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Fungal associations in Asphondylia (Diptera: Cecidomyiidae) galls from Australia and South Africa: implications for biological control of invasive acacias

Robin J. ADAIR[a],*, Treena BURGESS[b], Maryna SERDANI[c], Paul BARBER[b]

[a] Department of Primary Industries, PO Box 48, Frankston, Victoria 3199, Australia

[b] School of Biological Sciences & Biotechnology, Murdoch University, Murdoch 6150, WA, Australia

[c] Agricultural Research Council, Plant Protection Research Institute, Private Bag X5017, Stellenbosch, South Africa

Abstract

Gall-forming *Asphondylia* are well represented on Australian *Acacia* and have potential for biological control where Australian acacias cause ecological or economic harm, particularly South Africa. *Asphondylia* in Australia and South Africa are associated with communities of fungi in their galls. In Australia, *Botryosphaeria dothidea* (as its *Dichomera* synanamorph) is the most abundant and sometimes the only fungus present and is implicated as the primary species forming a mutualistic relationship with *Asphondylia.* In the combined analysis of ITS and elongation factor 1-α sequence data, isolates of *B. dothidea* from Australia and South Africa form distinct sub-clades. Female *Asphondylia* carry *B. dothidea* (as *Dichomera* conidia) in mycangia located posterior to sternite 7.

While conidia are always present on field-collected specimens, laboratory-reared females rarely carry conidia. The mechanism and location of spore collection remains unresolved, but needs to be understood if *Asphondylia* species are to be utilised for biological control of invasive Australian acacias. As *B. dothidea* is a polyphagous plant pathogen capable of infecting crops of economic importance, including *Acacia* plantations, the introduction of novel strains of *B. dothidea* associated with biological control of acacia is undesirable, however endemic forms of the fungus could possibly be exploited by introduced *Asphondylia*.

Keywords: Acacia; Asphondylia; Biological control; Botryosphaeria dothidea; Cecidomyiidae; Dichomera; Galls; Symbiosis

Introduction

Asphondylia is a cosmopolitan genus of gall-forming Cecidomyiidae with around 271 described species that occur on a broad range of host families, but particularly the Asteraceae, Fabaceae and Chenopodiaceae (Gagné 2004). Eighteen *Asphondyia* species are described from Australia with six species recorded from Australian acacias (Adair et al., 2000, Adair, 2004 and Veenstra-Quah et al., 2007). *Asphondylia* also utilise African *Acacia* species (now segregated into *Vachellia, Senegalia* and *Acacia*; Maslin 2008) as hosts and form galls on vegetative and reproductive organs (Harris, 1980, Gagné and Marhosay, 1993 and Adair, 2004). *Asphondylia* are always associated with fungi in galls and considerable debate has occurred on the nature of the cecidomyiid-fungus relationship.

Trelease (1884) first suggested that cecidomyiid and fungal interactions may be involved in gall formation in his study of *Asteromyia* on the Asteraceae hosts *Solidago* and *Aster*. Baccarini (1893) then alluded to a mutualistic association between a fungus and *Asphondylia capparides* in the floral galls of *Capparis spinosa*. Subsequently, symbiotic relationships have been proposed for a range of

cecidomyiids (Neger, 1910, Docters van Leeuwen, 1929, Docters van Leeuwen, 1939, Buchner, 1930, Puzanowa-Malysheva, 1935, Goidanich, 1941, Meyer, 1952, Borkent and Bissett, 1985 and Bissett and Borkent, 1988) with the first described from *Asphondylia* galls on the European genera *Cytisus* and *Coronilla* (Neger 1910). The presence of fungal communities in European 'ambrosia galls' prompted Mani (1964) to suggest true symbiosis is absent as no primary symbiont could be identified. Others concluded that fungal co-associates were inquilines and their presence in galls was purely incidental (Ross 1932) or relationships could not be resolved (Batra & Lichtwardt 1963). Compelling evidence for Baccarini's (1893) original proposal for symbiosis is provided by observations of *Cladosporium* conidia being deposited with eggs by *Asphondylia* on *Symplocos* (Docters van Leeuwin 1929), the isolation of a proposed symbiont (*Diplodia* sp.) from *Asphondylia* (=*Ischnonyx*) galls on *Prunus* (Goidanich 1941) and partial evidence of symbiosis in *Lasioptera* on *Eryngium* (Meyer 1952). The discovery of spore-containing mycangia on the abdomens or ovipositors of female cecidomyiids associated with fungi and recent studies on the interactions between plant and fungal tissues within galls provided support for mutualism between Asphondyliini and Lasiopterini and a range of coelomycetes fungi (Borkent and Bissett, 1985, Bissett and Borkent, 1988, Yukawa and Rohfritsch, 2005 and Rohfritsch, 2008).

Most fungi isolated or identified from cecidomyiid galls or mycangia belong to poorly differentiated coelomycete genera associated within the Botryosphaeriaceae, but particularly *Diplodia*, *Dothiorella*, *Fusicoccum* and *Macrophoma* (Bisset & Borkent 1988). Many of the genera that now make up the Botryosphaeriaceae (Phillips et al., 2008 and Crous et al., 2006) were, until recently, known in the genus *Botryosphaeria*. Fungi belonging to the Botryosphaeriaceae appear to be successful as endophytes and as latent opportunistic pathogens of woody hosts (Slippers and Wingfield, 2007 and von Arx, 1987). Many species with cosmopolitan distributions such as *Diplodia pinea* and *Botryosphaeria dothidea* are common opportunistic pathogens (Slippers and Wingfield, 2007 and Smith et al., 1996), while those with a limited host range may not be pathogenic to their hosts (Pavlic et al., 2008 and Taylor et al., in press).

Asphondylia larvae are mostly monophagous or stenophagous and feed on shoots, buds, flowers or fruits and are capable of reducing their host's fitness. As such, several species are under development as classical biological control agents for invasive plants, including Australian acacias (Adair 2008), particularly where control programs target the suppression of reproductive organs without adverse affects on vegetative growth of the host (Adair et al., 2000 and Adair, 2004). However, the biology of the cecidomyiid-fungus relationships within *Asphondylia* galls and their taxonomic status requires clarification before these insects can be utilised as biological control agents. In particular, possibly mutualistic fungi from Australian *Asphondylia* galls are likely to require independent risk assessment, if found to be non-indigenous in regions of intended introduction.

In this paper, the ecological and taxonomic relationships between Australian *Asphondylia* and their fungal associates are determined, particularly those from acacias. We focus on *Acacia mearnsii*, an aggressive invader of riparian ecosystems in South Africa and *Asphondylia glabrigerminis*, which can cause high mortality of *Acacia mearnsii* flower buds. The associations in South African *Asphondylia* are also examined to provide regional perspectives. Our results have broad utility because *Asphondylia* species are known from other invasive plant species where biological control programs are active or in development.

Materials and methods

Fungi from Asphondylia galls

Asphondylia galls were collected from a broad range of host plants in southern Australia and South Africa. Fresh galls were triple surface sterilised by rinsing in 70 % ethanol for 30 s, followed by 1 % NaOCL for 2 min, then again in 70 % ethanol for 15 s before fungal material was dissected from the inner lining of the gall chamber. Dissected tissue was placed in Petri dishes containing potato agar

(PA) made from 20 g potato, 20 g Biolab® agar and 1000 ml H_2O, or water agar (20 g Biolab® agar, 1000 ml H_2O) and cultured at 27 °C. After 1–5 d, small cubes of agar (5 mm × 5 mm) at the outer edge of colonies were transferred to fresh PA plates for sporulation and identification. Isolates were identified at Centraalbureau voor Schimmelcultures, Netherlands and the Biosystematics Division, Agriculture Research Council, Pretoria, South Africa. Dried voucher specimens of *Dichomera* synanamorphs of *Botryosphaeria* from *A. mearnsii* were lodged with the Victorian Department of Primary Industries mycological collection (VPRI 32031, 32032, 41369, 41370, 41373, 41376–41383, 41385–41387, 41394–41401).

Fungi isolated from buds of A. mearnsii

Ungalled flower bud clusters (from here referred to as buds) of *A. mearnsii* were sampled in Australia and South Africa to determine if fungi isolated from *A. glabrigerminis* galls were present as endophytes in the host tree. At nine sites in Australia and five sites in South Africa, five inflorescences with ungalled buds were haphazardly selected from each of five *A. mearnsii* trees. Five buds from each inflorescence were removed and triple surface sterilised using the method described above. Buds were cut in half with a sterile scalpel and plated onto PA or water agar and cultured at 25–27 °C. Mycelial growth was hyphal-tipped onto PA and cultured until identification was possible.

Gall development, anatomy and biology

The internal structure and tissue arrangement of *A. glabrigerminis* galls from *A. mearnsii* and *Acacia irrorata* were examined to help determine the relationship between the host and fungal co-associates. Fresh galls from a range of developmental stages were preserved in glutaldehyde/paraformaldehyde fixative prior to sectioning. Sectioning commenced by soaking galls in 70 %, 90 % and 100 % ethanol consecutively for 1 h to dehydrate tissue before clearing in xylol for 1 h. Galls were infiltrated with liquid paraffin wax at 60 °C for 2 h then embedded in wax before cutting 5 μm sections with a rotary

microtome. Sections were allowed to dry at 60 °C for 1 h before de-waxing in xylol for 10 min and hydrated (100 %, 90 %, 70 % alcohol) before staining with Mayer's hematoxylin and eosin. Sections were then dehydrated (70 %, 90 %, 100 % alcohol) and cleared in xylol before mounting with Entallen medium and covering with a glass coverslip. Tissue structure was examined using phase-contrast microscopy.

Dried mature galls from *A. mearnsii* were moist incubated in sealed containers at 24 °C and 12 h light and examined weekly for development of fruiting structures. Similarly, mature galls collected from *A. mearnsii* trees and litter were placed in nylon mesh bags (20 cm × 15 cm), and placed on the soil surface beneath the canopy of a mature *A. mearnsii* at Frankston (38°12′ S, 145°16′ E), Melbourne on 7 Jan. 2005. Galls were inspected for pycnidia using a stereomicroscope in Mar. and Aug. 2005.

Trees of Australian acacias with populations of *Asphondylia* galls were inspected for fungal fruiting structures by casually inspecting galls at various stages of development at 154 locations in southern Australia. Galls were inspected visually and prospective material was examined in closer detail under a stereomicroscope and followed by single spore isolations where fertile material was detected. At Campbelltown, NSW (33°59′ S, 150°50′ E), where a large population of *A. glabrigerminis* was present, 50 dehisced galls were also collected in Sep. 2004 from the litter layer beneath the canopy of *A. mearnsii* and inspected for fungal fruiting structures.

At Cranbourne, Victoria (38°7′ S, 145°16′ E); Knoxfield, Victoria (37°52′ S, 145°14′ E), Rowville, Victoria (37°54′ S, 145°14′ E) and Campbelltown, New South Wales, inflorescences with mature galls of *A. glabrigerminis* were collected from each of four trees. Haphazardly selected galls were dissected to determine the development stage of the cecidomyiid and the frequency and identity of fruiting fungal associates.

Fungi from Asphondylia adults

The external anatomy of *Asphondylia bursicola* mycangia reared from *Acacia decurrens* fruits from Holbrook were examined using SEM. Ethanol preserved abdomens were critical point dried, mounted on aluminium stubs with carbon glue and sputter-coated with gold palladium alloy then examined with a Leo S440 scanning electron microscope.

To determine the role of cecidomyiids as possible vectors of fungal spores, adults of the *A. glabrigerminis* were reared from mature galls collected from *A. mearnsii* at Cranbourne, Knoxfield and Rowville. At Mittagong (34°26′ S, 150°28′ E), galls were collected from *A. mearnsii* and the closely related *Acacia parramattensis*. Galls were held in plastic emergence cylinders (15 cm × 10 cm) that were sterilised with 70 % ethanol. Adults were collected every 1–12 h and held individually in sterile glass vials. Adults were dried, sexed and then plated onto water agar. Mycelial growth was hyphal-tipped and aseptically transferred to PA and cultured for identification. A total of 21 female and 26 male flies were sampled, together with 42 flies of undetermined sex. Vouchers of fungi were lodged with VPRI (41388–41393).

To determine if female *Asphondylia* carry fungal spores in their mycangia, wild adults were collected by pooting into sterile glass containers from host trees in eastern Australia. In addition, adults were reared from galls held in sterilised cages under laboratory conditions. The presence or absence of fungal fruit bodies on gall material was recorded. The abdomens from freshly caught female flies were removed with a sterile scalpel, cleared and then mounted on glass slides in Canada balsam following the method of Gagné (1989). Mycangia were examined for the presence of conidia using differential interference contrast microscopy. Fungi present in mycangia were identified by dissecting mycangia from wild-caught *A. glabrigerminis* collected either ovipositing or resting on *A. mearnsii* at

Campbelltown, New South Wales. Mycangia were plated onto PA and maintained at 25 °C in a controlled environment chamber with 12 h light. Mycelial growth was hyphal-tipped, aseptically transferred to PA and maintained in culture until sporulation. Voucher specimens were lodged with VPRI (VPRI 32352–32367, 1371,41372,41374,41375,41384,41402–41406).

DNA isolation, PCR amplification and phylogenetic reconstruction

For each isolate, approximately 50 mg of fungal mycelium was scraped from the surface of 7-day-old cultures, ground using a glass rod, suspended in 200 µl of DNA extraction buffer (200 mM Tris-HCL pH 8.0, 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) and incubated for 1 h at 70 °C. DNA was purified using the Ultrabind® DNA purification kit following the manufacturer's instructions (MO BIO Laboratories). Part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified for all isolates using the primers ITS-1F and ITS4 (White *et al.* 1990). In addition, part of the elongation factor 1-α was amplified for selected isolates using primers EF1-728F and EF1-986R (Carbone & Kohn 1999). The PCR reaction mixture, PCR conditions, the clean up of products and sequencing were as described previously (Burgess *et al.* 2005).

To compare the isolates in this study with related species, in particular other isolates of *B. dothidea*, sequences were obtained from GenBank (isolate code, identity and accession numbers for sequence data used are given in TreeBASE SN3376), (http://www.treebase.org/treebase/index.html). Parsimony analysis was performed on individual datasets in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Non-informative characters were removed prior to analysis and characters were unweighted and unordered. The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices)

were determined (Hillis & Huelsenbeck 1992). Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analysis was conducted on the same aligned and combined dataset as that used in the distance analysis. First, MrModeltest v. 3.5 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v. 3.1 (Ronquist & Heuelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1 000 000 generations. Trees were saved each 1000 generations, resulting in 10 001 trees. Burn-in was set at 50 001 generations (i.e. 51 trees), well after the likelihood values converged to stationery, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated.

Inoculation of A. mearnsii buds with conidia of Dichomera

To determine if *Dichomera* present in *Asphondylia* galls were capable of inducing gall formation in the absence of cecidomyiids, an isolate (RJA3001) of a *Dichomera* synanamorph of *B. dothidea* from *A. glabrigerminis* galls on *A. mearnsii* was cultured on PA with an infusion of *A. mearnsii* leaves at 20 °C in a controlled environment chamber. Pycnidia were collected, macerated then added to 5 ml of autoclaved distilled water to produce a spore suspension of 1×10^5 conidia ml^{-1}. In a quarantine glasshouse, 52 inflorescences of *A. mearnsii* with small to mature bud clusters were wiped with spore suspension using a sterile paintbrush. Treated inflorescences were immediately enclosed in clear plastic bags for 24 h and maintained under glasshouse conditions with natural lighting at 21–25 °C. Control and treated inflorescences were inspected for gall formation after 6 weeks.

In a second experiment, macerated pycnidia of the same isolate (RJA3001) were prepared into a wet paste with a drop of autoclaved distilled water. Entomological micro-pins (6 mm × 0.23 mm) were mounted on toothpicks and used as needles to pierce small to large buds of *A. mearnsii* on 13 inflorescences. The tapered ends of the micro-pins were dipped in spore paste immediately prior to piercing individual buds several times by inserting the sharp point just below the outer layer of fringed bracteoles. Inflorescences were bagged and maintained as above.

Oviposition tests with Asphondylia

Caged oviposition tests were used to determine if *Asphondylia* spp. were able to induce galls on their host acacias. Mature bud galls of *A. glabrigerminis* and *Asphondylia pilogerminis* were collected from *A. mearnsii* and *A. baileyana* in Victoria, respectively, and transferred to a quarantine insectary in South Africa where they were stored in clean emergence cages. Both *Asphondylia* species frequently occur on *A. mearnsii* in eastern Australia.

Adult cecidomyiids were collected every 1–2 d and released in cylindrical polyester mesh sleeves (80 cm × 30 cm, 417 µ gauge gauze) tied onto branches of *A. mearnsii* with inflorescences in bud or with developing fruit. Branches with fruit were tested as the closely allied *A. bursicola* develops in young pods. Sleeves were retained on the test plants for 6–11 d after which time all adults had died. The number of adults released in each sleeve ranged from 22 to 44 with a female:male ratio ranging from 1:1 to 2:1. Branches were labelled and retained on the host plant for 2–5 m, after which branches were removed and the number of galled inflorescences were counted. There were four replicates of *A. mearnsii* branches in fruit and 13 replicates of branches in bud. Oviposition tests were determined using field specimens of *A. mearnsii* at Stellenbosch (33^{o}0.56′ S, 18°50′ E) and Theewaterskloof Dam (33°58′ S, 19°8′ E), South Africa.

Results

Fungi from Asphondylia galls

Sixteen accessions of *Asphondylia* galls were made in southern Australia between 1999 and 2003 from 11 host plant species in six families. Nine accessions were from acacias and seven from non-Mimosaceae genera (Table 1). Australian accessions of *Asphondylia* galls yielded 21 fungal taxa in culture with 1–11 species occurring within galls of a single host. All isolations yielded the *Dichomera* synanamorph of *Botryosphaeria*, many at high incidence levels and this was the only fungus isolated from *Dodonaea viscosa* (Sapindaceae), *Acacia cyclops, A. irrorata* and fruit galls of *A. mearnsii*. Eighteen fungal taxa were isolated from *Asphondylia* galls collected from Australian acacias, and all were detected within bud galls from *A. mearnsii* (Table 1).

In South Africa, 10 *Asphondylia* accessions were made from 10 host species and nine families, with only one accession from an African *Acacia* (Table 2). South African accessions of *Asphondylia* galls yielded 11 fungal taxa with 1–5 species occurring within galls of a single host (Table 2). *Dichomera* sp. was frequent in South African *Asphondylia* galls, including galls induced by an undescribed *Asphondylia* sp. on *Acacia karroo*. Incidence levels of *Dichomera* sp. were lower than that occurring in Australian galls. Five fungal genera were common to both Australian and South African *Asphondylia* galls: *Dichomera, Epicoccum, Alternaria, Phoma* and *Fusarium*.

Fungi isolated from buds of A. mearnsii

Twenty-seven fungal taxa were isolated from surface-sterilised buds of *A. mearnsii* collected in Australia with a mean (±SEM) richness of 7.6 ± 1.4 fungal taxa per site (Table 3). Buds of *A. mearnsii* in South Africa had a less diverse fungal flora than buds from Australia, with nine fungal taxa recorded and a mean (±SEM) of 2.2 ± 1.0 fungal taxa per site (Table 3). All but two South African fungal genera (*Pycnostysanus, Chaetomium*) were present in buds from Australian accessions

of *A. mearnsii*. All fungi isolated from buds of *A. mearnsii* from Australia and South Africa occurred at low incidence levels. The pathogenic genera *Alternaria*, *Aureobasidium*, *Coniothyrium* and *Colletotrichum* were widespread in buds of *A. mearnsii* from Australia. A *Dichomera* sp. was isolated from *A. mearnsii* buds at three sites in eastern Australia (Table 3).

Gall development, anatomy and biology

Mature bud galls of *A. glabrigerminis* are globose contracting abruptly into a flattened to tapered apex with a distinct, centrally located nipple-like mucro formed from remnant perianth (Fig 1A). The exterior is glabrous to sparsely pubescent. Gall formation commences soon after oviposition (Sep.–Oct.) where slightly swollen buds occur amongst the withered perianth of flowers. Galls expand rapidly reaching full size within several months, although larval development is slow in the early stages of gall development. Larvae undergo accelerated development 1–2 m before emergence and occupy a central development chamber that encompasses most of the interior space of the gall.

Mycelia of fungal co-associates are evident early in gall development, although mostly localized in the distal region, probably around the point of inoculation or entry. Mycelia develop around, but not within, staminal tissue and line the inner wall of the bud gall (Figs 2A,B). In young galls, staminal tissues appear normal, but filaments soon become grossly thickened and enlarged with the expansion and development of mycelia (Fig 2C). The gall wall progressively becomes thickened through the growth of sclerenchyma cells and simultaneous compaction of inner parenchyma tissue. Fungal mycelia ramify in the apex of the gall and extend across the inner gall surface and amongst the stamen bundles, where cellular disintegration of staminal tissue and the outer parenchyma of the gall wall are evident. A larval cavity forms with advanced disintegration of staminal tissue, which is associated with compression of remnant staminal tissue against the inner gall wall amongst thin layers of mycelium (Figs 2D,E).

In a mature gall, the cecidomyiid larva occupies most of the gall cavity and is surrounded by a compacted layer of melanised fungal mycelium that forms a mantle over the entire inner gall wall (Fig 2F). Staminal remnants occur in the mantle, but intra-cellular penetration of intact host tissue by mycelium is absent in all stages of gall development. The pupa creates an emergence hole through the fungal mantle and gall wall by rotating and using its antennal horns as a cutting device (Fig 1B). Almost without exception, *Asphondylia* galls developing on buds of Australian acacias contain both an immature cecidomyiid (egg, larva or pupa) and fungal mycelium, although in many cases the cecidomyiid may succumb to parasitoids, disease or predators and may not be readily detected.

Moist incubation of mature *A. glabrigerminis* galls resulted in a proliferation of generalist saprotrophic fungi, but not *Dichomera* sp. Similarly, detached galls exposed to field conditions for 7 m were devoid of internal or external fruiting structures linked to the dominant fungal associate. At field sites in both Australia and South Africa, where *Asphondylia* were located, fungal fruiting structures on galls attached to host plants were extremely rare and sporadic. However, in Sep. and Oct. 2004, at three sites in eastern Melbourne (Knoxfield, Rowville and Cranbourne), erumpent pycnidia with cirri of *Dichomera* sp. were found on galls of *A. glabrigerminis* occurring on *A. mearnsii*. Pycnidia development coincided with the emergence of adult *Asphondylia* (Table 4). Pycnidia were solitary to aggregated, dark brown to black, globose and raised above the gall surface. The external gall wall often cracked to reveal developing or mature stroma (Fig 1C) and the gall contents consisted of a solid matrix of melanised hyphae (Fig 1D). In nearly all cases pycnidia were found on galls where the immature stages of *Asphondylia* had either died or were absent. Except for one case, galls with normal *Asphondylia* development (larvae or pupae present or emergence completed) were devoid of pycnidia (Table 4). Uredinia of the rust fungus *Uromycladium notabile* were found on four normal galls and one parasitised gall at the Campbelltown site.

Cirri from the pycnidia on galls of *A. glabrigerminis* produced two distinct forms of conidia, a *Dichomera*-type of muriform, pigmented and irregularly shaped conidia, (8.5–)10–18 (–22) × (5–)6–6.5(–7.5) μm [average length 13.6 μm, average width 6.6 μm], and a *Fusicoccum*-type of aseptate, hyaline and fusiform conidia (17.5–)20–23.7(–25) × (5–)7.5–8.7(–10) μm [average length 21.7 μm, average width 8.4 μm]. Monospore cultures of each conidium type produced identical *in vivo* growth characteristics resulting in the formation of pycnidia that again produced two forms of conidia. Isolations were subject to sequencing (see below) to clarify their identification. Growth characteristics and conidial morphologies were consistent with anamorphs of *Botryosphaeria* (Barber *et al.* 2005).

Fungi from Asphondylia adults

Mycangia of *Asphondylia* from Australian *Acacia* consist of a membranous sac posterior to the seventh abdominal sternite and are similar to the organs described from congeneric species (Bissett & Borkent 1988) (Figs 1E,F). The sac extends laterally to the margin of the sternite and opens broadly along the posterior margin. The apex is widely obtuse and a hairless, longitudinally grooved pad subtends the entrance to the mycangium (Fig 1F).

Laboratory-reared adults of *A. glabrigerminis* act as vectors of fungi. Nine fungal genera were isolated from adult *A. glabrigerminis* and all were mitosporic Ascomycota and predominantly cosmopolitan saprotrophs (Table 5). A *Cladosporium* sp. was most abundant on adults (37 %), followed by a *Dichomera* sp. (15.7 %). The location of fungal spores on the bodies of whole adult *Asphondylia* was not determined.

No laboratory-reared female *Asphondylia* from *A. baileyana, A. mearnsii* and *A. decurrens* contained visible fungal conidia in their mycangia when fruiting bodies were not present on the external gall surface. In the presence of *Dichomera* sp. fruiting bodies, up to 90 % of females contained muriform

conidia resembling those produced by *Dichomera* sp. within the mycangial pouch. Similarly, up to 95 % of wild-caught adults contained mycangial conidia (Table 6).

Seven fungal species were isolated from 47 excised mycangia from wild-caught *A. glabrigerminis* from *A. mearnsii*, but were dominated by *Dichomera* sp., which occurred in 74 % of individuals (*n* = 34). Less frequently occurring species were either common saproptrophs *Alternaria* sp. (*n* = 3), *Epicoccum* sp. (*n* = 2), potential pathogens *Diplodia* sp. (*n* = 3), common soil fungi *Trichoderma* sp. (*n* = 1), or were unidentified hyphomycetes (*n* = 2).

DNA isolation, PCR amplification and phylogenetic reconstruction

Dichomera spp. were consistently isolated from galls of Australian and South African *Asphondylia* and therefore proposed as the primary gall associate and possible symbiont. The phylogenetic relationship between *Dichomera* spp. collections from each region was determined using pure cultures maintained on PA.

Fifteen isolates of *Dichomera* from 10 species of *Asphondylia* from Australian and South African material were prepared. Isolates were collected from 11 host plant species including single spore isolates of *Fusicoccum*-type and *Dichomera*-type conidia from a single host gall (Table 7).

All isolates used were initially compared to each other and to closely related species based on the ITS sequence data alone (TreeBASE, SN3376), and grouped together in a strongly supported clade with other isolates of *B. dothidea* from around the world. The combined ITS and EF1-α sequence data consisted of 921 characters, of which 275 were parsimony informative and these were included in the analysis. The dataset contained significant phylogenetic signal compared to 1000 random trees

($P < 0.01$, g1 = −1.51). Heuristic searches of unweighted characters in PAUP resulted in four most parsimonious trees of 536 steps (CI = 0.78, RI = 0.89) (TreeBASE, SN3376). Bayesian analysis resulted in a tree with similar topology (TreeBASE, SN3376) to the parsimony tree (Fig 3). All isolates from this study group clustered together with other *B. dothidea* isolates from around the world in a strongly supported clade that is separated with strong support from *B. corticis* (Fig 3). There was more sequence variation among isolates of *B. dothidea* in the EF1-α gene region than in the ITS region (TreeBASE, SN3376). In the combined analysis, isolates collected from *Asphondylia* galls in South Africa form a distinct group within the *B. dothidea* clade that is also separate from other *B. dothidea* isolates collected from grape vines in South Africa.

Inoculation of A. mearnsii buds with conidia of Dichomera

A. mearnsii buds inoculated with concentrated *Dichomera* conidia, in suspension or as a paste, flowered normally within several weeks of treatment. After 6 weeks, no bud galls had formed. Galls normally become apparent at field sites within 5 weeks from emergence of adult *Asphondylia*.

Oviposition tests with Asphondylia spp.

A. glabrigerminis oviposit in immature buds 1/2–2/3 of full size, mostly in the late afternoon and deposit a single egg usually in the distal region between the top of the anthers and the inner perianth wall. Under caged conditions, adults were observed in mating positions and females performed oviposition rituals that involved short episodes of antennal probing at the potential oviposition site followed by foreleg tapping in a tight circle over the bud surface. Oviposition then commenced with the female acutely bending her abdomen beneath the thorax before probing and then inserting her ovipositor through bud tissue. However, in oviposition tests using sleeved branches of *A. mearnsii*, cage-reared adults of *A. glabrigerminis* and *A. pilogerminis* failed to induce gall formation after 2–

5 m from the introduction of adults. We were unable to determine whether females had been fertilised by males and that eggs were inserted during the oviposition ritual.

Discussion

Asphondylia galls on Australian *Acacia* invariably occur with fungi that proliferate in the early stages of gall development. In buds, fungal mycelia ramify and surround enlarged stamens that are eventually degraded in the presence of the developing insect or are compacted within melanised hyphae that surround the inner surface of the gall. Intra-cellular penetration seems to be absent. In fruit galls, mycelia surround the developing seed causing its abortion and eventual destruction. Adult *Asphondylia* emerge through the wall of the gall leaving their exuviae in the emergence holes cut by pupae. In galls from Australian *Acacia*, fruit bodies of fungal associates were extremely rare. However, sporadic formation of erumpent pycnidia of *B. dothidea* (*Dichomera* synanamorph) occurred in eastern Australia on galls where the cecidomyiid had perished and coincided with emergence of adult *A. glabrigerminis* from other healthy galls.

Botryosphaeria dothidea (as its *Dichomera* synanamorph) was the most abundant and widespread fungus isolated from *Asphondylia* galls from Australian *Acacia* species and in several cases the only fungus present. It has been previously shown that the *Fusicoccum* and *Dichomera* synanamorphs of *B. dothidea* can be produced within the same pycnidium (Barber *et al.* 2005), and although this was not observed in the present study, DNA-based sequence data showed both spore-types isolated from cirri of single galls were identical. *Dichomera*-type conidia predominate in mycangia of wild *Asphondylia* from Acacia and have identical ITS and EF1-α sequence characteristics to a *Dichomera* sp. isolated from bud and fruit galls of three species of Australian *Acacia, Dodonaea viscosa* (Sapindaceae), *Hybanthus floribunda* (Violaceae) and *Billardiera variifolia* (Pittosporaceae). This evidence suggests that *B. dothidea* is the primary fungus present in bud and fruit galls from Australian *Acacia* and is

possibly actively collected by female *Asphondylia* and utilised to foster larval development. Furthermore, *B. dothidea* is also present in South African *Asphondylia* galls. Bisset & Borkent's (1988) suggestion that there may be fewer species of fungus than cecidomyiids involved in ambrosia galls, that several cecidomyiids may use the same fungus, and that the primary fungal co-associate is *Botryosphaeria* sp., is upheld by this study. *Asphondylia* larvae in Australian galls are always associated with internal fungal mantles. We did not study the feeding behaviour of *Asphondylia* larvae, nor other possible developmental requirements linked to the presence of *B. dothidea*, but this is required to strengthen the case for symbiosis between the two organisms. *In vitro* culture of *Asphondylia* larvae in the presence of *B. dothidea* may elucidate the relationship between the two organisms.

The inadvertent introduction of plant pathogens, such as *Uromycladium* spp., with insects used for the biological control of Australian acacias in South Africa, is a concern shared by the biological control fraternity and commercial forestry industries in South Africa. Insects can act as vectors of plant pathogen spores (Favaro and Battisi, 1993, James et al., 1995 and Pakaluk and Anagnostakis, 1997) and direct-releases of insects have a greater risk of accidental introductions of pathogens than insects that are reared through one or more generations in a quarantine laboratory prior to release in the field. Laboratory-reared *Asphondylia* have a low diversity and incidence of phoretic fungi primarily because ecdysis occurs externally and because of the scarcity of sporulating structures on the buds and fruits of Australian *Acacia*. Most are common saprotrophs but potential pathogens may also occur. When pycnidia of *B. dothidea* are present on galls at ecdysis, adult *Asphondylia* transport conidia within mycangia and act as a fungal vector. If direct release of *Asphondylia* adults were considered in a biological control program for Australian *Acacia*, collection of galls without fruiting structures and subsequent surface sterilization prior to adult emergence would reduce the risk of accidental transmission of potential pathogens.

The Botryosphaeriaceae is a family rich in parasites, saprotrophs and endophytes with a world-wide distribution on a wide range of hosts, mainly woody plants, but also is found in the stems of grasses and thalli of lichens (Smith et al., 1994, Denman et al., 2000 and Slippers and Wingfield, 2007). *B. dothidea* is a common and economically important pathogen affecting a range of crops, but notably: apples, eucalypts, pines, pistachios and grape vines (Hodges, 1983, Pennycook and Samuels, 1985, Michailides, 1991, Parker and Sutton, 1992, Smith et al., 1994, Phillips, 1998 and Burgess et al., 2005). It is also reported as a pathogen of *A. mearnsii* in South Africa (Roux *et al.* 1997). More than 40 species of *Dichomera* have been described, mostly from outside Australia and from a wide range of hosts (Sutton 1980), often as pathogens or endophytes (Gregory, 1982, Butin, 1992, Yuan, 1999, Slippers et al., 2004 and Barber et al., 2005). In eastern Australia, a close relationship occurs between lesions of *Dichomera eucalypti* on eucalypt leaves and a species of eulophid wasp (Whyte 2003).

A *Camarosporium* sp. and *Neofusicoccum australe* have been recorded from Australian *Acacia* (Shivas, 1989, Barber et al., 2005, Philips et al., 2006 and Taylor et al., in press), however, these fungi have not been found associated with *Asphondylia* galls and, based on morphological features, are distinguishable from the *Dichomera* synanamorph of *B. dothidea* associated with *Asphondylia* galls. Although *B. dothedia* is a known endophyte (Smith et al., 1996, Slippers and Wingfield, 2007 and Taylor et al., in press), we could not show that it is endophytic in *A. mearnsii*. Despite extensive isolations from surface-sterilised buds, the incidence was very low from trees where *Asphondylia* galls were later found to be present. If *B. dothidea* is endophytic in *A. mearnsii* and other *Asphondylia* hosts, it would help explain the widespread occurrence of this fungus in galls where fruit bodies are rarely observed. The presence of *B. dothidea* in *Asphondylia* mycangia could then be interpreted as a secondary transport process, rather than one critically linked to functional symbiosis. More intensive sampling of *A. mearnsii* both in South Africa and Australia is required to clarify the trophic status of *B. dothidea* found within *Asphondylia* galls.

The occurrence of *B. dothidea* in *Asphondylia* galls across southern Australia and South Africa suggests this fungus is ubiquitous. However, the source of conidia collected by these flies remains obscure. Although pycnidia were found on galls from *A. mearnsii* where the cecidomyiid had perished, pycnidia were absent in most seasons and were not observed on other *Acacia* species or genera with *Asphondylia* galls. We speculate that *Asphondylia* larvae may regulate mycelial development and sporulation, and the development of pycnidia occurs, albeit sporadically, when *Asphondylia* larvae and their parasitoids fail to complete development in galls. Whether the fungi within galls act as antagonists to the development of *Asphondylia* larvae in some situations is unclear. It seems that in most seasons female *Asphondylia* from Australian hosts transport *B. dothidea* conidia from sources other than their galls prior to oviposition. Sporulation of *Macrophoma gallicola*, the fungal associate of *Asteromia carbonifera* galls from *Solidago*, occurs on infected leaves shed in the previous autumn (Bissett & Borkent 1988). However, we did not see a similar process for infected acacia galls positioned in the litter layer beneath trees of *A. mearnsii*. As *B. dothidea* is a known endophye and latent pathogen, it is possible that the fungus could be present on dead twigs or stem cankers and the insect uses these as a source of conidia. The exact method of spore collection by female *Asphondylia* is also unresolved, but may occur by scooping spores into the mycangium (Borkent and Bissett, 1985 and Rohfritsch, 2008). The furrowed pad at the entrance to the mycangium (Fig 1B), together with the obtuse apex renders such a mechanism feasible. Hairs surrounding the mycangial pouch may act as a 'scrub brush' to dislodge conidia and direct them towards the mycangial pouch (Rohfritsch 2008). While the process of introduction of conidia into host tissues remains unclear and requires clarification to confirm suggestions of deliberate insect-mediated spore transfer, Rohfritsch (2008) suggests conidia are dispersed into oviposition channels in a fluid consisting of collateral-gland secretions and plant fluids released from drilling by the ovipositor. However, fecal transfer of spores (Haridass 1987) also remains a possibility and also requires investigation. An additional possibility is that spores are deposited on the external gall surface and following germination, hyphae penetrate the gall chamber through oviposition channels. The failure of laboratory-reared *Asphondylia* to induce galls in sleeved cages in the field, suggest that the simultaneous presence of *Dichomera* sp. and the immature stages of *Asphondylia* may be required to

initiate gall development, where *Asphondylia* larvae may induce gall formation through salivary secretions and *Dichomera* sp. hyphae supplement the nutrition of the developing insect.

B. dothidea was isolated from *Asphondylia* galls in both Australia and South Africa and it is therefore reasonable to expect that Australian *Asphondylia* should be able to utilise South African *B. dothidea* for gall formation. Thus, "fungus-free" insects could be imported into South Africa and therefore obviate the need for the introduction of Australian genotypes of *B. dothidea,* which would require independent and comprehensive host specificity and risk assessment analysis. Furthermore, the role of *Asphondylia* as a vector of *B. dothidea* and implications for biosecurity of commercial acacia plantations in South Africa requires resolution. However, while the mechanism and location of spore collection by female *Asphondylia* is elusive and laboratory-reared *Asphondylia* fail to induce galls, a problem common to caged *Asphondylia* (Yukawa *et al.* 1983), matching South African *B. dothidea* isolates with Australian midges for biological control of invasive acacia will be difficult. Further understanding of the oviposition requirements of captive female *Asphondylia* is required to resolve this matter.

Asphondylia and fungal co-associate compatibility is an important issue in the development of these insects as biological control agents and may have been a contributing factor for the failure of *Asphondylia opuntiae* to establish on the cactus *Opuntia inermis* in Australia, despite the release of large numbers of insects (Hamlin, 1924 and Mann, 1969). These considerations will be important for use of *Asphondylia* as biological control agents for other weeds such as *Chromolaena odorata*, *Ligustrum* spp., *Prosopis* spp., *Lantana camara, Ulex europeus, Sida acuta, Sida rhombifolia* and *Cytisus scoparius*.

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Table 1.

Fungi isolated from *Asphondylia* galls collected in Australia from indigenous hosts

Cecidomyiid	Host family	Host species	Organ[a]	*n*	Fungi[b]
Asphondylia sp.	Mimosaceae	*Acacia littorea*	B	11	*Dichomera* sp.[c] (3), *Bipolaris* sp. (1), *Colletotrichum* sp. (1), *Phoma* sp. (1), *Septoria* sp. (2)
Asphondylia glabrigerminis	Mimosaceae	*Acacia irrorata*	B	11	*Dichomera* sp.[c] (9)
Asphondylia bursicola	Mimosaceae	*Acacia irrorata*	F	10	*Dichomera* sp.[c] (3)
Asphondylia seminis	Mimosaceae	*Acacia cyclops*	F	10	*Dichomera* sp.[c] (3)
Asphondylia bursicola	Mimosaceae	*Acacia mearnsii*	F	20	*Dichomera* sp.[c] (20)
Asphondylia glabrigermins	Mimosaceae	*Acacia mearnsii*	B	20	*Dichomera* sp.[c] (2)
Asphondylia pilogerminis	Mimosaceae	*Acacia mearnsii*	B	2	*Dichomera* sp.[c] (1)
Asphondylia glabrigerminis	Mimosaceae	*Acacia mearnsii*	B	40	*Dichomera* sp.[c] (2), *Alternaria* sp. (6), *Botryocrea* sp. (1), *Chaetomium globosum* (1), *Diplodia* sp. (6), *Drechslera biseptata* (1), *Fusarium lateritium* (6), *Heteropatella* sp. (1), *Phoma* sp. (2), *Xylaria* sp. (1), sterile grey (1)
Asphondylia pilogerminis	Mimosaceae	*Acacia mearnsii*	B	40	*Dichomera* sp.[c] (2), *Alternaria* sp. (1), *Apiosordaria verruculosa* (1), *Coniothyrium* sp. (1), *Diplodia* sp. (2), *Epicoccum purpureum* (1), *Phoma* sp. (2), sterile grey (2), yeast (3)
Asphondylia dodonaeae	Sapindaceae	*Dodonaea viscosa*	L, S	10	*Dichomera* sp.[c] (6)
Asphondylia anthocercidis	Solanaceae	*Anthocersis littorea*	F	14	*Dichomera* sp.[c] (2), *Alternaria* sp. (1), sterile white (1), sterile grey (5)
Asphondylia inflata	Chenopodiaceae	*Halosarcia pergranulata*	S	7	NI
Asphondylia floriformis	Chenopodiaceae	*Sarcocornia quinqueflora*	S	–	NI
Asphondylia sp.	Pittosporaceae	*Billardiera variifolia*	ST	10	*Dichomera* sp.[c] (17), *Phragmotrichum* sp. (1)
Asphondylia sp.	Pittosporaceae	*Billardiera heterophylla*	B, F, ST	10	*Dichomera* sp.[c] (15), *Phoma*. sp. (1)
Asphondylia sp.	Violaceae	*Hybanthus floribundus*	B	14	*Dichomera* sp.[c] (9), *Cladosporium* sp. (1), *Phoma* sp. (1), sterile white (1)

a Host organs infected by cecidiomyiid: L = leaves, F = fruits, B = buds, S = stems, ST = shoot tips, n = number of galls sampled.

b Numbers in parenthesis are the number of isolates obtained for each fungal taxon.

c Dichomera synanamorph of Botryosphaeria. NI = No fungi isolated from galls.

Table 2.

Fungi reared from *Asphondylia* galls collected in South Africa. All host species are indigenous to South Africa, except for *Ricinus communis*

Cecidomyiid	Host family	Host species	Organ[a]	*n*	Fungi[b]
Asphondylia sp.	Vitaceae	*Rhoicissus digitata*	F	5	*Dichomera* sp.[c] (5), *Alternaria* sp. (1)
Asphondylia sp.	Fabaceae	*Psoralea oligophylla*	F	12	*Dichomera* sp.[c] (9)
Asphondylia sp.	Fabaceae	*Virgilia oroboides*	B	19	*Dichomera* sp.[c] (8), *Fusarium* sp. (4), *Nigrospora* sp. (5), *Alternaria.* sp. (1), sterile black, yeast-like (1)
Asphondylia sp.	*Mimosaceae*	*Acacia karroo*	B	13	*Dichomera* sp.[c] (1), *Alternaria* sp. (1), *Epicoccum pupurascens* (1), *Phoma* sp. (1), sterile grey (4)
Asphondylia sp.	Ebenaceae	*Diospyros glabra*	S	15	*Fusicoccum* sp. (?)
Asphondylia sp.	Mesembryanthaceae	*Psilocaulon articulatum*	F	1	*Dichomera* sp.[c] (1)
Asphondylia ricini	Euphorbiaceae	*Ricinus communis*	B	13	*Macrophoma* sp. (6)
Asphondylia sp.	Tiliaceae	*Grewia occidentalis*	B	7	*Dichomera* sp.[c] (3), *Alternaria* sp. (1), *Phomopsis* sp. (1)
Asphondylia sp.	Chenopodiaceae	*Salsola* sp.	ST	4	*Epicoccum pururascens* (3)
?Asphondylia sp.	Santalaceae	*Osyris compressa*	B	38	*Dichomera* sp.[c] (1), *Epicoccum purpurascens* (1), sterile black, yeast-like (3)

a Host parts infected by the cecidiomyiid: L = leaves, F = fruits, B = buds, S = stems, ST = shoot tips, *n* = number of galls sampled.

b Numbers in parenthesis are the number of isolates obtained for each fungal species.

c *Dichomera* synanamorph of *Botryosphaeria*.

Table 3.

Fungi isolated from surface-sterilised ungalled buds of *Acacia mearnsii* from Australia and South Africa

Country	Site	State or Province[a]	Asphondylia spp. Present[b]	*n*	Fungi[c]
South Africa	Stellenbosch	WC	N	–	*Alternaria* sp. (1), *Colletotrichum gloeosporioides* (1), *Nigrospora* sp. (1), *Phomopsis* sp. (1), *Pycnostysanus* sp. (1), sterile grey (1)
	Villiersdorp	WC	N	25	Sterile white (1)
	Grabouw	WC	N	25	*Chaetomium* sp. (1), sterile white (1)
	Paarl	WC	N	25	NI
	Kylemore	WC	N	25	black yeast-like (1), sterile grey (1)
Australia	Campbelltown	NSW	Y	50	*Alternaria* sp. (2), *Aureobasidium* sp. (3), *Cladosporium* sp. (1), *Colletotrichum* sp. (1), *Nigrospora* sp. (1), *Truncatella* sp. (1), sterile grey (1)
	Armidale	NSW	N	20	Sterile grey (2)
	Marulan	NSW	N	25	Sterile white (1)
	Warrandyte	VIC	Y	25	*Dichomera* sp.[3] (3), *Cercospora* sp. (1), *Colletotrichum* sp. (2), *Coniothyrium* sp. (2), *Diplodia* sp. (2), *Epicoccum purpurascens* (1), *Graphium* sp. (2), *Phomopsis* sp. (5), *Torulla* sp. (1), *Ulocladium* sp. (2), sterile grey (1), sterile white (1), sterile brown (2)
	Bundoora	VIC	Y	25	*Alternaria* sp. (4), *Aureobasdium* sp. (2), *Dichomera* sp.[3] (3), *Colletotrichum* sp. (4), *Coniothyrium* sp. (2), *Fusarium* sp. (2), *Phoma* sp. (9), sterile grey (2), yeast-like (1)
	Brimbank	VIC	Y	25	*Alternaria* sp. (5), *Aureobasidium* sp. (4), *Phoma* sp. (3), *Steganosporium* sp. (1), *Stemphylium* sp. (1), sterile grey (1), sterile brown (1)
	Cranbourne	VIC	Y	25	*Alternaria* sp. (1), *Aureobasidium* sp. (2), *Curvularia* sp. (1), *Colletotrichum* sp. (2), *Coniothyrium sp.* (2), *Epicoccum purpurascens* (1), *Nigrospora* sp. (1), *Phoma sp.* (3), *Phomopsis* sp. (2), *Seimatosporium arbusti* (6), sterile brown (3)
	Lilydale	VIC	Y	25	*Alternaria* sp. (1), *Aureobasidium* sp. (1), *Coniothyrium* sp. (3), *Dichomera* sp.[3](2), *Curvularia* sp. (1), *Diplodia* sp. (1), *Epicoccum purpurascens* (2), *Fusarium* sp. (2), *Phomopsis* sp. (3), sterile grey (6)
	Denmark	WA	N	25	*Alternaria* sp. (1), *Colletotrichum* sp. (1), *Diplodia* sp. (1), *Phoma* sp. (5), *Seimatosporium* sp. (1), sterile white (2), sterile grey (4), black yeast (2), unidentified hyphomycete (8)

a NSW = New South Wales, VIC = Victoria, WA = Western Australia, WC = Western Cape.

b Asphondylia spp. present on Acacia mearnsii at collection site Y = Yes, N = No.

c Number in parenthesis is the number of isolations of each fungus. NI = no isolations. n = number of buds sampled per site.

Table 4.

Percentage occurrence of fruiting structures of *Dichomera* sp. on the bud galls of *Asphondylia glabrigerminis* at four locations in eastern Australia. *n* = sample size for each gall class

Site	Normal *Asphondylia* development		Parasitised *Asphondylia*		Immature *Asphondylia* dead or absent	
	n	**% pycnidia**	***n***	**% pycnidia**	***n***	**% pycnidia**
Knoxfield	49	0	11	18.1	83	79.5
Rowville	30	0	17	11.7	95	72.6
Cranbourne	38	2.6	35	0	10	10.0
Campbelltown	190	0	6	0	21	0

Table 5.

Fungi isolated from adult *Asphondylia glabrigerminis* reared under caged conditions

Site	***N*[a]**	**Fungi[b]**
Cranbourne	♂ 3, ♀ 2 ?18[c]	*Cladosporium* sp. (7). *Acremonium* sp. (1), *Chaetomium* sp. (2), *Penicillium* sp. (2) sterile white (3)
Knoxfield	♂ 10, ♀ 5	*Cladosporium* sp. (9), *Dichomera* sp. (2)
Rowville	♂ 13, ♀ 14	*Cladosporium* sp. (14), *Phoma* sp. (2), *Epicoccum purpureum* (2), *Dichomera* sp. (4), *Alternaria* sp. (1)
Mittagong	*? 24*	*Dichomera* sp. (8), *Penicillium* sp. (8), *Cladosporium* sp. (3)

a N = sex and number of adults sampled.

b Numbers in parenthesis are the number of isolates of each fungal species.

c Sex uncertain.

Table 6.

Incidence of spores present in mycangia *of Asphondylia* spp. from Australian *Acacia*

Cecidomyiid	Location (year)	Host	Source[a]	*n*	%♀'s with spores	Mean spore size (µm) L × W (*n*)[b]
Asphondylia glabrigerminis	NSW, Mittagong	*A. mearnsii*	L	20	0	–
Asphondylia glabrigerminis	Vic., Rowville[c]	*A. mearnsii*	L	42	80.9	
Asphondylia glabrigerminis	Vic., Knoxfield[c]	*A. mearnsii*	L	10	90.0	
Asphondylia pilogerminis	Vic., Pearcedale	*A. baileyana*	L	20	0	–
Asphondylia bursicola	NSW, Holbrook	*A. decurrens*	L	13	0	–
Asphondylia glabrigerminis	NSW, Campbelltown (2001)	*A. mearnsii*	W	21	90.4	10.8 × 7.7 (34)
Asphondylia glabrigerminis	NSW, Campbelltown (2002)	*A. mearnsii*	W	19	94.7	12.7 × 8.1 (17)

a W = wild-caught adults, L = laboratory-reared adults.

b Mean conidia size, L = spore length, W = spore width.

c Galls contained pycnidia of *Dichomera* sp. at time of emergence.

Table 7.

Isolates of *Botryosphaeria* from Australian and South African *Asphondylia* used for construction of phylogenetic trees

Culture number	Identity	Host	Host organ	Host plant	Location	Genbank accession no's	
						ITS	EF-1α
RJA3242	*B. dothidea*	*Asphondylia* sp.	Fruit	*Rhoicissus digitata*	Knysna, South Africa	EF614616	EF614636
RJA3179	*B. dothidea*	*Asphondylia* sp.	Flower bud	*Virgillea oroboides*	Knysna, South Africa	EF614617	EF614633
RJA 3279	*B. dothidea*	*Asphondylia* sp	Flower bud	*Osyris compressa*	Groenvlei, South Africa	EF614618	EF614635
RJA 3241	*B. dothidea*	*Asphondylia* sp.	Flower bud	*Grewia occidentalis*	Knysna, South Africa	EF614620	EF614637
RJA 3278	*B. dothidea*	*Asphondylia* sp.	Flower bud	*Acacia karroo*	Montague, South Africa	EF614619	EF614634
RJA 3253A	*B. dothidea*	*Asphondylia* sp.	Flower bud	*Hybanthus floribunda*	Adelaide, SA., Australia	EF614622	EF614639
RJA 3161	*B. dothidea*	*As. glabrigermins*	Flower bud	*Acacia mearnsii*	Campbelltown, NSW, Australia	EF614624	EF614640
RJA 3275	*B. dothidea*	*Asphondylia* sp.	Fruit	*Acacia cyclops*	Fremantle, WA., Australia	EF614625	EF614642
RJA 3253	*B. dothidea*	*As. dodonaeae*	Petiole	*Dodonaea viscosa*	Adelaide, SA, Australia	EF614621	EF614638
RJA 3247	*B. dothidea*	*As. glabrigerminis*	Flower bud	*Acacia irrorata*	Tenterfield, NSW, Australia	EF614623	EF614641
RJA 3261	*B. dothidea*	*Asphondylia* sp.		*Billardiera variifolia*	Denmark, WA, Australia	EF614626	EF614643
RJA 3319/C/1	*B. dothidea*[a]	*As. glabrigerminis*	Flower bud	*Acacia mearnsii*	Knoxfield, VIC, Australia	EF614630	EF686574
RJA 3319/F/4	*B. dothidea*[b]	*As. glabrigerminis*	Flower bud	*Acacia mearnsii*	Knoxfield, VIC, Australia	EF614629	EF686580
RJA 3320/M/8	*B. dothidea*	*As. glabrigerminis*	Mycangia		Knoxfield, VIC, Australia	EF614627	EF686582

a *Dichomera* spore type.

b *Fusicoccum* spore type

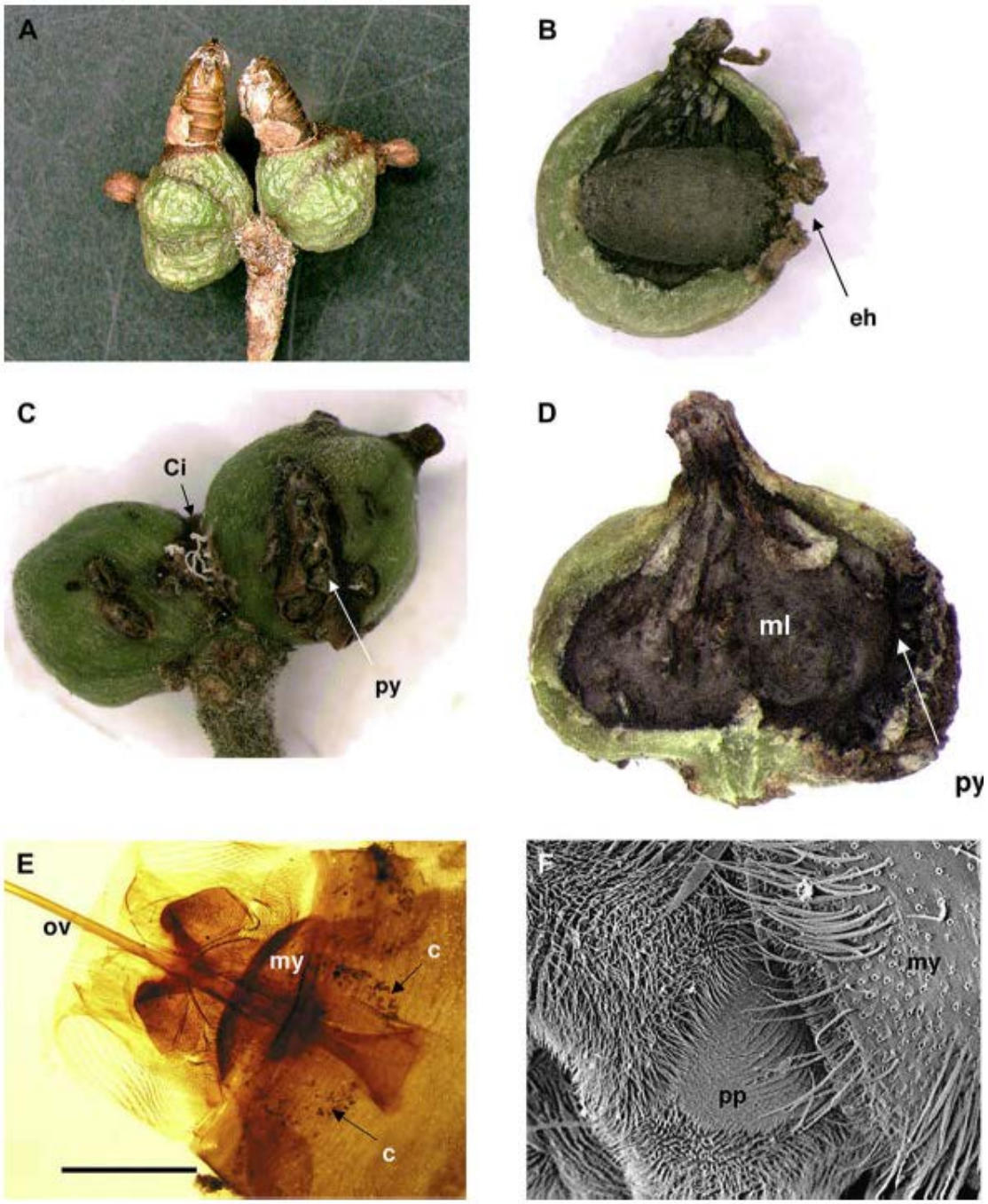

Fig 1.

Development of *Dichomera* invasion in galls of *Asphondylia glabrigerminis* from *Acacia mearnsii* and *A. irrorata*. A. Bud galls of *A. mearnsii* with exuviae of *A. glabrigerminis* present in emergence holes. B. Dissected bud gall showing development chamber and emergence hole used by *A. glabrigerminis*. C. Galled buds of *A. mearnsii* induced by *A. glabrigerminis* showing erumpent pycnidia and cirrhi emerging from surface lesions. D. Dissected bud gall showing melanised hyphae in the gall chamber and pycnidia emerging through the gall wall. E. Ventral view of abdominal sternites showing mycangium with conidia. F. Scanning electron micrograph of the abdominal sternite and entrance to mycangium of *Asphondylia*. Scale bar = 100 μm, c = conidia, ci = cirrhi, eh = emergence hole, ml = melanised hyphal layer, my = mycangium, ov = ovipositor, pp = posterior plate, py = pycnidia.

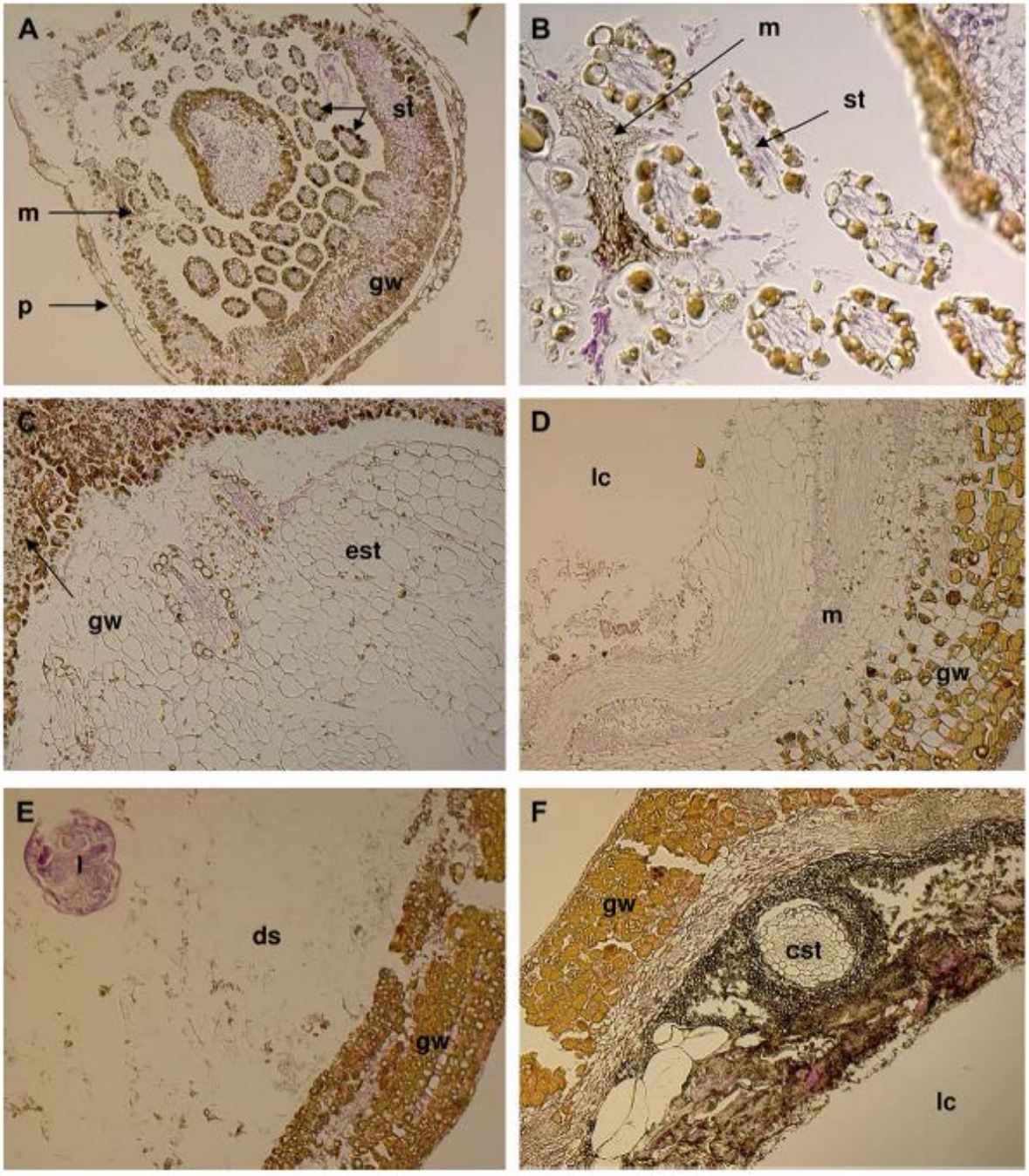

Fig 2.

Development of *Dichomera* invasion in galls of *Asphondylia glabrigerminis* from *Acacia mearnsii* and *A. irrorata*. Light micrographs of A. Transverse section of five-week gall from *A. mearnsii* with early infection on internal wall. B. Mycelium *of Dichomera* ramifying amongst developing stamens. C. Longitudinal section showing mycelium amongst abnormally enlarged stamens. D. Transverse section of gall from *A. irrorata* showing commencement of disintegration of stamen tissue, formation of larval cavity and thickening of mycelial layer against inner gall wall. E. Disintegration of stamen and ovary tissues within the gall chamber. F. Melanised hyphae forming a compacted layer against the internal gall wall with encased remnants of stamen tissue. cst = compacted stamen remnants, ds = disintegrating stamens, est = enlarged stamen, gw = gall wall, l = larva, lc = larval development chamber, m = mycelium, p = perianth, st = stamen.

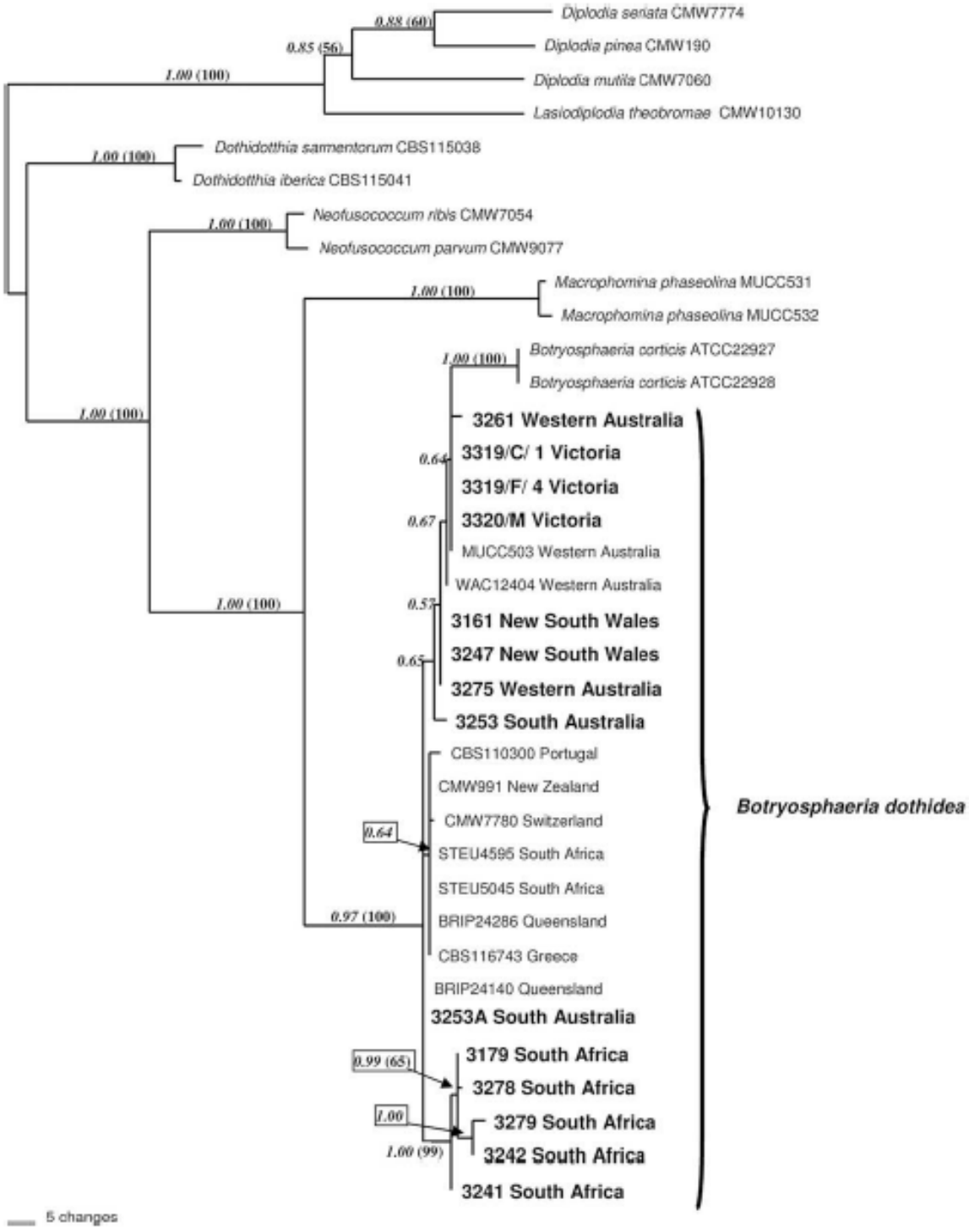

Fig 3.

One of 4 most parsimonious trees of 536 steps resulting from the analysis of the combined ITS and EF1-α sequence data. The posterior probabilities resulting from Bayesian analysis are given in italics and the bootstrap values of the branch nodes are in brackets. Isolates from this study are in bold.