 days, for five weeks. At each assessment, a 2 mm<sup>2</sup> sliver of mycelia was removed from the confrontation zone and examined under 100× phase contrast magnification for the presence of clamp connections. All pairings were scored for pigmentation and line formation after three weeks and again after eight weeks incubation.

Two major pairings were performed: i) single-spore isolates from a single basidiocarp (intra-basidiocarp pairings) were paired in every possible combination, i.e. ten homokaryons harvested from each of two *G. philippii*, one *G. mastoporium* and two *G. australe* basidiocarps; ii) single-spore isolates representatives of each mating type, as determined by (i) from each basidiocarp were paired with representatives of each mating type from the other basidiocarp of the same species (inter-basidiocarp, intra-species pairings). Successful anastomoses were characterized by complete fusion of hyphal walls, protoplasm continuity and occurrence of nuclei in the middle of hyphal bridges. Mating types were assigned to each monokaryon according to

patterns of compatibility (Esser 1962; Miller et al. 1999; Pilotti et al. 2002; Raper 1953).

## Fluorescence microscopy

Quantitative evaluation of the nuclear condition of the cells of isolate pairings was made using the fluorescent stains DAPI (4',6-diamidino-2-phenylindole, Sigma-F6057, Fluoroshield™ with DAPI; Sigma-Aldrich) and calcofluor (Fluorescent Brightener No. 28, Sigma-Aldrich). The DAPI illuminated the nuclei while the calcofluor illuminated the cell walls and cross-walls, facilitating the counting of nuclei in each cell.

Mycelial blocks of 2 mm<sup>2</sup> were placed 3.5 cm apart (to allow room for changing objectives) in the centre of glass slides covered with a thin layer of malt extract agar. Slides were sealed in petri dishes and incubated in the dark at 25 °C until hyphal contact and interaction between isolates were observed. Excess moisture was removed by incubating the slides for 20 min at 35 °C, the slides were then immersed in a 0.5 µg/ml aqueous solution of DAPI for not less than 30 min. Each slide was gently rinsed in distilled H<sub>2</sub>O, then counterstained for five seconds with a 0.25% aqueous solution of calcofluor, immediately before examination. The slide was again rinsed, then mounted in the DAPI solution and left for 15 min at room temperature before viewing (Prigione and Marchisio 2004). Fluorescence images were acquired using a Leica Leitz DM RBE fluorescence microscope (A4 filter cube for UV light excitation BP 340–380,400 LP 425) fitted with a Leica DC300F digital camera interfaced to the Leica AF software suite (Leica Microsystems GmbH, Zetzlar, Germany). Image acquisition was under auto-contrast with exposure times between 2 and 4 s.

## Results

### Germination and mycelial interactions

Spores of all *Ganoderma* species germinated after 24–48 h of incubation on agar media, except for those from the third *G. philippii* sporocarp, which were plated at lower density and took up to two weeks to germinate. DNA testing confirmed all putative single-spore isolates as either *G. philippii*, *G. mastoporum* or *G. australe* and were consistent with the basidiocarp from which the spores were collected. Isolates were examined for the presence of clamp connections and ten isolates lacking clamp connections were selected from each parent basidiocarp for mating studies. Each of these isolates

**Table 1** Mating reactions between sibling homokaryotic isolates 11–20 from *Ganoderma australe* fruiting body 1 (GAFB1)

GAFB1	A3B3			A4B4			A4B3		A3B4	
	11	16	13	17	12	18	14	19	15	20
11	–	–	–	+	+	+	–	–	–	–
16		–	–	+	+	+	–	–	–	–
13			–	+	+	+	–	–	–	–
17				–	–	–	–	–	–	–
12					–	–	–	–	–	–
18						–	–	–	–	–
14							–	–	+	+
19								–	+	+
15									–	–
20										–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A and/or B alleles). 13/45 = 0.29 (29% sibling compatibility)

had white mycelium and there was no or very little pigmentation in the agar medium after 2 weeks' growth.

### Intra-basidiocarp pairings

Ten to thirteen single-spore isolates from each individual basidiocarp were paired in all combinations. Six sets of pairings were performed; three from *G. philippii* basidiocarps, one *G. mastoporum* and two *G. australe*

**Table 2** Mating reactions between sibling homokaryotic isolates 1–10 from *Ganoderma australe* fruiting body 2 (GAFB2)

GAFB2	A1B1			A2B2				A1B2		A2B1
	6	3	5	10	4	9	2	8	7	1
6	–	–	–	+	+	+	+	–	–	–
3		–	–	+	+	+	+	–	–	–
5			–	+	+	+	+	–	–	–
10				–	–	–	–	–	–	–
4					–	–	–	–	–	–
9						–	–	–	–	–
2							–	–	–	–
8								–	–	+
7									–	+
1										–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A or AB alleles). 14/45 = 0.31 (31% sibling compatibility)



**Table 3** Mating reactions between sibling homokaryotic isolates 1–10 from *Ganoderma mastoporium* fruiting body 1 (GMFB1)

GMFB1	A5B5			A6B6				A5B6		A6B5
	8	4	1	9	3	6	2	7	10	5
8	–	–	–	+	+	+	+	–	–	–
4		–	–	+	+	+	+	–	–	–
1			–	+	+	+	+	–	–	–
9				–	–	–	–	–	–	–
3					–	–	–	–	–	–
6						–	–	–	–	–
2							–	–	–	–
7								–	–	+
10									–	+
5										–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A and/or B alleles). 14/45 = 0.31 (31% sibling compatibility)

(Tables 1, 2, 3, 4, 5 and 6). All pairings were examined for pigmentation and mycelial characteristics three and eight weeks after inoculation. The pairings of single basidiospore isolates from the third *G. philippii* sporocarp were conducted at a later date and observations were made weekly for 5 weeks.

The homokaryotic self-crosses showed consistent behaviour in each of the three replicates. As expected, self-pairings inevitably lacked line formation or colour

**Table 4** Mating reaction between sibling homokaryotic isolates 1–10 from *Ganoderma philippii* Fruiting Body 1 (GPFB1)

GPFB1	A7B7					A8B8		A7B8	A8B7	
	1	3	5	6	8	9	10	7	2	4
1	–	–	–	–	–	+	+	–	–	–
3		–	–	–	–	+	+	–	–	–
5			–	–	–	+	+	–	–	–
6				–	–	+	+	–	–	–
8					–	+	+	–	–	–
9						–	–	–	–	–
10							–	–	–	–
7								–	+	+
2									–	–
4										–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A and/or B alleles). 12/45 = 0.27 (27% sibling compatibility)

**Table 5** Mating reaction between sibling homokaryotic isolates 11–20 from *Ganoderma philippii* fruiting body 2 (GPFB2)

GPFB2	A9B9			A10B10				A9B10		A10B9
	13	16	20	18	12	19	11	14	15	17
13	–	–	–	+	+	+	+	–	–	–
16		–	–	+	+	+	+	–	–	–
20			–	+	+	+	+	–	–	–
18				–	–	–	–	–	–	–
12					–	–	–	–	–	–
19						–	–	–	–	–
11							–	–	–	–
14								–	–	+
15									–	+
17										–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A and/or B alleles). 15/45 = 0.33 (33% sibling compatibility)

change, with mycelia from the two agar plugs mingling freely. A wide variety of interactions was observed in the other pairings. Mycelial interactions between sibling monokaryons could be subjectively grouped using observations of isolate macromorphology and hyphal

**Table 6** Mating reaction between sibling homokaryotic isolates from *Ganoderma philippii* fruiting body 3 (GPFB3)

GPFB3	A11B11				A11B12			A12B11			A12B12			
	9-1	9-10	9-24	9-22	9-2	9-28	9-34	9-5	9-16	9-14	9-19	9-32	9-8	
9-1	–	–	–	–	–	–	–	–	–	–	–	–	+	+
9-10		–	–	–	–	–	–	–	–	–	–	–	+	+
9-24			–	–	–	–	–	–	–	–	–	–	+	+
9-22				–	–	–	–	–	–	–	–	–	+	+
9-2					–	–	–	+	+	+	+	–	–	–
9-28						–	–	+	+	+	+	–	–	–
9-34							–	+	+	+	+	–	–	–
9-5								–	–	–	–	–	–	–
9-16									–	–	–	–	–	–
9-14										–	–	–	–	–
9-19											–	–	–	–
9-32												–	–	–
9-8													–	–

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles

+ compatible; – incompatible (common A and/or B alleles). 20/68 = 0.29 (29% sibling compatibility)

interactions. Many pairings were characterised by a demarcation zone between the two isolates, starting as a zone of sparse growth. As the culture aged, mycelium covered the plate, but a ‘seam’ was apparent between the two isolates (Fig. 1). This demarcation was maintained after further subculture (Fig. 2). In some pairings, colony morphology changed, from fluffy, off-white mycelium to a mottled, crustose, golden-brown appearance with strong yellow to golden-brown pigmentation in the agar. The first signs of pigmentation were observed at 2 weeks after inoculation, starting as lemon yellow to pale golden-brown and by five weeks the colour had deepened to a strong golden-brown (Fig. 3).

Determination of compatibility based on the presence of clamp connections produced confusing results (data not shown), so the number of nuclei per cell was examined using fluorescent microscopy. This confirmed single nuclei per cell in single basidiospore isolates (Fig. 4a). Examination of the mycelium from the interaction zone of paired isolates revealed that anastomosis occurred between lateral swellings of two neighbouring hyphae (Fig. 4b; peg-to-peg fusion), two hyphal tips (tip-to-tip fusion), between a hyphal tip and a lateral hyphal wall (tip-to-side fusion), and between a hyphal tip and a lateral swelling of a hypha (tip-to-peg fusion). Upon fusion, clamp cells formed as projections of the hypha, enabling nuclear migration to occur by bridging

the cell septa (Fig. 4c, d). Clamp connections were absent in monokaryotic mycelia but were observed in many of the pairings.

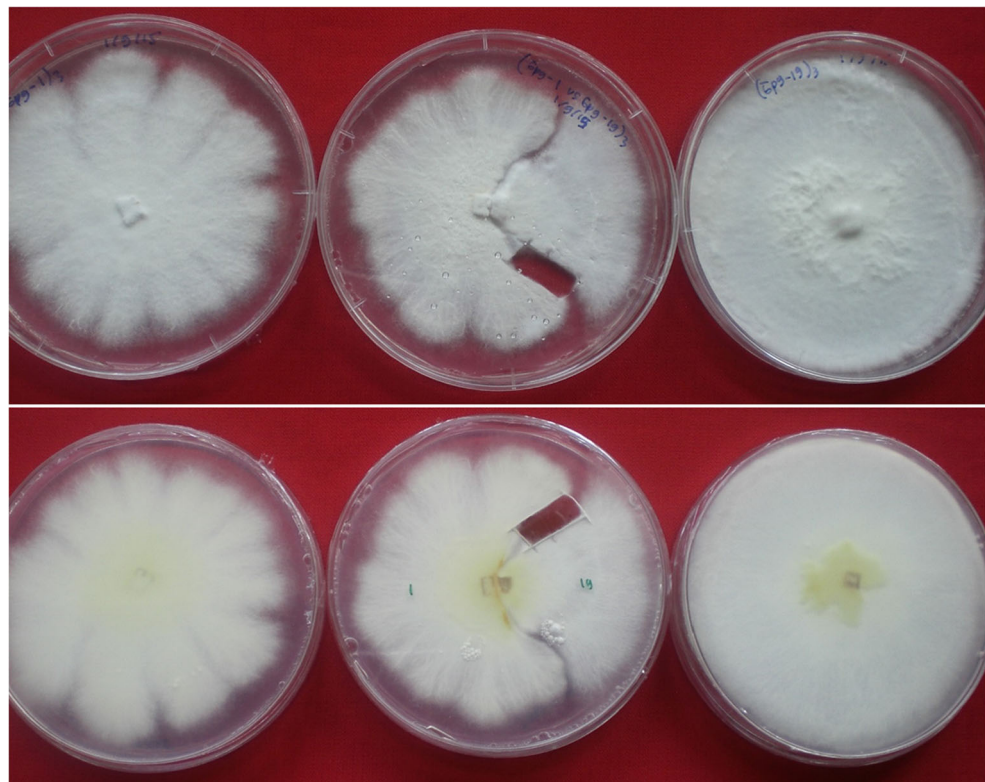
Some pairings showed partial compatibility, with clamp cell formation; however, nuclear migration did not occur to complete dikaryotisation (Fig. 4d). In fully compatible crosses, clamp cells formed, nuclei entered the mycelium of the opposite mating type (Fig. 4e), the hyphal septa appeared to dissolve and the nuclei migrated through the hyphae until they reached a tip cell.

Only the presence of clamp connections and two nuclei per hyphal cell after mating indicated full sexual compatibility. In all cases, monokaryons were self-sterile.

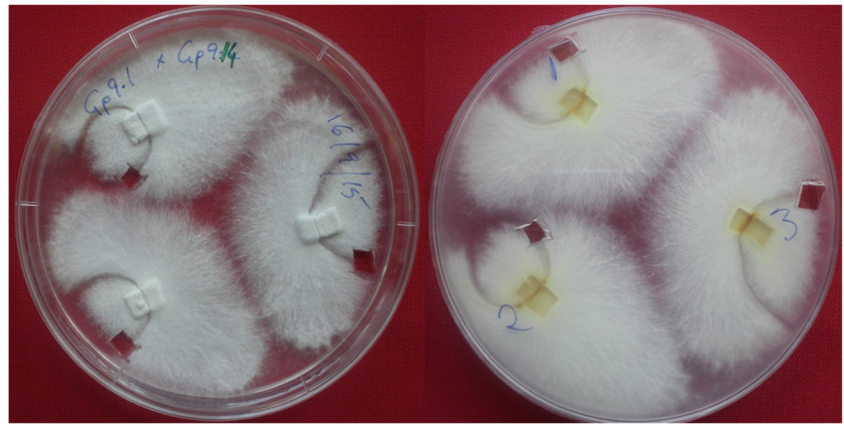
The ratio of compatible to incompatible matings observed was ~1:4 in all three species. The percentage of compatibility varied from 26 to 33%, and single spore isolates could be categorised into groups representing mating types from each basidiocarp (Tables 1, 2, 3, 4, 5 and 6). Sexually compatible monokaryotic parent isolates were arbitrarily assigned differing A and B mating type alleles. Partially compatible monokaryotic pairings were each assigned differing A alleles and identical B alleles. Incompatible monokaryotic parent isolates were assigned identical A and B mating type alleles (see Tables 1, 2, 3, 4, 5 and 6).

Monokaryon isolates were also paired with reconstituted dikaryon isolates from the same parent.

**Fig. 1** Pairing (centre) of incompatible *G. philippii* monokaryon isolates 9–1 (left) and 9–19 (right) as viewed from above (upper panel) and below (lower panel)



**Fig. 2** Three-week old subculture from the interaction zone of incompatible pairing 9-1 × 9-14, as seen from above (left) and below (right)



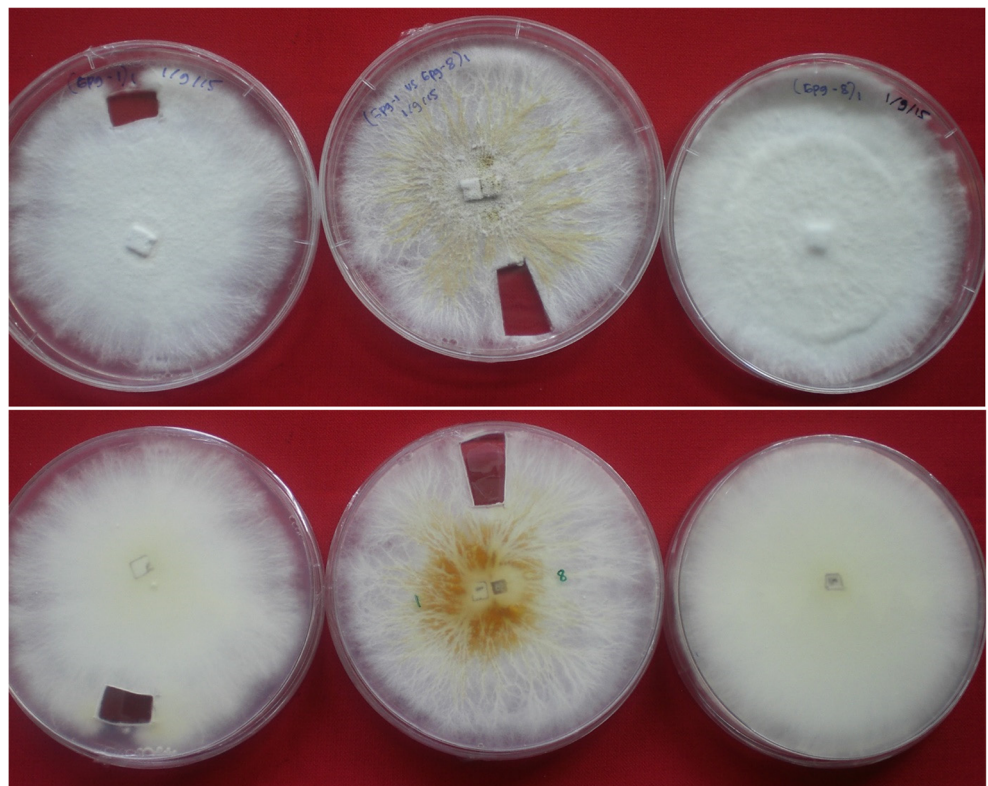
The monokaryon was dikaryotised when paired with a dikaryon containing a compatible nucleus. Gross morphological changes were similar to those observed in pairings between two monokaryons (Figs 5 and 6). The undikaryotised monokaryon grew more slowly than the dikaryon though a zone of sparse growth often separated an incompatible monokaryon/dikaryon pairing (Fig. 6).

Changes in gross colony morphology (esp. colour and texture) were common in compatible pairings but were not completely reliable as indicators of compatible pairings (Table 7). In compatible pairings, the upper

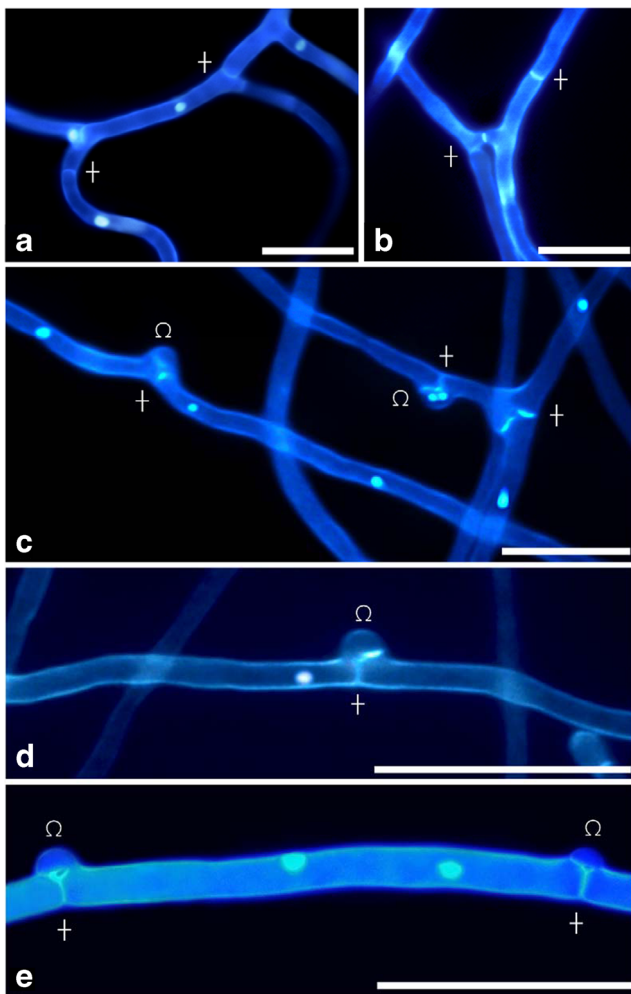
surface changed from a fluffy texture with fine mycelial strands to a grainy crust with thick, ropey mycelial strands. The colour changed from white to yellow or golden-brown. There was often a strong yellow to deep golden-brown pigmentation on the underside of the agar. In incompatible pairings, the colour change was rare, took longer to appear and was usually paler or much smaller in extent.

For each species, monokaryons representing each mating type from each basidiocarp, were out-crossed in all combinations. In all cases full sexual compatibility, as indicated by the formation of clamps and nuclear

**Fig. 3** Pairing (centre) of compatible *G. philippii* monokaryon isolates 9-1 (left) and 9-8 (right) as viewed from above (upper panel) and below (lower panel)







**Fig. 4** Photomicrographic overview of heterokaryon formation and nuclear status during axenic pairings of *G. philippii* single spore isolates. a, monokaryotic hyphae, showing a single DAPI-stained nucleus per cell. b, sibling monokaryotic hyphae; showing a site of hyphal anastomosis resulting from the meeting of two lateral outgrowths (peg-to-peg fusion). c, dikaryotic hyphae; showing the site of hyphal anastomosis, clamp connections and two DAPI-stained nuclei per cell subsequent to migration. d, incompatible pairing; showing a clamp connection but no nuclear migration (one DAPI-stained nucleus per cell). e, dikaryon generated from the mating of fully compatible monokaryons, showing clamp connections and two DAPI-stained nuclei per cell. Clamp cells ( $\Omega$ ), septa ( $\dagger$ ). Bar = 10  $\mu$ m

migration in culture (data not shown), was recorded. Thus, it would seem as if multiple alleles exist at both mating type loci.

## Discussion

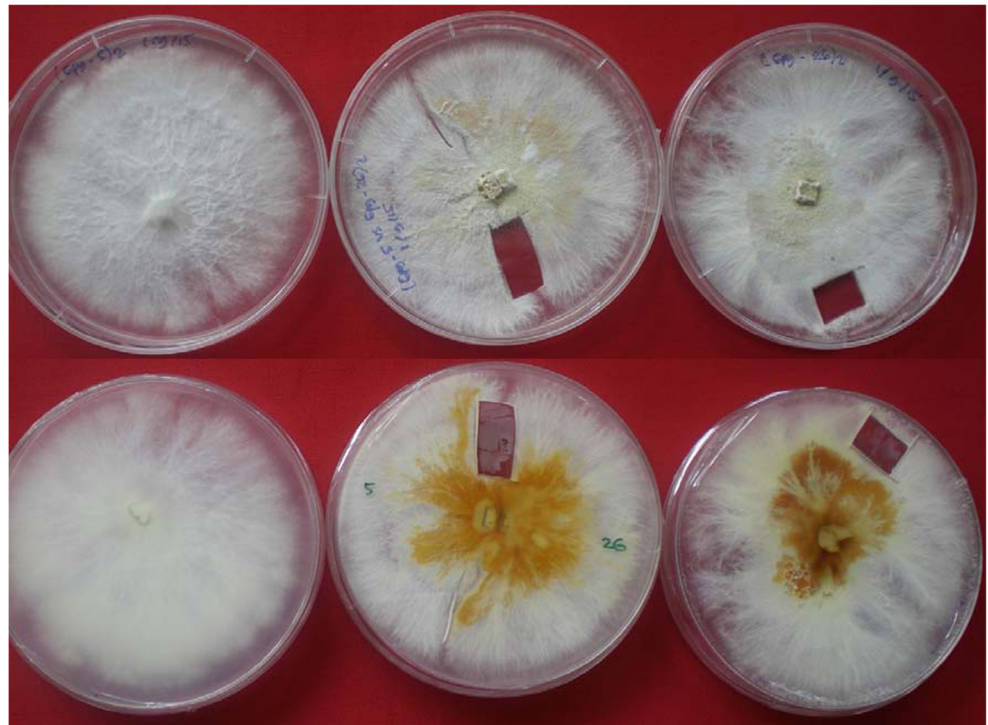
Visual assessment of macroscopic growth form provided a preliminary indication of likely compatibility, as did the presence of abundant clamp connections, but both were less consistent and accurate than direct assessment of nuclear status by fluorescence microscopy. All three

of the *Ganoderma* species in this study were heterothallic as demonstrated by changes in nuclear status and consistent with an estimated 90% of basidiomycete species (Kües et al. 2011). The pairing study confirmed this and the ~1:4 ratios of compatible to incompatible crosses observed between homokaryotic siblings are consistent with ratios found for other *Ganoderma* species (Adaskaveg and Gilbertson 1986; Hseu and Wang 1996; Pilotti et al. 2002; Triratana and Chaiprasert 1991). Though sample sizes in this study were smaller than in many other studies, segregation of monokaryon sibling isolates into two groups with sexual incompatibility between the two groups indicates a tetrapolar mating system with mating type alleles at two loci (Esser 1962, 1971; Miller et al. 1999; Pilotti et al. 2002; Raper 1953). Genomic analyses have clarified the genetic basis of mating system determination in the basidiomycota, and shown that the same genes are present in bipolar and tetrapolar species. These genes include the homeodomain (HD) encoding locus, also called the MAT-A locus, and the pheromone/receptor (P/R) locus, or MAT-B (James et al. 2013). A compatible mating requires heterozygosity at both of these loci and negative frequency-dependent selection results in high genetic variability of MAT alleles. The two loci are linked in bipolar species and unlinked in tetrapolar. Bipolarity is considered to be the ancestral state for fungi, whereas tetrapolarity is considered to be the ancestral state of Agaricomycetes, with bipolarity evolving on several occasions (James 2015; James et al. 2013). The assumption that this transition is irreversible (Raper 1953) has recently been questioned (James et al. 2013). The transition from tetrapolar to bipolar can occur by coalescence of the two MAT loci or by the evolution of a self-compatible pheromone/receptor pair, in either case all genes are still present allowing, theoretically, for reversion to tetrapolarity (James et al. 2013). A third cause of transition to bipolarity may be the formation of pseudogenes at incompatibility loci and reversion to tetrapolarity may be more difficult in this instance.

A tetrapolar mating system is the most complex of known fungal mating systems and is associated with both high outbreeding potential, and low inbreeding potential. Any one progeny can only mate with 25% of its siblings (Ni et al. 2011), affording an in-breeding restriction of 25% that favours out-crossing within a population (Ni et al. 2011). Outbreeding potential will depend on the number of alleles at each MAT locus in a population. In other tetrapolar basidiomycetes the number of alleles at the HD locus has been estimated from three for *Crucibulum vulgare* to 288 for *Schizophyllum commune* (James 2015). At the P/R locus, the number ranges from two (*Tremella mesentericus*) to 354



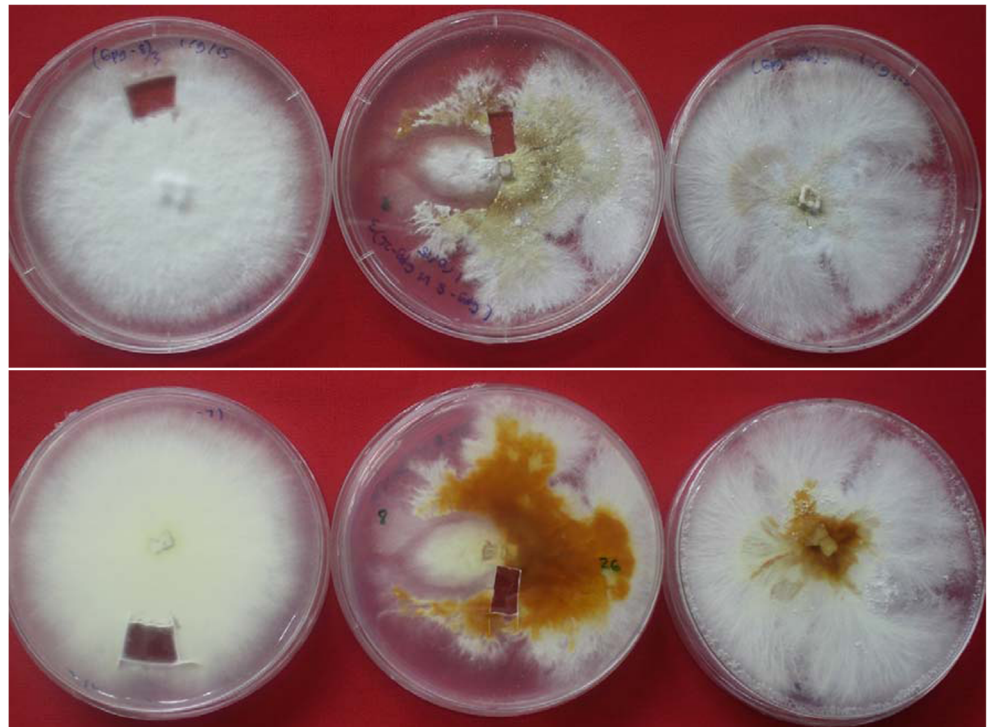
**Fig. 5** A compatible di-mon pairing between monokaryon 9-5 ( $A_{11}B_{12}$ , left) and dikaryon (right) reconstituted from monokaryons 9-14 ( $A_{11}B_{12}$ ) and 9-2 ( $A_{12}B_{11}$ ), as seen from above (upper panel) and below (lower panel)



(*Pleurotus populinus*) (James 2015). These can result in a high number of mating types, which can theoretically result in a very high outbreeding potential, but outbreeding is restricted by the locus with the fewer alleles. A total of 81 HD and 83 P/R alleles were

detected from 52 dikaryons in a population of *Ganoderma boninense* from an oil palm plantation (Pilotti et al. 2003), in a study that clearly demonstrated the involvement of basidiospores in disease dissemination. A similar study looking at populations of

**Fig. 6** An incompatible di-mon pairing (centre) between monokaryon 9-8 ( $A_{12}B_{12}$ , left) and dikaryon (right) reconstituted from monokaryons 9-14 ( $A_{11}B_{12}$ ) and 9-2 ( $A_{12}B_{11}$ ), as seen from above (upper panel) and below (lower panel)



**Table 7** Comparison of macromorphology and scoring of clamp connections with mating reactions between sibling homokaryotic isolates from *Ganoderma philippii* fruiting body 3 (GPFB3)

GPFB3	A11B11				A11B12			A12B11				A12B12	
	9-1	9-10	9-24	9-22	9-2	9-28	9-34	9-5	9-16	9-14	9-19	9-32	9-8
9-1	-	-	-	-	-	c	c	-	-	-	-	CC	CC
9-10		-	-	-	-	-	-	-	-	-	-	CC	CC
9-24			-	-	-	c	c	-	-	-	-	CC	CC
9-22				-	-	-	c	-	-	-	-	CC	CC
9-2					-	c	-	CC	CC	CC	CC	-	-
9-28						-	-	CC	CC	CC	CC	-	c
9-34							-	CC	c	CC	CC	-	-
9-5								-	-	-	-	-	-
9-16									-	-	-	c	-
9-14										-	-	-	-
9-19											-	-	-
9-32												-	-
9-8													-

CC indicates abundant clamp connections (>5 per field of view), c indicates few clamp connections, - indicates no clamp connections seen. Grey shading indicates pairings that resulted in granular crusty cultures with yellow to golden-brown pigmentation

*Ganoderma philippii* has potential to shed light on root-rot disease spread in *Acacia mangium* plantations in Indonesia, but fresh sporocarps of *G. philippii* are available for only a short period and most commonly produced on recently killed trees or those with advanced root disease. Sporocarps of *G. mastoporium* and *G. australe* are much more common than those of *G. philippii* in *Acacia* plantations in Indonesia (D. Puspitasari, pers. obs.) Multiple alleles appeared to be present in *Ganoderma philippii* and *G. australe* at both mating type loci, as shown by the mating compatibility of crosses between monokaryons from different basidiocarps. Given the relative ease of obtaining *G. philippii* isolates from infected roots (Yuskianti et al. 2014; Francis et al. 2014) compared to obtaining isolates from basidiospores, a population genetic study based on molecular markers is likely to be easier to implement than one based on pairing tests between monokaryons.

The three co-occurring *Ganoderma* species investigated in this study all produce a typical white rot of wood, though only *G. philippii* is an aggressive root pathogen. This species also varies in basidiospore production and germination. In addition to a lower abundance of sporocarps, and lower production of basidiospores per unit surface area (D. Page, pers. obs.) *G. philippii* is more particular in its germination requirements (Page et al. 2017).

Although spread and infection of *Ganoderma* in *A. mangium* plantations has historically been thought to occur

clonally (Mohammed et al. 2012), there may be significantly more input into these processes from basidiospores than has previously been considered. Population genetic studies are expected to provide further clarification, as has been the case for *Ganoderma* species in other crops such as oil palm (Pilotti et al. 2003).

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