Phylogeny and host relationships of the Australian gall-inducing fly *Fergusonina* Malloch (Diptera: Fergusoninidae)

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A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

DECLARATION

I, Michaela Fay Elizabeth Purcell, declare that this thesis is my own original work, except for the *Eucalyptus* phylogenies in Chapter 3 contributed by Andrew H. Thornhill. This thesis has not been submitted for consideration at any other academic institution.

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ABSTRACT

Fergusoninidae is a monogeneric family of mainly Australian flies. In a unique obligate mutualism with a nematode, these flies induce galls on plants in the family Myrtaceae, and have been recorded on seven genera of host plants, most commonly on the eucalypts. Most host plants are associated with multiple species of *Fergusonina*, usually galling different sites on the plant. Despite the abundance and diversity of Fergusoninidae and its tight association with Australia's most iconic flora, the host specificity and coevolutionary relationships of *Fergusonina* with its plant hosts have not previously been examined in depth.

I used a phylogenetic approach based on mitochondrial COI to examine the evolutionary relationships between *Fergusonina* species and their plant hosts, initially performing a Bayesian analysis of 41 putative species on flies from *Eucalyptus* plant hosts. This analysis revealed well-supported lineages of flies characterised by larval morphology and gall type, usually from the same plant host subgenus. The deeper phylogenetic relationships between groups of species remained unclear, so I performed a further analysis of an expanded dataset including flies from four host genera, using separate and concatenated COI and nuclear CAD sequences.

Having disparate evolutionary time scales, *Fergusonina* and their hosts cannot have codiverged early in the history of Myrtaceae, but current fly-plant host specificity suggested that there may be cospeciation at finer taxonomic levels. A fine-scale analysis of

flies collected from a clade of ten *Eucalyptus* species explored the plant-fly coevolutionary relationships in three clades of flies from different sites of the host plant: flower buds, leaf blades and vegetative shoot buds. The degree of host specificity displayed by the three fly groups varied markedly, with flower bud gallers exhibiting the most cophylogenetic history, and leaf blade gallers the least. These results suggest that host switching occurred often in the history of *Fergusonina* and Myrtaceae.

I compared molecular, morphological and ecological criteria for determining species limits, including a number of molecular species delimitation models. Delimiting species using a 2% pairwise distance was most consistent with other data such as larval and adult morphology, host and gall site. However, molecular methods were not adequate to clarify some ambiguous species limits, highlighting the need to integrate multiple criteria when identifying species in this group.

Over the course of the study, I discovered around 95 unrecorded host plant/gall site associations, indicating that the potential number of species in this family is very large. The definable morphological and ecological differences among the lineages of *Fergusonina*, supported by molecular evidence, argue for a revision of the genus along these lines. The type species for *Fergusonina,* collected in Sydney in 1924, is in poor condition and is not identifiable; there are no records of its host, gall type or larval morphology, and I could not extract and DNA from it. A neotype will need to replace the existing holotype, or the type species assigned to a probable group. After a comparison of morphological characters I concluded that the type species is likely to belong to a group associated with the host genus *Corymbia*.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview of Fergusoninidae

Fergusonina Malloch (Diptera: Fergusoninidae) is a genus of small, black and yellow flies, with a body length of around 2-3 mm and wingspan of 5-7 mm (Currie 1937). All species of Fergusoninidae induce galls on Myrtaceae in a mutual association with the nematode *Fergusobia* (Tylenchida: Neotylenchidae). To avoid ambiguity in this thesis, the genera *Fergusonina* and *Fergusobia* will be abbreviated as *Fn*. and *Fb*. respectively. Such an association is unique among insects and nematodes (Currie 1937; Davies and Giblin-Davis 2004; Davies *et al*. 2010a). The relationship is an obligate mutualism as the flies disperse the nematodes, and the nematodes are thought to initiate the galls in which the fly larvae develop (Taylor *et al*. 1996; Giblin-Davis *et al*. 2001; Davies and Giblin-Davis 2004). *Fergusonina* and *Fergusobia* occur mainly in Australia, but a small number of species have also been found in New Zealand, India, Papua New Guinea and the Philippines (Harris 1982; Siddiqi 1986; 1994; Taylor *et al*. 2007; Davies *et al*. 2010a). They have been recorded on seven genera of Myrtaceae: *Angophora*, *Corymbia*, *Eucalyptus*, *Leptospermum*, *Melaleuca*, *Metrosideros* and *Syzygium* (Currie 1937; Tonnoir 1937; Harris 1982; Davies and Giblin-Davis 2004; Taylor *et al*. 2007; Taylor and Davies 2008; Davies *et al*. 2017).

1.2 Aims

This thesis addresses some of the outstanding questions concerning the biology, evolutionary history and taxonomy of the common but little-known Australian fly *Fergusonina*. The aims of this project are as follows:

Chapter 2:

- Using a phylogenetic approach to examine the evolutionary relationships between a broad sample of *Eucalyptus*-galling species of *Fergusonina,* and their correspondence to host plant taxonomy, gall type and larval morphology.

Chapter 3:

- To narrow the scope of analysis, given varying patterns of host association between the lineages found in Chapter 2, by examining patterns of host-switching and codivergence between one inclusive clade of *Eucalyptus* containing ten species and their associated *Fergusonina* gallers, using a comprehensive sample of species from several gall types on target host plant species.

Chapter 4:

- To build upon the findings of Chapter 2, elucidating the deeper relationships between the *Fergusonina* lineages associated with particular gall and dorsal shield types indicated by the COI-based phylogeny. To do this I use mitochondrial COI and the nuclear gene CAD, and expand the dataset to flies from four genera of host plants, and provide more robust support at the base of the *Fergusonina* phylogeny by using a more slowly evolving nuclear gene.

- Given some ambiguous species boundaries identified in the previous chapters, I investigate molecular and morphological protocols for delineating species limits in

Fergusonina (Scheffer *et al.* 2004, Ye *et al.* 2007, Taylor and Davies 2010), some based on pairwise sequence divergence, and others based on the molecular coalescent.

Chapter 5:

- To examine the taxonomic complexity associated with this increasingly large genus, in particular the need to establish the identity of the type species *Fn. microcera* as a first step towards revising the genus and erecting new genera according to lineages defined in chapters 2 and 4 by concordant molecular, morphological and ecological criteria.

1.3 The *Fergusonina-Fergusobia* system

It is currently thought that each *Fergusonina* species is associated with its own species of *Fergusobia* (Davies and Lloyd 1996; Davies and Giblin-Davis 2004; Taylor 2004; Taylor *et al.* 2005), but this has not been comprehensively investigated, in part due to the difficulty of obtaining galls that yield both fly larvae and nematodes at several stages of development. Furthermore, with very rare exceptions (Goolsby *et al*. 2000; Nelson *et al*. 2014) each species pair forms galls on only one type of plant tissue (e.g. flower bud, leaf bud or leaf blade), and generally attacks only one or a small number of host plant species (Giblin-Davis *et al*. 2001; Taylor *et al*. 2005; Davies *et al*. 2010a). Given this host specificity, *Fn. turneri*, with its mutualist nematode *Fb. quinquenerviae* was considered an excellent biological control agent for its host, the invasive *Melaleuca quinquenervia*, which has

become a major weed in the Florida everglades (Center *et al.* 2012). Unfortunately, controlled releases between 2005 and 2007 failed to establish (Pratt *et al.* 2013).

Fergusonina galling is rarely heavy enough to cause significant damage to a host plant, but as nutrient sinks (Weis *et al.* 1988; Goolsby *et al.* 2000), multiple large galls may have a detrimental impact on young saplings, on which they frequently occur. *Fergusonina* galls are often found on plants that have been targeted by other gall-inducers such as psyllids or wasps (pers. obs.), which suggests the flies are exploiting or contributing to weakened immunity in the host. The reproductive output of adult trees is also affected to some extent; Currie (1937) observed that *E. camaldulensis* and *E. hemiphloia* flower buds may be very heavily galled, and that "whole branches may break under the weight of galls." Investigating reduced seed production in *Corymbia* (then *Eucalyptus*) *maculata* in the south coast in 1930, Morgan (1933) noted that approximately 10% of inflorescences included at least one bud galled by *Fergusonina*, and that inflorescences containing galled buds also dropped many more ungalled buds than unaffected inflorescences. He suggested that in dry periods when eucalypts lose a lot of buds, the additional losses caused by galling may result in significant reduction of seed production.

1.3.1 Taxonomy, morphology and diversity

Malloch (1924) erected the genus *Fergusonina* in the leaf miner subfamily Agromyzinae for the new species *Fergusonina microcera*, represented by a single "poorly preserved" (Malloch 1932) female specimen collected in New South Wales by E. W. Ferguson. Upon receiving more *Fergusonina* specimens he revised his description of *Fn. microcera* and described a further six species (Malloch 1925). Tonnoir (1937) described another 12

species of *Fergusonina* and devised a key that was mainly based on colouration. While he recognised the limitations of this system, he felt that with so few species yet described (19 in total) any grouping made at the time would be artificial, and his key at least allowed for a relatively quick means of identification without requiring the dissection of genitalia. He proposed that *Fergusonina* should be placed in its own subfamily, Fergusonininae, and subsequently Hennig (1958) removed the genus from Agromyzidae and established the monogeneric family Fergusoninidae. Their placement in the Diptera phylogeny has long been uncertain, but the most recent molecular evidence suggests that the closest relative to Fergusoninidae is Carnidae (Bayless 2016), commonly known as bird flies or filth flies due to their association with birds' nests, carrion and faeces (Sabrosky 1959; Grimaldi 1997).

Adult Fergusoninidae are distinguished from other families by unusual placement of the antennae, which are set in pits near the lower margin of the eyes (Malloch 1924) and by the dorsal and ventral sclerites of the sixth abdominal segment of females being fused into a single cone (Hennig 1958). The larvae have three instars, with characteristic features being the two pairs of black, strongly sclerotised spiracles at the head and posterior ends of the larva, and strong chewing mouthparts; most species also possess a unique structure known as the 'dorsal shield' (Currie 1937) on the tergites of the thoracic and abdominal segments of third instar larvae and puparia. The complexity of this shield can vary from a simple sclerotised plate or some raised bumps and nodules, to a complex structure with rake-like teeth (Currie 1937, Taylor 2004, Taylor and Davies 2010).

Only a fraction of the likely number of species of either *Fergusonina* or *Fergusobia* has so far been described (Tonnoir 1937; Scheffer *et al.* 2004; Taylor *et al.* 2005; Davies *et al.* 2010a) (Appendix I). They are widespread on eucalypts in eastern Australia, where

most of the sampling has been done, but their distribution is patchy and their abundance varies according to the time of year (Goolsby *et al.* 2000, Davies *et al.* 2010a).

The complex biology and host associations in the *Fergusonina-Fergusobia* system discussed in this thesis mean that any phylogeny of the group should be constructed in the context of morphology, behaviour, ecological relationships and genetic information. Unfortunately much of this information is lacking for species described in the early days of Fergusoninidae taxonomy, before genetic sequencing was available and some species, such as the aforementioned *F. microcera*, were described based solely on one life stage, and often gall and/or host plant information were lacking, meaning some named species are not identifiable. These problems highlight the value of using an integrative approach in taxonomy (Dayrat 2005, Padial *et al.* 2010, Yeates *et al*. 2011) and in systems such as this it is vital in making sense of species boundaries and relationships.

1.3.2 Fergusonina *life cycle*

Flower bud gallers' life cycles are timed around seasonal flowering, but whether all *Fergusonina* species have an annual cycle may depend on the species. It has been suggested that some species may produce more than one generation in a year, whereas some may be biennial or go through a stage of diapause (Currie 1937, Davies *et al.* 2001, Taylor *et al.* 2005, Head 2008).

Mating occurs within around 48 hours of emerging if the weather is warm, but may be delayed at lower temperatures (Currie 1937). Currie's paper (1937) is the only one that mentions mating being observed. In one study in which adult flies were caged twelve hours after emerging, it was noted that no mating was witnessed in the cage, which suggests it

might have taken place within twelve hours of emergence (Giblin-Davies *et al.* 2001). Oviposition, which generally takes place from mid-morning to mid-afternoon (Currie 1937), begins 2-3 days after emergence and may continue for two weeks or longer (Giblin-Davis *et al.* 2001). Adult flies may live for up to around 17 to 20 days (Currie 1937, Giblin-Davis *et al.* 2001). When the female fly finds suitable meristematic tissue on a host plant she injects her eggs and the juvenile nematodes she has been carrying since she emerged from her gall chamber (Fig. 1.1). With each oviposition the fly injects from one to 50 nematodes (Currie 1937). In cage experiments individual females may revisit and oviposit in the same gall over a period of days, but this behaviour has not been confirmed in the field (Giblin-Davis *et al.* 2001). Some galls may hold several hundred eggs, often laid by multiple females (Purcell *et al.* 2015). Total lifetime fecundity is still not known (Taylor and Davies 2010) but has been estimated at 183±42 in *Fn. turneri* (Giblin-Davies *et al.* 2001). The punctures caused by oviposition leave scars in the plant tissue as it ages, which may provide cues to other females, indicating host suitability or signalling that there are already eggs occupying the bud (Giblin-Davis *et al.* 2001) as there is a limit to how many larvae a gall will sustain, depending on bud size and location; overexploitation has been found to kill the bud (Giblin-Davis *et al.* 2004a, Giblin-Davis *et al.* 2004b).

The fly larvae hatch around one to two months later, generally around the time the nematodes become mature (Giblin-Davis *et al.* 2004a), and feed on cell sap or gall tissue thought to be created by the nematodes' feeding activity (Currie 1937, Giblin-Davis *et al.* 2004a). The mature nematodes are parthenogenetic females that live alongside the fly larvae, feeding on plant material (Fisher and Nickle 1968, Giblin-Davis *et al.* 2004a). *Fergusobia* is the only known genus of nematodes that has both a parthenogenetic and

amphimictic (sexual) phytophagous generation followed by an insect parasitic stage (Taylor 2004).

Each fly larva excavates a cavity between layers of hypertrophied cells, and nematodes aggregate around it. Where eggs are inserted and precisely how galls develop in axial bud and leaf bud galls is not clear (Giblin-Davis *et al.* 2004a). In flower bud galls, the plant cells fuse to form a locule around the larva and its attendant nematodes, which may or may not be bound by a membrane (Currie 1937, Giblin-Davis *et al.* 2004a). The number of locules varies with type and size of gall, from one to over 100 or even several hundred (Currie 1937, Taylor *et al.* 1996, Taylor *et al.* 2005, Taylor and Davies 2008). The larva will remain in this locule with its attendant nematodes until it eventually emerges as an adult (Currie 1937, Giblin-Davis *et al.* 2004a, Ye *et al.* 2007).

In some gall types, each larva tunnels towards the outer layer of the gall when ready to pupate, making a thin pupal "window". These windows consist of a thin layer of epidermal cells between the puparium and the outside of the gall which the larva creates by eating or scraping at the gall material prior to pupation (Giblin-Davis *et al.* 2004a, Head 2008). Some species of leaf and shoot bud gallers discharge a gelatinous substance prior to pupating, which attaches the posterior end of the pupa to the locule wall (Currie 1937, Taylor 2004). This probably serves to anchor the puparium while the fly emerges (Currie 1937).

When it emerges as an adult it leaves the gall by breaking through the puparium and the pupal window with its ptilinum, an eversible sac in the fly's head. Mature flies emerge from harvested galls kept in the lab within three weeks of collection, rarely more, and it can

take up to two weeks from first to last fly to emerge from the gall (pers. obs). This is often followed by the emergence of adult parasitoid and hyperparasitoid wasps.

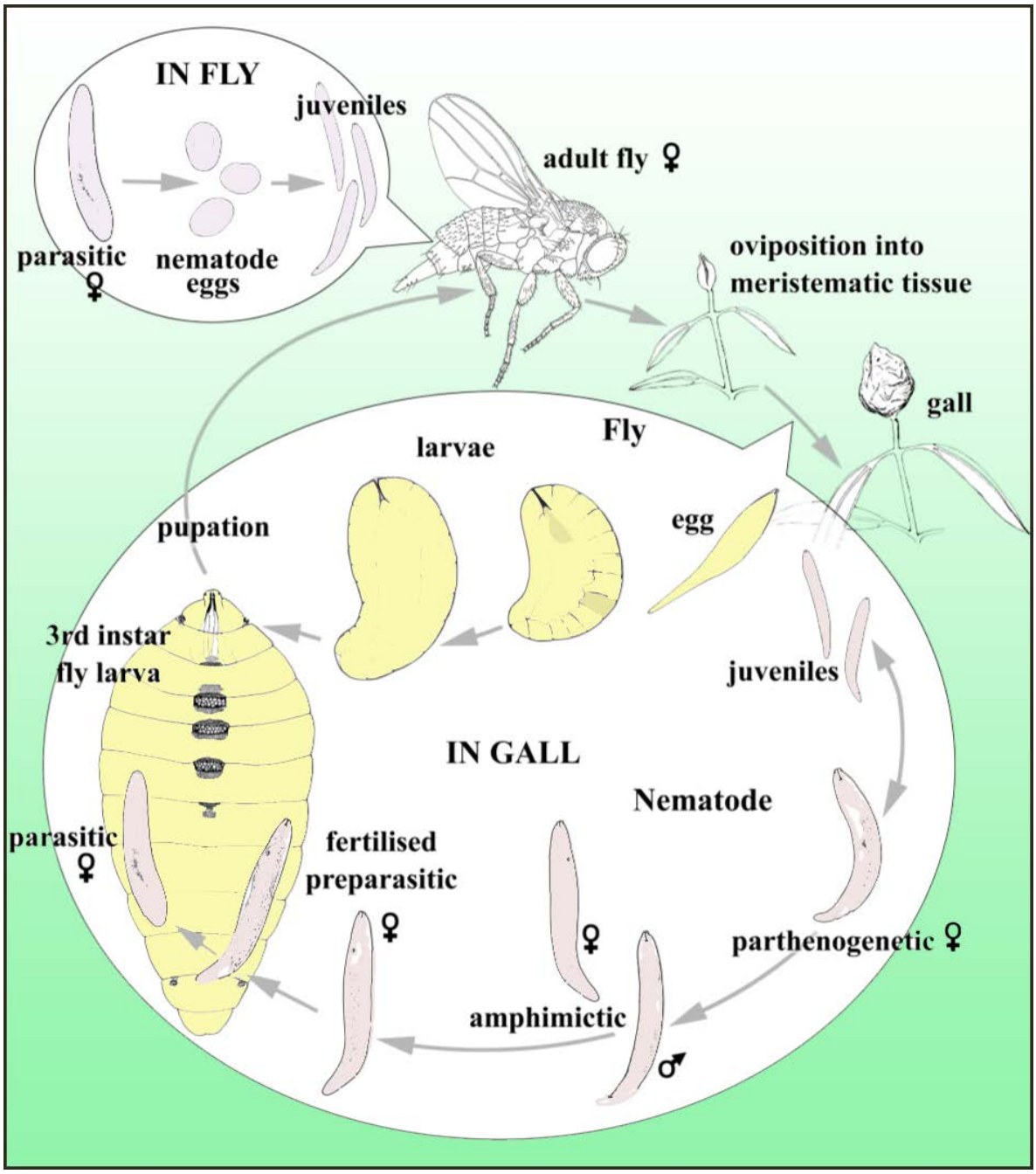


Fig. 1.1 Life cycles of *Fergusonina* and its associated *Fergusobia* nematode, from Taylor *et al.* 2005.

1.3.3 Gall initiation

In general, each species of fly is associated with one gall type and location (Davies *et al.* 2010a). To what extent gall morphology is determined by the behaviour of the fly, the nematode or the meristematic tissue on which the gall develops is not yet known (Giblin-Davis *et al.* 2004a) but the fact that similar gall morphology is found among different hosts and distinct clades of *Fergusonina/Fergusobia* suggests that gall shape is highly influenced by the meristem into which the fly oviposits (Giblin-Davis *et al.* 2004a, Davies *et al.* 2010a) and may also be affected by timing of oviposition relative to bud development (Giblin-Davis *et al.* 2004a).

Gall formation begins well before the fly larvae hatch (Giblin-Davis *et al.* 2001), and is presumed to be initiated by the feeding nematodes, perhaps by chemical secretions from the oesophageal gland (Taylor *et al*. 1996; Giblin-Davis *et al.* 2001; Nelson *et al.* 2014). Attempts to induce galls by artificially injecting either *Fergusonina* eggs, *Fergusobia* juveniles or both into host plant material have been largely unsuccessful to date (Currie 1937, Giblin-Davis *et al.* 2001, Ye *et al.* 2007). However, attempts to inject *Fergusobia* into *M. quinquenervia* buds yielded a single dead male about a month later, in bud tissue that was slightly abnormal; no other nematodes, nor marked signs of gall initiation, were found in any of the other injected buds (Giblin-Davis *et al.* 2001). Currie (1937) noted that gall development around infertile fly eggs was initiated but did not progress far, and the surrounding nematodes eventually died without reproducing.

While the fly eggs are developing, hypertrophied, thin-walled parenchymal cells begin to proliferate between developing leaves in shoot bud galls, or among stamenate primordia in flower buds (Currie 1937). The one or two innermost layers of cells

surrounding each locule are full of a granular cytoplasm (Giblin-Davis *et al.* 2004a) and secrete a thickish fluid, full of protoplasm, while less immediate cells are relatively watery (Currie 1937). As these inner cells build up, the outer surface of the gall also expands, and the gall is generally discernible after about a month (Currie 1937). The parthenogenetic nematode females have much stouter stylets than pre-parasitic females, and using these to pierce the cells (Giblin-Davis *et al.* 2003) causes them to release the rich protoplasm which is the presumed food source of the first and second instar fly larva (Currie 1937). When the fly is in its third instar it tears down and consumes the inner walls of the gall, and as the gall material dries up the free-living nematodes begin to die (Currie 1937, Davies *et al.* 2001).

1.3.4 Gall types

Each fly larva develops inside a single chamber or locule within the gall, surrounded by a number of nematodes. Whether a gall contains just one or several locules (and larvae) varies consistently according to fly-nematode species, and most gall types can be clearly divided into these subtypes, e.g. unilocular and multilocular flower bud galls are inhabited by different species (Nelson *et al.* 2014). Authors who have documented fergusoninid galls since the 1930s (Currie 1937) have had their own systems for naming the gall types, some of which can be ambiguous. For instance, it is unclear what Currie's "leaf galls" and "stem galls" specifically refer to. In this thesis I have mainly adhered to the system proposed in Nelson *et al.* (2014), but have replaced some terms like "leafy" leaf blade gall with "fused leaf gall" and have not distinguished between leaf pea galls and flat leaf galls, or axial and terminal shoot bud galls, as I have found that these characters are variable within species.

However, the gall morphology is not always well-defined, and fused leaf galls and shoot bud galls, for example, can sometimes be difficult to differentiate without dissecting the gall and looking at the distinguishing morphology of the larvae.

1.3.5 Fergusobia *life cycle*

The first eggs laid by the parthenogenetic *Fergusobia* females within the gall develop into males. When the fly larva is in its third and final instar the nematode then lays eggs that become infective (pre-parasitic) sexual females (Fisher and Nickle 1968, Davies and Lloyd 1997, (Davies *et al.* 2016)). These mate, and the fertilised females then enter the female fly larvae (Fisher and Nickle 1968). Males have occasionally been found in *Fergusonina* larvae (Davies and Lloyd 1996, Davies *et al.* 2001) but never in adult flies (Davies *et al.* 2010a). The number of parasitic nematodes inside a larva appears to vary among species (Giblin-Davis *et al.* 2003). It is not known how this number is regulated (Nelson *et al.* 2014). Stress may affect the fly's ability to cope with the nematode load, as there were cases among caged flies in Florida where nematodes invaded the thorax and head and seriously reduced the ovaries and other organs (Giblin-Davis *et al.* 2001).

Within the fly's haemocoel, the parasitic female nematodes permanently shed their cuticle and stylet. Their digestive tract atrophies, and as their reproductive tract swells, their soft, thickened epidermal layer, covered in microvilli, expands (Currie 1937, Fisher and Nickle 1968). It is presumed that this is to enable them to absorb nutrients directly from the host through their epidermis (Giblin-Davis *et al.* 2001, Davies *et al.* 2010a). The nematode lays its eggs into the haemolymph of the pupating fly (Currie 1937, Davies *et al.* 2001), and as the fly pupa develops, the new generation of nematodes hatches in its haemocoel and

migrates to its ovaries, to be deposited with the fly's eggs when they have been fertilised. These will become the next generation of parthenogenetic females. In some cases it appears that these juveniles may undergo at least one moult while inside the fly (Giblin-Davis *et al.* 2001) but this has not been confirmed.

Dissections and molecular studies have found evidence of *Fergusobia* nematodes only in female flies (Currie 1937; Fisher and Nickle 1968; Davies *et al.* 2001; Taylor 2004; Scheffer *et al.* 2013), although they occur in the locules of all fly larvae (Currie 1937). It may be that some chemical released by the larva, perhaps during moulting, either repels or stimulates the nematodes to enter (Giblin-Davis *et al.* 2003) depending on the sex of the fly. While it is possible that the nematodes penetrate males but are killed by immunological defences or come across physiological barriers and die, as occurs with some other entomophilic nematode species (Stoffolano 1973), no visible or molecular traces of nematodes have yet been recorded in male flies (Scheffer *et al.* 2013). This would mean that the nematodes accompanying male larvae, if they are unable to move between locules, are unable to reproduce (Nelson *et al.* 2014). Given that a *Fergusonina* fly deposits a number of sibling nematodes with her eggs, the reproductive success rate must outweigh these costs (Stoffolano 1973).

The site and method by which the nematode penetrates the fly larva are not known. Nematodes typically enter insect hosts either through natural openings such as the mouth, anus or spiracles, or by penetrating the cuticle directly (Stoffolano 1973, Ishibashi and Kondo 1990). Currie (1937) believed they probably entered through the tough cuticle, but neither he nor subsequent researchers have observed it occurring and an examination of 46 larvae yielded no evidence of scarring (Purcell 2012). Currie described the "heavily

chitinised" spiracles as having three slits, covered in elliptical perforations, raised on petal-like lips (1937). These openings are too small to allow entry by nematodes, and the anus is therefore the most likely route of entry. Attempts to verify this by dissecting the larvae and looking for nematodes in the digestive tract have so far been unsuccessful (Purcell 2012), but the method could be improved by using a stain that can differentiate nematode from insect cuticle, such as Mallory-Heidenhain stain (Winsor 1984) or Grenacher's Borax Carmine (Nickle and MacGowan 1992).

The means by which the adult flies emerge from their galls vary according to species and gall type. Species that inhabit membrane-bound locules within the anther chamber of flower bud galls emerge when the bud's operculum opens at flowering time (Giblin-Davis *et al.* 2004a). Those that live in non membrane-bound locules within floral disc tissue are released when the gall dries and cracks open (Currie 1937). *Corymbia ptychocarpa* was found to have a combination of the two, with non membrane-bound locules and a detachable operculum that cracks open (Giblin-Davis *et al.* 2004a). Some species that develop in terminal and axial shoot bud galls emerge through brittle pupal windows (Giblin-Davis *et al.* 2004a, Head 2008).

1.3.6 Natural enemies

Fergusoninid galls are regularly attacked by a number of parasitoid wasp species and phytophagous inquilines (other animals that exploit the gall by living within it and feeding on it) such as caterpillars and beetle larvae (Taylor *et al.* 1996; Goolsby *et al.* 2001; Taylor *et al.* 2005). These can cause significant damage to the gall, and may not discriminate between plant tissue and gall inhabitants when feeding (Head 2008, Taylor and Davies

2010). Fly larvae near the outer edges of the gall are at higher risk of parasitoid attack than those deeper within the gall tissue beyond reach of the wasp's ovipositor, giving an advantage to those laid early (Giblin-Davis *et al*. 2001, Taylor and Davies 2010). However, Currie (1937) suggested that larvae near the centre of the gall may become fatally trapped by competitive inquiline chalcid wasp larvae that inhabit the surrounding gall tissue and create tough walls around their chambers that the *Fergusonina* larvae cannot breach. Hymenopteran larvae are often found in a locule with the sclerotised dorsal shield parts the only remnants of the original fergusoninid occupant; sometimes these remains are balled up and cradled by the wasp larva (Goolsby *et al.* 2001; Taylor and Davis 2008) (Fig. 1.2). It is possible that these balls also contain nematode remains and plant material. Davies *et al.* (2001) dissected 175 galls from *M. quinquenervia* and found that 40% of the galls contained more wasps than *Fergusonina* flies, while 49% contained more flies than wasps. 17% contained lepidopteran inquilines, of which 33% had excavated most of the gall, killing its inhabitants.

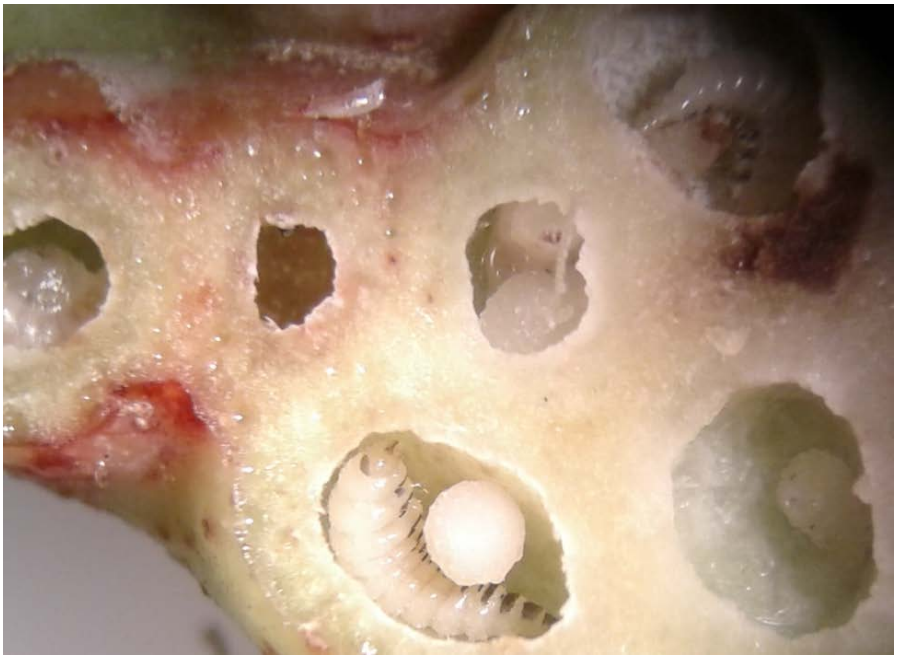


Fig. 1.2 Parasitised *Fergusonina* leaf blade gall showing wasp larvae with balls of fergusoninid remains.

In one study of galls on *E. camaldulensis* in South Australia, 45% of terminal leaf bud galls that had been initiated were destroyed, parasitised or failed to develop and 55% of flower bud galls disappeared altogether (Head 2008). Birds have not been observed feeding on the galls, but based on the appearance of damaged galls it is suspected that parrots, rosellas and lorikeets may attack them (Taylor *et al.* 2005). The number of associated parasitoid species varies with the fly or plant host species, and while 11 hymenopteran species have so far been associated with *Fergusonina* on *M. quinquenervia* (Goolsby *et al.* 2001) and 12 species on *E. camaldulensis* (Taylor *et al.* 1996), only one has been reared from *C. ptychocarpa* galls, and none have been reared from galls of *Fn. metrosiderosi*, the only fergusoninid found so far in New Zealand on *Metrosideros excelsa* (Taylor *et al.* 2007). One factor limiting parasitism could be the penetrability of the gall's outer layer, which may be quite hard and woody in some species (Taylor and Davis 2008).

1.4 Materials and methods – collection of specimens

Each chapter has its own section dealing with the methods particular to that part of the project, however the same collection methods were used throughout the study. Galls were hand-collected from host plants, sealed in a clear plastic zip-lock bag and labelled with site code, tree number and gall number. Mature galls containing pupae were checked daily for emergent adult flies, which were collected from the bags and placed in sealed vials in 95% ethanol. Younger galls were dissected as soon as possible after collection and the larvae removed and placed in 95% ethanol. GPS data were recorded at each collection site in decimal degrees. Where the tree host could not be identified on site, diagnostic material such as buds, fruits, leaves and bark was collected; trees were also photographed to assist

later identification. Where galls were collected from juvenile trees that were too young to bear buds or fruit, material was obtained from nearby adult trees, as an aid to identifying the host species. Hosts were identified with the aid of the *EUCLID* interactive key (Slee *et al*. 2006), field guides, and other sources (Chippendale and Wolf 1981; Brooker and Kleinig 2001; National Parks Association of the ACT 2007; Brooker and Nicolle 2013).

1.5 Publication of chapters from this thesis

Two chapters of this thesis have been published in peer-reviewed journals; the thesis is arranged to follow the evolving pattern of the publications. Chapter 2 has been published as:

Purcell MF, Wallenius TC, Yeates DK & Rowell DM. 2016. Larval dorsal shield morphology is highly correlated with gall type in the enigmatic gall-forming fly, *Fergusonina* Malloch (Diptera : Fergusoninidae). *Australian Journal of Zoology* **64**, 233-248.

Chapter 3 has been published as:

Purcell MF, Thornhill AH, Wallenius TC, Yeates DK & Rowell DM. 2017. Plant host relationships of three lineages of the gall-inducing fly *Fergusonina* Malloch (Diptera: Fergusoninidae) on *Eucalyptus* L'Hérit. *Arthropod-Plant Interactions*. doi:10.1007/s11829-017-9561-1

For this reason there is some inevitable repetition of some general aspects of the biology of the *Fergusonina-Fergusobia* system, and the materials and methods. Superficial modifications have been made to the text in the published chapters to make them consistent with the format of the thesis.

CHAPTER 2: RELATIONSHIP BETWEEN LARVAL DORSAL SHIELD MORPHOLOGY AND GALL TYPE

2.1 Abstract

The gall-inducing fly family Fergusoninidae, in association with a mutualist nematode, induces galls on Myrtaceae. Traditionally, each fly species has been thought to be host-specific and targets a particular site on its host plant. One host species may be host to as many as four fly species, each with different oviposition sites, giving rise to a range of gall types. Third instar fly larvae possess a distinctive sclerotised "dorsal shield" of unknown function, that vary morphologically across the genus. I use a phylogenetic approach to examine the relationship of the dorsal shield morphology to other elements of this complex system. A phylogeny of 41 species, estimated using Bayesian analysis of mtCOI sequences, indicated a strong correlation between dorsal shield morphology and the gall type associated with the larva. I discuss possible functions of the dorsal shield, and other factors which may have led to their phylogenetic distribution. In addition, I have identified cases where fly species have formed galls on more than one host species. In some instances it is possible that these associations are an opportunistic response to artificial tree plantings.

2.2 Introduction

Fergusonina Malloch (Diptera: Fergusoninidae) is a genus of small, acalyptrate flies that form galls on Myrtaceae, in a mutual association with the nematode *Fergusobia* (Tylenchida: Neotylenchidae). Such an association is unique among insects and nematodes (Currie 1937; Davies and Giblin-Davis 2004a; Davies *et al*.

2010a). The relationship is an obligate mutualism as the flies disperse the nematodes, and the nematodes are thought to initiate the galls in which the fly larvae develop (Taylor *et al*. 1996; Giblin-Davis *et al*. 2001; Davies and Giblin-Davis 2004a). *Fergusonina* and *Fergusobia* occur mainly in Australia, but a few species have also been found in New Zealand, India, Papua New Guinea and the Philippines (Harris 1982; Siddiqi 1986; 1994; Taylor *et al*. 2007; Davies *et al*. 2010a). They have been recorded on seven genera of Myrtaceae: *Angophora*, *Corymbia*, *Eucalyptus*, *Leptospermum*, *Melaleuca*, *Metrosideros* and *Syzigium* (Currie 1937; Tonnoir 1937; Harris 1982; Davies and Giblin-Davis 2004a; Taylor *et al*. 2007; Taylor and Davies 2008). Each *Fergusonina* species is associated with its own species of *Fergusobia* (Davies and Lloyd 1996), and furthermore, with very rare exceptions (Goolsby *et al*. 2000; Nelson *et al*. 2014) each species pair forms galls on only one type of plant tissue (e.g. flower bud, leaf bud or leaf blade), and generally attacks only one or a small number of host plant species (Taylor *et al*. 2005; Davies *et al*. 2010a).

2.2.1 Life cycle and gall formation

Juvenile female nematodes are deposited in suitable meristematic plant tissue by the foundress fly when she oviposits. Gall formation begins before the larva hatches (Currie 1937; Giblin-Davis *et al.* 2001) and is thought to result from the nematodes' feeding on the plant (Currie 1937; Taylor *et al.* 1996; Giblin-Davis *et al*. 2001). When the fly hatches it creates a small chamber or locule between layers of hypertrophied plant cells, and it develops within this cavity along with a number of nematodes (Currie 1937). Whether galls are unilocular or multilocular depends on the fly-nematode species; multilocular galls may contain anything from a few

flies to hundreds, and are often founded by more than one female (Purcell *et al*. 2015). The parthenogenetic nematodes mature and produce a sexual generation of males and females. After mating, a small number of fertilised nematodes enter third instar *Fergusonina* larvae and become parasitic, without obvious detriment to the fly (Currie 1937; Giblin-Davis *et al*. 2003a; Taylor 2004; Nelson *et al*. 2014). It is believed that they only enter female larvae, as nematodes have never been found in male flies (Scheffer *et al*. 2013). The nematodes lay their eggs in the haemocoel of the *Fergusonina* larva, and on hatching, move to the developing oviducts and are eventually injected into a plant host with the fly's eggs (Davies *et al.* 2001; Nelson *et al*. 2014). In many gall types, before the fly pupates it excavates a tunnel to the outer surface of the gall and creates a "pupal window"; a thin layer of plant cells that will serve as an exit for the mature fly (Giblin-Davis *et al*. 2004a; Head 2008). Some flower bud gallers, however, exit via the bud's operculum when it opens (Currie 1937; Giblin-Davis *et al*. 2004a; Davies *et al*. 2010a).

2.2.2 Larval morphology and the dorsal shield

Fergusonina larvae have three instars. Final instar larvae are white to cream and elongate- to ovate-pyriform. They have black, heavily sclerotised mouthparts and two distinctive pairs of dark spiracles at the posterior margin of the cephalothorax and on the second- or third-last abdominal segment**.** This instar also has projections that Currie (1937) called "paired papillae" or "thoracic tubercles" on the thoracic segments – sometimes two pairs on the cephalothorax. These may be conspicuously long and bristle-like (Fig. 2.1a, c, h), or tiny nodules. Species with relatively long thoracic projections inhabit galls with spacious locules in which the larvae have room to move around, such as axial "pea" galls (Fig. 2.2d).

The function of these tubercles is not known; they may be sensory or act as a physical buffer for the larva within its chamber, or both. The cuticular dorsal shield (Currie 1937) is a unique morphological feature of third instar fergusoninid larvae. Depending on species, this shield ranges in complexity from an unadorned patch of thickened chitin to an arrangement of plates, spicules and one or two rows of teeth or hooks (Currie 1937; Taylor *et al*. 2005). Once formed, the size of the dorsal shield remains fixed. However, the size of the third instar varies dramatically during development, and often varies between individuals even within the one gall.

The dorsal shield is common to most, but not all, *Fergusonina* species and is an important diagnostic character. While shield morphology varies among species groups, there are broadly defined shield forms that occur across several groups, such as the "bars" type (Davies *et al*. 2010a), having of a series of dark transverse bands of chitinous bumps or spicules (Fig. 2.1m, r, s), or the "dots" type (Davies *et al*. 2010a) (Fig 2.1c, h, k, o) consisting of two smooth plates of sclerotised cuticle or thickened patches of chitin, with other variables such as presence or absence of surrounding spicules, or the arrangement and shape of the plates.

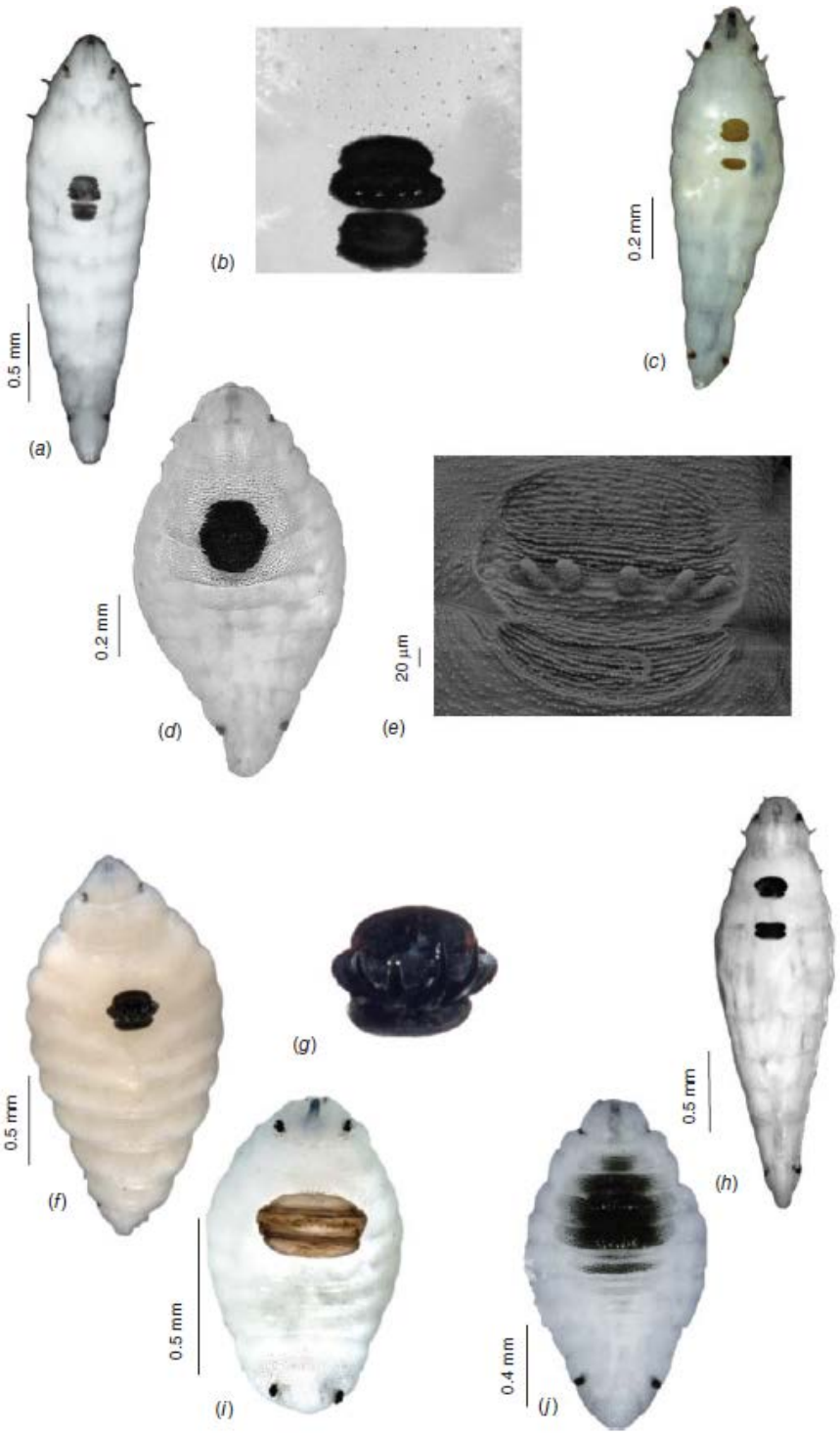


Fig. 2.1 *Fergusonina* larval dorsal shield types from (a) unilocular axial pea gall on *E. rubida*; (b) detail of shield from pea gall on *E. rubida*; (c) fused leaf gall on *E. elata*; (d) multilocular shoot bud gall on *E. blakelyi*; (e) SEM image of same; (f) multilocular pea gall on *E. leucoxylon*; (g) same, detail; (h) unilocular axial pea gall on *E. melliodora*; (i) multilocular flower bud gall on *E. stellulata*; (j) unilocular stem gall on *E. viminalis*; (k) unilocular flower bud gall on *E. lacrimans*; (l) same, detail; (m) multilocular shoot bud gall on *E. macrorhyncha*; (n) leaf blade gall on *E. radiata*; (o) leaf blade gall on *E. sideroxylon*; (p) leaf blade gall on *E. melliodora*, detail; (q) leaf blade gall on *E. macrorhyncha*; (r) leaf blade gall on *E. dives*; (s) detail of same.

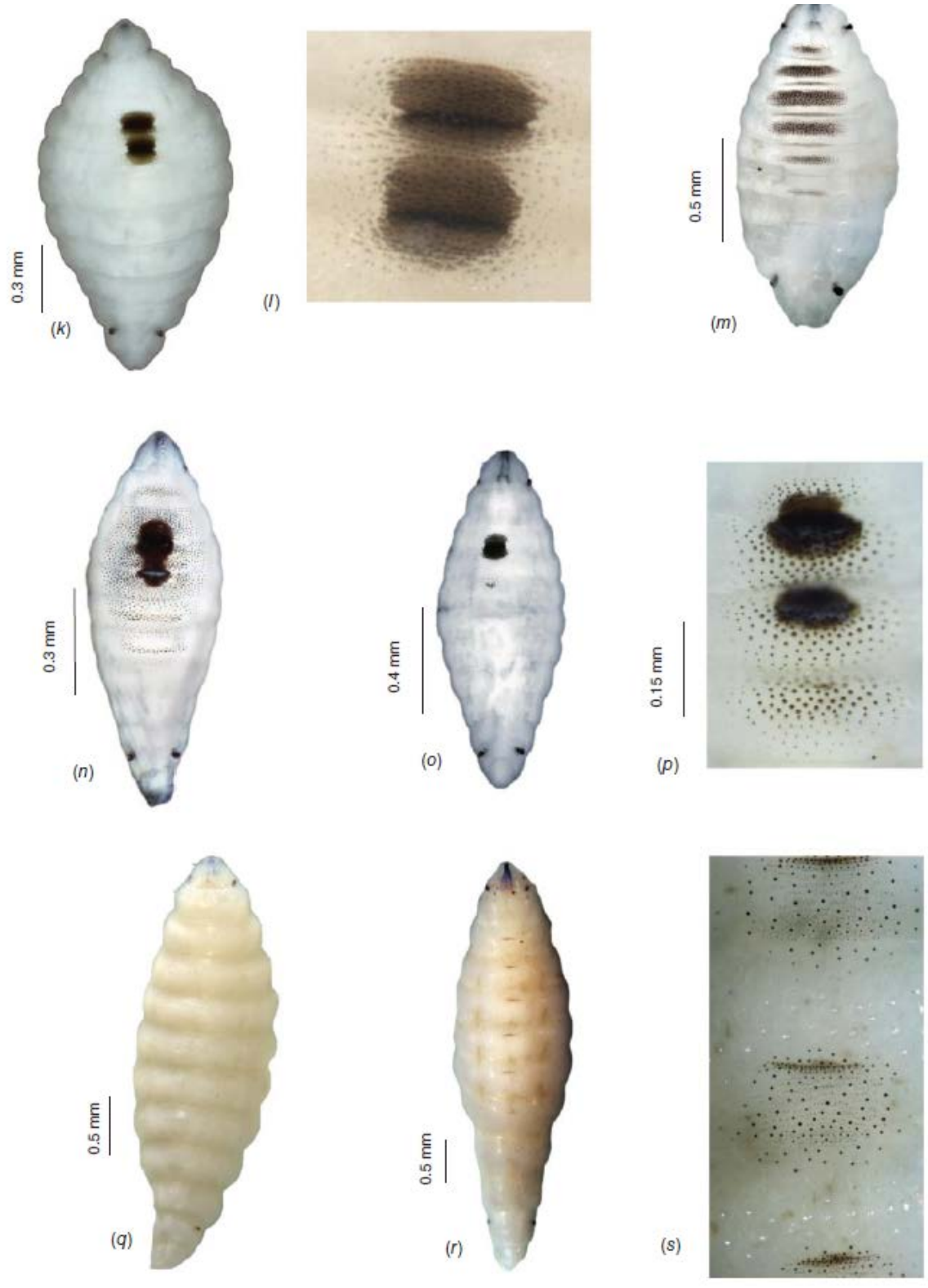


Fig. 2.1 (*continued*)

The function of the dorsal shield is unknown although it may be used to scrape at the interior of the gall to obtain edible plant material and enlarge the locule as the larva grows (Currie 1937; Taylor *et al.* 1996). Currie (1937) speculated that variation in the structure showed an evolutionary trend towards increased complexity and that shields with hooks and teeth are better adapted for scraping than plainer shields. A similar structure, the "sternal spatula" is present in the final instar larvae of gall midges (Diptera: Cecidomyiidae) and is thought to be used for burrowing, or excavating hard plant material or soil (Sen 1939; Milne 1961). In particular, Sen (1939) observed this spatula being used to excavate a tunnel to the outer layer of the gall prior to pupation.

Others have speculated that the dorsal shield may help to anchor the larva within the gall locule, serve some purpose in relation to the nematodes, or provide defence against parasitoids, which are a major cause of *Fergusonina* mortality (Giblin-Davis *et al*. 2003a; Taylor *et al*. 2005; Head 2008; Taylor and Davies 2010).

2.2.3 Gall morphology

There are four main gall types recognised in this system, broadly defined by their site on the host plant: i) terminal and axial shoot bud galls, ii) leaf blade galls, iii) stem galls and iv) flower bud galls (Giblin-Davis *et al*. 2004a; Davies *et al*. 2010a; Nelson *et al*. 2014). Within these categories there may be further specialisation (e.g. some flower bud galls are localised to the stigma), and all but the leaf blade galls can be subdivided into unilocular or multilocular types (Nelson *et al*. 2014).

Gall morphology is largely determined by the host tissue targeted and the timing of oviposition relative to bud development (Giblin-Davis *et al*. 2004a;

Davies *et al*. 2010a). Thus gall forms such as the multilocular shoot bud galls inhabited by diverse fly-nematode lineages have a very similar appearance on a wide range of host species across Myrtaceae (Giblin-Davis *et al*. 2004a; Ye *et al*. 2007; Davies *et al*. 2010a).

Multilocular galls on terminal or axial shoot buds (Fig. 2.2a), usually on young trees, are one of the most obvious and recognisable gall forms (Nelson *et al*. 2014); they are usually around 2 cm long but can be over twice that length. Unilocular axial pea galls (Fig. 2.2d, e) are distinctive but relatively small (3 - 4 mm) spherical growths, and house only a single larva and its nematodes. Flower bud galls (Fig. 2.2c, p) may be small and cryptic or large, malformed buds that stand out among the unparasitised buds in an umbel (Giblin-Davis *et al.* 2004a; Taylor *et al.* 2005). Leaf blade galls (Fig. 2.2j-m) consist of a small number of locules and may be flat and cryptic, or bear a cluster of pea-like lumps. The morphology of these gall types is described in detail in Giblin-Davis *et al.* (2004a) and Taylor *et al.* (2005).

Fig. 2.2 *Fergusonina-Fergusobia* gall types. (a) Terminal shoot bud gall from *E. radiata*; (b) dissected terminal shoot bud gall from *E. radiata* showing larvae in locules; (c) flower bud galls on *E. stellulata*; (d) unilocular axial pea gall on *E. polyanthemos*, cut open to show larva; (e) unilocular axial pea gall on *E. mannifera*; (f) fused leaf galls from *E. mannifera*; (g) dissected fused leaf gall from *E. mannifera*; (h) multilocular axial pea galls with pupal windows, on *E. leucoxylon*; (i) stem pea gall on *E. melliodora*; (j) *E. sideroxylon* leaf blade gall; (k) the same gall, dissected; (l) leaf blade gall on *E. elata*; (m) dissected leaf blade gall and larvae from *E. dives*; (n) stem galls on *E. mannifera*; (o) unilocular terminal shoot bud galls on *E. viminalis*; (p) unilocular flower bud galls on *E. lacrimans*, with all buds galled.

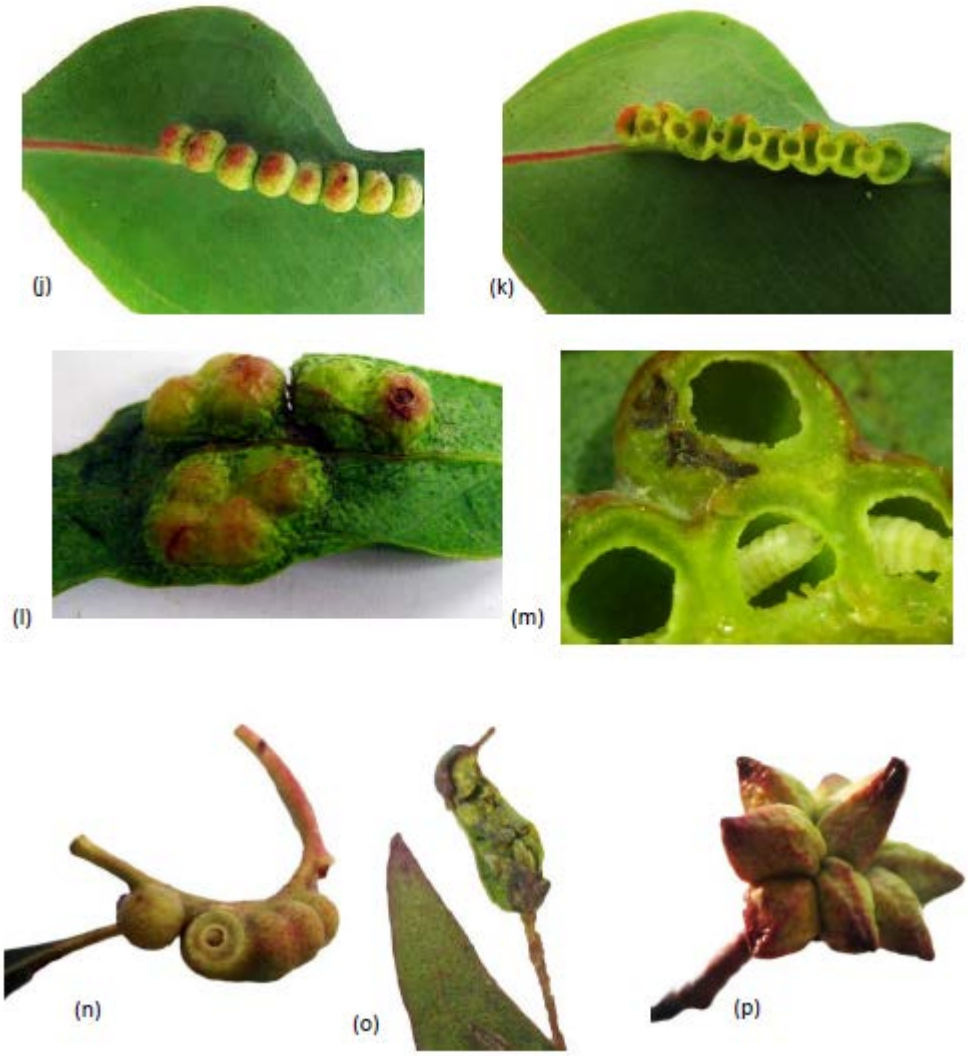


Fig. 2.2 (*continued*)

Fergusonina collection records indicate an apparent relationship between dorsal shield types and gall types, e.g. barred shield types being commonly associated with terminal and axial shoot bud galls (Davies *et al*. 2010a; 2013a). Molecular studies of *Fergusobia* nematodes have provided some phylogenetic support for this relationship (Ye *et al*. 2007; Davies *et al*. 2010a).

The purpose of this study was to further elucidate the relationship between gall type and shield type, by performing a phylogenetic analysis of a region of the mitochondrial gene cytochrome c oxidase subunit I (mtCOI) sequenced from over

170 flies from a range of plant host species and comparing dorsal shield type and gall type as it varied across the phylogeny. Many of the host and gall associations detailed here were previously unrecorded, and some new gall types are described.

2.3 Materials and methods

2.3.1 Collection methods

Galls were hand-collected from 33 species of *Eucalyptus* in the Australian Capital Territory (ACT), New South Wales (NSW), Queensland (Qld) and Victoria (Vic) between 2011 and 2015 (Table 2.1). GPS data were recorded at each collection site in decimal degrees. Where the tree host could not be identified on site, diagnostic material such as buds, fruits, leaves and bark was collected; trees were also photographed to assist later identification. Where galls were collected from juvenile trees that were too young to bear buds or fruit, material was obtained from nearby adult trees, as this was helpful in identifying the host species. Hosts were identified with the aid of the *EUCLID* interactive key (Slee *et al*. 2006), field guides, and other sources (Chippendale and Wolf 1981; Brooker and Kleinig 2001; National Parks Association of the ACT 2007; Brooker and Nicolle 2013).

Table 2.1 Locality information for *Fergusonina* specimens collected for DNA analysis.

***Eucalyptus host* species**	**Subgenus**	**Section**	**GPS Coordinates**	**Collection location**
E. andrewsii	*Eucalyptus*	*Cineraceae*	-28.1859, 153.0969	Beaudesert, QLD
			-28.1421, 153.1125	Lamington NP, QLD
E. blakelyi	*Symphyomyrtus*	*Exsertaria*	-35.3496, 149.0326	Canberra, ACT
			-35.2763, 149.0804	
E. burgessiana	*Eucalyptus*	*Eucalyptus*	-35.1541, 150.7076	Jervis Bay, JBT
			-35.1536, 150.7061	
E. cunninghamii	*Eucalyptus*	*Eucalyptus*	-33.6209, 150.3278	Blue Mountains, NSW
E. dalrympleana	*Symphyomyrtus*	*Maidenaria*	-35.3500, 148.8196	Namadgi NP, ACT
E. delegatensis	*Eucalyptus*	*Cineraceae*	-35.3886, 148.8090	Namadgi NP, ACT
			-35.3904, 148.8020	

			-35.3367, 148.8304	
			-37.1100, 148.9023	Mt. Delegate, VIC
E. dives	*Eucalyptus*	*Aromatica*	-35.3500, 148.8200	Namadgi NP, ACT
			-35.3223, 148.8380	
			-35.7330, 149.0015	
			-35.8662, 148.9915	
E. elata	*Eucalyptus*	*Aromatica*	-35.3038, 149.1297	Canberra, ACT
			-35.2903, 149.1383	
			-36.8573, 149.6902	Myrtle Mountain, NSW
E. fastigata	*Eucalyptus*	*Eucalyptus*	-36.1240, 149.5105	Badja, NSW
			-35.4455, 149.5877	Tallaganda, NSW
			-36.5964, 149.4102	Brown Mountain, NSW

			-36.6100, 149.4168	
E. lacrimans	*Eucalyptus*	*Cineraceae*	-35.2920, 149.0624	Canberra, ACT
			-36.0328, 148.8002	Adaminaby, NSW
E. leucoxylon	*Symphyomyrtus*	*Adnataria*	-35.2665, 149.1188	Canberra, ACT
E. ligustrina	*Eucalyptus*	*Capillulus*	-34.6746, 150.7128	Barren Grounds, NSW
E. macarthurii	*Symphyomyrtus*	*Maidenaria*	-35.2702, 149.1136	Canberra, ACT
E. macrorhyncha	*Eucalyptus*	*Capillulus*	-35.2737, 149.1130	Canberra, ACT
			-35.2759, 149.0991	
			-35.2636, 149.1085	
E. mannifera	*Symphyomyrtus*	*Maidenaria*	-35.2718, 149.1170	Canberra, ACT
			-35.3496, 149.0326	
E. melliodora	*Symphyomyrtus*	*Adnataria*	-35.2769, 149.1136	Canberra, ACT

			-35.3272, 149.1151	
			-35.2686, 149.1100	
			-35.3496, 149.0326	
E. notabilis	*Symphyomyrtus*	*Latoangulatae*	-28.1421, 153.1125	Lamington NP, QLD
E. obliqua	*Eucalyptus*	*Eucalyptus*	-36.6100, 149.4168	Brown Mountain, NSW
E. olsenii	*Eucalyptus*	*Nebulosa*	-35.2781, 149.1085	Canberra, ACT
E. pauciflora	*Eucalyptus*	*Cineraceae*	-36.0748, 148.8347	Adaminaby, NSW
			-36.0464, 148.7355	
			-35.2763, 149.0804	Canberra, ACT
			-35.2718, 149.1170	
			-35.5646, 148.7785	Namadgi NP, ACT
E. piperita	*Eucalyptus*	*Cineraceae*	-33.7042, 150.2883	Blue Mountains, NSW

E. polyanthemos	*Symphyomyrtus*	*Adnataria*	-35.3496, 149.0326	Canberra, ACT
E. racemosa	*Eucalyptus*	*Cineraceae*	-33.5732, 150.2935	Blue Mountains, NSW
			-33.6993, 150.4903	
			-33.7709, 150.3762	
			-35.0713, 150.1688	Budawang Range, NSW
E. radiata	*Eucalyptus*	*Aromatica*	-35.6473, 149.5065	Tallaganda, NSW
			-35.3294, 149.8769	Braidwood, NSW
			-35.8662, 148.9915	Namadgi NP, ACT
E. rossii	*Eucalyptus*	*Cineraceae*	-35.2636, 149.1085	Canberra, ACT
E. sideroxylon	*Symphyomyrtus*	*Adnataria*	-35.2750, 149.1143	Canberra, ACT
			-35.3468, 149.0434	
E. sieberi	*Eucalyptus*	*Cineraceae*	-34.6746, 150.7128	Barren Grounds, NSW

E. smithii	*Symphyomyrtus*	*Maidenaria*	-35.9402, 149.5743	Snowball, NSW
E. stellulata	*Eucalyptus*	*Longitudinales*	-35.2686, 149.1100	Canberra, ACT
E. stricta	*Eucalyptus*	*Eucalyptus*	-33.5479, 150.2609	Blue Mountains, NSW
			-33.6007, 150.3339	
			-34.6753, 150.7117	Barren Grounds, NSW
E. tereticornis	*Symphyomyrtus*	*Exsertaria*	-35.5460, 150.3700	Kioloa, NSW
E. viminalis	*Symphyomyrtus*	*Maidenaria*	-36.1409, 149.4851	Badja NP, NSW
			-36.1765, 149.4628	
			-35.4130, 149.5354	Tallaganda, NSW
			-35.7548, 149.5183	

Each gall was sealed in a clear plastic zip-lock bag and labelled with site code, tree number and gall number. Mature galls containing pupae were checked daily for emergent adult flies, which were collected from the bags and placed in sealed vials in 95% ethanol. Younger galls were dissected as soon as possible after collection and the larvae removed and placed in 95% ethanol.

Where only adults were obtained from a gall, dorsal shield morphology was inferred from the empty puparia.

Images of larvae and their dorsal shields were taken using a Nikon ShuttlePix P-400Rv Digital microscope and a Leica Application Suite Imaging System, and compiled using Zerene Stacker v1.04 (Zerene Systems, LLC) and Adobe Photoshop CS6 version 13.0.1 (Adobe Systems Inc.). The tree was annotated using Fig Tree v. 1.4.2 and Adobe Illustrator CS6 version 16.0.3.

One specimen of an undetermined species of *Fergusonina* from a shoot bud gall on *E. blakelyi* was prepared for scanning electron microscopy (SEM) by immersing overnight in 100% ethanol, then in fresh 100% ethanol for a further 12 hours. It was then critical point dried using a Tousimis Autosamdri-815 Series A fully automatic critical point dryer, mounted on a single carbon SEM mount, gold coated using an Emitech K550X Gold Coater, and scanned with a Zeiss EVO LS 15 Scanning Electron Microscope.

2.3.2 Ingroup sampling and outgroup

While *Fergusonina* and *Fergusobia* occur on several genera within Myrtaceae, this study focused on the species-rich and abundant genus *Eucalyptus*. Recently published research on *Eucalyptus* indicates it is monophyletic, sister to a clade consisting of *Corymbia* and *Angophora* (Parra-O *et al.* 2006). Current molecular data from the flies

and nematodes on *Melaleuca* and *Eucalyptus* suggest that the two host-associated groups are reciprocally monophyletic (Ye *et al*. 2007; Davies *et al*. 2010a; 2012; 2013a; 2014a). Very few species of *Fergusonina* have been collected from the non-*Eucalyptus* host genera, apart from a small group of shoot bud gallers on *Melaleuca*, and targeted sampling of the other genera would be needed to obtain a more comprehensive *Fergusonina* phylogeny.

Sites were selected for their accessibility and diversity of host species, and galls were also collected opportunistically from any hosts encountered throughout the study period; most of the flies came from localities within or close to the ACT. Due to the high rate of parasitism by wasps and damage from lepidopteran inquilines, many galls contained no usable material. Most of the samples were collected from multilocular shoot bud galls, as these galls are common, and often contain many larvae.

Recent molecular evidence has identified the opomyzoid families Neurochaetidae and Agromyzidae as probable close relatives of Fergusoninidae (Wiegmann *et al*. 2011). Hence *Melanagromyza virens* Leow (Diptera: Agromyzidae) and *Neurochaeta inversa* McAlpine (Diptera: Neurochaetidae) were chosen as outgroups in this analysis.

2.3.3 Assignment of species status

Except where indicated, most species included in this study are undescribed. Species of *Fergusonina* are cryptic and useful diagnostic characters within this group are still being refined. Moreover, for many of the specimens collected here, adults were not available. Consequently, in this study operational taxonomic units (OTU's) were assigned on the basis of the host species from which galls were collected, larval

morphology and gall type. On generation of sequence data, the relationship between this classification and true species status was subsequently assessed (see Discussion).

2.3.4 Molecular methods

Where possible, fly DNA from each OTU was extracted from several individuals over more than one extraction session.

DNA was extracted from whole, 95% ethanol-preserved adults and larvae using a DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA, USA) following the protocols outlined by the manufacturer. A region of COI of approximately 830 base pairs (bp) was amplified using the primers C1-J-2183 (Jerry) 5' CAACATTTATTTTGATTTTTTGG 3' and TL2-N-3014 (Pat) 5' TCCATTGCACTAATCTGCCATATTA (Simon *et al*. 1994). This region is a slightly more sensitive marker in Fergusoninidae than the Folmer barcoding region (Purcell *et al*. 2015).

The polymerase chain reaction (PCR) was run using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Foster City, CA, USA) set to the following parameters: 94° C for 2 minutes; 35 cycles at 94° C for 30 seconds, 50° C for 30 seconds and 72° C for 1 minute, then incubation at 72° C for 3 minutes. PCR results were confirmed by gel electrophoresis. The PCR product was purified using a mix of 0.4 µl Exonuclease 1 (Genesearch Australia Pty Ltd), 1.6 µl Shrimp Alkaline Phosphatase (SAP) (USB Affymetrix, CA, USA) and 3 µl MilliQ water per sample, and submitted to the Biomolecular Resource Facility of the John Curtain School of Medical Research, Australian National University for further preparation and sequencing.

The DNA sequences were assembled, aligned and trimmed to 663 bp in Geneious 8.0.2 (Biomatters Ltd, Auckland, NZ) using the Geneious global alignment with free end gaps, and by eye.

Where corresponding COI sequences for additional *Fergusonina* and outgroup species were available from GenBank, these were included in the phylogeny; the accession numbers are listed in Table 2.2 and the associated papers describing the larval morphology of these species are listed below: *Fn. daviesae* Nelson and Yeates (Nelson *et al.* 2011a); *Fn. taylori* Nelson and Yeates (Nelson *et al.* 2011a); *Fn. tasmaniensis* Nelson and Yeates (Nelson *et al.* 2012); *Fn. lockharti* Tonnoir (Currie 1937; Taylor and Davies 2010); *Fn. nicholsoni* Tonnoir (Tonnoir 1937; Scheffer *et al*. 2004; Davies *et al*. 2010a).

Table 2.2 Accession numbers of *Fergusonina and Melanagromyza* sequences obtained from GenBank.

Fn. daviesae	*Fn. taylori*	*Fn. tasmaniensis*	*Fn. lockharti*	*Fn. nicholsoni*	*M. virens*
JF437655 JF437657-660 JF437662 JF437664 JF437666-667 JF437669-671 JF437670	JF437681-683 JF437685-686 JF437688-689 JF437692-695 JF437697-699 JF437701-702 JQ609282	JQ609284-286 JQ609292 JQ609294	AY687933	AY687934	EF104660

Bayesian analyses of 167 unique sequences were performed using the General Time Reversible substitution model (Tavaré 1986) with a gamma-distributed rate variation with invariant sites (GTR+I+G) applied to all three codon positions, within the MrBayes Geneious plugin version 2.2.2 (Huelsenbeck and Ronquist 2001). This was

determined by PartitionFinder (Lanfear *et al*. 2012) to be the most appropriate partitioning scheme and model for this dataset. The chains were run for 4,000,000 generations and the burn-in set at 25%. Trees were sampled every 500 generations. Convergence was assessed based on the standard deviation of split frequencies, and was considered to have occurred when this value was below 0.01 (Ronquist *et al*. 2012).

To test for the presence of nuclear mitochondrial DNA (numts), the aligned DNA sequences were translated into amino acid sequences and checked for stop codons. Sequences were deposited in GenBank under accession numbers KX950501–KX950600. Extracted DNA, adults, and third instar larvae where possible, were retained for vouchering and lodged in the Australian National Insect Collection, Canberra (ANIC). The voucher numbers are listed in Table 2.3.

2.4 Results

These analyses revealed 14 well-supported clades (posterior probability 0.77 – 1.0) with strongly corresponding gall type and dorsal shield type (Fig. 2.3). The gall types and shield types associated with each clade are listed and described in Table 2.4. While some gall types were associated with multiple clades (Table 2.4), of the 16 distinct forms of dorsal shield recorded in this study, none except for the absence of a dorsal shield occurred in more than one clade or was associated with more than one gall type.

2.4.1 Gall type – shield type relationships

Two clades were recorded from flower bud galls: multilocular galls where several locules occur among the developing stamens of the bud (Fig. 2.2c) and unilocular galls, in which the locule occurs in place of the ovules in a healthy bud (Fig. 2.2p). Only *E. pauciflora* and *E. lacrimans* hosted both flower bud gall types.

Axial unilocular pea galls may be stalked or sessile (Fig. 2.2d, e). This variation is not correlated with fly or plant host species. These galls appeared three times in the phylogeny, in clades 2, 5 and 9, and in every instance the larvae were elongate rather than ovate. Clade 2 was limited to hosts in the subsection *Terminales*, while clade 5 was associated with a range of hosts from two subgenera, *Symphyomyrtus* and *Eucalyptus.* The larvae in these two clades have two dark spots made of four smooth plates in clade 2 (Fig. 2.1h), and bearing teeth in clade 5 (Fig. 2.1a, b).

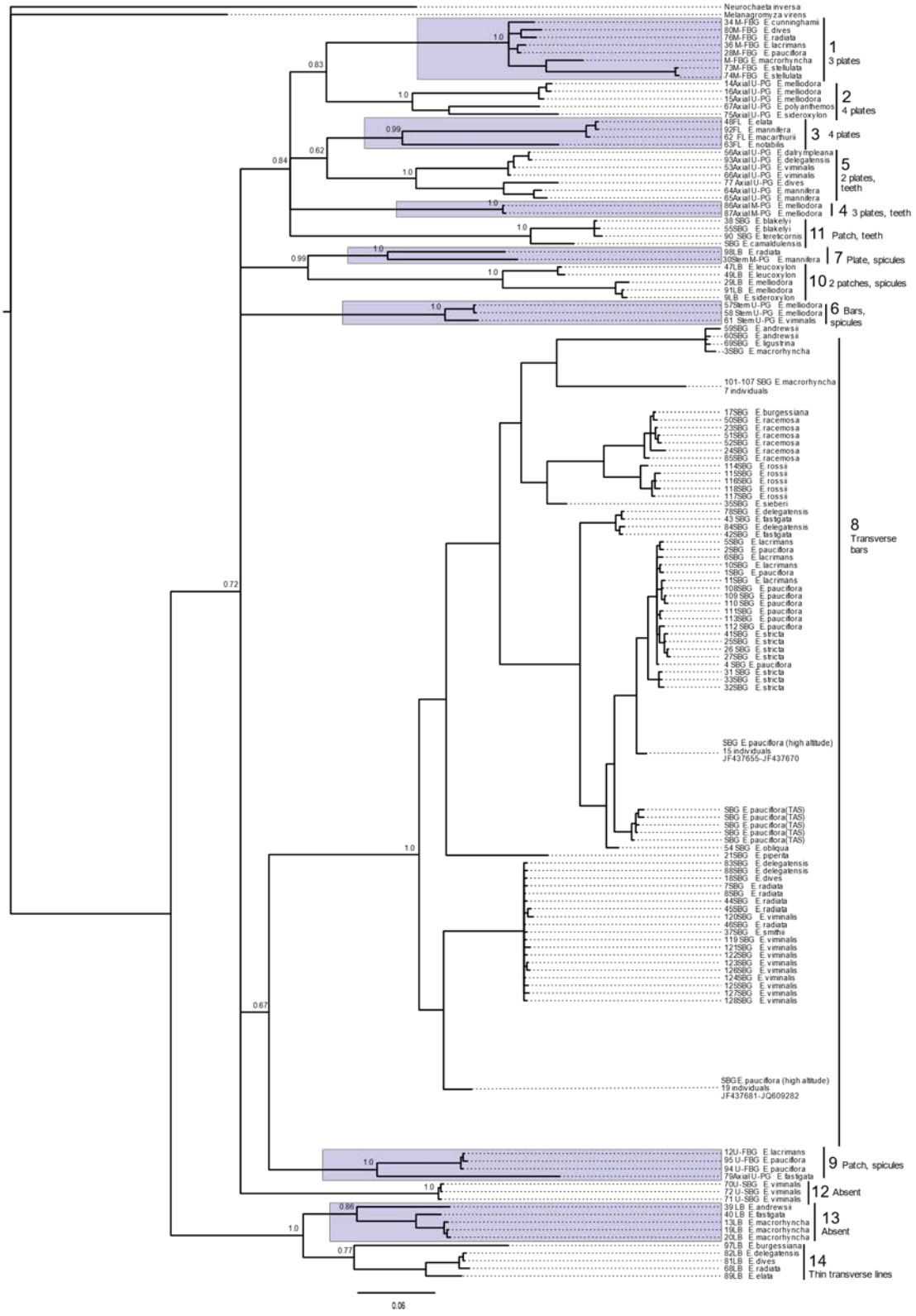


Fig. 2.3 Bayesian tree inferred from mtCOI sequences, with numbered clades showing the relationships between gall type, host and dorsal shield type. Alternate clades are shaded for clarity. The specimen numbers correspond to ANIC voucher numbers (Table 2.3). Values at nodes represent the Bayesian posterior probabilities.

Table 2.3 Voucher numbers of *Fergusonina* and *Neurochaeta* specimens and DNA lodged at ANIC, with the corresponding sequence numbers used in Fig. 2.3.

Sequence	Voucher								
1	29-039388	**27**	29-039414	**53**	29-039440	**79**	29-039466	**105**	29-039492
2	29-039389	**28**	29-039415	**54**	29-039441	**80**	29-039467	**106**	29-039493
3	29-039390	**29**	29-039416	**55**	29-039442	**81**	29-039468	**107**	29-039494
4	29-039391	**30**	29-039417	**56**	29-039443	**82**	29-039469	**108**	29-039495
5	29-039392	**31**	29-039418	**57**	29-039444	**83**	29-039470	**109**	29-039496
6	29-039393	**32**	29-039419	**58**	29-039445	**84**	29-039471	**110**	29-039497
7	29-039394	**33**	29-039420	**59**	29-039446	**85**	29-039472	**111**	29-039498
8	29-039395	**34**	29-039421	**60**	29-039447	**86**	29-039473	**112**	29-039499
9	29-039396	**35**	29-039422	**61**	29-039448	**87**	29-039474	**113**	29-039500
10	29-039397	**36**	29-039423	**62**	29-039449	**88**	29-039475	**114**	29-039501
11	29-039398	**37**	29-039424	**63**	29-039450	**89**	29-039476	**115**	29-039502
12	29-039399	**38**	29-039425	**64**	29-039451	**90**	29-039477	**116**	29-039503
13	29-039400	**39**	29-039426	**65**	29-039452	**91**	29-039478	**117**	29-039504
14	29-039401	**40**	29-039427	**66**	29-039453	**92**	29-039479	**118**	29-039505
15	29-039402	**41**	29-039428	**67**	29-039454	**93**	29-039480	**119**	29-039506
16	29-039403	**42**	29-039429	**68**	29-039455	**94**	29-039481	**120**	29-039507
17	29-039404	**43**	29-039430	**69**	29-039456	**95**	29-039482	**121**	29-039508
18	29-039405	**44**	29-039431	**70**	29-039457	**96**	29-039483	**122**	29-039509
19	29-039406	**45**	29-039432	**71**	29-039458	**97**	29-039484	**123**	29-039510
20	29-039407	**46**	29-039433	**72**	29-039459	**98**	29-039485	**124**	29-039511
21	29-039408	**47**	29-039434	**73**	29-039460	**99**	29-039486	**125**	29-039512
22	29-039409	**48**	29-039435	**74**	29-039461	**100**	29-039487	**126**	29-039513
23	29-039410	**49**	29-039436	**75**	29-039462	**101**	29-039488	**127**	29-039514
24	29-039411	**50**	29-039437	**76**	29-039463	**102**	29-039489	**128**	29-039515
25	29-039412	**51**	29-039438	**77**	29-039464	**103**	29-039490		
26	29-039413	**52**	29-039439	**78**	29-039465	**104**	29-039491		

Table 2.4 Summary of the clades showing gall type, larval dorsal shield type and host associations

	GALLS	LARVAE	HOSTS
CLADE 1	**Multilocular flower bud galls** (Fig. 2.2c). Larvae enclosed in small pods among developing stamens of flower; galled buds often deformed and greatly enlarged.	Small, ovate. Shield consists of three plain dark brown to black cuticular plates extending from posterior half of T3 to anterior of A2, meeting at segmental margins. Middle plate broadest (Fig. 2.1i).	*E. dives*, *E. stellulata* (ACT), *E. cunninghamii*, *E. lacrimans*, *E. pauciflora*, *E. stricta* (NSW), *E. macrorhyncha* (SA).
CLADE 2	**Unilocular axial pea galls** (Fig. 2.2d). Spherical, thin-walled, stalked or sessile growths in axils of leaves. Each gall contains a single, spacious locule.	Elongate-pyriform. Four smooth, black plates. A hemispherical plate, anteriorly convex, lies on posterior edge of T3 and abuts a smaller plate on anterior margin of A1. Another plate on posterior of A1 abuts a fourth, slightly longer one on A2. All plates apart from T3 elongate-elliptical (Fig. 2.1h).	*E. melliodora*, *E. polyanthemos*, *E. sideroxylon* (ACT).
CLADE 3	**Fused leaf galls** (Fig. 2.2f, g). Large (~ 5 cm; may grow to over 10 cm). Also known as 'leafy' bud galls (Taylor *et al.* 2005). Locules arranged in rows between multiple leaf blades, fusing them together. Leaf edges often visible running down length of gall.	Elongate-pyriform with a pair of bristle-like projections on each thoracic segment. Shields are composed of four simple plates of chitin arranged in two pairs across T3 to A1 and A1 to A2. The shield of the *E. notabilis* larva was inferred from empty puparia (Fig. 2.1c).	*E. elata*, *E. macarthurii*, *E. mannifera* (ACT), *E. notabilis* (QLD). Also recorded from *E. bridgesiana and E. viminalis* (Davies *et al*. 2014b)

CLADE 4	**Multilocular axial pea galls** (Fig. 2.2h). Small, dark pink clusters of spherical pea galls on axial shoot buds.	Ovate-pyriform. Possess three plates extending from T3 to A2, joined at segmental margins, with two to four anterior-facing hooks on posterior margin of plate on A2 (Fig. 2.1f).	*E. melliodora* (ACT). Also found on *E. sideroxylon* and *E. leucoxylon* in the ACT, but have not been sequenced.
CLADE 5	**Unilocular axial pea galls** (Fig. 2.2e). As with clade 2, but sometimes with a conical peak. May be paired, with one gall on each opposing axil.	Elongate with bristle-like thoracic projections. Shields consist of a pale, broad scattering of spicules on T3 and two dark patches of thick cuticle on A1 to A2, and A2 to A3. On posterior edge of anterior patch is a row of five or six short, rounded teeth; number varies between individuals (Fig. 2.1a,b).	*E. delegatensis*, *E. dives*, *E. mannifera* (ACT), *E. dalrympleana*, *E. viminalis* (NSW). Also found on *E. rubida* but have not been sequenced.
CLADE 6	**Unilocular stem pea galls** (Fig. 2.2i). Small (~ 2 - 3 mm), sessile on stems, and crowned with nodules.	Ovate-pyriform. Shield consists of a scattering of raised speckles on T1, a bar of spicules on T2, and broad bars of spicules and ridges coalesced into solid dark patch from T3 to A2, narrow bars on A3 and A4, and faint grey spicules on last and second last abdominal segments (Fig. 2.1j).	*E. melliodora* (ACT), *E. viminalis* (NSW).
CLADE 7	**Multilocular stem pea galls** (Fig. 2.2n)**/flat leaf blade galls.** Stem galls a series of connected locules running along plant stem. Leaf blade galls are very flat, with fewer than ten locules, barely discernible on leaf surface.	Shield consists of a central plate across T3 to A2, surrounded by clusters of spicules, which are rounded (Fig. 2.1n), or pointed and face inwardly towards the plate. Bands of spicules on A3 and A4. Shield coated in a clear, thick substance	*E. mannifera (ACT), E. radiata (NSW).* Larvae with a similar shield type have also been found in a flat leaf blade gall on *E. melliodora*, but not sequenced.

CLADE 8	**Multilocular shoot bud galls** (Figs 2.2a, b). Conspicuous, roughly rounded, chilli- or teardrop-shaped growths on the tips of terminal or axial stems. One or two leaves may sometimes be integrated with gall and extend beyond it. Round, irregularly-spaced locules are interspersed throughout gall.	Ovate-pyriform larvae with dorsal shields consisting of a series of five to nine transverse bars of dark, thickened cuticle, from second or third thoracic segment (T2 or T3) to third to sixth abdominal segment (A3 – A6). Some have thin grey lines between bars in intersegmental creases (Fig. 2.1m). Number of abdominal bars varies between individuals.	*E. delegatensis*, *E. dives*, *E. macrorhyncha*, *E. olsenii*, *E. pauciflora*, *E. rossii* (ACT), *E. burgessiana*, *E. fastigata*, *E. lacrimans*, *E. ligustrina*, *E. obliqua*, *E. pauciflora*, *E. piperita*, *E. racemosa*, *E. radiata*, *E. smithii*, *E. stricta*, *E. viminalis* (NSW), *E. andrewsii (*QLD), *E delegatensis* (VIC). Two genetically distinct OTUs were collected from a single *E. macrorhyncha* individual.
CLADE 9	**Unilocular flower bud galls** (Fig. 2.2p)/**Unilocular axial pea galls**. Flower bud galls occur on snow gums *E. pauciflora* and *E. lacrimans*, with single locules in lower half of the bud, and the inner structures of flower absent. Axial pea gall is identical to other galls of this type described above.	Shields consist of narrow area of brown chitin from T3 to A2, thickened across the two junctions between the segments. Larvae from flower bud galls have numerous brown spicules and ridges surrounding these patches (Fig. 2.1k,l); larvae from *E. fastigata* have only a few spicules anterior to each thick patch, with a small area of spicules on A2.	*E. pauciflora* (ACT), *E. lacrimans*, *E. fastigata* (NSW).
CLADE 10	**Leaf blade galls** (Fig. 2.2j, k). One or two rows of connected locules running along leaf midvein; these alternate between large empty and small occupied locules.	Larvae elongate. Shield is two dark patches of thickened cuticle, surrounded by black spicules (Fig. 2.1o). Larvae from *E. melliodora* also possess a scattering of dark spicules on A3 (Fig. 2.1p).	*E. leucoxylon*, *E. melliodora*, *E. sideroxylon (ACT).*

CLADE 11	**Multilocular shoot bud galls.** Morphology as per those in Clade 8.	Elongate-ovate with an shield of three joined, sclerotised patches, extending from T3 to A2 with a row across the middle patch of four to seven anterior-facing teeth (Fig. 2.1d,e).	*E. blakelyi* (ACT), *E. tereticornis* (NSW), *E. camaldulensis* (SA).
CLADE 12	**Unilocular terminal shoot bud galls** (Fig. 2o). Elongate galls that resemble two young terminal leaves fused together. Contain a single larva inside spacious locule.	Elongate, with no dorsal shield.	*E. viminalis* (NSW).
CLADE 13	**Leaf blade galls** (Fig. 2.2l, m). The locules are spacious and may be irregularly arranged along the leaf blade, or clustered at the leaf tip or base (Head 2008).	Large, elongate, with no dorsal shield (Fig. 2.1q). The shield of the *E. andrewsii* larva was inferred from empty puparia.	*E. macrorhyncha* (ACT), *E. fastigata* (NSW), *E. andrewsii* (QLD).
CLADE 14	**Leaf blade galls.** Morphology as per clade 13.	Elongate, with a series of ~7 narrow, often faint transverse stripes near the apex of each segment, beginning at T2, with small, sparsely-scattered spicules between them (Fig. 2.1r,s).	*E. delegatensis*, *E. dives*, *E. pauciflora* (ACT), *E. burgessiana* (JBT), *E. elata*, *E. radiata* (NSW), *E. delegatensis* (VIC).

An instance of this unilocular gall type also appeared in a relatively under-sampled clade (clade 9) with the unilocular flower bud galls. The species within this clade had similar dorsal shields, except for the number and arrangement of spicules around the sclerotised patches (Table 2.4). Only one example of this gall-shield type was found, i.e., from *E. fastigata*.

The multilocular fused leaf gallers (Fig. 2.2f, g) in clade 3 have elongate larvae with long bristle-like thoracic projections, and two-dot shields made of smooth plates, almost identical to the pea gallers in clade 2 but dark brown rather than black (Fig. 2.1c). There are also minor differences in the shapes of the plates and the extent to which they abut at the segmental margins.

Multilocular axial pea gallers (clade 4; Fig. 2.2h) possess shields with three plates with prominent, curved, anterior-facing teeth (Fig. 2.1f, g). These were found only on *E. sideroxylon*, *E. leucoxylon* and *E. melliodora* (Section *Adnataria*, Series *Melliodorae*) but as yet I do not have genetic data for the flies from the first two hosts. There are few samples in this clade, and their position in the phylogeny is currently unresolved.

Clade 6 is an unplaced clade of flies that inhabit unilocular galls on plant stems (Fig. 2.2i). I have examples only from two hosts, *E. melliodora* and *E. viminalis* (subgenus *Symphyomyrtus*). The surface of the gall features bumps and lobes that may be leaf buds. The larvae from these galls are ovate and possess a broad, grey-black area of sclerotised spicules (Fig. 2.1j). They are small relative to the interior of the locule.

The multilocular terminal and axial shoot bud galls (Fig. 2.2a, b) are common and relatively conspicuous. These gall types may be large and can contain at least 200 larvae, though more commonly they house 15 – 40. Clade 8 has the highest number of representatives here, with ovate larvae bearing shields of bars of pigmented spicules

(Fig. 2.1m). Sampling to date generally associates this clade with the subgenus *Eucalyptus*, with the exceptions of *E. smithii*, *E. dalrympleana* and *E. viminalis* from *Symphyomyrtus*. The smaller clade 11 has a rough patch of chitin with a row of anterior-facing teeth (Fig. 2.1d, e). The three associated hosts known so far are all from the *Symphyomyrtus* Section *Exsertaria*.

Leaf blade galls (Fig. 2.2j-m) are very susceptible to parasite attack and are frequently taken over by parasitoid and hyperparasitoid wasps. The results show four clades of flies (clades 7, 10, 13 and 14) that occur on leaf blades, two of which (clades 13 and 14) form a larger clade that is sister to the rest of the fergusoninids in this study. The larvae in clade 13 have no dorsal shields, and in clade 14 the shield comprises a number of fine, transverse lines between sparse scatterings of spicules (Fig. 2.1r, s). In some individuals the shield is not obvious as the pigmentation of the spicules can be very faint. The larvae are elongate and relatively large for the genus, and inhabit spacious locules.

The leaf blade galls in clade 10 (Fig. 2.2j, k) were found only on trees in *Eucalyptu*s section *Adnataria*, series *Melliodorae* and differ structurally from other leaf blade galls. They are composed of alternating large and small chambers, but only the large locules are visible externally, appearing as one or two rows of regular pea-like bumps, while the larvae and nematodes occupy the small, hidden locules. These galls and larvae resemble flat leaf galls from *E. siderophloia* (section *Adnataria*, series Siderophloiae) described and illustrated in Giblin-Davis *et al*. (2004a) and Davies *et al*. (2013b). The fourth lineage of leaf blade gallers appears in clade 7, which is comprised of stem galls (Fig. 2.2n) consisting of a series of connected locules running along the plant stem and petiole, and leaf blade galls. These may both be variants of Currie's (1937) "leaf and leaf stem" galls on *E. stuartiana* (now *E. bridgesiana*). Their

relationship is relatively distant (12.4% divergence) but the larvae share a feature that has not yet been found in other clades. The shield, an intricate arrangement of spicules around a sclerotised plate, is coated in a clear, mucous substance which becomes opaque and breaks up on contact with ethanol. These galls are relatively obscure and few have been collected. The stem gall from *E. mannifera* described here is the only one of its kind collected from this host; of the flat leaf galls, one has been collected from *E. radiata* and is included in this study, and one has been found on *E. melliodora* but no successful sequences obtained.

Clade 12 contains a small sample of a single species of fly, and a gall type to date only found on *E. viminalis*. These galls (Fig. 2.2o) superficially resemble small multilocular terminal shoot bud galls, but contain only one locule. The larvae have no dorsal shields.

2.4.2 Host relationships

The mean pairwise base pair distance between all unique *Fergusonina* haplotypes (n = 165) was 13.54% with a maximum distance of 20.51%. The mean distance between the unique haplotypes of each OTU on a host species was 0.68%.

Some individual galls contained multiple COI haplotypes, while some haplotypes occurred across multiple host plants (not always of the same tree species) and sometimes across a large geographic range (over 200 km apart). For instance, among the leaf blade gallers (clade 14) one sequence was derived from galls from *E. elata*, *E. dives*, *E. delegatensis* and *E. lacrimans*, and another was shared by *E. delegatensis*, *E. elata* and *E. pauciflora*. From clade 1, the multilocular flower bud gallers, one single haplotype was collected from two closely-related species of mallee ash, *E. stricta* and *E. cunninghamii*, in the Blue Mountains, NSW. From clade 8, one

haplotype was found on *E. burgessiana* and *E. racemosa*, and from clade 5 one was shared by *E. dalrympleana* and *E. viminalis*.

Also in clade 8, a species morphologically and genetically identified as *Fn. manchesteri* (0.15% - 1.06% bp pairwise distance between haplotypes) was collected from the subgenera *Symphyomyrtus* and *Eucalyptus*, on species frequently found growing naturally together in mixed stands (*E. viminalis, E. smithii, E. radiata, E. dives* and *E. delegatensis*). Additionally, flies from two species of eucalypt growing together, naturally-occurring *E. macrorhyncha* and cultivated *E. olsenii* outside its natural range, had identical haplotypes.

All haplotypes within each OTU had a pairwise bp distance of under 2% (0.15% - 1.96%), while between OTUs the distance was over 2% (2.11% - 20.51%), with the following exceptions. There was a small distance of 0.75% - 1.21% between shoot bud gallers *Fn. omlandi* on *E. pauciflora* and those from *E. stricta* in the Blue Mountains, NSW, but flies from *E. stricta* in Barren Grounds, NSW differed from *Fn. omlandi* (including those from *E. stricta* from the Blue Mountains) by 1.36% - 2.26%. The flies from the two *E. stricta* populations differed from each other by 1.96% - 2.41% and are reciprocally monophyletic. Base pair differences between the reciprocally monophyletic *Fn. daviesae* and *Fn. omlandi,* which occur on different subspecies of *E. pauciflora,* varied by a wide range of 1.96% - 3.02%. with the sequences from *E. macrorhyncha* and *E. andrewsii* each being less than 2% distant from the flies from *E. ligustrina* (1.66% and 0.9% - 1.96% respectively) but differing from each other by between 1.66% and 2.71%.

In clade 3 there was little variation among the haplotypes of fused leaf gall flies from *E. elata, E. macarthurii* and *E. mannifera* in the ACT (0.3% - 1.06% pairwise bp distance) but they differed by 14.3% - 15.3% from those from *E. notabilis,* the other

host in this clade, collected in Qld. Leaf blade galls were also collected from *E. delegatensis* at both sites, and the flies from these were genetically and morphologically identical to each other and to those from the same gall type from *E. dives* in the ACT and *E. elata* in NSW (Table 2.1). Two species (pairwise distance 11.92% - 12.82%) were collected from one individual *E. macrorhyncha*; one of these is commonly associated with the host and the other was only collected in a single gall.

Some dorsal shield forms in different lineages were superficially similar, such as the "dots" shield types in clades 2, 3 and 10 associated with axial pea galls, fused leaf galls and leaf blade galls respectively. There were slight, but consistent, intraspecific differences in the form of those from clade 10, depending on host. Though the COI haplotypes from *E. melliodora* and *E. sideroxylon* flies differed by only 0.3% - 1.51%, the larvae from *E. melliodora* possessed a scattering of dark spicules on the third abdominal segment that was absent from any collected from *E. sideroxylon.*

2.5 Discussion

Having a high rate of variation, COI provides good resolution at species level and below. To further resolve phylogenetic relationships at deeper nodes, I am assembling a nuclear dataset that will allow me to make stronger inferences about the evolution of the dorsal shield types (in prep).

2.5.1 Phylogenetic patterns

These results show a strong correlation between dorsal shield morphology and gall type, to the extent that confident predictions about larval morphology and gall type can be made based on relationships inferred from the phylogeny.

The appearance of some gall types in multiple fly lineages suggests that the formation of this type of gall has evolved independently several times and is consistent with previous findings (Ye *et al*. 2007; Davies *et al*. 2010a).

The results lend support to Currie's (1937) hypothesis of a general progression from absence of dorsal shields or those consisting of a simple sparse scattering of sclerotised spicules (a minor modification of the spicules that cover the bodies of many dipteran larvae) such as we see in clades 13 and 14, to more complex structures of coalesced patches or bars of spicules (clades 6 to 11), and thick plates of cuticle (clades 1 to 5), perhaps the result of a group of spicules amalgamated into a single structure. The teeth or hooks appear both on the plate-type shields (clades 4 and 5) and on the spiculose shields (clade 11). It is important to note, however, that some of the support levels at the deeper nodes are low, and I cannot confidently infer the phylogenetic relationships at these levels.

2.5.2 Host relationships

Unlike the gall type-shield type relationships, host associations vary less reliably with the phylogeny; while some *Fergusonina* clades here are associated with a single subgenus of host (e.g. clade 11 of shoot bud gallers in clade 1*)* and some were found within a single section (for instance, the shoot bud gallers in clade 11 associated with Section *Exsertaria*) others utilise a more diverse range of hosts when those plants occur in sympatry (e.g. clades 2, 3, 8 and 10 associated with two subgenera, *Eucalyptus* and *Symphyomyrtus*). Equally, while many OTUs were collected from only one host species, others occurred on closely related hosts (e.g. the flower bud gallers in clade 9 from the snow gums *E. pauciflora* and *E. lacrimans*), or on sympatric but distantly related hosts (e.g. the species from *E. olsenii* and *E. macrorhyncha*, *or Fn. manchesteri*,

in clade 8). These examples indicate some degree of lability in host selection. The ability to exploit novel hosts would be beneficial where erratic seasonal patterns may affect the growth of suitable oviposition sites on natural host populations (Nelson *et al*. 2014).

2.5.3 Species delimitation

As a consistent heuristic, flies were considered putative conspecifics when their sequences differed by less than 2%, as this threshold was most consistent with characterisation by morphology, host and gall type. However, given the range of COI variation within species, some apparent species limits within the shoot bud gallers in clade 8 straddled this threshold, such as the morphologically distinct and reciprocally monophyletic *Fn. omlandi* and *Fn. daviesae,* and the species occurring on *E. macrorhyncha*, *E. ligustrina* and *E. andrewsii*. There is a slight difference in dorsal shield morphology between the leaf blade gallers on *E. melliodora* and *E. sideroxylon,* suggesting they are distinct species, but they fall well within the 2% species limit. Consistent with other findings in other Diptera (Meier *et al.* 2006) my results suggest that species delimitation by such an arbitrary measure may not always be adequate for this genus, and indicate further work is required to determine reliable diagnostic features within *Fergusonina*.

2.5.4 Function of the dorsal shield

The strong correlation observed between gall and shield morphology within clades initially suggests a functional relationship. If the purpose of the shield was only to help excavate a tunnel or scrape at the plant material, as may be the case with cecidomyiids, the more elaborate shields would occur on larvae that live in tissue-dense galls (e.g.

multilocular shoot bud galls), and larvae with simple or no shields would be found in galls with a thin wall and a very spacious locule, such as pea galls. The reverse would be expected if the shield served to anchor the larva within the locule. However, there is no overall consistency in gall and shield structure across the phylogeny; *Fergusonina* shield complexity does not strictly correspond to either gall thickness or locule spaciousness relative to the larva. Larvae from axial pea galls may have elaborate shields with teeth, or two small, smooth patches of thickened cuticle. The two shoot bud gall shield types are simple bars of spicules, and a sclerotised patch with a row of teeth. Furthermore, similar shield types occur in dissimilar gall types, with the two simple patches being associated with capacious pea galls as well as with fused leaf galls with a very convoluted internal structure and deep but narrow locules. Where locules are spacious, larvae with plain or absent shields might have alternative means of buffering or anchoring them within the gall, such as long thoracic projections, but there is no consistent correlation here either.

The nature and source of the substance on the larvae from clade 7 have yet to be established, and chemical analysis such as gas chromatography mass spectrometry (GCMS) could be valuable, to assess its composition. The galls and dorsal shields resemble *Fergusonina* sp. 7 from *E. bridgesiana* (in Currie 1937). Currie (1937) observed these larvae arching and apparently defecating on their shield, which could explain the occurrence of the substance. Whether this feeds the symbiotic nematodes, protects against desiccation, or serves some other purpose remains a matter for speculation. However, this behaviour may have been unrelated to the substance on the shield, as many fergusoninid larvae, when dissected from their galls, react by squirming and arching.

The dorsal shield is unlikely to be a defence against parasitoid wasps, as it does not cover a large area of the body, and parasitism very often occurs before the larvae reach the third instar prior to the development of the dorsal shield. The observation that some species of *Fergusonina* lack dorsal shields suggests that the function of the shield is not absolutely critical to survival and development of the larvae. These inconsistencies suggest that there is no single overall function for these shields, but it may be that different shield types serve different and possibly multiple purposes for each species.

CHAPTER 3: PLANT HOST RELATIONSHIPS OF THREE LINEAGES OF *FERGUSONINA* ON *EUCALYPTUS* L'HÉRIT.

3.1 Abstract

The gall-inducing fly family Fergusoninidae, in association with a mutualist nematode, induces galls on Myrtaceae. Each fly species typically targets a particular site on its host plant, giving rise to a range of gall types, and one plant species may host at least four fly species. While incongruent fly-host evolutionary time scales preclude early cospeciation, it is possible that Fergusoninidae have been diverging with their host plants more recently at correspondingly finer taxonomic levels, such as within host subgenera. To test this possibility, I reduced the scale of my analysis and focussed on a clade of ten *Eucalyptus* species, sampling intensively and using a phylogenetic approach to compare the relationships between these plant hosts and their associated flies. I also took advantage of the fact that three different gall types, each with its own clade of *Fergusonina* flies, could be sampled on this focal host clade, in effect giving me three different host/fly association tests on the one set of hosts. The phylogenies of flies from the three different gall types were estimated using Bayesian analysis of mtCOI sequences and compared with an existing phylogeny of the eucalypt host clade. While each gall type showed a different pattern of host relationships, heuristic and quantitative analysis showed there was little correspondence between plant and fly phylogenies and I conclude that host-switching is prevalent in this system. There was more host fidelity in the flower bud gallers on this group of eucalypts, and there was least in the leaf blade gallers, with the shoot bud gallers demonstrating an intermediate level of host fidelity. I discuss possible factors which may have led to their patterns of

host association. This is the first study of *Fergusonina* to focus on one clade of *Eucalyptus* L'Hérit. (Myrtaceae) with intensive sampling, and shows that each host plant species is commonly used by multiple fergusoninid species. This has provided me with the opportunity to study in detail the host relationships of three separate clades of *Fergusonina* from different plant tissue types, and has revealed many previously unrecorded host plant/gall site associations.

3.2 Introduction

Gall inducing insects have complex and highly specialised relationships with their host plants. Gall initiation involves alteration of the plant's cells and manipulation of its physiology at the galled site to provide nutrients and protection for the inhabitants (Abrahamson and Weis 1987; Meyer 1987; Shorthouse and Rohfritsch 1992; Hartley 1998; Inbar *et al.* 2004). Such intimacy with the host plant's physical and chemical traits necessitates a degree of fidelity not just to the host taxon but often to a particular site on the plant, such as a flower bud or petiole (Meyer 1987; Shorthouse and Rohfritsch 1992; Williams 1994; Nelson *et al.* 2014). Consequently, a high level of phylogenetic congruence might be expected between the gallers and their hosts, either through codivergence or through host switching to similar, closely related species.

However, many evolutionary relationships between specialist herbivores such as gall inducers and their hosts are not so simple or predictable, and the mechanisms behind host selection and successful exploitation of novel hosts can be complex, and are poorly understood in many systems (Strong *et al.* 1984; Thompson 1994; Ronquist and Liljeblad 2001; de Vienne *et al.* 2013).

3.2.1 The Fergusonina-Fergusobia *system*

Members of the genus *Fergusonina* are small acalyptrate flies that induce galls solely on the plant family Myrtaceae with an obligate mutualist nematode, *Fergusobia* spp. Currie (Sphaerularioidea: Tylenchida). To date they have been recorded from seven genera: *Angophora*, *Corymbia*, *Eucalyptus*, *Leptospermum*, *Melaleuca*, *Metrosideros* and *Syzygium* (Currie 1937; Tonnoir 1937; Harris 1982; Davies and Giblin-Davis 2004; Taylor *et al.* 2007; Taylor and Davies 2008) almost entirely in Australia, with a small number of collections from New Zealand, India, Papua New Guinea and the Philippines (Harris 1982; Siddiqi 1986; 1994; Taylor *et al.* 2007; Davies *et al.* 2010b). As far as is known, this mutualism between insects and nematodes is unique (Davies and Giblin-Davis 2004).

The tightly integrated life cycles of *Fergusonina* and *Fergusobia* have been described in detail elsewhere (Currie 1937; Fisher and Nickle 1968; Taylor *et al.* 2005; Nelson *et al.* 2014), but in summary, the nematodes are carried in adult female flies and deposited in plant material when the fly oviposits. These nematodes are parthenogenetic females, whose feeding action on the plant material is believed to stimulate cellular changes in the plant material that lead to gall growth. These females produce a generation of males and females while the fly larva develops. A small number of fertilised nematodes enter the female larvae prior to pupation, and lay eggs inside the fly's body, the resultant juveniles being carried by the adult fly to the next plant host.

The plant host species, gall site, and whether galls contain single or multiple fly larvae vary depending on the fly species. Galls may be in flower buds, at the ends of terminal or axial shoots, along leaf blades or leaf tips, or other areas of new growth (Currie 1937; Giblin-Davis *et al.* 2004a, Purcell *et al.* 2016). Thus, the *Fergusonina-Fergusobia*

system provides a fascinating model for examining coevolution at three levels, by investigating the relationships between the flies, their mutualist nematodes, and their host plants.

This system has generally been considered to be highly host-specific, where one fly-nematode species pair will gall a particular site on a single plant species or a small number of close relatives (Scheffer *et al.* 2000; 2004; Taylor 2004; Taylor *et al*. 2005; Davies *et al.* 2010b; Nelson *et al.* 2011a; Nelson *et al.* 2014), with some exceptions (Purcell *et al.* 2016). However, in the eucalypt gallers this has generally been inferred from molecular studies on *Fergusobia* (Giblin-Davis *et al.* 2003b; Ye *et al.* 2007; Davies *et al.* 2010a; 2010b) and what is true for the nematode is not necessarily so for the fly. Despite the tightness of the association we cannot be certain that there is strict vertical transmission of the nematodes. Some gall types commonly have multiple foundresses, which could allow horizontal transfer of nematodes between conspecific fly lineages and, potentially, between different fly species galling the same site on the same host (Nelson *et al.* 2014; Purcell *et al.* 2015; Davies *et al.* 2016).

The extent to which this mutualism has evolved in concert with host plant speciation or by host-shifts, or a combination of the two, has long been a question of interest (Giblin-Davis *et al.* 2004b; Taylor *et al.* 2005; Scheffer *et al.* 2013; Nelson *et al.* 2014; Davies *et al.* 2016). Fergusoninidae are not represented in the fossil record and the crown age of the family is unknown, but is estimated to be less than 42 million years (Wiegmann *et al.* 2011; Nelson *et al.* 2014), around half the estimated crown age of Myrtaceae (Thornhill *et al.* 2012; 2015). This age discrepancy means that the relationship between Myrtaceae and Fergusoninidae was most likely established long after significant

evolutionary events of the host, when there were already many genera of Myrtaceae and multiple lineages within the genus *Eucalyptus sensu latu*. Fergusoninidae are now widespread on Myrtaceae, so it is most likely that the mutualism developed on one lineage, then moved on to the major lineages of Myrtaceae by host switching (Ye *et al.* 2007; Davies *et al.* 2010b; Nelson *et al.* 2014). However, more recent codivergence between the flies and Myrtaceae hosts at a finer taxonomic level could possibly be identified as co-evolutionary events (Davies *et al.* 2016), and current records suggest that some *Fergusonina* and *Fergusobia* clades are allied with groups of closely related host plants (Davies *et al.* 2010b; Nelson *et al.* 2014, Purcell *et al.* 2016).

To investigate whether timed co-evolution between *Fergusonina* and Myrtaceae has occurred at a finer evolutionary scale, I obtained a narrow but comprehensive dataset targeting a well-defined representative clade of ten *Eucalyptus* species, and collected all the *Fergusonina* galls found on these plants. In addition, *Fergusonina* consists of reciprocally monophyletic clades corresponding to the various gall types (e.g. flower bud galls or leaf blade galls) (Purcell *et al.* 2016), permitting multiple tests of coevolutionary hypotheses within the focus clade across the gall type lineages. The level of host fidelity within these clades appeared to vary between lineages, from highly host-specific species to those with a quite broad host range. I expected that as host specificity declined I would find evidence of a corresponding decline in fly-host plant co-evolution.

3.3 Materials and methods

3.3.1 Hosts plants

A clade of ten *Eucalyptus* species (Table 3.1) was targeted for gall collection based on its

convenient size, the strong support for its internal phylogeny, and its geographical accessibility; all of the species occur in South Eastern Australia, and most are reasonably abundant within their range.

3.3.2 Collection methods

Galls were hand-collected from ten species of *Eucalyptus* within the focus clade in the Australian Capital Territory (ACT), Jervis Bay Territory (JBT), New South Wales (NSW), and Victoria (Vic) between 2011 and 2015 (Table 3.2). Hosts were identified with the aid of the *EUCLID* interactive key (Slee *et al.* 2006), field guides, and other sources (Chippendale and Wolf 1981; Brooker and Kleinig 2001; Boland *et al.* 2006; National Parks Association of the ACT 2007; Brooker and Nicolle 2013).

Several collection trips were made for each eucalypt species, as gall numbers were found to fluctuate seasonally and from year to year, as well as among host populations. Galls were collected from all sites on the host; flower buds, leaf blades, and terminal and axial shoots (Table 3.1).

Each gall was sealed in a clear plastic zip-lock bag and labelled with site code, tree number and gall number. Mature galls containing pupae were checked daily for emergent adult flies, which were collected from the bags and placed in sealed vials in 95% ethanol. Younger galls were dissected as soon as possible after collection and the larvae removed and placed in 95% ethanol. Where only adults were obtained from a gall, larval morphology was inferred from the empty puparia, which retain the external characters of the final larval stage.

Table 3.1 Total number of individuals sequenced from each gall type on each host, and the number (in parentheses) of unique COI haplotypes obtained**.** Figures separated by a slash belong to separate species.

***Eucalyptus* host**	**Gall type**						**Total *Fergusonina* spp. per host sp.**
	M-SBG	**M-FBG**	**U-FBG**	**Leaf blade**	**U-PG**	**Fused leaf**	
E. olsenii L.A.S. Johnson and Blaxell	2 (1)*	4 (1)	0	0	0	0	**2**
E. lacrimans L.A.S. Johnson and K.D. Hill	12 (4)	3 (1)	4 (1)	2 (2)	0	0	**4**
E. elata Dehnh..	6 (2)	1 (1)	0	1 (1)* / 2 (2)	0	8 (2)	**5**
E. radiata Sieber ex DC	14 (5)	2 (1)	0	4 (1) /3 (1)	0	0	**4**
E. pauciflora subsp. *pauciflora* Sieber ex Spreng.	64 (8)	1 (1)	3 (2)	2 (1)	0	0	**4**
E. burgessiana L.A.S. Johnson and Blaxell	11 (2)	3 (2)	2 (1)	2 (1)	0	0	**4**
E. cunninghamii Sweet	2 (1)	4 (1)	0	0	0	0	**2**
E. delegatensis R.T. Baker	6 (2) / 2 (2)	0	0	5 (2)	1 (1)	0	**4**
E. fastigata H. Deane and Maiden	7 (2)	2 (2)	1 (1)	1 (1)	1 (1)	0	**5**
E. fraxinoides H. Deane and Maiden	4 (2)	4 (1)	0	4 (1)	0	0	**3**

** From cultivated plant*

Gall type abbreviations:
M-SBG: Multilocular terminal or axial shoot bud gall; M-FBG: Multilocular flower bud gall; U-FBG: Unilocular flower bud gall;
U-PG: Unilocular axial pea gall

3.3.3 Ingroup and outgroup sampling

Three *Fergusonina* lineages were selected for analysis. A separate analysis was performed for each of three *Fergusonina* lineages. Each lineage is composed of members that are morphologically distinctive and are associated with particular gall types: terminal and axial shoot bud gallers (clade 8 in chapters 2 and 4), leaf blade gallers (clade 14), and multilocular flower bud gallers (clade 1). These three lineages are presumed to be monophyletic based on a combined analysis of morphology, host plant, gall type and molecular data. (Purcell *et al*. 2016). The shoot bud galler lineage included 31 unique sequences, the leaf blade galler lineage included 11 unique sequences, and the flower bud gallers included 11 unique sequences. These three clades were chosen as they were found on almost all host plants (Table 3.1). Any groups that occurred on fewer than half of the host plants were eliminated from the study. Two genetically and morphologically distinct species of leaf blade gallers were found on *E. radiata*. I included only the specimens from the clade common to the other host plant species in the study. A third distinct type of leaf blade galler collected from *E. fastigata,* not belonging to the study clade, was also excluded. This was the only species of leaf blade galling fly collected from this plant species. No live specimens were obtained from shoot bud galls on *E. cunninghamii* as the galls were either spent, or destroyed by caterpillars; therefore, DNA was extracted from two puparia from one of these galls, following the protocols outlined below.

Table 3.2 Locality information for *Fergusonina* specimens collected for DNA analysis.

Eucalyptus host	GPS Coordinates	Collection location	Sequence ID/ANIC Voucher number
E. burgessiana (Faulconbridge mallee ash)	-35.1541, 150.7076	Jervis Bay, JBT	**FBG 8** /29-039521; **FBG 9**/29-039522; **SBG 30**/29-039533; **SBG 31**/29-039404
	-35.1536, 150.7061		**LBG 7**/29-039484
E. cunninghamii (cliff mallee ash)	-33.6209, 150.3278	Blue Mountains, NSW	**FBG 5**/29-039421; **SBG22**/29-039532
E. delegatensis (alpine ash)	-35.3886, 148.8090	Namadgi NP, ACT	**LBG 3**/29-039527; **SBG 2**/29-039470; **SBG 3**/29-039475
	-35.3904, 148.8020		**LBG 5**/29-039469
	-37.1100, 148.9023	Mt. Delegate, Vic	**SBG 25**/29-039471; **SBG 26**/29-039465
E. elata (river peppermint)	-35.3038, 149.1297	Canberra, ACT	**LBG 6**/29-039476
	-35.2804, 149.11214		**SBG 28**/29-039530
	-36.8573, 149.6902	Myrtle Mountain, NSW	**LBG 3**/29-039526; **LBG 5**/29-039483
E. elata (cont.)	-36.9094, 149.6095	Wyndham, NSW	**FBG 7**/29-039520
	-36.8995, 149.6073		**SBG 29**/29-039531

E. fastigata (brown barrel)	-36.5964, 149.4102	Brown Mountain, NSW	**SBG 24**/29-039429
	-36.6100, 149.4168		**SBG 27**/29-039430
	-35.4510, 149.5838	Tallaganda, NSW	**FBG 10**/29-039517; **FBG 11**/29-039518
E. fraxinoides (white ash)	-35.9622, 149.5794	Gourock NP, NSW	**FBG 1**/29-039516; **LBG 1**/29-039525; **SBG 9**/29-039529; **SBG 10**/29-039528
E. lacrimans (weeping snow gum)	-35.2920, 149.0624	Canberra, ACT	**SBG 14**/29-039397; **SBG 18**/29-039392; **SBG 21**/29-039398
	-36.0328, 148.8002	Adaminaby, NSW	**FBG 3**/29-039423; **SBG 15**/29-039393
	-36.0464, 148.7356		**LBG 3**/29-039523; **LBG 4**/29-039524
E. olsenii (Woila gum)	-35.2781, 149.1085	Canberra, ACT	**SBG 8**/29-039534
	-36.6042, 149.6794	Numbugga Walls, NSW	**FBG 6**/29-039519
E. pauciflora (snow gum)	-36.0866, 148.8710	Adaminaby, NSW	**SBG 16**/29-039391
	-36.0464, 148.7355		**FBG 4**/29-039415
	-35.2763, 149.0804	Canberra, ACT	**SBG 12**/29-039498

	-35.2718, 149.1170		**SBG 11**/29-039500; **SBG 13**/29-039388; **SBG 17**/29-039389; **SBG 19**/29-039496; **SBG 20**/29-039497; **SBG 23**/29-039499
	-35.5646, 148.7785	Namadgi NP, ACT	**LBG 5**/29-039486
E. radiata (narrow-leaved peppermint)	-35.6473, 149.5065	Tallaganda, NSW	**LBG 2**/29-039455
	-35.3294, 149.8769	Braidwood, NSW	**FBG 2**/29-039463
	-35.8662, 148.9915	Namadgi NP, ACT	**SBG 1**/29-039432; **SBG 4**/29-039394; **SBG 5**/29-039395; **SBG 6**/29-039431; **SBG 7**/29-039433

Fergusonina turneri, which induces shoot bud galls on *Melaleuca quinquenervia*, was chosen as the outgroup for these analyses, as molecular evidence indicates that flies and nematodes from *Melaleuca* and the eucalypts form reciprocally monophyletic clades (Ye *et al.* 2007; Davies *et al.* 2010b). The *Fn. turneri* sequence used here was obtained from GenBank (accession number AY687948) (Scheffer *et al.* 2004). Additionally, I included a eucalypt-associated species that induces unilocular shoot bud galls on *E. viminalis*. These flies form a monophyletic group within *Fergusonina*'s eucalypt-galling lineage (Purcell *et al.* 2016).

3.3.4 Assignment of Operational Taxonomic Unit (OTU)

Almost all *Fergusonina* included in this study are undescribed, and often only larvae were obtained. The third instar larvae of most *Fergusonina* species bear a unique sclerotised structure known as the "dorsal shield" (Currie 1937) which can be used to identify an individual to a species group and gall type (Purcell *et al.* 2016). However, finer taxonomic distinctions are not possible based on larval morphology. Moreover, reliable diagnostic adult features are yet to be determined, as the flies exhibit intraspecific morphological variation in size, markings and even chaetotaxy.

A thorough examination of species boundaries in *Fergusonina* requires a larger and more comprehensive dataset than that used in this narrow focus study, but as a consistent heuristic, delimitation of species by a COI haplotype distance of 2% or higher is almost always congruent with characterisation by morphology, host plant and gall type (Purcell *et al.* 2016). Consequently, the OTUs assigned here have been based on a combination of larval morphology, host plant, gall type and molecular data.

3.3.5 Molecular methods

Where possible, the DNA of flies from each host plant species was extracted from several individuals over more than one extraction session.

DNA was extracted from whole, 95% ethanol-preserved adults, larvae and puparia using a DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA, USA) following the protocols outlined by the manufacturer. A region of COI of approximately 830 base pairs (bp) was amplified using the primers C1-J-2183 (Jerry) 5' CAACATTTATTTTGATTTTTTGG 3' and TL2-N-3014 (Pat) 5' TCCATTGCACTAATCTGCCATATTA (Simon *et al.* 1994). This region is a slightly more sensitive marker in Fergusoninidae than the Folmer barcoding region (Purcell *et al.* 2015).

For these, the polymerase chain reaction (PCR) was run using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Foster City, CA, USA) set to the following parameters: 94° C for 2 minutes; 35 cycles at 94° C for 30 seconds, 50° C for 30 seconds and 72° C for 1 minute, then incubation at 72° C for 3 minutes. PCR results were confirmed by gel electrophoresis, and the product purified using a mix of 0.4 µl Exonuclease 1 (Genesearch Australia Pty Ltd), 1.6 µl Shrimp Alkaline Phosphatase (SAP) (USB Affymetrix, CA, USA) and 3 µl MilliQ water per sample before being submitted to the Biomolecular Resource Facility of the John Curtain School of Medical Research, Australian National University for further preparation and sequencing.

To maximise sequencing success, tailed primers were used for some of the samples: Jerry 5' CAGGAAACAGCTATGACC CAACATTTATTTTGATTTTTTGG 3' and Superpat 5' TGTAAAACGACGGCCAGT GCACATWTCTGCCATATTAGA 3'. These were

amplified with a Dyad Peltier Thermocycler, using a touchdown PCR program as outlined in Regier (2007) as follows: 94° C for 30 seconds, 25 cycles of 55° C for 30 seconds (minus 4° C per cycle), 72° C for 1 minute plus two seconds per cycle; 12 cycles of 94° C for 30 seconds, 45° C for 30 seconds, 72° C for 2 minutes plus 3 seconds per cycle; 72° C for ten minutes. The PCR product was submitted to LGC Genomics GmbH in Berlin, Germany, for sequencing with the primers M13REV 5' CAGGAAACAGCTATGACC 3' and M13(-21) 5' TGTAAAACGACGGCCAGT 3'.

The DNA sequences were assembled, aligned and trimmed to 660 bp using Geneious 8.0.2 (Biomatters Ltd, Auckland, NZ).

3.3.6 Phylogenetic analysis

All three of the separate Bayesian analyses were performed with the same parameters. I used the General Time Reversible substitution model (Tavaré 1986) with a gamma-distributed rate variation with invariant sites (GTR+I+G) applied to all three codon positions, within the MrBayes Geneious plugin version 2.2.2 (Huelsenbeck and Ronquist 2001). This was determined by PartitionFinder v.1.1.1 (Lanfear *et al.* 2012) to be the most appropriate partitioning scheme. The chain was run for 1,000,000 generations and the burn-in set at 25%. Trees were sampled every 200 generations. Convergence was inferred by the standard deviation of split frequencies reaching a value below 0.01 (Ronquist *et al.* 2012).

To test for the presence of nuclear mitochondrial DNA (numts), the aligned DNA sequences were translated into amino acid sequences and checked for stop codons. Duplicate haplotypes collected from different host plants were included in the analyses, but duplicates from the same hosts were discarded, as retaining them

did not affect the topology of the trees. Sequences have been deposited in GenBank under the accession numbers MF695818–MF695841. Adults and third instar larvae were retained for vouchering and lodged in the Australian National Insect Collection, Canberra (ANIC).

The *Eucalyptus* host clade is a subset of a recent species level phylogeny of the eucalypts (Gonzalez-Orozco *et al.* 2016).

3.3.7 Cophylogenetic analysis

Cophylogenetic analysis was performed using JANE 4.01, which uses an event-cost method to reconstruct host and parasite cophylogenies (Conow *et al.* 2010). This method assigns a cost to each of five possible coevolutionary events: codivergence (host and parasite diverge together), duplication (the parasite diverges and both lineages remain associated with the same host), host switching (parasite diverges and one species switches to a new host species), loss (host loses a previously associated parasite) and failure to diverge (host diverges and parasite follows both lineages) (Charleston 1998; Conow *et al.* 2010). It then seeks the least costly solution that can be obtained given these cost parameters.

Where necessary, multiple conspecifics were removed from the fly trees and each tip was treated as an independent OTU. Using a population size of 150 with 300 generations according to the ratio suggested for small datasets (Conow *et al.* 2010), I compared a range of cost schemes from 0 to 2 for each parameter, prioritising either codivergence or host switching, or giving an equal cost to both. Based on these results, I used two schemes that gave the lowest overall cost reconstructions for all three gall types: Codivergence = 1, duplication = 0, host switching = 0, loss = 1 and failure to diverge = 0; and codivergence = 2,

duplication = 0, host switching = 0, loss = 1 and failure to diverge = 0. Statistical analyses were performed within JANE to test whether the costs of the reconstructions were significantly lower than might occur by chance.

The tanglegrams were compiled and annotated using Fig Tree v. 1.4.2 and Adobe Illustrator CS6 version 16.0.3.

3.4 Results

The gall types collected from each host plant are summarised in Table 3.1. All host plant species were associated with multiple *Fergusonina* species. Of the three clades of gallers used in the phylogenetic analyses, only the shoot bud galls were found on all host species.

Moreover, I found that many *Fergusonina* species utilise two or more host plants. Of the ten host species, all but *E. fraxinoides* and *E. olsenii* bore fly species also found on another host within or outside the focus clade.

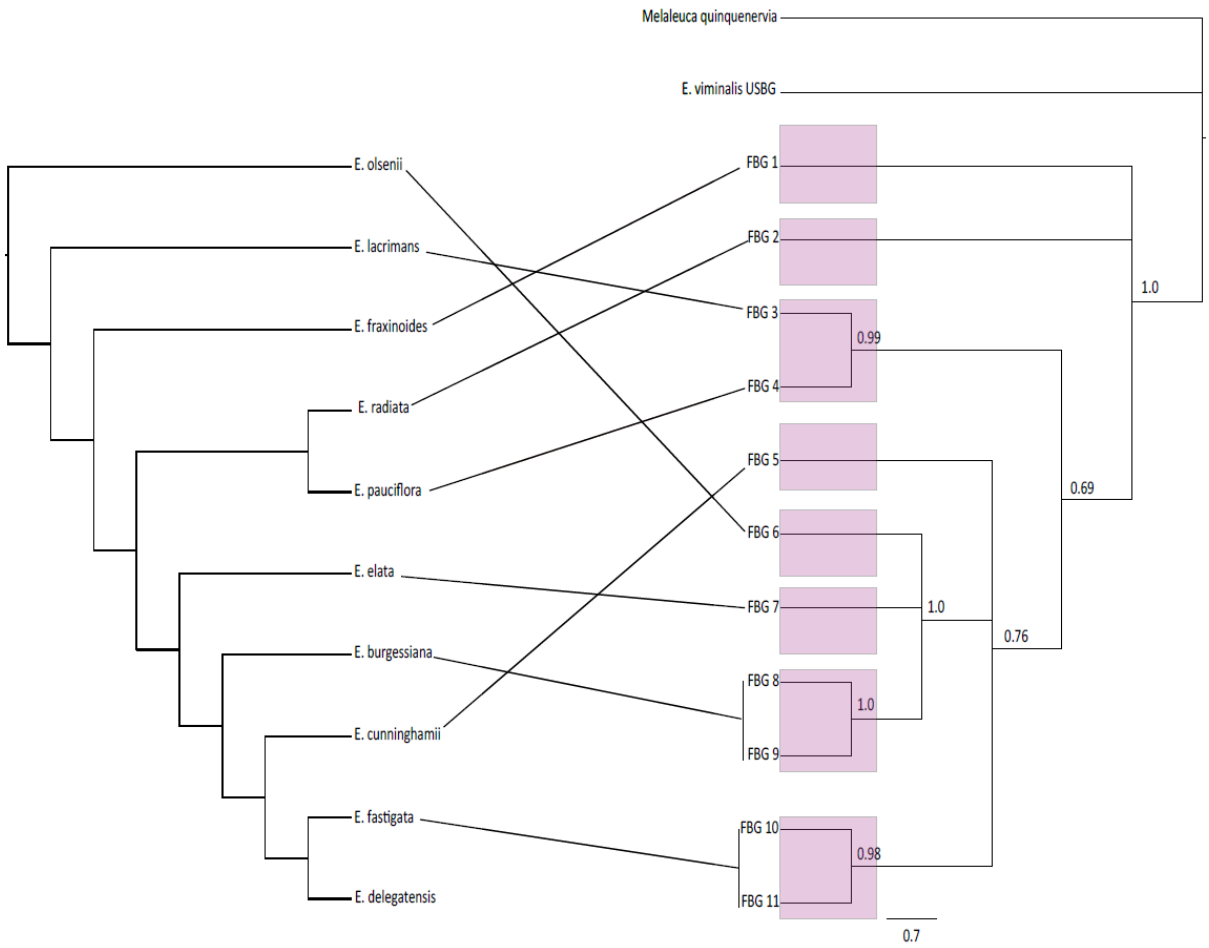
3.4.1 Patterns of host associations

The patterns of the host-fly affiliations differed between the three groups, as follows.

i) Flower bud galls

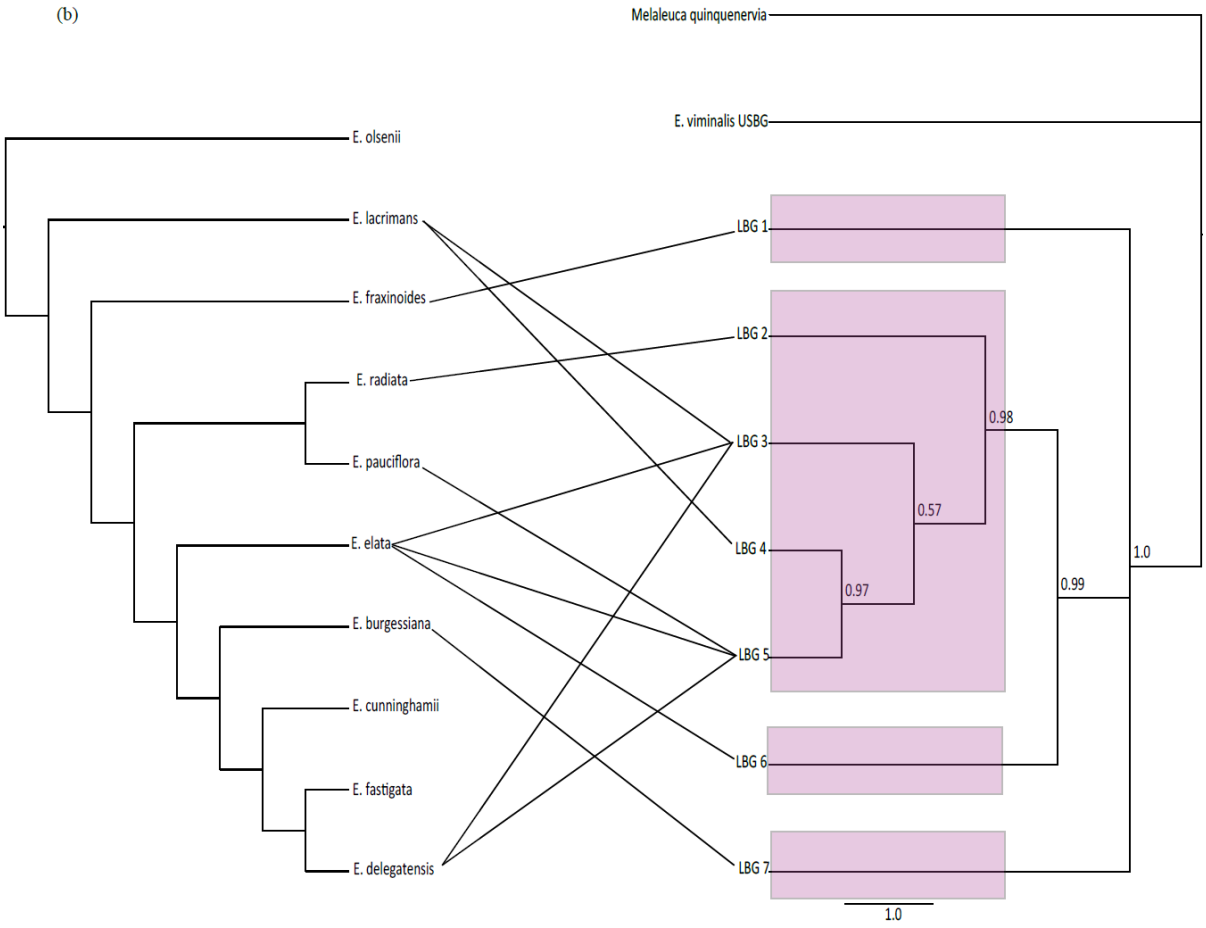
The flower bud gallers showed the greatest host specificity among the three groups (Fig. 3.1a); eight species were collected from nine plant host species; within this group, only *E. pauciflora* and *E. lacrimans* hosted the same *Fergusonina* OTU. The same fly species from *E. cunninghamii* also occurs on *E. stricta* in the same locality (*E. stricta* is not in the focus clade, but both are mallee ashes in the series Strictae), an identical

haplotype being collected from both host plants. There is more phylogenetic congruence between the flies and their hosts in this clade than in the other two gall types studied.



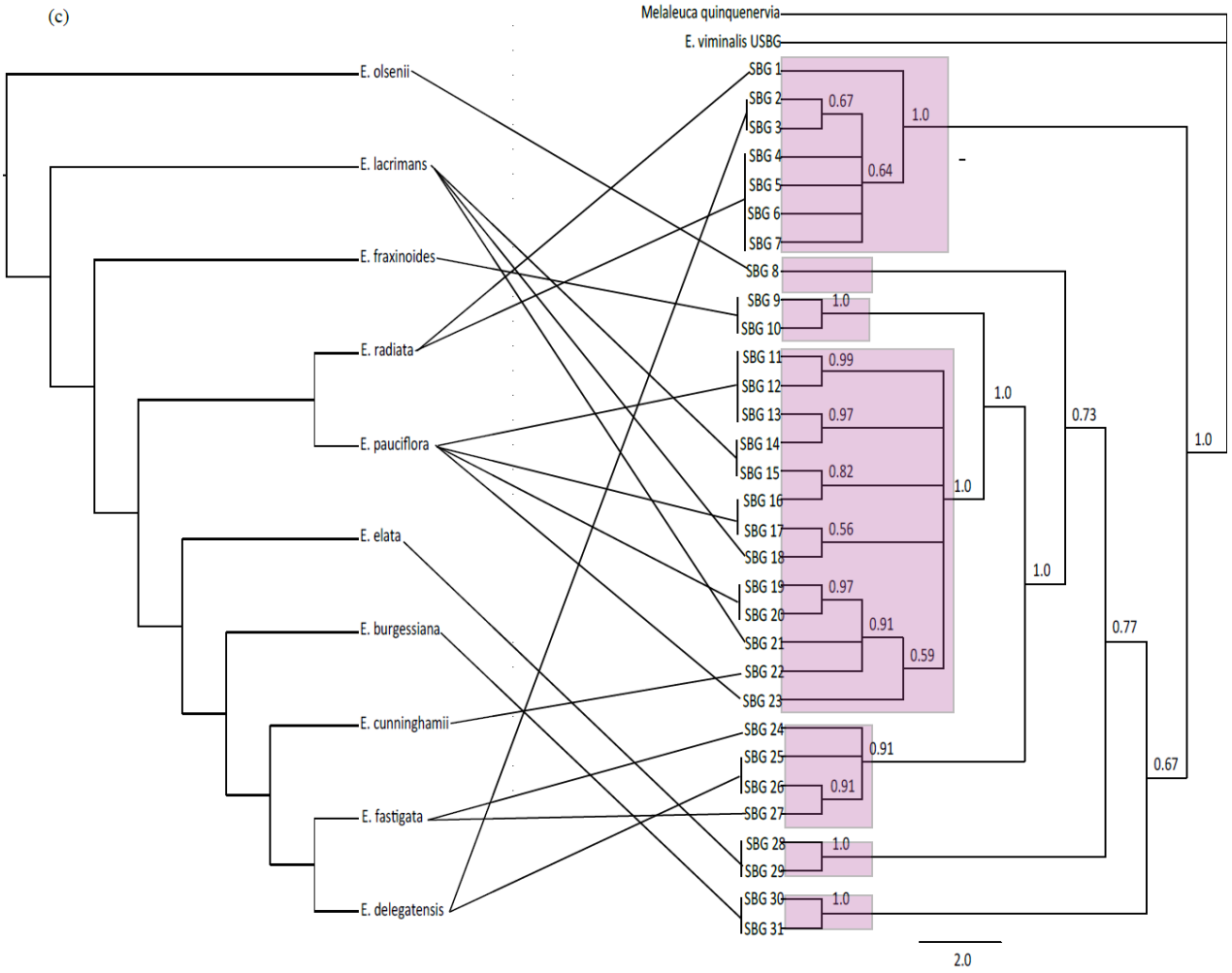
(a)

Fig. 3.1 Tanglegrams of *Eucalyptus* hosts and their associated *Fergusonina* flies from a) multilocular flower bud galls, b) leaf blade galls and c) multilocular shoot bud galls. Values at nodes represent Bayesian posterior probabilities. The tips of the *Fergusonina* trees represent unique COI haplotypes, with the OTUs demarcated by shaded boxes.



(b)

Fig. 3.1 (*continued*)



(c)

Fig. 3.1 (*continued*)

ii) Leaf blade galls

Four *Fergusonina* species were collected from seven host species. The leaf blade gallers showed the least host specificity (Fig. 3.1b), with a single fly species occurring on six host species: *E. delegatensis*, *E. elata*, *E. radiata*, *E. pauciflora* and *E. lacrimans* as well as *E. dives* outside the focus host clade. Within these, one haplotype occurred on *E. pauciflora*, *E. delegatensis* and *E. elata*, and another (differing by 0.15% bp distance) on *E. lacrimans*, *E. delegatensis* and *E. elata*. Two species with a 3.77-3.92% bp

distance from each other were collected from *E. elata* in different localities; to date the species collected in Canberra has only been found on *E. elata*, but the species collected from Bemboka also occurs on four other host species within the target group (*E. radiata, E. pauciflora, E. lacrimans,* and *E. delegatensis).*

iii) ***Shoot bud galls***

Shoot bud galls (Fig. 3.1c) yielded the most specimens, being relatively common and often containing many flies. Seven *Fergusonina* species were collected from ten *Eucalyptus* host species. Shoot bud galls were found on all hosts (Table 3.1) but those found on *E. olsenii* were collected from cultivated specimens in the Australian National Botanic Gardens and were of a COI haplotype that occurs commonly on the locally abundant *E. macrorhyncha*. As no shoot bud galls were found on *E. olsenii* in its native range, I was unable to confirm whether this is a natural association or an anomaly.

Fergusonina omlandi, described from shoot bud galls on *E. pauciflora subsp. pauciflora* (Nelson *et al.* 2011a), was also collected from *E. lacrimans* and *E. cunninghamii;* in one case the same haplotype occurred on both *E. pauciflora* and *E. cunninghamii*. It has also been collected from *E. stricta* (Purcell *et al.* 2016). The species collected from *E. elata* also occurs on *E. sieberi*, and that from *E. burgessiana* has also been found on *E. racemosa.*

Two species were collected from *E. delegatensis* in different localities; the pairwise base pair difference between the flies from the two localities was 12.22% - 12.52 %. Moreover, the species associated with *E. delegatensis* at Mt Delegate was also collected from *E. fastigata* in Glenbog State Forest between Nimmitabel and Bemboka NSW, and the species from the ACT also occurred on *E. radiata* in Namadgi NP. The species collected in the ACT occurs on other hosts (*E. viminalis*, *E. smithii*, *E. stellulata*

and *E. dives*), but to date has not been recorded further south than the Adaminaby area (-35.94311, 148.63253).

3.4.2 Codivergence analysis

A visual inspection of the tanglegrams (Fig. 3.1) did not suggest any congruence between the fly and host plant phylogenies in any of the clades and therefore gave no indication of codivergence of the fly and host lineages. This is supported by the results of the analysis using JANE. There were two schemes that yielded the lowest cost reconstructions in all gall types, the only difference between the two being the cost of codivergence, which was either one or two. For both schemes, the cost of duplication, host switching and failure to diverge was zero each, and for the cost of loss was one. The lowest cost reconstructions for all gall types had no instances of codivergence or duplication. The lowest cost flower bud galler reconstructions involved seven host switches, three losses and two failures to diverge, with a total cost of three. The lowest cost reconstructions for the leaf blade gallers had three host switches, four losses and four failures to diverge, with a total cost of four; and the shoot bud gallers' least costly reconstructions consisted of six host switches, ten losses and four failures to diverge, with a total cost of ten. Within each gall type, the cost of codivergence (whether it was one or two) did not affect the number of occurrences of each event, though the topological mapping of the reconstructions sometimes differed. In all cases the costs of the reconstructions were significantly lower than might be expected by chance (P=0.0).

3.5 Discussion

While a few plant hosts were already known to have more than one associated *Fergusonina* species (Currie 1937; Scheffer *et al.* 2004; Davies *et al.* 2010b; Nelson *et*

al. 2011a; Nelson *et al.* 2014; Purcell *et al.* 2016), I found this to be the case with every host I examined, suggesting that it may in fact be usual for each host plant species to accommodate several species of *Fergusonina* (Table 3.1). Usually the various fly species occur in different plant tissues or geographical ranges of the plant. The two geographically separated species of shoot bud gallers identified on *E. delegatensis* suggest that fly species may have distinct ranges within the range of the host plant. Similarly, two species were collected on *E. elata* leaf blades in different localities, Bemboka, NSW and Canberra, ACT (Table 3.2). The latter were collected from trees planted in an urban environment, and may have switched from another local host species, though to date they have not been collected from any other host species.

The eucalypts on which I found the fewest gall types were *E. olsenii* and *E. cunninghamii,* which both have very restricted ranges on exposed, rocky sites (Slee *et al.* 2006). *E. cunninghamii* has very small, narrow leaves which may not be suitable for leaf blade galling fergusoninids. Flies collected from shoot bud galls on an artificially planted specimen of *E. olsenii* in Canberra may have switched from a local host plant, *E. macrorhyncha,* as they carried an identical COI haplotype (Purcell *et al.* 2016). The fact that only flower bud galls were found on the small natural population of *E. olsenii* may have been due to the timing of sampling, or the host plant may lack the diversity of fly species found on trees with larger ranges, perhaps due to fire, drought or other events causing a local extinction, and the fragmentation of *E. olsenii* populations presenting a barrier to recruitment.

There is almost no correspondence within any of the gall type clades between the *Fergusonina* and *Eucalyptus* phylogenies, and the results strongly indicate that the evolution of this group has been characterised by host switching rather than codivergence. This is in keeping with evidence that long-term cospeciation of hosts and

symbionts is relatively rare (Ronquist and Liljeblad 2001; Inbar *et al.* 2004; de Vienne *et al.* 2013). In contrast to host affiliation, gall type is highly conserved among the fly lineages (Purcell *et al.* 2016) indicating that fergusoninids move more readily between host plant species than between tissue types on the same species. This implies that different bud tissues on the same plant, such as flower buds and shoot buds, differ more from each other than from their counterparts on other host plants, and the flies/nematodes are tissue specialists. The adaptation may relate to the bud structure, the cellular composition, or to the chemical compounds in the tissue, affecting the parasites' ability to manage or manipulate the plant's defences, initiate cell hypertrophy or optimise nutrient availability (Meyer 1987; Shorthouse and Rohfritsch 1992; Hartley 1998; Giblin-Davis *et al.* 2004a). In *Fergusonina*, as with some other gall-inducing organisms (Williams 1994) the targeted sites can be very specific. For instance, flower bud gallers occur in particular parts of the bud: some species attack the stigma and others, such as the ones in the study, occur among the stamens (Currie 1937; Giblin-Davis *et al.* 2004a; Nelson *et al.* 2014). Moreover, maintaining these site-specific niches reduces interspecific competition for galling sites on the same host plants (Inbar et al. 2004). An exception to this is *Fn. turneri*, which galls both flower buds and shoot buds on *M. quinquenervia* (Giblin-Davis *et al.* 2001; Scheffer *et al.* 2004; Taylor 2004; Head 2008; Wright *et al.* 2013).

3.5.1 What governs host plant affiliation?

While the different fly lineages are primarily tissue specialists, they are not host plant generalists. As there is no evidence of host-fly cospeciation within this group, other factors must be determining the host ranges of these flies. Some of the *Fergusonina* species occurring on the focus clade host plants have also been collected from

Eucalyptus species outside this clade, and where this occurs, the primary determinant appears to be host sympatry; for example, the presence of shoot bud gallers of the local *E. macrorhyncha* on the non-local *E. olsenii* at the Australian National Botanic Gardens in Canberra.

In no-choice oviposition tests on *Fn. turneri* (Wright *et al.* 2013), while the females favoured the buds of their natural host plants, *M. quinquenervia*, they also oviposited and nemaposited in non-target host species in other genera of Myrtaceae, although not on any non-myrtaceous plants. However, galls only successfully developed on *M. quinquenervia*. The extent to which either the nematodes or the fly larvae are responsible for establishing and maintaining the gall is unknown. Gall formation begins well before the fly larvae hatch (Giblin-Davis *et al.* 2001), and is presumed to be caused by the feeding nematodes, perhaps by chemical secretions from the oesophageal gland (Taylor *et al*. 1996), but there may be some other stimulus. It is possible that the process is initiated by some chemical injected into the plant material by the adult fly at the time of oviposition (Fisher and Nickle 1968, Taylor *et al.* 1996, Davies *et al.* 2001). Currie (1937) noted that gall development around infertile fly eggs was initiated but did not progress far, and the surrounding nematodes eventually died without reproducing. Attempts to induce galls by artificially injecting either *Fergusonina* eggs, *Fergusobia* juveniles or both into host plant material have been unsuccessful to date (Currie 1937, Giblin-Davis *et al.* 2001, Ye *et al.* 2007).

If gall success is dictated by the nematodes' ability to bypass the plant's defences, those laid in a novel host species might successfully exploit it if the plant has relatively low chemical defences (Whiffin and Bouchier 1992) or its immunity has been compromised, and from these weak individuals eventually spread through the population. There would be selective pressure on the nematodes to adapt to novel plant

host species where the flies are prone to ovipositing in them, as they depend on the flies reaching maturity to complete their life cycle. It is common to find that some young host plants have relatively high gall density while others of the same approximate size and age in the same population have none (Purcell *et al.* 2015). While chemotypic variation in the invasive paperbark *M. quinquenervia* affects host choice in two of its biological control agents, the weevil *Oxyops vitiosa* and the psyllid *Boreioglycaspis melaleucae* (Wheeler 2005; Wheeler and Ordung, 2005; Padovan *et al.* 2010), the role (if any) of chemotype in host selection or gall success in this system is yet to be investigated. It is also unknown whether *Fergusonina-Fergusobia* exploit or manipulate plant chemistry by increasing or decreasing levels of nutrients and defensive secondary compounds in the gall tissue relative to the surrounding plant tissue, as has been shown with many other gall inducers (Abrahamson and Weis 1987; Hartley and Lawton 1992; Hartley1998).

One would expect the fly to be highly selective and avoid wasting eggs and energy ovipositing fruitlessly in non-host plants. However, an individual female almost certainly spreads her eggs over several sites. The lifetime fecundity of the flies is not known (Davies *et al.* 2001) and egg production can be impaired by the parasitic nematodes (Currie 1937; Giblin-Davis *et al.* 2001) but fecundity has been estimated at 183 ± 42 in *Fn. turneri*, and oviposition is thought to continue throughout the three weeks or so of adult life (Giblin-Davis *et al.* 2001). Spreading egg clutches across multiple sites minimises the chance of losing all offspring to parasitism, gall failure or gall destruction, and when the numbers of available oviposition sites on host plants are low or there is limited time to search, it might be advantageous to explore some unsuitable or non-target plants despite the relatively low chance of success (Craig *et al.* 1994; Larsson and Ekbom 1995; Inbar *et al.* 2004; Nelson *et al.* 2014). Sometimes it

may simply be a case of accidental oviposition in the "wrong" host, perhaps as a result of the fly's aging; age-related mobility and behavioural changes have been recorded in flies (Carey *et al.* 2006) and other invertebrates (Anotaux *et al.* 2016).

Host plant hybridisation may be another mechanism by which host switching or expansion occurs. Hybrids may be less resistant to attack (Morrow *et al.* 1994) while in disturbed areas they grow rapidly and may form hybrid swarms that expand into the parent populations (Pryor 1959) making them potentially attractive alternatives to the parent hosts. However, there is no indication that this occurs often, if at all, in this system. While *E. dives, E. elata* and *E. pauciflora* all hybridise or intergrade with *E. radiata* (Whiffin 1981; Johnson and Hill 1990) and share a species of leaf blade galler, this is the only such example in this study. Moreover, the same *Fergusonina* species also occurs on *E. delegatensis,* which has no recorded hybrids.

3.5.2 How and why do host relationships vary between gall types?

The propensity for host-switching appears to vary between the *Fergusonina* groups, with some lineages attacking a wide range of hosts within (and occasionally across) host subgenera, while others are more restricted (Purcell *et al.* 2016). The same patterns are reflected at species level, with some attacking a number of hosts (such as the leaf blade gallers) and others not (the flower bud gallers).

The flower bud gallers showed the greatest host specificity among the three clades. This might be largely because flowering times vary between *Eucalyptus* species, restricting the flies' oviposition options and leading to reproductive isolation after a host shift (Craig *et al.* 1994). Additionally, flies feeding on nectar may stay close to the flowering branches they emerged from and be less likely to stray to novel host plants in search of food. It may also be due to the relative complexity and species-dependent

variation in the flower bud structure compared with a leaf bud. In laboratory no-choice tests on *Fn. turneri*, Wright *et al.* (2013) observed that while the flies probed both leaf buds and flower buds of non-host species, they did not oviposit in the non-host flower buds. However, they tested far fewer plant species with flower buds (three) than with leaf buds (21).

It may be that the various gall type lineages have simply developed different biochemical strategies for gall initiation unrelated to the plant tissue type, and the mechanism used by the flower bud galler is more host specific than that of the leaf blade galler regardless of the bud site.

If *Fergusobia* from different lineages are able to interbreed in multilocular galls (which presupposes that the nematodes move between locules), the consequent genetic diversity could make them more adaptable to new host plants than species from unilocular galls with no opportunity for genetic mixing (Nelson *et al.* 2014). This might account for the relatively large host plant range of the shoot bud galling species compared to the flower bud gallers; the flower buds contain few larvae and are likely to be founded by a single fly. Conversely, the nematodes from leaf blade galls, whose fly species have a large host range, are probably similarly constrained; most galls on a given leaf blade are likely to be singly founded (though this has not been confirmed) and often the locules are discrete, precluding any movement between them.

While this study found no evidence of codivergence between the plant hosts in this clade and their associated *Fergusonina* species, this does not necessarily extend to all groups within the genus, such as those that occur only on hosts within a single section (Purcell *et al.* 2016) and quite different patterns might have emerged had another clade of plant hosts or flies from different gall types been examined.

The factors determining host relationships in this system remain to be

discovered and are likely to be complex and context-dependent. The plant properties that enable a nematode to initiate galls and the cues used by female *Fergusonina* seeking a host are still unknown and probably vary depending on the bud tissue galled. To obtain a more complete understanding of the processes at play in this system, a similar study of the *Fergusobia* associated with these study species would be necessary.

CHAPTER 4: SPECIES BOUNDARIES, PHYLOGENETIC RELATIONSHIPS AND PLANT HOST ASSOCIATIONS

4.1 Abstract

Flies in the monogeneric family Fergusoninidae induce galls on Myrtaceae in a mutual association with a nematode. COI-based phylogenies have shown that this group is comprised of distinct lineages, and these lineages are also supported by similar larval morphology, gall site and host genus, but the relationships between these groups are poorly resolved. In addition to poor resolution of the deeper relationships, fergusoninid species within, and sometimes among lineages are difficult to distinguish morphologically, and identification to species level can involve comparing adult chaetotaxy and genital morphology. In this paper I use an expanded COI data set (228 sequences) from a wider host range, augmented by 52 CAD sequences to investigate the relationships among the lineages. I also investigated the feasibility of using molecular methods to identify species boundaries in *Fergusonina* by assessing the consistency between COI data and morphological, geographical and ecological information. With a 663 base pair region of COI I compared the results of species delimitation using bPTP and ABGD as well as uncorrected pairwise distances. In most cases, bPTP, ABGD, and a $\leq 2\%$ uncorrected pairwise distance threshold sorted the haplotypes into groups that corresponded to other features such as gall site and host plant, but none of these approaches agreed consistently on every species. Of the models used, the 2% threshold provided the most plausible results. However, while COI data may identify many species boundaries in *Fergusonina,* genetic distance methods alone cannot be relied on to resolve the challenges of species delimitation in this group and an integrative approach should be used.

4.2 Introduction

4.2.1 The Fergusonina-Fergusobia *system*

Fergusonina Malloch (Diptera: Fergusoninidae) is a large genus of acalyptrate flies occurring almost exclusively in Australia. In a unique mutualism with a nematode, *Fergusobia* Currie (Sphaerularioidea: Tylenchida) they induce galls on Myrtaceae and have been recorded from seven genera to date: *Angophora*, *Corymbia*, *Eucalyptus*, *Leptospermum*, *Melaleuca*, *Metrosideros* and *Syzygium* (Currie 1937; Tonnoir 1937; Harris 1982; Davies and Giblin-Davis 2004; Taylor *et al*. 2007; Taylor and Davies 2008; Davies *et al*. 2017). Fly-nematode species pairs are host specific on one or a few host species, and target a particular site on the plant, such as flower buds or leaf blades. Depending on the species, the galls may contain either one or multiple larvae, and potentially several hundred (Ye *et al*. 2007), each in an individual chamber or locule. Adult female fergusoninids carry the nematodes and deposit them in the meristematic tissue of the plant host with their eggs. The presence of the egg and the nematode are both necessary for a gall to develop. As the fly larvae develop within the gall, these plant-feeding parthenogenetic nematodes produce a sexual generation, and a small number of fertilised females enter the female fly larva and lay eggs, which hatch within the larva to be deposited in a new plant host. These processes have been described in detail elsewhere (Currie 1937; Fisher and Nickle 1968; Taylor *et al*. 2005; Nelson *et al*. 2014).

Almost all molecular studies published to date on the eucalypt-feeders in this system have concentrated on the nematodes (Ye et al. 2007; Davies *et al*. 2010a; 2012; 2013a; 2013b; 2014b). Evidence for phylogenetic congruence between the flies and the

nematodes has been presented in several studies (Giblin-Davis *et al*. 2003; Scheffer *et al*. 2004; Davies *et al*. 2010a; 2010b). These authors have argued for coevolution on the basis of correspondence between the nematode molecular phylogeny and *Fergusonina* third instar larval morphology, and also a preliminary study using molecular data from flies and nematodes from ten host plant species (Giblin-Davis *et al.* 2003b). Nevertheless, the degree of congruence between fly-nematode phylogenies is still unknown, and some anomalies suggest the relationship is not always straightforward (Davies *et al*. 2010a). In addition, I have found one instance of two different types of gall (a leaf blade gall and a shoot bud gall) containing fly species from two different lineages (confirmed by morphological and molecular evidence) exploiting the same leaf on *E. pauciflora*, and sharing tissue (Fig 6.3). This behaviour, while unusual, presents a potential route for nematodes to move between fly host species. Therefore, to understand this system it is necessary to investigate the evolutionary histories of both the flies and the nematodes.

4.2.2 Phylogenetic analysis

My previous study on the phylogenetic relationship between gall type and dorsal shield morphology of flies from *Eucalyptus* (Purcell *et al*. 2016) revealed strongly supported gall type/shield type lineages, but lost resolution at deeper nodes. Here I present a larger COI data set which includes samples from four host genera (*Eucalyptus*, *Corymbia*, *Melaleuca* and *Angophora*), as well CAD from a subset of these samples to elucidate the relationships between the lineages and gain some insight into the pattern of evolution of the gall and larval shield morphologies. Recently Scheffer *et al*. (2017) published a phylogeny of a diverse sample of *Fergusonina* species from all 7 known host genera, collected between

1999 and 2012. Unfortunately, the enormous diversity and potentially vast number of undiscovered *Fergusonina* species means that any large-scale phylogeny of the family at this stage will be missing important taxa, particularly as sampling has usually been opportunistic rather than targeted. Work on the diversity and host associations of this system is ongoing, and new species are regularly found (Purcell *et al*. 2016; Scheffer *et al*. 2017). Most of the flies used in the present study were collected between 2012 and 2016, and seven new gall type/shield type lineages have emerged and were included in this study.

4.2.3 Species delimitation

The symbiosis between *Fergusonina* and *Fergusobia* invites questions about coevolution and cospeciation, and how this might relate to their associations with their host plants and gall site. It is valuable, therefore, to identify species boundaries in these taxa, but species delimitation and species-level taxonomy are problematical. Adults and larvae show limited morphological variation within lineages while at the same time showing within-species variation for the few characters that would be expected to be diagnostic (Tonnoir 1937). In addition, features that appear to be diagnostic in one clade, whether they are differences in markings, or number and length of setae, are not necessarily so in other clades. To date, the most reliable species diagnosis requires knowledge of host species, gall type, larval morphology and adult morphology (Purcell *et al*. 2016), but the only certain way to link adults, larvae and galls is to collect galls, remove some larvae at appropriate stages of development, while rearing others to adulthood. Unfortunately this leaves too few of each stage to reliably document within-species variation, especially because assemblages of larvae and adults collected in this way are likely to be closely related and so not represent

the range of genetic variation within a given species. There is some evidence that adult genital morphology may be diagnostic, but for the reasons given above, it is difficult to determine if this character is consistently reliable, particularly between closely-related species. Detailed adult morphology has been recorded for relatively few species; only thirty-nine species of *Fergusonina* have been described, and several of these lack adult or larval descriptions, or important information such as gall type and host, or were described from single specimens, sometimes in bad condition (Malloch 1924; 1925; 1932; Tonnoir 1937). The type species, *Fn. microcera* Malloch was described from a single, net-caught female adult specimen and we have no knowledge of its host, gall type or larval morphology (Malloch 1924).

This challenge is not unique to the Fergusoninidae, and is a potential problem for gall-inducing insects in general. For example, the genus *Apiomorpha* (Eriococcidae) presents a very similar problem (Cook 2000). In this genus, gall morphology is an important diagnostic feature, as is host species and adult morphology. In the case of *Apiomorpha*, however, karyology has been a powerful tool, as there is considerable chromosomal variation and it has been well documented (Cook 2000). While the extent of chromosomal variation in *Fergusonina* is unknown, the use of genetic variation is another possible avenue of investigation. As discussed in chapter 2, within a 663 base pair region of COI, a $\leq 2\%$ uncorrected pairwise distance is generally consistent with delimitation by larval and adult morphology, host species and gall type (Purcell *et al*. 2016; Scheffer *et al*. 2017). However, in some cases this 2% criterion is inadequate, as some interspecific distances, such as that between *Fn. omlandi* and *Fn. daviesae*, cross the cut-off boundary.

In an effort to clarify these ambiguities and assess the usefulness of using COI to

identify species boundaries in Fergusoninidae, I subjected an enlarged COI dataset to several different species delimitation protocols. I compared the results obtained from three models within ABGD (Automatic Barcode Gap Discovery) (Puillandre *et al*. 2012), bPTP (Bayesian implementation of Poisson Tree Processes) (Zhang *et al*. 2013), and the uncorrected pairwise distances calculated using the Geneious molecular analysis program (Biomatters Ltd, Auckland, NZ). Where possible, the results from these analyses were compared with larval and/or adult morphology, and other features such as plant host and gall type.

4.2.4 Host relationships

While the host plant species is an important diagnostic character in this system (Taylor 2004), the results of chapters 2 and 3 have suggested that the level of host specificity in *Fergusonina* depends on the lineage. While there may be some history of cospeciation between *Fergusonina-Fergusobia* and their hosts, there has almost certainly been a high degree of plant host switching, as evidenced by the multiple unrelated species on the same host plant species (Davies *et al*. 2010a). Members of the major *Fergusonina* clades are generally associated with hosts of the same subgenus but there are many exceptions (Purcell *et al*. 2016) and often a lack of congruence between fly/nematode and host phylogenies below subgenus (Davies *et al*. 2016; Purcell *et al*. 2017). Individuals from one clade of *Fergusobia* nematodes have been collected from shoot bud galls on *Angophora floribunda* and *E. acmenioides* (Davies *et al*. 2010a) in the only example to date of a *Fergusobia* lineage occurring on more than one host genus. There are no genetic data for

the flies from this clade, but it was inferred from the spent puparia that the larvae were morphologically similar (Davies *et al*. 2010a).

4.3 Aims

The aims of this study are two-fold. The first is to address the shortcomings of the COI-based phylogeny identified in Chapter 2 in order to better understand the evolution of host plant relationships and dorsal shield morphology. The second aim is to assess the usefulness of molecular species delimitation methods to identify *Fergusonina* species boundaries.

4.4 Materials and methods

Flies were collected from three genera, *Eucalyptus*, *Corymbia* and *Angophora* in five states and territories across Australia (Table 4.1). Collection and preparation methods have been described in previous chapters. In addition, sequences of species associated with *Melaleuca* were obtained from GenBank (Table 4.2). As some of the collecting was opportunistic, there was inevitably some sampling bias towards the relatively conspicuous gall types such as the fused leaf and shoot bud galls, *versus* the small and obscure pea galls, and the often cryptic flower bud galls that occur in the canopies of mature trees.

Table 4.1 Locality and voucher information for *Fergusonina* specimens collected for DNA analysis.

***Host* species**	**GPS Coordinates**	**Collection location**	**Specimen ID/ANIC Voucher number**
A. floribunda	-36.5993, 149.6610	Numbugga Walls, NSW	**160-161**/29-039547-48
C. gummifera	-33.6993, 150.4904	Linden, NSW	**156-157**/29-039543-44
E. andrewsii	-28.1859, 153.0969	Beaudesert, Qld	**39**/29-039426
	-28.1421, 153.1125	Lamington NP, Qld	**59-60**/29-039446-47
E. blakelyi	-35.2763, 149.0804	Canberra, ACT	**55**/29-039442
	-35.3496, 149.0326		**38**/29-039425
E. botryoides	-35.5460, 150.3701	Kioloa, NSW	**173**/29-039560
E. bridgesiana	-35.2817, 149.1753	Canberra, ACT	**168-170**/29-039555-57
E. burgessiana	-35.1541, 150.7076	Jervis Bay, JBT	**17**/29-039404; **134-135** /29-039521-22; **146**/29-039533; **151-152**/29-039538-39
	-35.1536, 150.7061		**97**/29-039484
E. camaldulensis	-32.6951, 115.77085	Forrest Hwy, WA	**171**/29-039558
E. cunninghamii	-33.6209, 150.3278	Blackheath, NSW	**34**/29-039421; **145**/29-039532
E. dalrympleana	-35.35, 148.8196	Namadgi NP, ACT	**56**/29-039443
E. delegatensis	-35.3886, 148.8090	Namadgi NP, ACT	**83**/29-039470; **88**/29-039475; **140**/29-039527
	-35.3904, 148.8020		**82**/29-039469
	-35.3367, 148.8304		**93**/29-039480
	-37.1100, 148.9023	Mt. Delegate, VIC	**78**/29-039465; **84**/29-039471
E. dives	-35.3500, 148.8200	Namadgi NP, ACT	**80**/29-039467
	-35.3223, 148.8380		**81**/29-039468
	-35.7330, 149.0015		**18**/29-039405
	-35.8662, 148.9915		**77**/29-039464
E. elata	-35.3038, 149.1297	Canberra, ACT	**89**/29-039476

	-35.2804, 149.11214		**143**/29-039530
	-36.8573, 149.6902	Myrtle Mountain, NSW	**96**/29-039483; **139**/29-039526
	-36.9094, 149.6095	Wyndham, NSW	**133**/29-039520
	-36.8995, 149.6073		**144**/29-039531
	-35.2906,149.1375	Canberra, ACT	**48**/29-039435
E. fastigata	-36.5964, 149.4102	Brown Mountain, NSW	**42**/29-039429
	-36.6100, 149.4168		**43**/29-039430
	-35.4510, 149.5838	Tallaganda, NSW	**130-131**/29-039517-18
	-35.5251, 149.5378		**174-175**/29-039561-62
	-35.4455, 149.5877		**79**/29-039466
	-36.1240, 149.5105	Badja, NSW	**40**/29-039427
E. fraxinoides	-35.9622, 149.5794	Gourock NP, NSW	**129**/29-039516; **138**/29-039525; **141-142**/29-039528-29
E. haemastoma	-33.7837, 151.2516	North Balgowlah, NSW	**172**/29-039559
E. lacrimans	-35.2920, 149.0624	Canberra, ACT	**5**/29-039392, **10-11**/29-039397-98;
	-36.0328, 148.8002	Adaminaby, NSW	**6**/29-039393; **12**/29-039399; **36**/29-039423
	-36.0464, 148.7356		**136-137**/29-039523-24
E. leucoxylon	-35.2330, 149.1186	Canberra, ACT	**47**/29-039494; **49**/29-039436
	-35.2753, 149.1153		**159**/29-039546
E. ligustrina	-34.6746, 150.7128	Barren Grounds, NSW	**69**/29-039456
E. macarthurii	-35.2702, 149.1136	Canberra, ACT	**62**/29/039449
E. macrorhyncha	-35.2781, 149.1085	Canberra, ACT	**13**/29-039400
	-35.2759, 149.0991		**19-20**/29-039406-07; **102-104**/29-039489-91; **107**/29-039494

	-35.2738, 149.1130		**3**/29-039390
	-35.2637, 149.1085		**101**/29-039488; **105-107**/29-039492-94
E. mannifera	-35.3496, 149.0326	Canberra, ACT	**92**/29-039479
	-35.2718, 149.1170		**30**/29-039417; **64-65**/29-039451-52
E. melliodora	-35.3496, 149.0326	Canberra, ACT	**57-58**/29-039444-45
	-35.2769, 149.1136		**29**/29-039416; **91**/29-039478
	-35.2686, 149.1100		**86-87**/29-039473-74
	-35.2763, 149.0804		**14-16**/29-039401-03
E. notabilis	-28.1421, 153.1125	Lamington NP, Qld	**63**/29-039450
E. obliqua	-36.6100, 149.4168	Brown Mountain, NSW	**54**/29-039441
E. olsenii	-35.2781, 149.1085	Canberra, ACT	**147**/29-039534
	-36.6042, 149.6794	Numbugga Walls, NSW	**132**/29-039519; **l76**/29-039563
E. pauciflora	-36.0866, 148.8710	Adaminaby, NSW	**4**/29-039391
	-36.0464, 148.7355		**28**/29-039415
	-35.2763, 149.0804	Canberra, ACT	**94-95**/29-039481-82; **111**/29-039498
	-35.2718, 149.1170		**1**/29-039388; **2**/29-039389; **108-110**/29-039495-97; **112-113**/29-039499-500
	-35.5646, 148.7785	Namadgi NP	**99**/29-039486; **177**/29-039564
E. piperita	-33.7042, 150.2883	Blackheath, NSW	**21**/29-039408
E. polyanthemos	-35.3496, 149.0326	Canberra, ACT	**67**/29-039454
E. polybractea	-33.927, 147.0860	Nr West Wyalong, NSW	**164**/29-039551
E. racemosa	-33.6993, 150.4903	Linden, NSW	**22**/29-039409; **24**/29-039411

	-33.7709, 150.3762	Kings Tableland, NSW	**23**/29-039410
	-35.0713, 150.1688	Budawang Range, NSW	**50-52**//29-039437-39
	-33.5732, 150.2935	Victoria Falls, NSW	**85**/29-039472
	-33.8026, 151.2640	Balgowlah Heights, NSW	**158**/29-039545
E. radiata	-35.6473, 149.5065	Tallaganda, NSW	**68**/29-039455
	-35.3294, 149.8769	Braidwood, NSW	**76**/29-039463; **98**/29-039485
	-35.8662, 148.9915	Namadgi NP, ACT	**7**/29-039394; **8**/29-039395; **44-46**/29-039431-33
E. rossii	-35.2636, 149.1085	Canberra, ACT	**114-118**/29-039501-05
E. rubida	-35.7488, 148.9993	Namadgi NP, ACT	**148-148**/29-039535-36
E. sideroxylon	-35.2750, 149.1143	Canberra, ACT	**9**/29-039396
	-35.3468, 149.0434		**75**/29-039462
E. sieberi	-34.6746, 150.7128	Barren Grounds, NSW	**35**/29-039422
	-36.8573, 149.6902	Myrtle Mountain, NSW	**155**/29-039542
E. smithii	-35.9402, 149.5743	Jinden, NSW	**37**/29-039424
E. stellulata	-35.2686, 149.1100	Canberra, ACT	**73-74**/29-039460-61; **178**/29-039565
	-35.9431, 148.6325	Tantangara, NSW	**154**/29-039541
E. stricta	-33.6007, 150.3339	Blackheath, NSW	**25-27**/29-039412-14; **162**/29-039549
	-33.5479, 150.2609	Hartley Vale, NSW	**41**/29-039428
	-34.6753, 150.7117	Barren Grounds, NSW	**31-33**/29-039418-20
E. tereticornis	-35.5460, 150.3700	Kioloa, NSW	**90**/29-039477

E. viminalis	-36.1409, 149.4851	Badja, NSW	**150**/29-039537; **165**/29/039552
	-36.1765, 149.4628		**61**/29-039448; **70-72**/29-039457-59
	-35.3294, 149.8769	Braidwood, NSW	**167**/29-039554; **163**/29-039550
	-35.4130, 149.5354	Tallaganda, NSW	**119-128**/29-039506-15; **166**/29-039553
	-35.7548, 149.5183		**53**/29-039440
	-35.4130, 149.5354		

Table 4.2. Accession numbers of *Fergusonina* and outgroup sequences obtained from GenBank.

Fn. daviesae	*Fn. taylori*	*Fn. tasmaniensis*	*Fn. lockharti*	*Fn. nicholsoni*	*Fn. goolsbyi*	*Fn. burrowsi*
JF437655 JF437657-60 JF437662 JF437664 JF437666-67 JF437669-71 JF437670	JF437681-83 JF437685-86 JF437688-89 JF437692-95 JF437697-99 JF437701-02 JQ609282	JQ609284-86 JQ609292 JQ609294	AY687933	AY687934	AY687954	AY687978
Fn. turneri	*Fn. centeri*	*Fn. makinsoni*	*Fn. purcelli*	*Fn. schefferae*	From *C. tessellaris*	From *M. stenostachya*
EF104819 AY687935 AY687937 AY687941 AY687945 AY687947 AY687950-51 AY687966 AY687970	AY687965 AY687983 AY687985	AY687958 AY687960-61	AY687980-81	AY687963-64	EF104733 EF104820	AY687987
M. virens	*M. nigrofasciata*	*N. inversa*				
EF104660 EF104745	KF688194-95	GU299271				

4.4.1 Choice of outgroups

While the sister group to Fergusoninidae remains uncertain, I chose three other families within Opomyzoidea to represent the outgroups; Agromyzidae, Chloropidae and Neurochaetidae. Current molecular evidence suggests that these families together are monophyletic and closely related to Fergusoninidae (Wiegmann *et al*. 2011).

4.4.2 Molecular methods

Where possible, to control for misleading results from contamination, fly DNA from each putative species was extracted from several individuals over more than one extraction session. DNA was extracted from whole, 95% ethanol-preserved adults and larvae using a DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA, USA) following the protocols outlined by the manufacturer. A region of COI of approximately 830 base pairs (bp) was amplified using the primers C1-J-2183 (Jerry) 5' CAACATTTATTTTGATTTTTTGG 3' and TL2-N-3014 (Pat) 5' TCCATTGCACTAATCTGCCATATTA (Simon *et al*. 1994). This region is a slightly more sensitive marker in Fergusoninidae than the Folmer barcoding region (Folmer *et al*. 1994; Hebert *et al*. 2003; Scheffer *et al*. 2004; Purcell *et al*. 2015). A region of the nuclear gene CAD of approximately 1,000 bp was amplified using the tailed primers 54F 5'. CAGGAAACAGCTATGACCGTNGTNTTYCARACNGGNATGGT 3' and 405R 5' TGTAAAACGACGGCCAGTGCNGTRTGYTCNGGRTGRAAYTG 3' (Moulton and Wiegmann 2004). For the COI region, the polymerase chain reaction (PCR) was run using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Foster City, CA, USA) set to the following parameters: 94° C for 2 minutes; 35 cycles at 94° C for 30 seconds, 50° C for 30 seconds and 72° C for 1 minute, then incubation at 72° C for 3 minutes. PCR results were confirmed by gel electrophoresis. The PCR

product was purified using a mix of 0.4 µl Exonuclease 1 (Genesearch Australia Pty Ltd), 1.6 µl Shrimp Alkaline Phosphatase (SAP) (USB Affymetrix, CA, USA) and 3 µl MilliQ water per sample, and submitted to the Biomolecular Resource Facility of the John Curtain School of Medical Research, Australian National University for further preparation and sequencing.

To maximise sequencing success, tailed primers were used for some of the samples: Jerry 5' CAGGAAACAGCTATGACC CAACATTTATTTTGATTTTTTGG 3' and Superpat 5' TGTAAAACGACGGCCAGT GCACATWTCTGCCATATTAGA 3'. These were amplified with a Dyad Peltier Thermocycler, using a touchdown PCR program as outlined in Regier (2007) as follows: 94° C for 30 seconds, 25 cycles of 55° C for 30 seconds (minus 4° C per cycle), 72° C for 1 minute plus two seconds per cycle; 12 cycles of 94° C for 30 seconds, 45° C for 30 seconds, 72° C for 2 minutes plus 3 seconds per cycle; 72° C for ten minutes. The PCR product was submitted to LGC Genomics GmbH in Berlin, Germany, for sequencing with the primers M13REV 5' CAGGAAACAGCTATGACC 3' and M13(-21) 5' TGTAAAACGACGGCCAGT 3'.

I obtained CAD sequences for representatives from each COI clade except those from *Angophora floribunda*, *Corymbia gummifera*, stem pea galls from *E. racemosa* and the *Melaleuca*-associated basal rosette galler *Fn. goolsbyi.* The CAD region was amplified with a Dyad Peltier Thermocycler, using a touchdown PCR program as outlined in Regier (2007) as follows: 94° C for 30 seconds, 25 cycles of 55° C for 30 seconds (minus 4° C per cycle), 72° C for 1 minute plus two seconds per cycle; 12 cycles of 94° C for 30 seconds, 45° C for 30 seconds, 72° C for 2 minutes plus 3 seconds per cycle; 72° C for ten minutes. The PCR product was run out in 1% agarose gel mixed with 2 µl gel red, and the bands excised under fluorescence. The gel bands were cleaned using a MO BIO Ultraclean Gelspin DNA Extraction Kit using the

protocols outlined by the manufacturer and a reamplification performed using the primers M13REV and M13(-21) following protocols from Regier (2007): 21 cycles of 94° C for 30 seconds, 50° C for 30 seconds, 72° C for 1 minute, plus 2 seconds per cycle; and 72° C for ten minutes.

The DNA sequences were aligned and trimmed to 663 bp (COI) and 652 bp (CAD) using Geneious 9.1 and Geneious 10 (Biomatters Ltd, Auckland, NZ). Aligned CAD and COI sequences were concatenated using Geneious 10.

Where corresponding COI or CAD sequences for outgroup species and additional *Fergusonina* species were available from GenBank, these were included in the phylogeny (Table 4.2).

To test for the presence of nuclear mitochondrial DNA (numts), the aligned DNA sequences were translated into amino acid sequences and checked for stop codons. Sequences were deposited in GenBank under accession numbers MG323931-MG324003. Adults and third instar larvae were retained for vouchering and lodged in the Australian National Insect Collection, Canberra (ANIC) (Table 4.1).

4.4.3 Phylogenetic analysis

Bayesian analyses of 228 unique COI sequences were performed using the General Time Reversible substitution model (Tavaré 1986) with a gamma-distributed rate variation with invariant sites (GTR+I+G) applied to all three codon positions, within the MrBayes Geneious plugin version 2.2.2 (Huelsenbeck and Ronquist 2001). The chain was run for 8,000,000 generations and the burn-in set at 25%. Trees were sampled every 500 generations. 45 CAD sequences were analysed with the MrBayes plugin using the GTR+G applied to all three codon positions with the chains run for 2,000,000 generations, sampled every 250 generations, and burn-in set at 25%. The concatenated

CAD and COI sequences were partitioned, with GTR+I+G applied to the COI region and GTR+G applied to the CAD region. The partitioning and substitution models used were determined by PartitionFinder v.1.1.1 (Lanfear *et al*. 2012) to be the best schemes for each gene dataset. Convergence was inferred by the standard deviation of split frequencies reaching a value below 0.01 (Ronquist *et al*. 2012). The trees were annotated using Fig Tree v. 1.4.2 and Adobe Illustrator CS6 version 16.0.3.

4.4.4 Species delimitation

ABGD

Given a dataset of aligned sequences, this program assesses putative species boundaries based on genetic pairwise distances between and within species. Sequences are partitioned into groups or operational taxonomic units (OTUs), which are further refined by recursively applying the inferred limits to these groups. I used the online version of ABGD to sort the dataset into OTUs using the available Jukes-Cantor (Jukes and Cantor 1969), Kimura 2-P (Kimura 1980), and simple distance models using the default divergence parameters of minimum prior intraspecific divergence (Pmin) = 0.001 and maximum prior intraspecific divergence (Pmax) = 0.01, and a relative gap width of 1.0. These parameters gave the most plausible results consistent with morphological and other data.

bPTP

Species boundaries were inferred from my rooted COI tree using bPTP with the following parameters: Number of MCMC generations: 500,000; burn-in: 0.25; thinning 100 (default). Convergence was inferred from the trace file.

4.5 Results

4.5.1 Phylogenetic analysis

The COI analysis (Fig. 4.1) revealed 15 clades consistent with host genus, gall type and larval dorsal shield morphology. As most of these correspond to those in chapter 2 I have retained the same clade numbers. All of these had very strong support (posterior probability = 0.99 - 1.0) with the exception of clade 13 (0.71), which in the CAD analysis (Fig. 4.2) is within clade 14 (1.0) despite morphological differences between the larvae in the two lineages (Purcell *et al*. 2016). This exception aside, the clades in the phylogenies inferred from CAD and the concatenated genes (Fig. 4.3) are consistent with those in the COI phylogeny.

Additionally, there were some branches consisting of single OTUs that did not belong within these gall type/shield type clades, as they were from under-sampled gall types or host genera, such as the pea galls from *A. floribunda*, the shoot bud galls from the two *Corymbia* species, and the stem pea galls from *E. racemosa*. No clades were associated with more than one host genus, and 4 of the 15 clades contained flies collected from more than one host subgenus. All three phylogenies contained a number of polytomies or branches with relatively low support (< 0.85) at deep nodes.

In the COI phylogeny, the *Melaleuca* shoot bud gallers formed a monophyletic clade which was sister to the *Angophora* pea gallers with reasonably high support (0.93). The rosette galler from *M. nervosa* was sister to the large group containing the majority of the eucalypt and *Corymbia* gallers, but with only moderate branch support in the COI phylogeny (0.83) and no CAD sequence, its placement here is uncertain. The two species of shoot bud gallers on *Corymbia* are morphologically dissimilar (Davies *et al.* 2010b) and are not grouped together.

Clades 1 – 5 formed a larger clade of larvae with dorsal shields composed of cuticular plates. Toothed structures on the dorsal shields were present in three lineages (clades 4, 5 and 11), all within this group and its sister clade. Another clade of plate-shielded larvae (clade 7) occurred within a well-supported group with two clades (8 and 10) of larvae with spiculose shields. The remaining clades contained flies with patches or bars of spicules. Elongate leaf blade gallers with absent or very lightly spiculose shields were grouped together in sister clades 13 and 14.

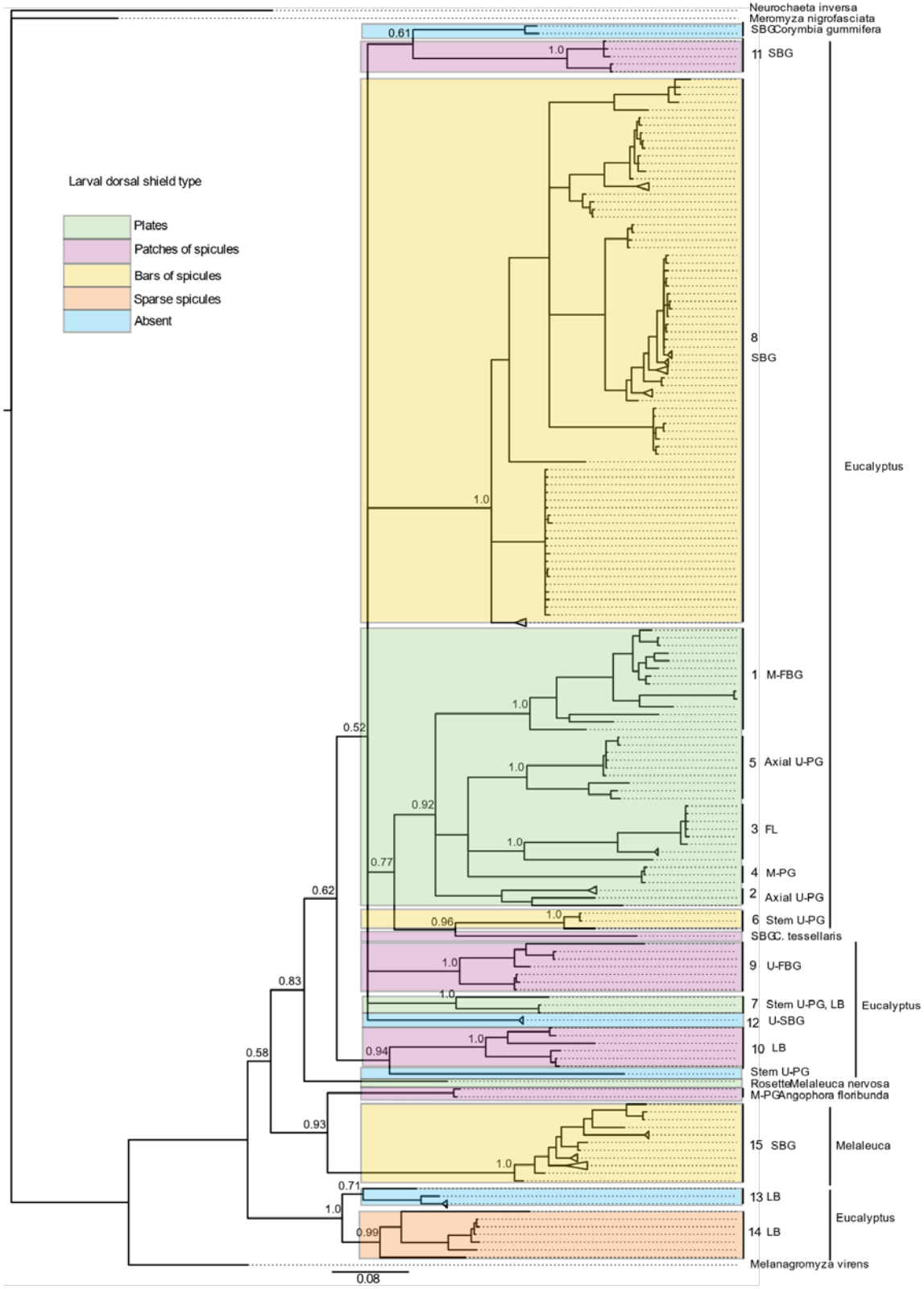


Fig. 4.1 Simplified Bayesian tree inferred from mtCOI sequences, with numbered clades showing the gall type/shield type groups and host genera. Values at nodes represent the Bayesian posterior probabilities. Gall type abbreviations as in previous chapters.

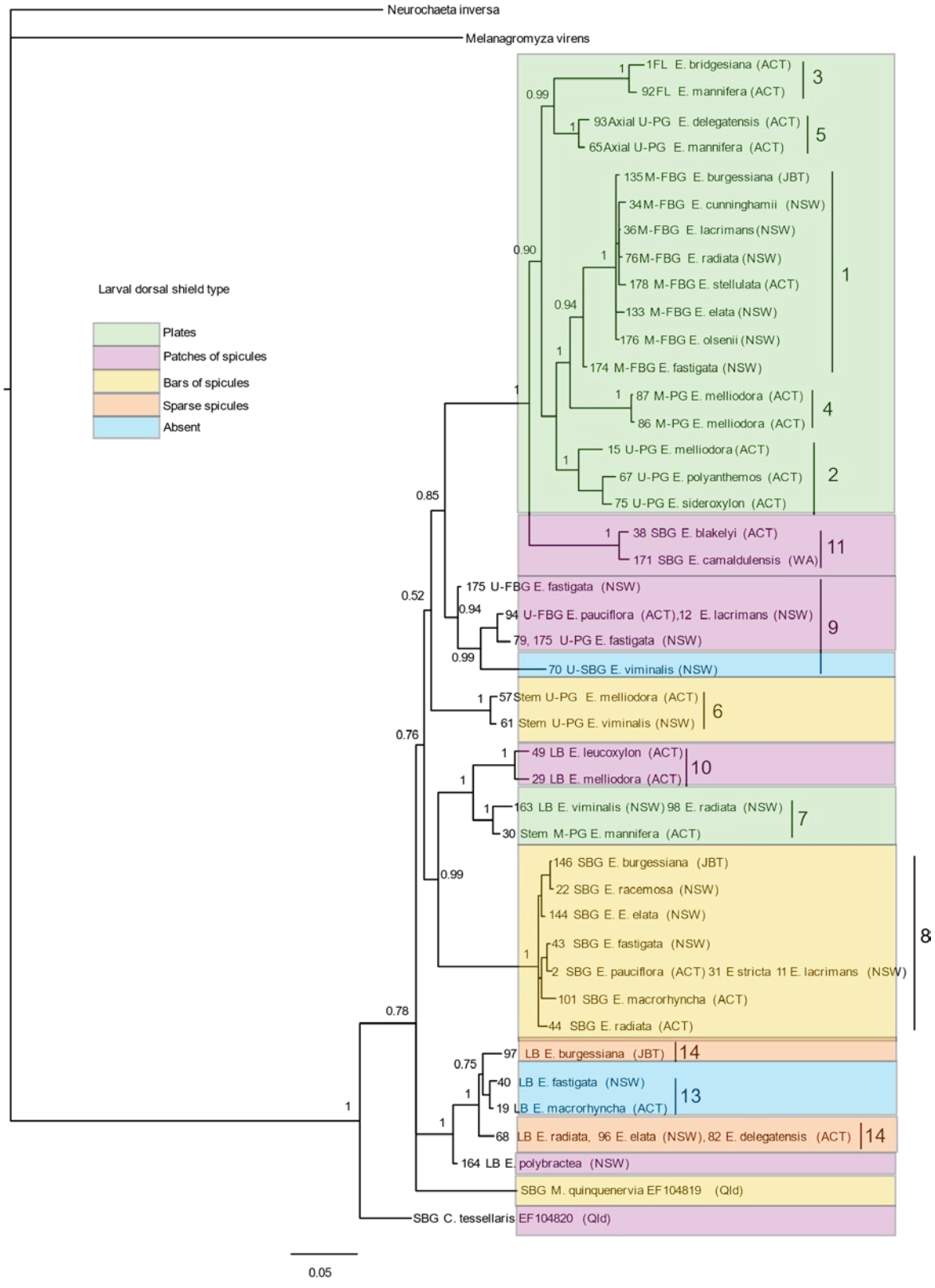


Fig. 4.2 Bayesian tree inferred from CAD sequences, with numbered clades showing the gall type-shield type groups. The specimen numbers correspond to ANIC voucher numbers (Table 4.1). Values at nodes represent the Bayesian posterior probabilities.

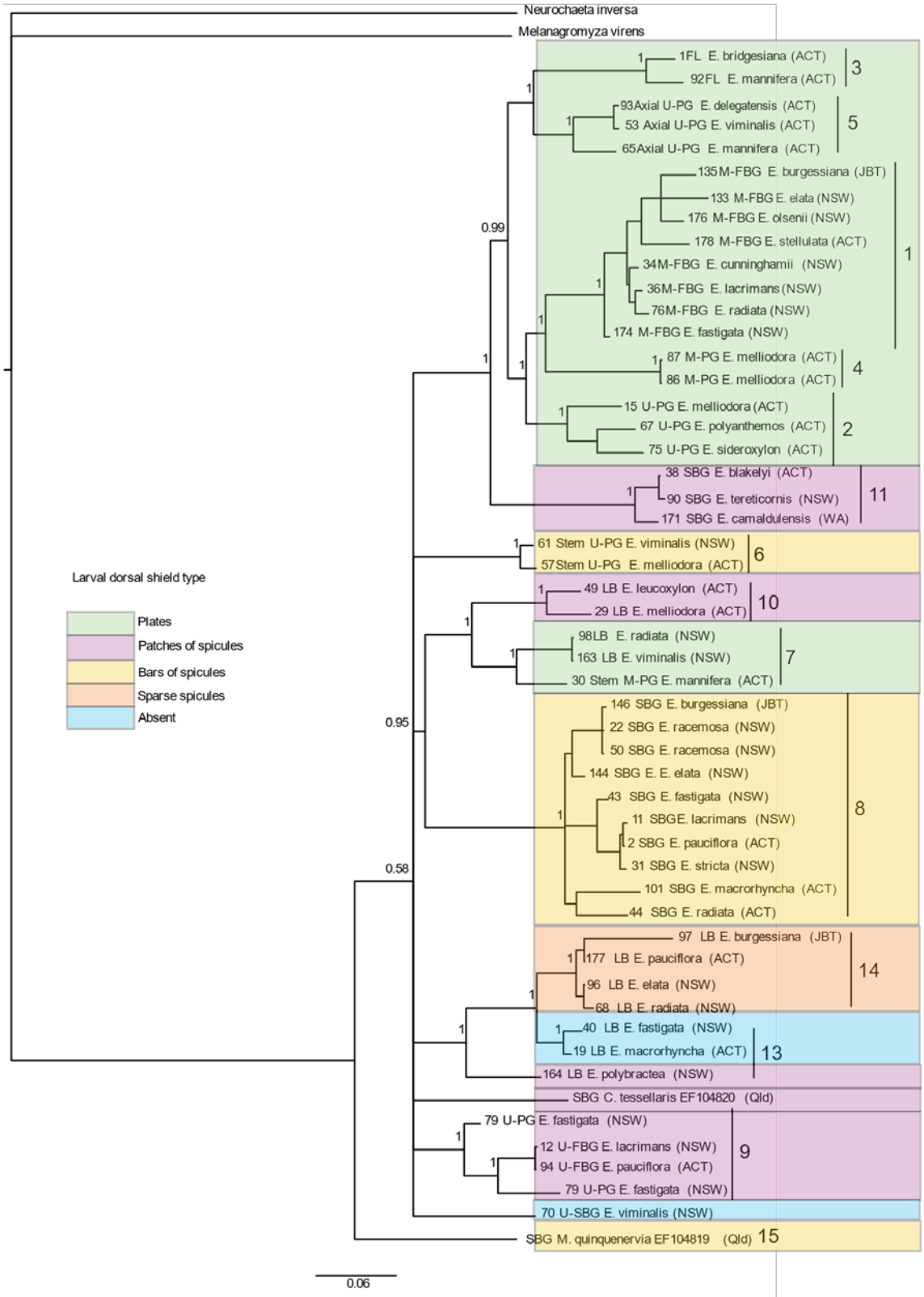


Fig. 4.3 Bayesian tree inferred from concatenated CAD and COI sequences, with numbered clades showing the gall type/shield type groups. The specimen numbers correspond to ANIC voucher numbers (Table 4.1). Values at nodes represent the Bayesian posterior probabilities.

OTUs with ambiguous relationships

In both the CAD and concatenated phylogenies, the leaf blade gallers collected from *E. polybractea* (subgenus *Symphyomyrtus*) occurred with high support (1.0) as sisters to a group of leaf blade gallers (clades 13 and 14). However, the COI tree put this species within a different clade of leaf blade gallers (clade 10) with high support (1.0) where all species, including those from *E. polybractea*, have similar dorsal shield morphology (Fig 2.1o) and all hosts are of the Section *Adnataria.*

The unilocular shoot bud gallers from *E. viminalis*, which lack dorsal shields, were unplaced in the COI and concatenated gene trees (Fig. 4.1, 4.3), but in the CAD phylogeny (Fig. 4.2) they belonged to clade 9, which includes unilocular flower bud gallers and axial pea gallers (posterior probability = 0.94). However, the larvae are dissimilar to the other members of the group, which have dark, spiculose shields (Fig. 2.1k,l). The leaf blade gallers in clade 13 also lack dorsal shields, but the adults lack the distinct dark markings on the thorax and abdomen of the unilocular shoot bud gallers. No adults were obtained for the unilocular flower bud gallers and axial bud gallers in this clade.

Gallers on *C. tessellaris* in Qld appeared as sisters to all the other fergusoninid lineages in the CAD tree (1.0) and were unplaced in the concatenated gene tree. In the COI tree they were sisters to the unilocular stem gallers in clade 6 (0.96). The larvae from this clade have a broad area of black spicules on the thoracic and abdominal segments (Fig. 2.1j); those from *C. tessellaris* have a weakly sclerotised patch of spicules on the second thoracic segment (Davies *et al*. 2010a).

Inconsistencies

Clades 13 and 14 were reciprocally monophyletic in the COI and concatenated trees

(Fig. 4.1, 4.3) but in the CAD tree (Fig. 4.2) the larvae without shields were nested within the clade of sparsely spiculose larvae. This group of leaf blade gallers was the sister group to the rest of the taxa in the COI phylogeny (1.0), including the specimens from non-*Eucalyptus* hosts. However, they were unplaced in the CAD and concatenated gene trees, forming part of a trichotomy.

Clade 9 was unplaced in the concatenated tree. In the CAD tree it was sister to a large group containing clades 1-5 and 11, whose larvae all have dorsal shields composed of sclerotised plates except clade 11, which has a patch of spicules. However, the support for this relationship was only moderately high (0.85). The same was true of the COI phylogeny, but without clade 11, which occurred with low support (0.61) as sister group to the shoot bud gallers from *C. gummifera.*

Clade 6 was unplaced in the concatenated tree and in the CAD tree was sister to the group consisting of clades 1 – 5, 11 and 9, but the support was low (0.52). As discussed above, in the COI tree clade 6 was sister to the species from *C. tessellaris,* forming the sister clade to the group of plate-shield flies (clades 1-5).

Representing the group from the *Melaleuca* shoot bud gallers (clade 15), *Fn. turneri* from *M. quinquenervia* was unplaced in the CAD tree, and in the concatenated tree replaced the species from *C. tessellaris* as sister to the rest of the groups in the dataset (0.1). In the COI tree it was sister to all except the leaf blade gallers in clades 13 and 14 (0.93).

4.5.2 Species delimitation

There were 72 putative species or OTUs when the COI sequences were delimited according to an uncorrected pairwise distance of ≤ 2%. bPTP sorted the dataset into 73 OTUs. ABGD sorted them into 57 OTUs using the Jukes-Cantor model, 58 OTUs using

Kimura 2-P, and 47 OTUs using Simple Distance. Overall, the methods agreed on the limits of 40 species. The likely species limits, given these results integrated with morphological and ecological data, are set out in Table 4.3.

2% uncorrected pairwise distance threshold

As discussed above, on the whole this method sorted COI haplotypes into OTUs that corresponded to other features such as morphology and gall type; however, there were some groups whose genetic distances ranged across the 2% threshold, which prevented clear resolution (OTU numbers 31, 42, and 43 in Table 4.3). The uncorrected distances between the sequences from *E. macrorhyncha* and *E. andrewsii* overlapped the threshold (1.7% - 2.7%) but the distance between each of these and the haplotype from *E. ligustrina* fell below the threshold (1.7% and 0.9–2.0%) (Purcell *et al*. 2016). All were treated as a single OTU by ABGD, but bPTP divided the two haplotypes from *E. andrewsii* and placed one with *E. ligustrina* and assigned the other to its own OTU, though the support for these divisions was low to moderate (0.74-0.9). The haplotypes from *E. gummifera* have a 2% bp distance which lies on the threshold. They were treated as two OTUs by bPTP but as a single OTU using the other methods.

bPTP

The maximum likelihood solution and highest Bayesian supported solution were identical. The sequences were sorted into 72 groups or OTUs, most of which were well supported (0.85 – 1.00). Support was low (< 0.70) in groups 6 and 26. Where the bPTP solutions differed from all the other models (groups 6, 31 and 72) it was by sorting haplotypes into smaller putative species.

ABGD

Conversely, ABGD models tended to lump disparate haplotypes into single species. The

results differed from the 2% distance threshold delimitation and bPTP where the axial pea galls from *E. dives* and *E. mannifera* in clade 5 (OTU 5 and 6 in Table 4.3) were assigned to a single OTU by all three models within ABGD, as were the flower bud galler OTUs 7 – 13 (clade 1) and 54 - 55 (clade 9), leaf blade gallers 51 – 52 (clade 13) and the shoot bud gallers 42 - 44 in clade 8. Shoot bud galls from the closely related hosts *E. camaldulensis*, *E. blakelyi* and *E. tereticornis* were placed in one group by Kimura 2-P, but under all other models the flies from *E. camaldulensis* were sorted into a discrete OTU. The Simple Distance model within ABGD placed all the shoot bud gallers from *Melaleuca* (clade 15) into one OTU. ABGD also lumped the haplotypes from the multilocular flower bud gallers from a number of disparate hosts from the ACT, NSW and SA into one OTU. I did not obtain adults from any of the galls within this group so unfortunately cannot compare the genetic data with adult morphology.

4.6 Discussion

4.6.1 Phylogenetic analysis

Apart from the few taxa whose closest relationships are unclear, the three trees agreed on the composition of the dorsal shield/gall type lineages. COI provided excellent resolution at this level (Fig. 4.1), but was inadequate for showing the relationships among these groups. The CAD and concatenated trees (Fig 4.2, 4.3) provided strong support for some clades, but the presence of several unresolved branches meant that there were inconsistencies, as outlined above.

Table 4.3 Probable *Fergusonina* species using integrated molecular, morphological and other data. The column on the right integrates data used to delimit the species (Dayrat 2005). Support values refer to Bayesian support according to bPTP. Gall type abbreviations as used in previous chapters.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
3	Distinctive gall type, adult markings and larval/pupal dorsal shield morphology.	1		FL *E. elata* **48** FL *E. macarthurii* **62** FL *E. mannifera* **92** FL *E. viminalis* **165-167**	All OTU-sorting models agree, pairwise distance of haplotypes within group < 2%. Moderate support (0.87). Distinctive adult morphology.
		2	*Fn. carteri*	FL *E. bridgesiana* **168-170**	All models agree. > 2% pairwise distance from OTUs from other hosts. Moderate support (0.86). Distinctive adult morphology.
		3		FL *E. notabilis* **63**	All models agree. >2% pairwise distance from OTUs from other hosts. Support 1.0. Distinctive adult morphology.
5	Distinctive gall type, adult markings and larval/pupal dorsal shield morphology.	4		UPG *E. rubida* **148-149** UPG *E. viminalis* **53, 66, 150** UPG *E. dalrympleana* **56** UPG *E. delegatensis* **93**	All models agree. Pairwise distance <2% within OTU, >2% from OTUs from other hosts. Support 0.94.
		5		UPG *E. dives* **77**	>2% pairwise distance from OTUs from other host plants. Support 1.0. No adult specimens.
		6		UPG *E. mannifera* **64-65**	>2% pairwise distance from OTUs from other host plants. Low support (0.59). No adult specimens.
1	Distinctive gall type, adults and larval/pupal dorsal shield morphology.	7		MFBG *E. cunninghamii* **34** MFBG *E. stricta* **162**	All models agree; support 0.99. Pairwise distance <2% within OTU, >2% from OTUs from other hosts. No adult specimens. Sympatric. Host plants mallee ashes.
		8		MFBG *E. dives* **80**	>2% pairwise distance from other OTUs. Support 0.96. No adult specimens.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
	Plant hosts belong to subgenus *Eucalyptus*.	9		MFBG *E. fastigata* **130-131, 174**	>2% pairwise distance from other OTUs. Support 0.98. No adult specimens.
		10		MFBG *E. fraxinoides* **129**	Pairwise distance <2% within OTU, >2% from OTUs from other hosts. Support 0.98.
		11		MFBG *E. lacrimans* **36** MFBG *E. pauciflora* **28**	>2% pairwise distance from OTUs from other host plants, <2% distance within OTU. Support 0.94. No adult specimens. Host plants snow gums.
		12	*Fn. nicholsoni*	MFBG *E. macrorhyncha*	>2% pairwise distance from other OTUs. Support 1.0. No adult specimens.
		13		MFBG *E. radiata* **76**	>2% pairwise distance from other OTUs. Support 0.96. No adult specimens.
		14		MFBG *E. elata* **133**	>2% pairwise distance from other OTUs. All models agree; support 1.0. No adult specimens.
		15		MFBG *E. stellulata* **73-74**	>2% pairwise distance from other OTUs. All models agree; support 0.99. No adult specimens.
		16		MFBG *E. burgessiana* **134**	>2% pairwise distance from other OTUs. All models agree; 1.0 support. No adult specimens.
		17		MFBG *E. olsenii* **132, 163, 176**	>2% pairwise distance from other OTUs. All models agree; support 1.0. Adults morphologically distinct.
4	Distinctive larval/pupal dorsal shield and gall type. Hosts are from *Eucalyptus* subseries *Leucoxylon.*	18		MPG *E. leucoxylon* **159** MPG *E. melliodora* **86-87**	All models agree; support 0.98. >2% pairwise distance from OTUs from other host plants, <2% distance within OTU. Adults from *E. leucoxylon* only.
2	Distinctive larval/pupal dorsal shield. Same gall	19	*Fn. greavesi*	UPG *E. polyanthemos* **67**	>2% pairwise distance from OTUs from other host plants. All models agree; support 1.0. No adult specimens (*Fn. greavesi* was described from larva (Currie 1937).

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
	type within clade. Host plants all *Eucalyptus* subsection *Terminales.*	20		UPG *E. sideroxylon* **75**	All models agree; support 1.0. >2% pairwise distance from other OTUs. No adult specimens.
		21		UPG *E. melliodora* **14-16**	All models agree; support 0.94. >2% pairwise distance from other OTUs. Adults have distinctive markings, but no adults from other hosts in clade are available for comparison.
11	Distinctive larval/pupal dorsal shield and adult morphology. Host plants all from *Eucalyptus* section *Exsertaria.*	22		SBG *E. blakelyi* **38, 55** SBG *E. tereticornis* **90**	>2% distance from other OTUs, <2% distance within OTU. Support 0.9. Adults from *E. blakelyi* only. Distinctive adult morphology.
		23	*Fn. lockharti*	SBG *E. camaldulensis* **171** (n=2)	>2% distance from other OTUs. Support 0.99. Distinctive adult morphology (Taylor and Davies 2010).
6	Distinctive larval/pupal dorsal shield and gall morphology. Host plants are subgenus *Symphyomyrtus.*	24		Stem UPG *E. melliodora* **57-58**	>2% distance from other OTUs. Support 0.99. No adult specimens.
		25		Stem UPG *E. viminalis* **61**	>2% distance from other OTUs. Support 1.0. No adult specimens.
10	Distinctive larval/pupal dorsal shield, and same gall site. All host plants in *Eucalyptus* section *Adnataria.*	26		LB *E. melliodora* **29, 91** LB *E. sideroxylon* **96**	>2% distance from other OTUs and <2% distance within OTU. Minor difference in larval morphology depending on host plant. Distinctive adult morphology, but no adults from other hosts in clade are available for comparison.
		27		LB *E. leucoxylon* **47, 49**	>2% distance from other OTUs. All models agree. Support 0.96. No adult specimens.
		28		LB *E. polybractea* **164**	>2% distance from other OTUs. All models agree. Support 1.0. No adult specimens.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
7	Distinctive larval/pupal dorsal shield, coated in clear substance.	29		Stem UPG *E. mannifera* **30**	>2% distance from other OTUs. All models agree. Support 1.0. No adult specimens.
		30		LB flat *E. radiata* **98** LB flat *E. viminalis* **163**	>2% distance from other OTUs. All models agree. Support 0.99. No adult specimens.
8	Distinctive larval/pupal dorsal shield morphology; gall type shared by all OTUs in clade. Distinctive adult morphology.	31		SBG *E. andrewsii* **59-60** SBG *E. ligustrina* **69** SBG *E. macrorhyncha* **3**	>2% distance from other OTUs. 1.7%-2.7% distance within OTU. All models but bPTP agree (support 0.74-0.9). Distinctive adult morphology.
		32		SBG *E. burgessiana* **17, 146** SBG *E. haemastoma* **172** SBG *E. racemosa* **22-24, 50-52, 85**	>2% distance from other OTUs and <2% distance within OTU. All models agree. Support 0.96. Distinctive adult morphology.
		33		SBG *E. delegatensis* (Vic) **78, 84** SBG *E. fastigata* **42-43**	>2% distance from other OTUs and <2% distance within OTU. All models agree. Adults from *E. fastigata* morphologically distinctive. No adult specimens from *E. delegatensis*.
		34	*Fn. herbaservus*	SBG *E. delegatensis* (ACT) **83, 88** SBG *E. stellulata* **154**	Adult morphology distinct from *Fn. manchesteri*, despite all COI-based sorting methods placing the two into one OTU.
		35	*Fn. manchesteri*	SBG *E. dives* **18** SBG *E. radiata* **7-8, 44-46** SBG *E. smithii* **37** SBG *E. viminalis* **119-128**	Adult morphology distinct from *Fn. herbaservus*, despite all COI-based sorting methods placing the two into one OTU.
		36		SBG *E. macrorhyncha* **101-107** SBG *E. olsenii* **147**	>2% distance from other OTUs and <2% distance within OTU. All models agree. No adult specimens.
		37		SBG *E. piperita* **21**	>2% distance from other OTUs. All models agree. Support 1.0. Distinctive adult morphology.
		38		SBG *E. botryoides* **173**	>2% distance from other OTUs. All models agree. Support 1.0. No adult specimens.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
		39		SBG *E. elata* **143-144** SBG *E. sieberi* **35, 155**	>2% distance from other OTUs and <2% distance within OTU. All models agree. No adults from *E. elata*. Those from *E. sieberi* have distinctive morphology.
		40		SBG *E. rossii* **114-118**	
		41	*Fn. taylori*	SBG *E. pauciflora debeuzevillei and niphophila* (high elevation) (n=19)	>2% distance from other OTUs. Support 0.83. Hosts are snow gums occurring at high elevations. Distinctive adult morphology.
		42	*Fn. omlandi*	SBG *E. lacrimans* **5-6, 10-11** SBG *E. pauciflora* subsp. *pauciflora* **1-2, 4, 108-113** SBG *E. cunninghamii* **145** SBG *E. stricta* **31-33, 41, 412-414**	≥2% distance from other OTUs. 0.2%-2.4% distance within OTU. Adults morphologically distinctive.
		43	*Fn. daviesae*	SBG *E. pauciflora* subsp. *debeuzevillei and niphophila* (high altitude) (n=15)	≥2% distance from other OTUs. Adults morphologically distinctive. Host are subspecies of snow gums occurring at high elevations.
		44		SBG *E. fraxinoides* **141-142**	>2% distance from other OTUs. Support 0.90. Distinctive adult morphology.
		45	*Fn. tasmaniensis*	SBG *E. pauciflora* (TAS) (n=6)	>2% distance from other OTUs. Support 0.96. Distinctive adult morphology.
		46		SBG *E. obliqua* **54**	>2% distance from other OTUs. Support 0.98. No adult specimens.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
14	Distinctive larval/pupal dorsal shield and adults, and same gall type. Plant hosts in subgenus *Eucalyptus.*	47		LB *E. delegatensis* **82, 140** LB *E. elata (*NSW) **96, 139** LB *E. pauciflora* **99, 177** LB *E. dives* **81** LB *E. lacrimans* **136-137** LB *E. radiata* **68**	All models agree; support 0.88. >2% pairwise distance from other OTUs and <2% within OTU. No adult specimens from *E. elata*, E. *pauciflora* or *E. lacrimans*. Those from *E. radiata* differ from the others in distance between wing cross veins.
		48		LB *E. burgessiana* **97**	All models agree; support 1.0. >2% pairwise distance from other OTUs. No adult specimens.
		49		LB *E. elata* (ACT) **89**	All models agree; support 1.0. >2% pairwise distance from other OTUs.
		50		LB E. *fraxinoides* **138**	All models agree; support 1.0. >2% pairwise distance from other OTUs. 1 adult specimen only.
13	Shared larval/pupal dorsal shield and gall type. Plant hosts in subgenus *Eucalyptus.*	51		LB *E. fastigata* **40**	>2% distance from other OTUs, high bPTP support (1.0). Distinctive adult morphology.
		52		LB *E. macrorhyncha* **13, 19-20**	>2% distance from other OTUs, high bPTP support (0.98). No adults available.
		53		LB *E. andrewsii* **39**	All models agree. >2% distance from other OTUs. Distinctive adult morphology.
9	Shared larval/pupal dorsal shield and gall type. Plant hosts in subgenus *Eucalyptus.*	54		UFBG *E. burgessiana* **151-152**	>2% distance from other OTUs. Support 0.99. No adult specimens.
		55		UFBG *E. fastigata* **153**	>2% distance from other OTUs. Support 1.0. No adult specimens.
		56		UFBG *E. lacrimans* **13** UFBG *E. pauciflora* **94-95**	>2% distance from other OTUs, <2% within OTU. All models agree. Support 0.99. No adult specimens.
		57		UPG *E. fastigata* **79, 175**	>2% distance from other OTUs. All models agree. Support 1.0. No adult specimens.
	Distinctive larval/pupal dorsal	58		USBG *E. viminalis* **70-72**	> 2% pairwise distance from other OTUs.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
	shield, adult markings and gall type. Only representative in the lineage.				
	Distinctive larval/pupal dorsal shield, adult morphology and gall type. Only representative in the lineage.	59	*Fn. goolsbyi*	Rosette *M. nervosa*	> 2% pairwise distance from other OTUs.
	Distinctive larval/pupal dorsal shield and gall type. Only representative in the lineage.	60		Stem UPG *E. racemosa*	All models agree. Support 1.0. >2% pairwise distance from other OTUs. No adult specimens.
	Distinctive larval/pupal dorsal shield and gall type. Only OTU in this dataset with host plant in genus *Angophora.* Only representative in the lineage.	61		MPG *A. floribunda*	All models agree. Support 0.92. >2% pairwise distance from other OTUs.
15	Distinctive larval/pupal dorsal	62	*Fn. burrowsi*	SBG *M. viridiflora*	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.

Clade		OTU	Species name	Gall type, host and specimen ID	Molecular and morphological criteria
	shield and same gall type. Hosts are all broad-leafed Melaleucas.	63	*Fn. centeri*	SBG *M. leucadendra* (n=3)	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.
		64	*Fn. makinsoni*	SBG *M. dealbata* (n=3)	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.
		65	*Fn. purcelli*	SBG *M. cajaputi* (n=2)	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.
		66	*Fn. schefferae*	SBG *M. nervosa* (n=2)	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.
		67	*Fn. turneri*	SBG *M. fluviatilis*	Support 1.0. >2% pairwise distance from other OTUs. Distinctive adult morphology? (Taylor 2004)
		68		SBG *M. fluviatilis*	Support 1.0. >2% pairwise distance from other OTUs. Distinctive adult morphology? (Taylor 2004)
		69		SBG *M. quinquenervia* (n=7)	Support 0.98. >2% pairwise distance from other OTUs. Distinctive adult morphology.
		70		SBG *M. stenostachya*	All models agree. >2% pairwise distance from other OTUs. Distinctive adult morphology.
	Distinctive larval/pupal dorsal shield and gall type. Only representative in the lineage.	71		SBG *C. gummifera* **156-157**	>2% pairwise distance from other OTUs. Distinctive adult morphology.
	Distinctive larval/pupal dorsal shield and gall type. Only representative in the lineage.	72		SBG *C. tessellaris* **72**	Support 1.0. >2% pairwise distance from other OTUs. All models agree. Support 1.0

There was no overall pattern of evolution in gall type, such as a progression from leaf blades to shoot buds or from unilocular to multilocular galls. Rather, most gall types evolved multiple times, with gall morphology usually related to the site of the gall rather than fly or host species.

Unfortunately, it is not possible to make a direct comparison between these phylogenies and the most recent *Fergusobia* nematode phylogeny (Davies *et al*. 2014b), as most of the host and gall type groups included do not correspond. However, there are some equivalent taxa such as the monophyletic clade of broad-leafed *Melaleuca* shoot bud gallers. In the nematode phylogeny this was the sister group to all the lineages from other host genera, as it was in the fly concatenated gene tree. The nematodes from *C. tessellaris* shoot bud galls were separate from those from the flower bud gallers from *C. ptychocarpa* and lay outside the taxa from *Eucalyptus, Angophora, Metrosideros, Syzygium* and *Corymbia*, reflecting the fly CAD tree.

4.6.2 Species delimitation

The molecular data provided a reasonable guide for species delimitation, and 40 of the OTUs (56% of those identified by the 2% criterion) were sorted identically by all models. However, there were some difficult species boundaries, outlined below, which produced varying results depending on the method employed.

Ambiguous species boundaries

Fergusonina turneri and its associated nematode *Fb. quinquenerviae* have been described from both *Melaleuca quinquenervia* and *M. fluviatilis*, but it is uncertain whether the two plants really host the same species (Davies and Giblin-Davis 2004; Taylor 2004; Scheffer *et al*. 2004; 2017). Taylor (2004) reports small but consistent

morphological differences between the adult flies according to host species, and my models all divide the corresponding haplotypes into separate OTUs, consistent with the results in Scheffer *et al*. (2004; 2017). Moreover, the two *Fn. turneri* haplotypes from eight individuals from *M. fluviatilis* are treated by all models as distinct OTUs, though at 2.9% the uncorrected pairwise distance between them is not large (Scheffer *et al*. 2004; 2017). There are minor morphological differences between fly and nematode specimens from *M. fluviatilis* from Home Hill, Qld *versus M. fluviatilis* in Townsville, Qld (Taylor 2004) which are less than 100 km apart, but the two fly haplotypes do not correspond to locality or morphology. No adult males were obtained from Townsville (Taylor 2004).

Fergusonina omlandi and *Fn. daviesae* are two reciprocally monophyletic and morphologically distinct species that form shoot bud galls on two different subspecies of *E. pauciflora* occurring at different elevations (Nelson *et al*. 2011a; 2011b). All of the models except the ≤ 2% distance criterion assigned *Fn. omlandi* to the same species as *Fn. daviesae*, and support from the ≤ 2% distance criterion was weak (2.0% - 3.0% uncorrected pairwise distance). However, bPTP gave this *Fn. omlandi-Fn. daviesae* group low support (0.44). Further, the ABGD Jukes-Cantor and Simple Distance models included *Fn. tasmaniensis* in this same group. Given the geographical and genetic distance (3.2 – 4.5%) between *Fn. tasmaniensis* and the mainland species this is an implausible grouping. Based on all the evidence, *Fn. omlandi* and *Fn. daviesae* and *Fn. tasmaniensis* should be viewed as distinct species.

There were two cases where there were inconsistencies between the morphological and molecular data. *Fn. herbaservus* and *Fn. manchesteri* have been described from shoot bud galls on *E. stellulata* and *E. viminalis* respectively (Purcell *et al*. 2013). However, while no COI haplotypes occurred on more than one host species, a

number of close haplotypes (0.2% - 1.0% pairwise distance) were associated with a wide range of other hosts (*E. radiata*, *E. dives*, *E. delegatensis* and *E. smithii*) and all models assigned them to a single group encompassing both *Fn. herbaservus* and *Fn. manchesteri*, albeit with relatively low Bayesian support (0.87) according to bPTP. Additionally, using the same delimitation criteria, Scheffer *et al*. (2017) linked this group (referred to in that paper as species 11) with the hosts *E. amygdalina* and *E. coccifera* as well as *E. stellulata*, with a maximum pairwise distance of 1.9% (Scheffer *et al*. 2017). Nonetheless, there are morphological differences in chaetotaxy and male genitalia in the two described species, which can be distinguished without need for dissection by the number of short hairs on the genae. Those from *E. viminalis*, *E. smithii*, *E. dives* and *E. radiata* have 15 - 20 hairs, and those from *E. stellulata* and *E. delegatensis* have >20 – 25. No morphological information is available for the specimens from *E. amygdalina* and *E. coccifera*.

Similarly, all delimitation methods grouped the leaf blade gallers from the closely related *E. sideroxylon* and *E. melliodora* in the same OTU, with an uncorrected pairwise distance of 0.3%-1.5%. However, there is a consistent difference in the dorsal shield between the larvae from the two hosts, with those from *E. melliodora* possessing a small patch of spicules (Fig. 2.10,p) that is absent from the larvae from *E. sideroxylon.*

Differences between models

ABGD tended to lump haplotypes that were split by bPTP and the 2% delimitation threshold, most notably the Simple Distance model which sorted the dataset into only 47 OTUs, as opposed to 71 or 73 OTUs using ≤ 2% and bPTP respectively. Some of these ABGD groupings were implausibly indiscriminate, such as assigning all the *Melaleuca* shoot bud gallers to a single OTU.

The results of the bPTP analysis corresponded to delineation by a 2% bp threshold except when this threshold was unclear due to some boundary overlap within the OTU, such as the four haplotypes from three host plants in group 31, clade 8.

Threshold overlaps within the same species and identical barcode haplotypes (Hebert *et al*. 2003) between heterospecifics are not unusual among Diptera (Meier *et al*. 2006). While the region of COI used here is a more sensitive marker in Fergusoninidae than the barcode region, it nevertheless presented some of the same problems and inconsistencies. However, using uncorrected pairwise distances is appropriate for this dataset as I am looking at short sequences from close relatives (Collins *et al*. 2012; Srivathsan and Meier 2012; Scheffer *et al*. 2017) and delimitation by this method corresponded with other characters more consistently than the solutions derived using the other methods tested in this study.

Despite the shortcomings mentioned above, DNA-based taxonomy using genetic data alone has been employed in other systems (for example, Hebert *et al*. 2004, Williams *et al*. 2012, Murphy *et al*. 2015) particularly with organisms lacking apparent distinguishing morphology (Cook *et al*. 2010, Murphy *et al*. 2015) but for these species to be meaningful units they must at least have some distinct ecological, behavioural or other features (Santos & Faria 2011). However, such a narrow line of inquiry is inappropriate for Fergusoninidae, which has a number of features that can, and should, be examined along with genetic data when considering the taxonomy of this group.

4.6.3 Integrative Taxonomy

While using the 2% delimitation threshold eliminates many of the ambiguities noted above, it seems clear that genetic distance methods are not always adequate to identify and distinguish reliably between *Fergusonina* species, and other information such as

larval and adult morphology, host plant, associated nematode species, locality and gall type must be considered in conjunction with genetic data. In recent years the concept of integrating different lines of evidence to delimit species boundaries has become more common (Dayrat 2005), and it is possible to apply this technique to the taxonomy of *Fergusonina* species by integrating evidence from adult and larval morphology, the extended phenotype (gall morphology), and molecular markers. Using this approach we can decide on species limits by taking advantage of all different lines of evidence, and also be specific about which evidence, and the strength of evidence, supporting a species limit. The likely species limits based on combined evidence and strength of support are listed in Table 4.3, with the far right column showing the delimitation criteria used. In most cases, morphological and ecological evidence is sufficient in Fergusoninidae to identify species. Where there are multiple plant hosts, COI sequence data provides additional evidence, and the congruence between the 2% uncorrected pairwise distance and morphological and other evidence is such that, in the absence of larvae, adults and host information, genetic evidence is a reliable method.

CHAPTER 5: TAXONOMIC CONSIDERATIONS

5.1 Introduction

The Fergusoninidae is a large monogeneric family of gall-inducing flies with 39 described species, but over 200 (Nelson *et al*. 2014) currently unnamed species recorded from diverse gall types and hosts within Myrtaceae. Thorough searching of a given *Eucalyptus* species usually reveals at least one associated *Fergusonina* species, and often several, on different sites on the plant or in different geographical ranges (Nelson *et al*. 2014). As there are 700-800 described species of *Eucalyptus*, and *Fergusonina* has been found on a further six genera within Myrtaceae, the number of *Fergusonina* species could conceivably be in the thousands. Amongst the known species, there are a number of distinct lineages which show consistent differences in adult and larval morphology, gall type and, to some extent, plant host relationships. Depending on the lineage, the association might be as broad as host genus, or as narrow as Section (Purcell *et al*. 2016). For example, in a lineage of axial unilocular pea gallers from hosts in the genus *Eucalyptus*, larvae have dorsal shields consisting of chitinous plates bearing curved teeth, and adults have distinctive dark markings, including a black katepisternum and lateral stripes on the thorax and abdomen.

While these lineages can be clearly distinguished, species delimitation is often impossible without data from a number of different sources. For example, adult fergusoninid flies have few morphological characters that are diagnostically informative among closely related species (Taylor 2004; Purcell *et al*. 2016), while there is also high intraspecific and sometimes sex-based variation, particularly in size, colour and markings (Tonnoir 1937; Purcell *et al*. 2013). Male genital structure in Fergusoninidae appears to be useful in distinguishing between species, but dissection of the male genitalia has been

performed on few undescribed *Fergusonina* species (Taylor 2004) as the process is too laborious and time-consuming to be performed routinely. Female genital morphology is generally uninformative, as it may be shared by groups of closely related species (Tonnoir 1937) while also being variable among conspecifics.

Taken together, larval morphology, host plant, and the galled site on the host are useful diagnostic indicators (Taylor 2004), but as discussed in chapter 4, even these are not always sufficient, and molecular sequencing of COI may be necessary to distinguish between some species.

Given the size of this genus, its diversity and clear delimitation between lineages, *Fergusonina* could be more practically treated as a number of separate genera within the family Fergusoninidae. While such a revision is beyond the scope of this thesis, a first step would be to determine which of the putative generic-level lineages should bear the name *Fergusonina*. To do this it is necessary to determine to which grouping the type species of *Fergusonina*, *Fn. microcera* Malloch, belongs, however this is complicated by the wide range of data necessary to identify individual species.

5.2 Species diagnosis

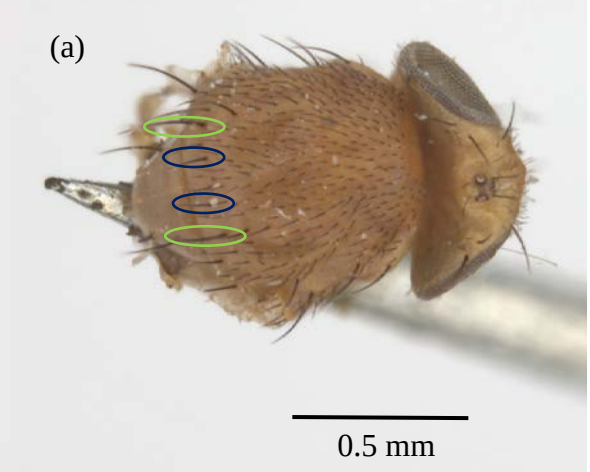
In the first step towards a revision of *Fergusonina,* I attempted to identify the type species *Fergusonina microcera* by examining the holotype and assembling all available information on it, and comparing it with other available species of the genus. *Fn. microcera* was described by J. R. Malloch when he erected the genus within Agromyzidae in 1924, based on a single “rather poorly preserved” (Malloch 1932) adult female collected by fellow dipterist E. W. Ferguson (Malloch 1924). Unfortunately, given the condition of the

specimen and the absence of information on morphological variation across the genus, the brief description is insufficient to distinguish this species from most congeners described subsequently. Moreover, the family's gall-inducing habits were not discovered until 1930, and as the holotype was caught in a sweep net, no gall morphology or host association was recorded, and nothing of its larval or pupal stages, which have since been shown to carry diagnostic characters. Subsequently, Malloch received an adult male fergusoninid and assigned it to the same species (Malloch 1925) based tenuously on colouration. However, there is no evidence that they are the same species (Tonnoir 1937). At that time, the extent of *Fergusonina* diversity was not known or suspected, and Malloch had only seen three other individuals from the genus to compare it with (Malloch 1925). Malloch redescribed *Fn. microcera* in 1932, still based on the sole female specimen, when he had more adults of other species for comparison. Five years later, A.L. Tonnoir redescribed the female in his revision of the genus (Tonnoir 1937). Malloch's and Tonnoir's descriptions of *Fn. microcera* have been included in Appendix II. Unfortunately, the type specimen, deposited in the Australian National Insect Collection (ANIC) in Canberra, is missing the abdomen, wings, and two pairs of legs (Fig. 5.1a).

Given the difficulties in distinguishing species, the morphology of the type specimen alone is insufficient to identify the type species, and more data are required.

I attempted to obtain DNA non-destructively from the type specimen using an Invitrogen Chargeswitch Forensic DNA Purification kit (Invitrogen Life Technologies, Carlsbad, CA), aiming to sequencing a region of COI and comparing it with a dataset of approximately 70 *Fergusonina* species. The DNA was measured using an Agilent D2200 TapeStation system (Agilent Technologies, Santa Clara, CA), which reported a very low

signal intensity without peaks, indicating that the extracted DNA was not of sufficient quality or quantity for sequencing. The holotype was kept intact, and was subsequently remounted and returned to the collection.



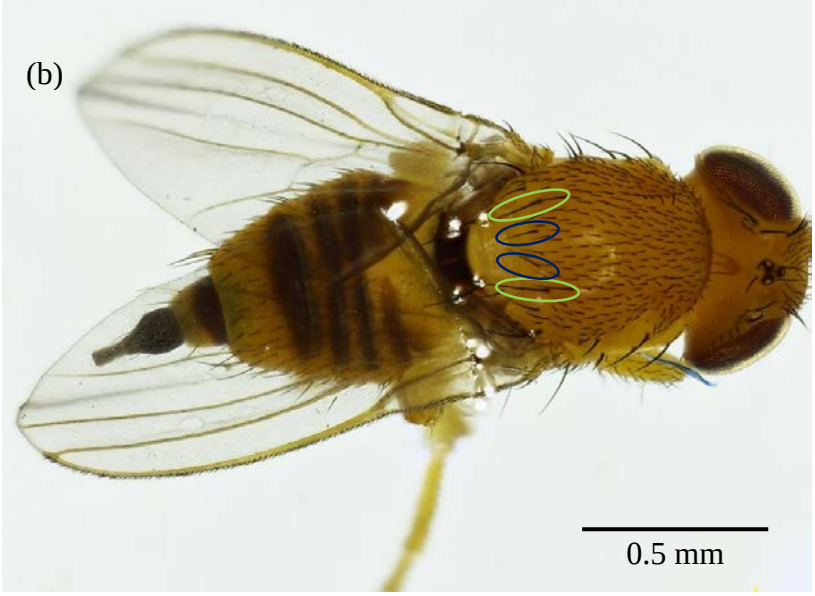


Fig. 5.1 Dorsal view of a) *Fn. microcera* holotype, and adult female shoot bud gallers from b) *E. racemosa* and c) *C. gummifera* showing relative lengths of acrostical (circled in blue) and longest dorso-central setae (circled in green). The species from *C. gummifera* differed from the other species and holotype in having relatively short acrosticals. Note also the discrete (b) vs coalesced (c) banding on the abdominal tergites.

With all attempts to identify the specimen by the descriptions, examination of the holotype and DNA sequencing being unsuccessful, *Fergusonina microcera* must be considered a *nomen dubium*. In such a situation, a case may be submitted to the International Commission on Zoological Nomenclature (ICZN) to designate a neotype under article 75.5: "Replacement of unidentifiable name-bearing type by a neotype" which states that

> "When an author considers that the taxonomic identity of a nominal species group taxon cannot be determined from its existing name-bearing type (i.e. its name is a *nomen dubium*), and stability or universality are threatened thereby, the author may request the Commission to set aside under its plenary power [Art. 81] the existing name-bearing type and designate a neotype. (International Commission on Zoological Nomenclature 2000)."

5.3 Attempts to collect a neotype

Perhaps the most useful information on the first described female *Fergusonina* is the type locality. The original collection locality for *Fn. microcera* is noted as North Harbour, Sydney, New South Wales (Tonnoir 1937). I take this to be what is now Sydney Harbour National Park on Dobroyd Head, which extends into the North Harbour inlet. Collecting trips were undertaken in May and September 2016 to this locality. As I have no records of the eucalypts in this area from the 1920's I searched all trees belong to *Eucalyptus*, *Angophora* and *Corymbia* for fergusoninid galls. My search was also extended to neighbouring areas within a 3 km radius, however much of the area is now suburban, and

little of the original native vegetation remains. Nevertheless, galls were successfully collected from *E. racemosa* and *E. haemastoma* and three unidentified species of juvenile eucalypts. Nineteen larvae and no adults were collected from the *E. haemastoma* shoot bud galls, and all but two were 2nd instar, which lack distinguishing morphological features. While I had no adults from *E. haemastoma*, the COI sequence obtained from these larvae using the methods outlined in chapter 2 had an uncorrected pairwise distance of 0.2% to 0.9% from the haplotypes of flies from *E. racemosa* from the Blue Mountains and Budawang Range in NSW and from *E. burgessiana* in Jervis Bay, and were therefore considered to belong to the same putative species (Table 4.3). However, this remains to be confirmed by morphological analysis. I had a number of adults from this species from the above hosts and localities, whose morphology could be compared with the *Fn. microcera* descriptions.

A more distant relative from a less commonly seen gall type (stem pea gall) was collected from *E. racemosa* at the same site. Again, only larvae were obtained. Of the flies from the three unidentified host plants, I obtained adults and larvae from fused leaf galls, larvae only from leaf blade galls, and adults from a shoot bud gall.

5.4 Morphological comparisons

While there is a lack of valuable information on the type species, there are a few key characters on this animal that can be used to exclude other species (Table 5.1). The most obvious distinguishing feature is the absence of the brown or black thoracic markings that are present in many *Fergusonina* species, though in some groups it may be faint or absent in females. After eliminating most available species with thoracic markings on the females,

I compared the holotype and Malloch's and Tonnoir's descriptions of *Fn. microcera* with superficially similar adults from leaf blade galls on *E. andrewsii* from Lamington National Park in Qld (n = 33 ♀, 27 ♂) shoot bud galls on *E. sieberi* from Barren Grounds, NSW, *E. racemosa* from Budawang Range (n = 116 ♀, 110 ♂) and the Blue Mountains, NSW (n = 34 ♀, 33 ♂), *E. burgessiana* from Jervis Bay (JBT) (n = 9 ♀, 11 ♂), *C. gummifera* (n = 3 ♀, 3 ♂) and an unknown host species from the type locality (n = 39 ♀, 30 ♂). Shoot bud gallers from *E. piperita* from the Blue Mountains (n = 2 ♀, 1 ♂), and stem pea gallers from *E. racemosa* from Manly, NSW were also included as all these host species occur in or near Sydney Harbour National Park (Benson 2011); however, while the fly species associated with particular hosts can have a large range there can be some geographical variation in host plant associations (Purcell *et al*. 2017; Scheffer *et al*. 2017). The holotype was photographed using a Leica Application Suite Imaging System, and the flies from *E. racemosa* and *E. gummifera* were photographed with a Nikon ShuttlePix P-400Rv Digital microscope and compiled using Zerene Stacker ver. 1.04 (Zerene Systems, LLC).

5.4.1 Head

One of the most distinctive features the *Fn. microcera* holotype is that it has only one well-developed orbital bristle (Fig. 5.2a,b), while the other *Fergusonina* specimens Malloch examined had two (Malloch 1932). Tonnoir (1937) noted that a second, relatively thick, outwardly-turned seta behind it may be interpreted as a second orbital bristle. In individuals from *E. racemosa* the lengths of the posterior bristles were variable, but while the posterior orbital bristle was always clear, the anterior bristle in some individuals was sometimes much shorter, being the same size as a small seta between the two, which may be a third

orbital bristle (Fig. 5.2c). The specimens from *C. gummifera* were the only ones examined where the posterior orbital bristle was significantly shorter than the anterior (Fig. 5.2d).

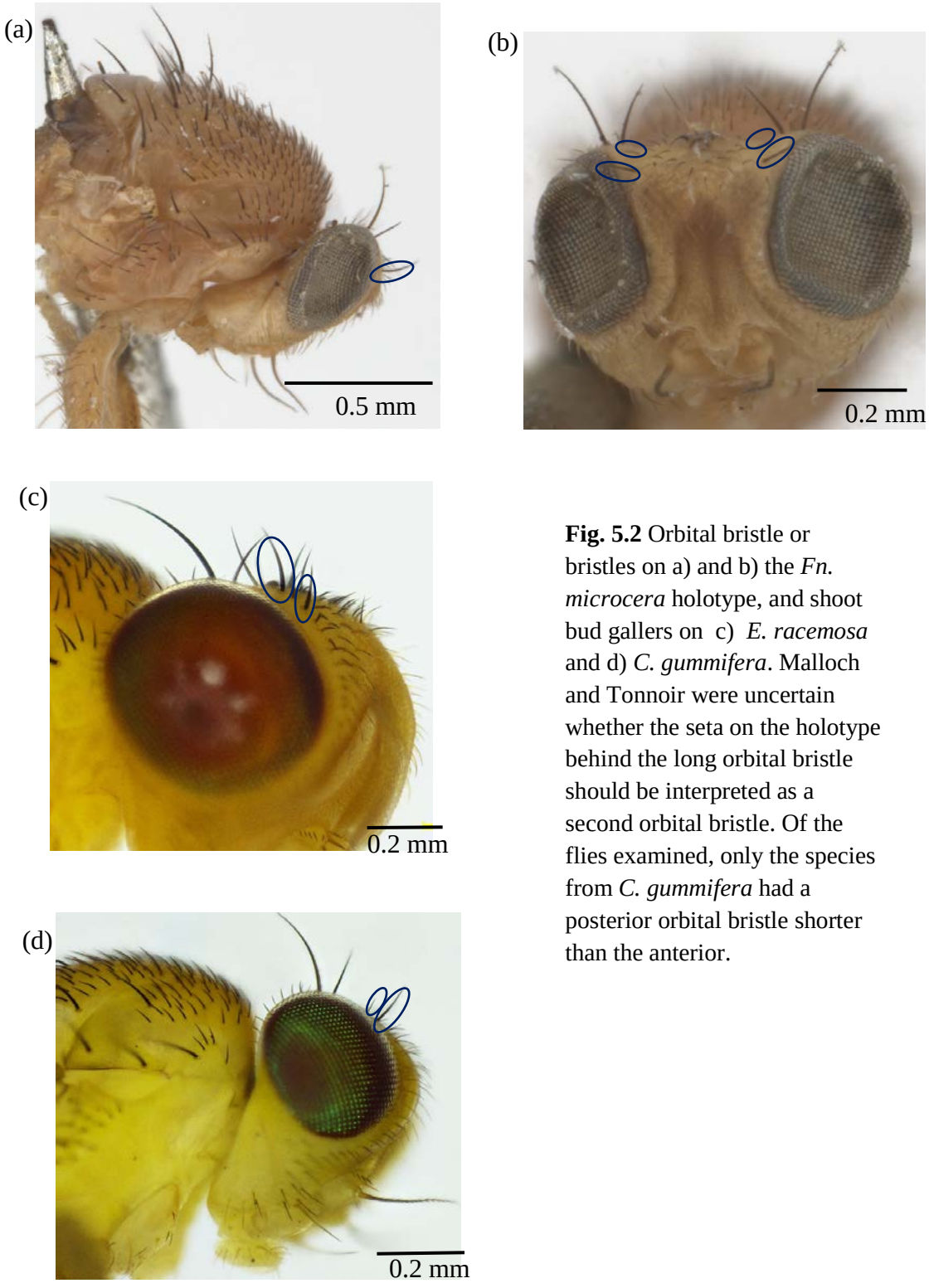


Fig. 5.2 Orbital bristle or bristles on a) and b) the *Fn. microcera* holotype, and shoot bud gallers on c) *E. racemosa* and d) *C. gummifera*. Malloch and Tonnoir were uncertain whether the seta on the holotype behind the long orbital bristle should be interpreted as a second orbital bristle. Of the flies examined, only the species from *C. gummifera* had a posterior orbital bristle shorter than the anterior.

Table 5.1 Morphological comparison of the *Fn. microcera* holotype with species that are morphologically similar or use plant species that occur in or near the type locality. Only larvae were available from *E. haemastoma* and the stem pea galls from *E. racemosa*.

	Holotype	**Sp. 1**			**Sp. 2**	**Sp. 3**	**Sp. 4**	**Sp. 5**	**Sp. 6**	**Sp. 7**
Plant host	?	*E. haemastoma*	*E. racemosa*	*E. burgessiana*	*E. piperita*	*C. gummifera*	*E. sieberi*	?	*E. andrewsii*	*E. racemosa*
Gall type	?	Shoot bud	Shoot bud	Shoot bud	Shoot bud	Shoot bud	Shoot bud	Shoot bud	Leaf blade	Stem pea gall
Larval dorsal shield	?	Barred	Barred	Barred	Barred	Absent	Barred	?	Absent	Absent
Collection locality	Sydney NSW	Sydney NSW	Budawang Range & Blue Mts NSW	Jervis Bay JBT	Blue Mts NSW	Blue Mts NSW	Barren Grounds NSW	Sydney NSW	Lamington NP QLD	Sydney NSW
Head (Fig. 5.2)										
Antennae yellow, black arista	✓	?	✓	✓	✓	✓	x	✓	✓	?
Ocellar triangle yellow with narrow brown-black ring around each ocellus	✓	?	✓	✓	✓	✓	✓	✓	x	?
1 or 2 orbital bristles. If 2, posterior much smaller	✓	?	x	x	x	✓	x	x	x	?
Thorax (Fig. 5.1)										
Rufous yellow, no markings	✓	?	✓ (♀)	✓ (♀)	x	✓	✓	✓	✓	?
Row of 5 dorso-central setae, anterior two v small	✓	?	x	x	x	x	x	x	x	?
Prescutellar acrosticals somewhat more than half as long as largest dorso-central	✓	?	x	x	✓	x	x	✓	x	?
Wing (Fig. 5.3)										
Costa extends only just over R2+3	✓	?	✓	✓	x	✓	✓	✓	✓	?
Two branches of radial cell distinctly divergent	✓	?	x	x	x	x	x	x	x	?
Posterior cross vein obsolete or incomplete	✓	?	x	x	x	✓	x	x	x	?
Distance between cross veins equal to length of posterior one	✓	?	x	Variable	✓	x	x	x	x	?

	Holotype	**Sp. 1**			**Sp. 2**	**Sp. 3**	**Sp. 4**	**Sp. 5**	**Sp. 6**	**Sp. 7**
Plant host	?	*E. haemastoma*	*E. racemosa*	*E. burgessiana*	*E. piperita*	*C. gummifera*	*E. sieberi*	?	*E. andrewsii*	*E. racemosa*
Abdomen (Fig. 5.1, 5.4)										
Tergites 1-5 fuscous, posterior border of 5th & 6th dull orange	✓	?	x	x	✓	✓	✓	✓	✓	?
Tergite 6 (♀) : 6 submarginal bristles, outer two larger than median	✓	?	x	x	Variable	Variable	x	x	Variable	?
Segments 1-6: Numerous small bristles on dorsal and ventral surfaces	✓	?	x	x	x	✓	x	x	x	?
Segment 6: Dull orange	✓	?	x	x	x	✓	x	x	x	?
Segment 7 (♀): Many small bristles on whole surface proximal to pairs of long subapical bristles	✓	?	x	x	x	✓	x	x	x	?

5.4.2 Thorax

The thorax of *Fn. microcera* is without markings and was described by Tonnoir (1937) as “rufous yellow”, which distinguishes it from lineages of *Fergusonina* with very dark or distinct thoracic vittae such as the fused leaf gallers. However in some species of shoot bud gallers (and possibly others) the males have clear markings which on females are indistinct or absent.

Malloch (1924) described two pairs of dorso-central bristles. Examination of the holotype revealed two pairs that were much longer than the surrounding setae, and two or three more pairs anterior to these that were much shorter and difficult to distinguish from the surrounding setae. Tonnoir considered there to be three clear pairs and two pairs that were indistinct (Tonnoir 1937). Of the other examined species, all had 3 – 4 pairs of dorso-central setae. The size of the acrostical setae relative to the posterior dorso-central bristles was consistent within species. In the holotype the acrostical setae were “longer than usual, somewhat half as long as the largest dorso-central” (Tonnoir 1937). All specimens examined had acrostical setae roughly half the size of the largest dorso-central setae except that from *C. gummifera*, which most closely resembled the holotype in most characters but had considerably shorter acrostical bristles.

5.4.3 Wing

The wings of the holotype are now missing. Tonnoir noted that the “two branches of the radial sector [were] distinctly divergent” (Tonnoir 1937) which is consistent with the illustration by Malloch (1925) (Fig. 5.3c) though this illustration was of the male that he assumed to be *Fn. microcera* and is probably a different species, which would explain the distinct outer cross vein shown, which is lacking in descriptions of the female. Couplet 3 in his key (Malloch 1932) notes the two branches are “not narrowed at apex”.

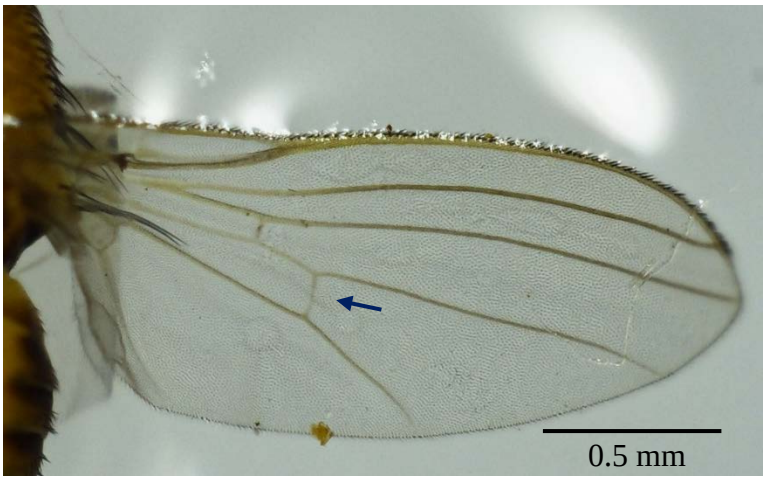
This is in contrast with all the adult specimens examined, where these veins were convergent or roughly parallel towards the apex (Fig. 5.3a); however, those from *C. gummifera* were very slightly divergent (Fig. 5.3b).

The posterior or outer cross vein (Fig. 5.3) is described as incomplete on one wing of the holotype, and obscure on the other (Malloch 1924; 1925; Tonnoir 1937) though Malloch has included it in his illustration of the venation (Fig. 5.3c) . This vein is complete and present in all specimens from *Eucalyptus* examined for this study, and in known gallers from *Melaleuca* (Taylor 2004). It may be that the venation in this specimen was anomalous. However, the specimens from *C. gummifera* had only indistinct or apparently absent posterior cross veins (Fig. 5.3b).

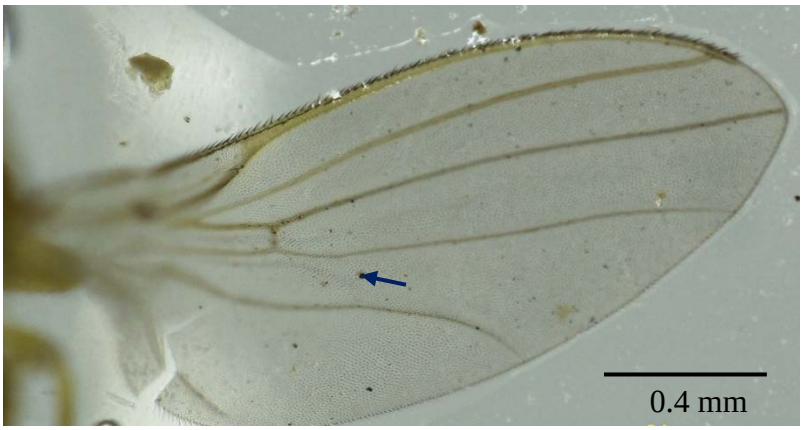
5.4.4 Abdomen

Malloch (1924) and Tonnoir (1937) described the first five abdominal tergites as fuscous, which suggests that the dark markings on these tergites (Fig. 5.1) appeared coalesced into a large patch rather than as discrete bars. While the extent to which these markings cover each segment can vary between conspecifics to some degree, the majority will appear either discrete or coalescent depending on the species. These were coalescent on the specimens from *C. gummifera*, the unknown eucalypt from the type locality and the leaf blade gallers from *E. andrewsii*. No dark markings are recorded on abdominal segment 6 of *Fn. microcera*, but do occur on the dorsal side of females from the unknown host from the type locality and the leaf blade gallers from *E. andrewsii*, and a grey-black ring circles all or most of the segment on females from *E. racemosa* (Fig. 5.1b, 5.4a) and *E. piperita*.

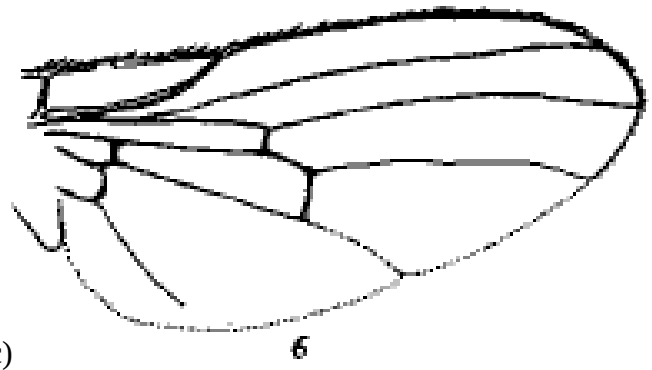
Of all the specimens examined, only the species from *C. gummifera* bore the many short bristles on segments 6 and 7 described by Tonnoir (1937) (Fig. 5.4).



(a)



(b)



(c)

Fig. 5.3 Wings of adult female shoot bud gallers from a) *E. racemosa*, with distinct posterior cross vein, and b) *C. gummifera*, without this vein, the latter consistent with the description of *Fn. microcera*, but not with c) Malloch's 1925 illustration, which is probably a different species.

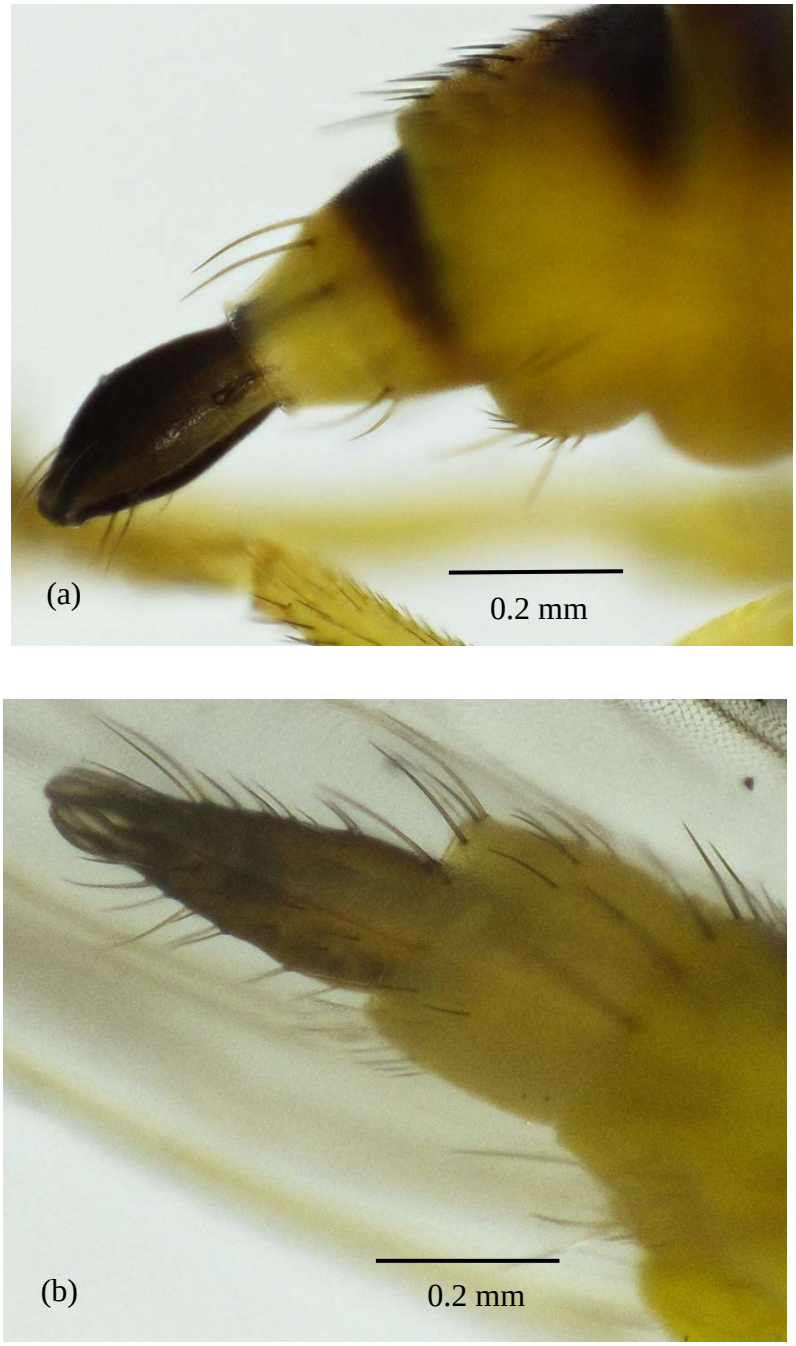


Fig. 5.4 Sixth and seventh abdominal segments of adult female shoot bud gallers from a) *E. racemosa* and b) *C. gummifera*, showing the latter's lack of dark markings, and setae consistent with the "many small bristles" described by Tonnoir on *Fn. microcera*.

5.5 Conclusions

It is not possible to place the type species with confidence in any recognised lineage, but some groups with clear morphological differences can be discounted, such as the fused leaf gallers. Of the eucalypt-galling specimens examined here, and those recorded from *Melaleuca* (Taylor 2004), the specimens that most closely resembled the descriptions and remnants of the holotype of *Fn. microcera* were reared from shoot bud galls on *C. gummifera.* Unlike the other species examined, this species matched *Fn. microcera* on the setation of female segments 6 and 7, the single orbital bristle, and the faint posterior cross vein; however it can be excluded from being conspecific on the basis of marked differences in the length of the prescutellar acrostical setae, a feature that is consistent within species. Similarly, *Fn. giblindaviesae* and *Fn. thomasi,* which gall flower buds on *Corymbia,* also lack the posterior cross vein, and possess numerous small setae on the ovipositor (Taylor and Davies 2008, Davies *et al*. 2017). I have no molecular data for these species, but they are likely to belong to the same genetic lineage. The only other recorded species lacking this vein is *Fn. madidum* from *Leptospermum* (Davies *et al*. 2017).

Galls were collected from *E. haemastoma* from the type locality, but these yielded no adults. COI sequence data suggested that these flies were the same species as those reared from *E. racemosa* and *E. burgessiana*. Adults of these two species were available, and compared to the holotype, but were distinctly different in a number of key morphological features from the head, thorax and abdomen. Either the flies from the type locality galling *E. haemastoma* are not the same species as Malloch's type specimen, or if they are, this species is not the same as the flies from *E. racemosa* or *E. burgessiana.* It would be important to attempt to obtain adults from the type locality

from *E. haemastoma* and compare them to the holotype. Without such specimens this linkage, based on COI data alone, may not be biologically accurate, rendering the comparison spurious.

The area where the holotype was collected in the 1920s has changed markedly in the intervening years, having undergone extensive urbanisation and land clearing (Daniel *et al*. 2006), and the military development that began in the 19th Century was still taking place when the holotype was collected, and continued until 1945 (NSW National Parks and Wildlife Service 2004). Moreover, the eucalypts around Sydney Harbour have also been affected by die-back caused by pollutants and the pathogen *Phytophthora cinnamomi* (Anderson *et al*. 1981; Daniel *et al*. 2006; Benson 2011; NSW National Parks and Wildlife Service 2012). Potential host plant species that may have been more abundant at the time, such as *E. haemastoma*, have dwindled in sites more prone to urban development (D. Benson 2016, pers. comm.). Fire regimes in the park have been either too frequent or too rare, with sections of the Park remaining unburned for decades (NSW National Parks and Wildlife Service 2004; Benson 2011; NSW National Parks and Wildlife Service 2012) and others being adversely affected by frequent arson (NSW National Parks and Wildlife Service 2004). These burning regimes have resulted in "changes to habitat including altered vegetation structure and species composition, weed invasion, senescent vegetation and loss of species" (NSW National Parks and Wildlife Service 2012). Appropriate burning intervals for the Park are still being determined (NSW National Parks and Wildlife Service 2012). Burning encourages flushing of new growth that is exploited by Fergusoninidae, while fires may also cause temporary local extinctions of *Fergusonina* that would become permanent if there is no recruitment from nearby populations, for instance when habitat has become

fragmented by human development (NSW National Parks and Wildlife Service 2012). It is therefore possible that *Fn. microcera* is now extinct in the area.

The unresolved identity and potential extinction of *Fn. microcera* leaves Fergusoninidae taxonomists with the options of either expanding the search area in the hope of collecting adults that resemble the type description, or of revising the taxon without identifying *Fn. microcera* to species but instead assigning it to its most probable group, which will retain the name *Fergusonina*, and revising the family on this basis. Given the evidence here it is likely that *Fn. microcera* belongs in one of the groups of flies associated (exclusively or otherwise) with the host plant genus *Corymbia*, but further collection of galls from this host genus, particularly around the Sydney area, would be necessary to confirm this, and adults should be obtained from *E. haemastoma* from the type locality and compared with the holotype before going ahead with the revision.

CHAPTER 6: MISCELLANEOUS OBSERVATIONS

During the course of this study I made some observations that were not central to this research and were therefore not followed up; nevertheless I feel they should be recorded and perhaps pursued in the future, as they could add valuable information to our understanding of the *Fergusonina-Fergusobia* system.

6.1 Seasonality

There has been no thorough investigation of seasonality and generation times in *Fergusonina*. Larval longevity and the number of annual generations of *Fergusonina* probably depend on species and gall type. While some species complete multiple generations per year, others, such as flower bud gallers, which must coordinate with the annual flowering of their hosts, may go through a period of diapause (Taylor *et al*. 1996; 2005; Head 2008). However this has not been documented, and questions such as at what developmental stage this occurs, if at all, and how it is coordinated between the flies and the nematodes have not been addressed. Taylor *et al.* (1996) observed that it took between two and four weeks for shoot bud galls on *E. camaldulensis* to reach full size following initiation, while Goolsby *et al*. 2001 reported that the period from egg to adult for shoot bud gallers from *M. quinquenervia* was around 90 days. The large time difference in these two studies may indicate a difference in developmental rate, or a period of stasis.

In the present study, the gall type, host plant and developmental stage of all flies collected between 2012 and 2016 were compiled to gain an overall picture of the seasonal patterns of galling (Table 6.1.1). It must be noted that this was not a focus of the study, and

the data are strongly influenced by the timing of collecting trips and range of host plants and bud types sampled. Nevertheless it is clear that for the major gall types, active galls were collected in every season, except the unilocular axial pea galls, which were not collected in the summer months. While no data on temperature tolerances of *Fergusobia* are available, it has been reported that temperatures around 38° Celsius are lethal to fig nematodes (Davies *et al*. 2016). Consequently, it is possible that *Fergusobia* nematodes in the small, thin-walled axial pea galls (Fig. 2.2d, e) may be more vulnerable to the heat than those in multilocular or fleshy galls.

Multilocular shoot bud galls were collected in every month. Flower bud galls and leaf blade galls were collected all year except March and July respectively, however given the low numbers collected, this may not indicate their absence. Similarly, fused leaf galls were not found in September or December. Axial pea galls were not collected over the hottest months from December to February. Overall, the greatest number of fly species were collected in April and the fewest in January, but this may be an artefact of sampling; *Fn. omlandi*, which inhabits large, conspicuous shoot bud galls on the snow gums *E. pauciflora* and *E. lacrimans* in the ACT and NSW, was collected in every month except October.

Gall abundance is variable for different species and at different sites. My collecting was targeted towards good coverage of the focus host clade, and given time limitations, collecting at any one site was carried out only until enough galls were collected for phylogenetic analysis. Consequently it was not practical to collect exhaustive demographic data over the 3 years.

Table 6.1 Seasonal timing of galls collected for this study. Each record is a count of putative species based on host plant and gall type. 2L/3L = 2nd/3rd instar larvae; P = pupae; A = adults. The gall types are illustrated in Fig. 2.2.

Gall type	Life stage	Spring			Summer			Autumn			Winter			Total
		Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
Fused	2L												1	
leaf	3L		1	1		1		2	2	2	4	1	2	
	P		1					1						
	A		1				1	1	2		2		1	
	Total	**0**	**3**	**1**	**0**	**1**	**1**	**4**	**4**	**2**	**6**	**1**	**4**	**27**
Leaf	2L		1			1								
blade	3L	1	1	2	2	4	2	3	8	6	4		1	
	P		1	1					4	1			1	
	A			1		4	3	3					2	
	Total	**1**	**3**	**4**	**2**	**9**	**5**	**6**	**12**	**7**	**4**	**0**	**4**	**57**
Multilocular	2L	3		1	1		1		3	1			2	
flower bud	3L	5	2	1	1		1		1	2	3	2		
	P		2	1	2									
	A			2	1									
	Total	**8**	**4**	**5**	**5**	**0**	**2**	**0**	**4**	**3**	**3**	**2**	**2**	**38**
Unilocular	2L													
flower bud	3L	1					1		1	1				
	P													
	A													
	Total	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**0**	**0**	**4**
Multilocular	2L													
pea gall	3L						1					1	2	
	P													
	A		1										2	

Gall type	Life stage	Spring			Summer			Autumn			Winter			Total
	Total	**0**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**4**	**7**
Unilocular	2L										1			
pea gall	3L	2	2						2		6	2	3	
(axial)	P			4							2		2	
	A	1	1	2					1		1		3	
	Total	**3**	**3**	**6**	**0**	**0**	**0**	**0**	**3**	**0**	**10**	**2**	**8**	**35**
Unilocular	2L													
pea gall	3L	1								1	1	1		
(stem)	P													
	A													
	Total	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**4**
Multilocular	2L		1	2	4		2	4	8	5		1	1	
shoot bud	3L	1		4	2	2	3	4	9	10	5	1	5	
	P		1		3	1	1	2	5	1	1		1	
	A	3	1		4	3	5	2	6	5	6	3	2	
	Total	**4**	**3**	**6**	**13**	**6**	**11**	**12**	**28**	**21**	**12**	**5**	**9**	**130**
Unilocular	2L													
shoot bud	3L	1												
	P													
	A	1												
	Total	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**
Total		**20**	**17**	**22**	**20**	**16**	**21**	**22**	**52**	**35**	**36**	**12**	**31**	

Nevertheless, there was chronological variation – for example there was a change in *Fn. omlandi* gall density across the years of this study, as these galls were very common in 2013 and rarely seen in 2016. Other workers have also noted annual fluctuations, perhaps due to variations in temperature, humidity, rainfall, fire, parasitism, bud growth of their host plants or other environmental factors affecting either the flies or nematodes directly (Davies *et al*. 2016). Currie (1937) noted that gall abundance on *E. macrorhyncha* fluctuated a great deal from year to year, most markedly on flower buds, possibly because shoot and leaf buds are more vulnerable to parasitism which keeps numbers at a reasonably constant low level. The abundance of buds, timing of budding, and proximity of suitable trees are the factors most likely to limit *Fergusonina* flower bud galls from year to year (Currie 1937).

6.2 MicroCT scanning

Until recently it has not been possible to look inside a *Fergusonina* gall without dissecting it, which disrupts the behaviour of animals inside. With MicroCT scanning it is possible to take a snapshot of the interactions between the flies, nematodes and other inquilines and perhaps resolve some outstanding questions concerning the biology of the system. In 2011, some multilocular shoot bud galls from *E. rossii* were frozen in liquid nitrogen and scanned at the Research School of Physics and Engineering at the ANU (LA Nelson, PD Cooper pers. comm). It was unknown whether freezing the plant material would produce clear images, but the method was successful and produced excellent scans of the internal structure of the galls (Figs. 6.1, 6.2, 6.3) and demonstrated that soft-bodied insects within galls can be imaged (Fig. 6.3). I compiled and analysed over 2,200 individual images looking for evidence of nematodes or fly larvae, but unfortunately these galls were spent and no larvae or nematodes

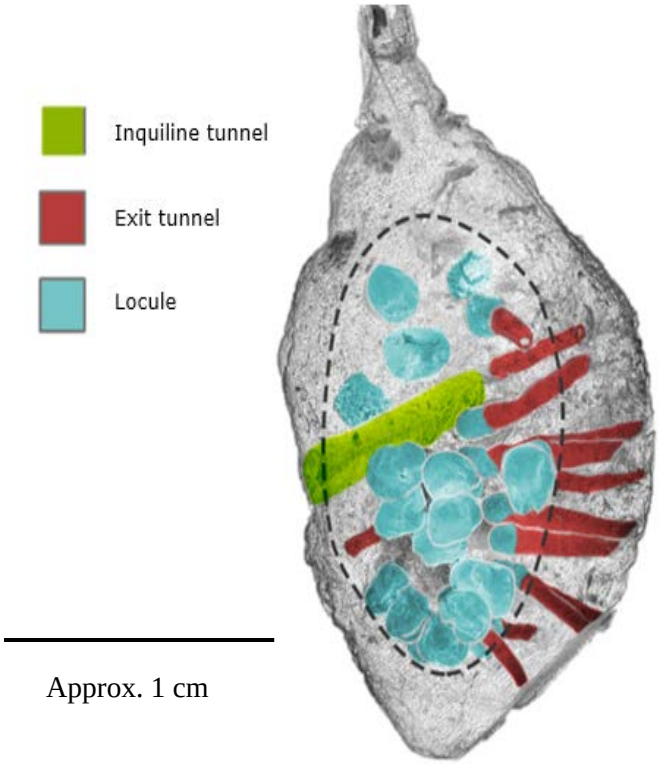


Fig. 6.1
3D reconstruction of MicroCT scanned shoot bud gall, showing the tunnels made by the fly larvae prior to pupation, and a tunnel excavated by a lepidopteran inquiline.

Image by Dr Ajay Limaye, VizLab, ANU Supercomputer Facility (ANUSF)

remained, though some locules contained fly pupae. Nevertheless, the scans offered some insights into the internal organisation of multilocular shoot bud galls, such as the long tunnels the larvae make to the outer edge of the gall, before pupating near the centre (coloured red in Fig. 6.1). Subsequently, I collected more multilocular galls for scanning. They have been frozen but not yet scanned. It would be valuable to pursue this, and even perhaps attempt time course study of live material by chilling the gall to slow down the animals inside, scanning them at intervals.

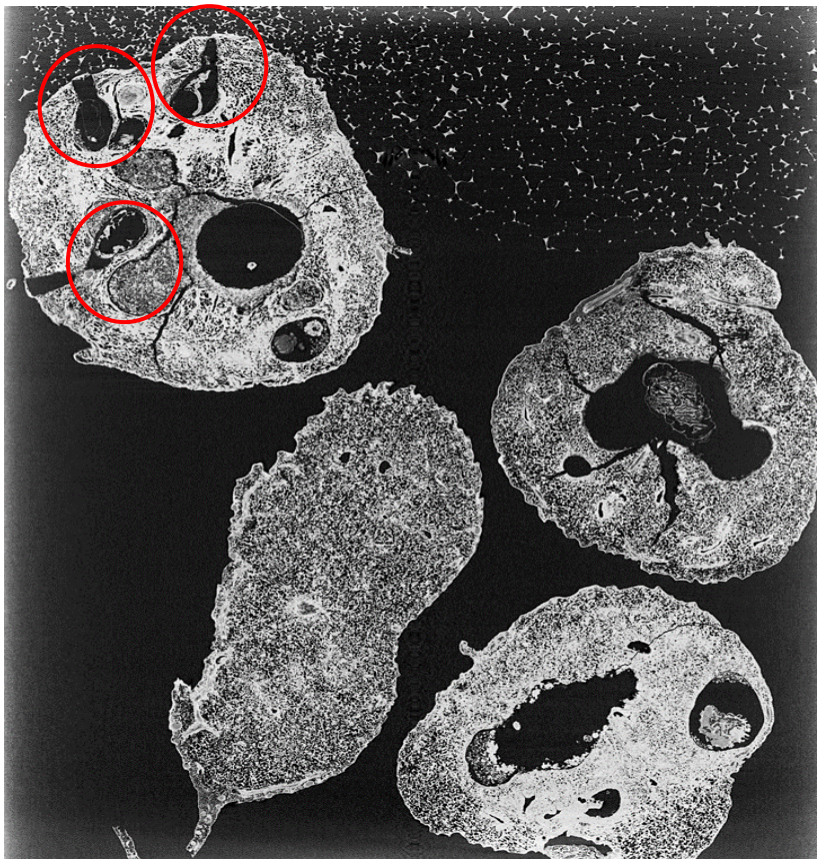


Fig. 6.2
A MicroCT scanned section of some shoot bud galls showing the prevalence of damage caused by destructive inquilines. The gall in the top left still contains some *Fergusonina* pupae (circled in red). Note the thin pupal windows through which the mature flies emerge from the gall.

Volume resolution 1µM

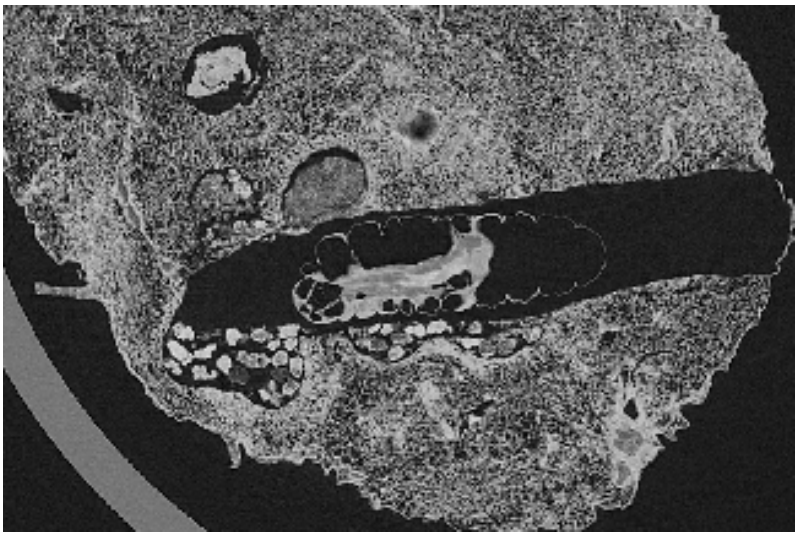


Fig. 6.3
Section of gall showing a lepidopteran inquiline and plant debris within a tunnel.

Volume resolution 1µM

6.3 Multiple species sharing gall site

The involvement of both nematodes and flies in the gall-inducing process adds complexity to questions of host specificity, host switching and coevolution. As nematodes are totally dependent on their fly vector, there is no obvious way in which they could switch eucalypt host species unless the flies also did. However where different *Fergusonina* species share the same host, there is the potential for nematodes to move from one fly species to another. In Chapter 3 I presented evidence that flies are able to utilise novel hosts, where the species commonly associated with the locally abundant *E. macrorhyncha* galled a cultivated specimen of *E. olsenii* in the Australian National Botanic Gardens, and the phylogenies presented in Chapter 4 clearly indicate historic host switching. The combination of "leaky" host fidelity of the fly and different species of flies utilising the same host provides a pathway for host-shifting in the nematodes. While no evidence has yet emerged of more than one *Fergusonina* species occupying the same gall, I have found one instance of two species from different lineages of gall-formers (confirmed by morphological and molecular evidence) exploiting the same leaf on *E. pauciflora*, with the locules of a leaf blade gall on the protruding leaf tip of a shoot bud gall (Fig. 6.4). This behaviour, while unusual, presents a potential route for nematodes to move to new fly host species, and may occur occasionally on host plant bud tissue, particularly leaf buds, targeted by multiple *Fergusonina* species.

Fig 6.4 Two species in one leaf: a leaf blade gall (circled in red) at the tip of a multilocular shoot bud gall (circled in blue).

CHAPTER 7: GENERAL DISCUSSION

This study has focused primarily on the evolutionary history of flies belonging to the family Fergusoninidae, their larval morphology in relation to gall type, and their association with plant hosts. It showed that larval morphology, in particular the form of the dorsal shield, characterises lineages of flies from the same gall type, and form distinct clades based on COI sequences. However, the same gall types occur in several different lineages, but dorsal shield morphology differs between every lineage that has them (Purcell *et al*. 2016). Among the species that gall the eucalypts, host association is more variable than gall type. Some clades display a tight association with closely related host species, and others can occur across different subgenera, though more usually they are restricted to a single subgenus (see Chapter 4). This variability in host fidelity also occurs at the species level, with some species galling only one or two closely related host plant species, and others occurring on multiple hosts, sometimes distantly related, within the same geographical range (Purcell *et al*. 2016; 2017).

Investigating host specificity raised questions of species limits in Fergusoninidae, and this study also compared a number of different analytical protocols for assessing these limits. With adults and larvae exhibiting both intraspecific morphological variability and interspecific uniformity within groups of closely related species, identifying these flies to species level is not straightforward, and requires integrating features such as host species, gall type, nematode species, and molecular sequence data. There is a risk of circularity in diagnosing a species by a single character when we define that character as species-specific based on its association with that putative species. Therefore it is extremely important to use a combination of

characteristics – morphological, geographical, ecological and genetic - in determining species boundaries (see Chapter 4).

The morphologically, genetically and ecologically distinct groups revealed by the phylogenetic analyses highlighted the need to revise this increasingly large genus, the only one in the family Fergusoninidae, on the basis of morphological and ecological distinctions (such as gall type and host plant genus). As the holotype of the type species of the only genus in the family is unidentifiable, this revision will first require either nominating a neotype or placing the holotype in a group to which it probably belongs (Chapter 5).

7.1 Diversity

During the course of this project, representatives of several new lineages, gall types and dorsal shield types were collected and sequenced, such as the unilocular terminal shoot bud gallers and the nodular stem gallers (Purcell *et a*l. 2016). To date, there are around 270 recorded host-gall type associations (listed in Appendix I), approximately 95 of which I discovered in this study. While some flies can utilise multiple hosts, it is probable that many represent new species. Of the 72 putative species used in the present study (Table 4.3), 54 were collected from a single host plant species, thus 75% of species in this study were host specific. Host and gall types were considered new or distinct if there were no other records of flies with the same dorsal shield morphology from that gall type on that host plant. I used a Fergusoninidae gall taxonomy used by other recent researchers (Nelson *et al*. 2014), with slight modification. Some existing records lack information about larval morphology, or the galls have ambiguous names (such as Currie's "leaf" galls (Currie 1937)) or names that encompass several sub-types (e.g. "shoot bud galls" may include fused leaf galls). Where flies could not be

definitively matched with any previously recorded species they were added as new records, although some may represent duplicates (Appendix I). As it is common to find galls that are spent or heavily parasitised, not all newly discovered galls could be included in the phylogenetic analyses.

Because of the large geographical range and the large number of species investigated in this study, much of the collecting was opportunistic, and new plant hosts were regularly discovered this way. When a number of plant species were sampled comprehensively, several *Fergusonina* species were uncovered on each, associated with different bud sites on the plants (Chapter 3). To date, sampling has been within a narrow geographical range. There have been few collections from outside eastern Australia (Head 2008; Davies *et al*. 2010a; Scheffer *et al*. 2017) or from other genera of Myrtaceae beyond *Melaleuca* and the eucalypts. The most species rich fly groups we have records for are from the most conspicuous galls (Nelson *et al*. 2014); large shoot bud galls and fused leaf galls on roadside trees may contain over a hundred flies and can be spotted from a moving car. More cryptic or obscure gall types require careful searching of the host plant and contain fewer or single larvae. Galls of the recently described species found on *Leptospermum* (Davies *et al*. 2017) could only be distinguished from healthy buds by squeezing them (K. Davies pers. comm). Similarly, leaf blade galls may only be evident as a thickening and discolouration of the leaf blade, and flower bud galls, too, can be indiscernible from healthy buds, and obtaining larvae in such cases necessitates collecting whole inflorescences and dissecting each bud. Most of the flower bud gallers included in chapter 3 were collected this way, as the host plants were targeted particularly for the study. If flower buds were collected and dissected from each potential host species encountered, many new *Fergusonina* species would doubtless be discovered. Given that there are over seven hundred species of

Eucalyptus species *sensu lato*, and many more species in the Myrtaceae, there are potentially thousands of undescribed species of *Fergusonina* in Australia.

7.2 Plant host associations

The associations between Fergusoninidae and its host plants are not straightforward, and the lack of a clear co-evolutionary signal reported in chapter 3 is consistent with findings in other plant-insect systems (Ronquist and Liljeblad 2001; Inbar *et al*. 2004; de Vienne *et al*. 2013). Some species occurred on multiple, closely related host plants and may have diverged with the hosts, whereas others were collected from very distantly related plants, suggesting a host switch has occurred. Targeting one clade of *Eucalyptus* revealed the prevalence of host switching in their associated fly groups (Purcell *et al*. 2017). However, there are indications that some groups of flies and host plants are more tightly associated than the ones studied, such as the clade of leaf blade gallers found on four hosts in the Section Adnataria (Brooker 2000) (Clade 10 in Chapters 2 and 4, Purcell *et al*. 2016). Clearly, while host switching in *Fergusonina* does occur, it is a relatively rare event given that there is still a high degree of host specificity within the family, and no species occur across host genera (Scheffer *et al*. 2017). Of the three groups in the study there was variability in host fidelity between the groups, from highly host specific flower bud gallers (clade 1) to the less host specific shoot bud and leaf blade gallers (clades 8 and 14). These patterns are not necessarily dependent on the site or morphology of the gall. For instance, the broad host ranges of the leaf blade gallers in clade 14, at both species and clade level, contrasts with those in the highly constrained clade 10, in which six of the seven species are from host plants within the *Eucalyptus* section Adnataria (Chapters 2 and 4); the seventh was collected from *E. obliqua* (Scheffer *et al*. 2017), in a different subgenus.

Thirty of the species used in this study were multilocular shoot bud gallers, of which 21 were from a single host species. I hesitate to classify these as monophagous, as new plant host associations are regularly discovered (see Appendix I) and some species boundaries are still questionable. It should also be noted that the multilocular galls are the most conspicuous of all the gall types, and therefore likely to be collected from a greater range of host plants than small or cryptic galls.

Nevertheless, multilocular gallers appear to have the greatest propensity for galling multiple host species, with over half of the recorded oligophagous species being multilocular shoot bud gallers or fused leaf gallers (Chapter 4; Scheffer *et al*. 2017). *Fergusonina* species (defined by < 2% COI pairwise distance) that feed on multiple hosts have also been found to have the greatest intraspecific genetic variation (Scheffer *et al*. 2017). Some caution should be used here, as the closer the variation is to the 2% threshold, the less certain the species boundary is likely to be, if based on COI evidence alone. Nevertheless, if this pattern of genetic diversity also applies to the nematodes, it could allow greater adaptability and flexibility in host use (Nelson *et al*. 2014; Purcell *et al*. 2017; Scheffer *et al*. 2017) and could explain the relatively large host range of some multilocular gallers (see Chapter 4).

7.3 Species delimitation in Fergusoninidae

Meaningful analysis of host specificity rests on making reliable species diagnoses, which cannot rely on molecular data alone (see Chapter 4). The larger the dataset and the greater the range of haplotypes, the more poorly defined the boundaries can become, because new haplotypes can occur on branches between other haplotype clusters, and some boundaries that were clear within this study were inconsistent with results in Scheffer *et al*. (2017) using the same 2% threshold criteria and the same gene region.

Specifically, leaf blade gallers on *E. leucoxylon* from clade 10 are split into three different species according to the latter paper, while the similar haplotypes of the shoot bud gallers on *E. camaldulensis, E. blakelyi* and *E. tereticornis* are sorted differently. Flies from *E. camaldulensis* are distinct from the species from *E. tereticornis* and *E. blakelyi* in my study, based on a 2% threshold and morphological characters, but are the same as those from *E. blakelyi* in Scheffer *et al*. (2017) based on molecular data alone. The flies from the three hosts possibly represent a single species, and morphological differences, mainly in length of the dorso-central setae, may be intraspecific variation. In Scheffer *et al*. (2017) species boundaries based on a 2% pairwise distance were assumed to be correct and used to make observations about host specificity and intraspecific genetic variation; however the reliability of these boundaries was not examined or compared with morphology.

7.4 Future directions

The discrepancies in molecular species limits discussed above highlight the need to find morphological characters in *Fergusonina* that are reliably and consistently diagnostic, and can be used in conjunction with genetic and other data to determine species limits. Therefore, a comprehensive analysis of adult morphology needs to be undertaken to determine whether or not there are identifying characters consistent across the family that can be used to distinguish between species. As has been discussed previously, this presents particular difficulties in the case of the Fergusoninidae and would represent a large PhD project in itself. A Fergusoninidae transcriptome is currently being sequenced and assembled (A. Zwick, pers. comm) and may reveal more informative markers for detecting molecular species limits.

To gain a greater understanding of the system, it will be necessary to create a phylogeny of the nematode species associated with the same fly lineages included in existing Fergusoninidae phylogenies. To be certain of the true *Fergusonina-Fergusobia* species associations, the flies and nematodes used for phylogenetic comparison should be taken from the same gall. A difficulty is that as galls mature, the nematodes begin to die, and it is often impossible to obtain taxonomically informative morphological data from both organisms from the same gall, particularly in the case of the nematodes where several life stages are required (Nelson *et al*. 2014). However, both nematode and fly DNA have been extracted and sequenced successfully from female flies carrying nematodes internally, using both fly- and nematode-specific primers (Scheffer *et al*. 2013) so it would be feasible to construct a large and informative *Fergusonina-Fergusobia* molecular co-phylogeny. This would also be possible using high throughput sequencing of females. This could then be compared with a phylogeny of the host plants to reveal possible differences in plant association patterns between the flies and the nematodes.

A previous study by Davies *et al*. (2010) found some discrepancies between fly and associated nematode genotypes that may indicate exceptions to the strict nematode-fly host specificity. Given that the tightly-linked life cycles of these organisms must limit the nematodes' ability to move between fly host species, transmission between fly species is probably extremely rare, but may be possible in certain circumstances if flies from two species co-founded a gall. It is not known whether any cues from the first foundress deter other species from ovipositing at the same site, but I have found a single leaf galled by two different species, as noted in Chapter 6.

As discussed above, *Fergusonina-Fergusobia* gall sampling has been wide-ranging but scattered, with comprehensive sampling only within a few regions in

eastern Australia. As new plant hosts and gall types are frequently discovered, there are likely to be important groups still missing from the Fergusoninidae phylogeny that could shed more light on the evolution of such features as host plant choice, gall type and dorsal shield morphology.

7.4.1 Outstanding questions

Many other important features of the *Fergusonina-Fergusobia* system are still matters for conjecture, and while some answers are elusive, chemical analysis could perhaps identify differences in male and female larvae that might repel or attract the pre-parasitic female nematodes, or elucidate the composition of the mucous substance found coating the larvae in one lineage of leaf blade gallers. The means by which the infective nematodes enter the female fly larvae are still unclear (Davies *et al*. 2016), but could be determined by dissection and staining, or possibly be observable *in vitro* (Davies *et al*. 2016) or using MicroCT scanning, as discussed in chapter 6. It is unknown why male nematodes sometimes penetrate the flies, albeit rarely (Davies and Lloyd 1996; Davies *et al*. 2001).

Other important questions include:

- What determines female oviposition choice, and to what extent is the successful colonisation of a novel plant host driven by either the flies' oviposition behaviour or the nematodes' ability to induce galls on an unfamiliar plant?
- How far can they disperse?
- Are nematode numbers within the fly larvae regulated, and if so, how?
- Can and do the nematodes move between locules as the gall is forming?
- How do the larvae use the dorsal shield, and does it play a part in their interactions with the nematodes?

- Are some gall types less tolerant of environmental stresses and how might climate change affect them?

Common but rarely seen, these little-known gallers of Australia's most iconic flora deserve more attention. With their plant hosts and nematode mutualists they provide a unique model for studying coevolutionary relationships on three trophic levels (perhaps more, if one were to include the parasitoids and hyperparasitoids commonly found within *Fergusonina-Fergusobia* galls) as well as offering insights into the fascinating but still poorly-understood behaviour of gall-inducing organisms.

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APPENDIX I

All recorded *Fergusonina* host associations and gall types to date. Gall types may be listed more than once on the same host if there is distinguishing larval morphology or insufficient information to determine whether they are the same species. Those highlighted in green were discovered during the course of this study.

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
Angophora								
A. apocynifolia						?	QLD	**14**
A. bakeri						M-SBG	QLD	**29**
A. costata						LB	QLD	**29, 32**
A. floribunda					*Fb. floribundae*	SBG	NSW	**18,28,34**
A. floribunda					*Fb. colbrani*	LB	NSW	**30**
A. floribunda						M-PG stem	NSW	**30**
A. floribunda						Stem	NSW	**30**
A. floribunda						FL	NSW	**30**
A. floribunda						M-PG	NSW	MF Purcell unpub. data
A. subvelutina						M-SBG	QLD	**14, 29**
Corymbia								
C. abbreviata		Rufaria	..	*Fn. thomasi*		M-FBG	WA	**16, 34**
C. citriodora ssp. variegata		Politaria	..			M-SBG	QLD, NSW	**14, 34**
C. gummifera		Rufaria	..			LB	NSW	MF Purcell unpub. data
C. gummifera		Rufaria	..			M-SBG	NSW	MF Purcell unpub. data
C. hylandii			..			LB	QLD	MF Purcell unpub. data
C. intermedia		Rufaria	..			?	?	**32**
C. maculata		Politaria	..	*Fn. biseta*		M-FBG	NSW	**2, 3, 4, 16**
C. maculata		Politaria	..	*Fn. eucalypti*		M-FBG	NSW	**3, 4, 10**
C. maculata		Politaria	..	*F. gurneyi*		M-FBG	NSW	**2, 3, 4, 16**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
C. maculata		Politaria	..			M-SBG (prob. FL)	NSW	**4**
C. maculata		Politaria	..			M-SBG	NSW	MF Purcell unpub. data
C. maculata		Politaria	..			M-SBG	VIC	**19**
C. maculata		Politaria	..			LB	QLD	**34**
C. maculata		Politaria	..			M-SBG	NSW	**34**
C. papuana		Blakearia	..	*?*	?	?	PNG	**14**
C. ptychocarpa		Rufaria	..	*Fn. giblindavisi*	*Fb. ptychocarpae*	M-FBG	QLD	**11,16, 18, 34**
C. tessellaris		Blakearia	..		*Fb. magna*	M-SBG	QLD	**6, 11, 18, 19, 34**
C. torelliana		Cadagaria	..			M-SBG	QLD	**34**
C. trachyphloia		Apteria	..			?	QLD	**14**
C. variegata			..			M-SBG	NSW	DK Yeates pers. comm
Eucalyptus								
E. acmenoides	Eucalyptus	Amentum	..			M-SBG	QLD	**34**
E. aggregata	Symphyomyrtus	Maidenaria	Foveolatae			?	?	**32**
E. ?aggregata	Symphyomyrtus	Maidenaria	Foveolatae			FL	ACT	MF Purcell unpub. data
E. albens	Eucalyptus	Adnataria	Buxeales			LB	NSW, SA, VIC	**14**
E. amygdalina	Eucalyptus	Aromatica	..	*Fn. carteri ?*	*Fb. tumifasciens*	M-SBG		**3, 4, 18**
E. amygdalina	Eucalyptus	Aromatica	..	*Fn. frenchi*		leaf		**3, 4**
E. amygdalina	Eucalyptus	Aromatica	..	*Fn. pescotti*		leaf	VIC	**3, 4**
E. amygdalina	Eucalyptus	Aromatica	..	*?*		M-SBG	TAS	**18**
E. andrewsii	Eucalyptus	Cineraceae	Psathyroxylon			M-SBG	QLD	**33**
E. andrewsii	Eucalyptus	Cineraceae	Psathyroxylon			LB	QLD	**33**
E. aromaphloia	Symphyomyrtus	Maidenaria	Acaciiformes			FL	SA, VIC	**14**
E. baueriana	Symphyomyrtus	Adnataria	Heterophloiae			?	?	**32**
E. baxteri	Eucalyptus	Capillulus	..	*Fn. williamensis*		M-SBG	VIC	**21**
E. baxteri	Eucalyptus	Capillulus	..			M-FBG	VIC	**34**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. ?biturbinata	Symphyomyrtus	Latoangulatae	Lepidotae-Fimbriatae			M-PG	QLD	MF Purcell unpub. data
E. blakelyi	Symphyomyrtus	Exsertaria	Erythroxylon	*Fn. tillyardi*	*Fb. curriei*	M-FBG	ACT	**3, 4, 11**
E. blakelyi	Symphyomyrtus	Exsertaria	Erythroxylon			M-FBG	ACT	MF Purcell unpub. data
E. blakelyi	Symphyomyrtus	Exsertaria	Erythroxylon			M-SBG	ACT	**33**
E. blakelyi	Symphyomyrtus	Exsertaria	Erythroxylon			LB	ACT	MF Purcell unpub. data
E. botryoides	Symphyomyrtus	Latoangulatae	Annulares			M-SBG	NSW	MF Purcell unpub. data
E. brevifolia	Symphyomyrtus	Platysperma	..			Petiole	WA	**14, 18**
E. bridgesiana	Symphyomyrtus	Maidenaria	Bridgesianae	*Fn. carteri*	*Fb. tumifasciens*	FL	ACT, NSW	**3, 4, 10, 18**
E. bridgesiana	Symphyomyrtus	Maidenaria	Bridgesianae			Leaf and petiole	ACT	**4**
E. bridgesiana	Symphyomyrtus	Maidenaria	Bridgesianae			M-FBG	ACT	MF Purcell unpub. data
E. bridgesiana	Symphyomyrtus	Maidenaria	Bridgesianae			Axial UPG	ACT	MF Purcell unpub. data
E. burgessiana	Eucalyptus	Eucalyptus	Strictae			M-SBG	NSW	**33, 35**
E. burgessiana	Eucalyptus	Eucalyptus	Strictae			LB	JBT	**33, 35**
E. burgessiana	Eucalyptus	Eucalyptus	Strictae			M-FBG	JBT	**35**
E. burgessiana	Eucalyptus	Eucalyptus	Strictae			U-FBG	JBT	MF Purcell unpub. data
E. camaldulensis	Symphyomyrtus	Exsertaria	Rostratae	*Fn. lockharti*	*Fb. brittenae*	M-SBG	SA	**3, 4, 17, 18**
E. camaldulensis	Symphyomyrtus	Exsertaria	Rostratae	*Fn. tillyardi*	*Fb. curriei*	M-FBG	SA	**3, 5, 10, 11, 18**
E. camaldulensis	Symphyomyrtus	Exsertaria	Rostratae		*Fb. camaldulensae*	Stem	SA	**18, 23**
E. camaldulensis	Symphyomyrtus	Exsertaria	Rostratae		*Fb. schmidti*	M-SBG	SA	**31, 34**
E. camaldulensis	Symphyomyrtus	Exsertaria	Rostratae			Stem		**29**
E. cladocalyx	Symphyomyrtus	Sejunctae	..			M-SBG		**18**
E. cladocalyx	Symphyomyrtus	Sejunctae	..			Stem & leaf		**18**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. cloeziana	Idiogenes	..	..			LB	ACT	MF Purcell unpub. data
E. coccifera	Eucalyptus	Aromatica	..			LB	TAS	**30, 32**
E. confluens	Symphyomyrtus	Platysperma	..			M-SBG	WA	**14**
E. coolabah	Symphyomyrtus	Adnataria	Aquilonares			?	SA	**14**
E. cosmophylla	Symphyomyrtus	Incognitae	..		*Fb. cosmophyllae*	M-SBG	SA	**18, 28, 34**
E. crebra	Symphyomyrtus	Adnataria	Siderophloiae	*Fn. brimblecombei*		M-FBG	QLD	**3, 4, 11**
E. cunninghamii	Eucalyptus	Eucalyptus	Strictae			M-FBG	NSW	**33, 35**
E. cunninghamii	Eucalyptus	Eucalyptus	Strictae			M-SBG	NSW	**35**
E. cupularis	Symphyomyrtus	Exsertaria	Subexsertae			?	?	**32**
E. dalrympleana	Symphyomyrtus	Maidenaria	Viminales	*Fn. thornhilli*		FL	NSW	**21**
E. dalrympleana	Symphyomyrtus	Maidenaria	Viminales			Axial U-PG	ACT	MF Purcell unpub. data
E. dalrympleana	Symphyomyrtus	Maidenaria	Viminales			M-SBG	NSW	MF Purcell unpub. data
E. dealbata	Symphyomyrtus	Exsertaria	Erythroxylon			M-SBG	NSW	**14**
E. deglupta	Telocalyptus		Degluptae		*Fb. brevicauda*	FBG	Philippines	**8, 10, 18**
E. deglupta	Telocalyptus		Degluptae		*Fb. philippinensis*	FBG	Philippines	**8, 10, 18**
E. delegatensis	Eucalyptus	Cineraceae	Fraxinales		*Fb. delegatensae*	M-SBG	TAS	**18, 28**
E. delegatensis	Eucalyptus	Cineraceae	Fraxinales			M-SBG	ACT	**35**
E. delegatensis	Eucalyptus	Cineraceae	Fraxinales			Axial U-PG	ACT	**33**
E. delegatensis	Eucalyptus	Cineraceae	Fraxinales			LB	VIC	**35**
E. diversifolia	Eucalyptus	Longistylus	..		*Fb. diversifoliae*	M-SBG	SA	**11, 18, 28**
E. diversifolia	Eucalyptus	Longistylus	..			M-SBG	SA	**34**
E. dives	Eucalyptus	Aromatica	Radiatae			M-SBG	NSW, ACT	**33**
E. dives	Eucalyptus	Aromatica	Radiatae			Axial U-PG	ACT	**33**
E. dives	Eucalyptus	Aromatica	Radiatae			M-FBG	ACT	**33**
E. dives	Eucalyptus	Aromatica	Radiatae			LB	ACT	**33**
E. dorrigoensis	Symphyomyrtus	Maidenaria	Microcarpae			M-FBG	ACT	MF Purcell unpub. data
E. elata	Eucalyptus	Aromatica	Radiatae			FL	ACT	**33**
E. elata	Eucalyptus	Aromatica	Radiatae			M-FBG	ACT	**35**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. elata	Eucalyptus	Aromatica	Radiatae			M-SBG	ACT	**35**
E. elata	Eucalyptus	Aromatica	Radiatae			LB	ACT	**33, 35**
E. elata	Eucalyptus	Aromatica	Radiatae			LB	NSW	**35**
E. elata	Eucalyptus	Aromatica	Radiatae			M-FBG	ACT & NSW	**35**
E. eugenioides	Eucalyptus	Capillulus	..		*Fb. eugenioidae*	M-FBG	ACT	**18, 24**
E. fasciculosa	Symphyomyrtus	Adnataria	Heterophloiae		*Fb. fasciculosae*	Stylet gall	SA	**18**
E. fastigata	Eucalyptus	Eucalyptus	Regnantes			M-SBG	NSW	**33, 35**
E. fastigata	Eucalyptus	Eucalyptus	Regnantes			LB	NSW	**33**
E. fastigata	Eucalyptus	Eucalyptus	Regnantes			Axial U-PG	NSW	**33**
E. fastigata	Eucalyptus	Eucalyptus	Regnantes			U-FBG	NSW	MF Purcell unpub. data
E. fibrosa ssp. fibrosa	Symphyomyrtus	Adnataria	Siderophloiae		*Fb. morrisae*	M-FBG	QLD	**18, 24**
E. fraxinoides	Eucalyptus	Cineraceae	Fraxinales			LB	NSW	**35**
E. fraxinoides	Eucalyptus	Cineraceae	Fraxinales			M-FBG	NSW	**35**
E. fraxinoides	Eucalyptus	Cineraceae	Fraxinales			M-SBG	NSW	**33, 35**
E. globoidea	Eucalyptus	Renantheria	Capitellatae			M-FBG	NSW	MF Purcell unpub. data
E. globulus	Symphyomyrtus	Maidenaria	Globulares			M-SBG	ACT	MF Purcell unpub. data
E. gomphocephala	Symphyomyrtus	Bolites	..	*Fn. newmani*	*Fb. gomphocephalae*	Leaf and stem PG	WA	**3, 4, 18, 34**
E. haemastoma	Eucalyptus	Cineraceae	..			M-SBG	NSW	**34**
E. hemiphloia	Symphyomyrtus	Adnataria	Buxeales	*Fn. brimblecombei*		M-FBG	VIC	**4, 11**
E. hemiphloia	Symphyomyrtus	Adnataria	Buxeales	*Fn. morgani*		M-FBG		**3, 4**
E. interstans	Symphyomyrtus	Liberivalvae	..			?	SA	**14**
E. intertexta	Symphyomyrtus	Adnataria	Buxeales			?	?	**32**
E. johnstonii	Symphyomyrtus	Maidenaria	Semiunicolores			U-PG	TAS	**14, 30**
E. lacrimans	Eucalyptus	Cineraceae	Pauciflorae	*Fn. omlandi*		M-SBG	ACT, NSW	**33, 35**
E. lacrimans	Eucalyptus	Cineraceae	Pauciflorae			LB	NSW	**35**
E. lacrimans	Eucalyptus	Cineraceae	Pauciflorae			U-FBG	NSW	**33**
E. lacrimans	Eucalyptus	Cineraceae	Pauciflorae			M-FBG	NSW	**33, 35**
E. largiflorens	Symphyomyrtus	Adnataria	Buxeales			M-FBG	SA	**14**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. lesouefii	Symphyomyrtus	Dumaria	Rufispermae			?	?	**32**
E. leucoxylon hybrid	Symphyomyrtus	Adnataria	Melliodorae		*Fb. fisheri*	LB	?	**7, 11,18**
E. leucoxylon	Symphyomyrtus	Adnataria	Melliodorae		*Fb. sporangae*	Axial U-PG	?	**18**
E. leucoxylon	Symphyomyrtus	Adnataria	Melliodorae		*Fb. leucoxylonae*	M-PG	ACT	**18, 31, 33**
E. leucoxylon	Symphyomyrtus	Adnataria	Melliodorae			M-SBG	?	**18**
E. leucoxylon	Symphyomyrtus	Adnataria	Melliodorae			LB	ACT	**34**
E. leucoxylon	Symphyomyrtus	Adnataria	Melliodorae			LB	ACT	**33**
E. ligustrina	Eucalyptus	Capillulus	Pachyphloius			M-SBG	NSW	**33**
E. lockyeri	Symphyomyrtus	Exsertaria	Phaeoxylon			?	?	**32**
E. loxophleba	Symphyomyrtus	Bisectae	Loxophlebae			?	?	**32**
E. macarthurii	Symphyomyrtus	Maidenaria	Foveolatae			FL	ACT	**33**
E. macarthurii	Symphyomyrtus	Maidenaria	Foveolatae			Axial U-PG	ACT	MF Purcell unpub. data
E. macrorhyncha	Eucalyptus	Capillulus	Pachyphloius	*Fn. curriei*		M-SBG	ACT	**3, 4**
E. macrorhyncha	Eucalyptus	Capillulus	Pachyphloius	*Fn. nicholsoni*	*Fb. juliae*	M-FBG	SA, ACT	**3, 4, 11, 18, 24**
E. macrorhyncha	Eucalyptus	Capillulus	Pachyphloius			M-SBG	ACT	**4**
E.macrorhyncha	Eucalyptus	Capillulus	Pachyphloius			M-SBG	ACT	**33**
E. macrorhyncha	Eucalyptus	Capillulus	Pachyphloius			LB	ACT	**33**
E. mannifera	Symphyomyrtus	Maidenaria	Microcarpae			M-FBG	NSW	MF Purcell unpub. data
E. mannifera	Symphyomyrtus	Maidenaria	Microcarpae			FL	ACT	**33**
E. mannifera	Symphyomyrtus	Maidenaria	Microcarpae			Axial UPG	ACT	**33**
E. mannifera	Symphyomyrtus	Maidenaria	Microcarpae			petiole and stem	ACT	**33**
E. mannifera subsp. maculosa	Symphyomyrtus	Maidenaria	Microcarpae			Leaf	ACT	**4**
E. marginata	Eucalyptus	Longistylus	..			LB	WA	**34**
E. melanophloia	Symphyomyrtus	Adnataria	Siderophloiae	*Fn. brimblecombei*		M-FBG	QLD	**3, 4, 11**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae	*Fn. evansi*		leaf	ACT	**3, 4**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			LB	ACT	**33, 34**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			LB (v. flat)	ACT	**33**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			Axial U-PG	ACT	**33**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			LB	ACT, VIC, NSW	**4**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			U-PG	ACT	**34**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			Stem U-PG	ACT	**33**
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			M-SBG	ACT	MF Purcell unpub. data
E. melliodora	Symphyomyrtus	Adnataria	Melliodorae			M-PG	ACT	**33**
E. microcarpa	Symphyomyrtus	Adnataria	Buxeales			Pea gall	?	**18**
E. microcarpa	Symphyomyrtus	Adnataria	Buxeales			M-FBG	?	**18**
E. microcarpa	Symphyomyrtus	Adnataria	Buxeales		*Fb. microcarpae*	LB	?	**18**
E. microcarpa	Symphyomyrtus	Adnataria	Buxeales			LB	SA	**34**
E. ?microcarpa	Symphyomyrtus	Adnataria	Buxeales			Axial U-PG	SA	**34**
E. nicholii	Symphyomyrtus	Maidenaria	Acaciiformes			Axial U-PG	ACT	MF Purcell unpub. data
E. nitida	Eucalyptus	Aromatica	Insulanae			M-SBG	TAS	**14, 18**
E. notabilis	Symphyomyrtus	Latoangulatae	Annulares			FL	QLD	**33**
E. notabilis	Symphyomyrtus	Latoangulatae	Annulares			M-SBG	QLD	MF Purcell unpub. data
E. obliqua	Eucalyptus	Eucalyptus	..			M-FBG	SA	**11, 18, 34**
E. obliqua	Eucalyptus	Eucalyptus	..			M-SBG	SA, NSW	**30, 33**
E. odorata	Symphyomyrtus	Adnataria	Buxeales	*Fn. brimblecombei*		M-FBG	SA	**4, 11**
E. ?odorata	Symphyomyrtus	Adnataria	Buxeales			M-Flat leaf	SA	**34**
E. olsenii	Eucalyptus	Nebulosa	Olsenianae			M-SBG	ACT	**35**
E. olsenii	Eucalyptus	Nebulosa	Olsenianae			M-FBG	NSW	**35**
E. ovata	Symphyomyrtus	Maidenaria	Foveolatae			?	?	**32**
E. ??paniculata	Symphyomyrtus	Adnataria	Rhodoxylon			M-SBG	NSW	MF Purcell unpub. data
E. parramattensis	Symphyomyrtus	Liberivalvae	..			?	?	**32**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae	*Fn. taylori*		M-SBG	VIC, ACT	**4, 20**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae			U-FBG	ACT	**4, 34, 33**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae			LB	?	**4, 34**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae			LB	NSW	**33**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae			M-FBG	NSW	**33, 35**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae			M-FBG	TAS	**34**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae	*Fn. omlandi*		M-SBG	NSW	**21, 33, 35**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae	*Fn. tasmaniensis*		M-SBG	TAS	**22**
E. pauciflora	Eucalyptus	Cineraceae	Pauciflorae	*Fn. daviesae*		M-SBG	NSW, ACT	**20, 33**
E. piperita	Eucalyptus	Cineraceae	Piperitales			M-SBG	NSW	**33**
E. planchoniana	Eucalyptus	Insolitae	..		*Fb. planchionianae*	FL	QLD	**18, 30**
E. platyphylla	Symphyomyrtus	Exsertaria	Subexsertae			M-SBG	QLD	MF Purcell unpub. data
E. platypus	Symphyomyrtus	Bisectae	Erectae			?	?	**32**
E. polyanthemos	Symphyomyrtus	Adnataria	Heterophloiae	*Fn. greavesi*		Axial U-PG	ACT, NSW	**2, 4, 30, 33**
E. ? polyanthemos	Symphyomyrtus	Adnataria	Heterophloiae			LB	ACT	**34, 33**
E. polybractea	Symphyomyrtus	Adnataria	Buxeales			?	?	**32**
E. polybractea	Symphyomyrtus	Adnataria	Buxeales			LB	NSW	MF Purcell unpub. data
E. populnea	Symphyomyrtus	Adnataria	Buxeales			?	?	**32**
E. porosa	Symphyomyrtus	Adnataria	Buxeales			M-SBG		**18**
E. porosa	Symphyomyrtus	Adnataria	Buxeales		*Fb. porosae*	LB	SA	**18, 27, 34**
E. pruinosa	Symphyomyrtus	Adnataria	Buxeales			M-FBG	WA	**34**
E. racemosa	Eucalyptus	Cineraceae	Psathyroxylon			M-SBG	QLD, NSW	**11, 18, 33**
E. racemosa	Eucalyptus	Cineraceae	Psathyroxylon			Stem U-PG	NSW	MF Purcell unpub. data
E. rossii	Eucalyptus	Cineraceae	Psathyroxylon			M-SBG	ACT	**33**
E. rossii	Eucalyptus	Cineraceae	Psathyroxylon			M-FBG	NSW	MF Purcell unpub. data
E. rossii	Eucalyptus	Cineraceae	Psathyroxylon			Axial U-PG		MF Purcell unpub. data
E. radiata	Eucalyptus	Aromatica	Radiatae			M-SBG	ACT	**33, 35**
E. radiata	Eucalyptus	Aromatica	Radiatae			LB	NSW	**33, 35**
E. radiata	Eucalyptus	Aromatica	Radiatae			M-FBG	NSW	**33, 35**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. radiata	Eucalyptus	Aromatica	Radiatae			Axial U-PG	NSW	MF Purcell unpub. data
E. radiata	Eucalyptus	Aromatica	Radiatae			LB	NSW	**33**
E. rubida	Symphyomyrtus	Maidenaria	Viminales			Axial U-PG	ACT	MF Purcell unpub. data
E. robusta	Symphyomyrtus	Latoangulatae	Annulares			M-SBG	SA	**34**
E. rudis	Symphyomyrtus	Exsertaria	Singulares	*Fn. lockharti*	*Fb. brittenae*	M-SBG	?	**3, 4, 17**
E. siderophloia	Symphyomyrtus	Adnataria	Siderophloiae			LB	?	**11, 12, 18, 23, 34**
E. siderophloia	Symphyomyrtus	Adnataria	Siderophloiae			FL	?	KA Davies unpub. data
E. ?siderophloia or ?fibrosa	Symphyomyrtus	Adnataria	Siderophloiae			LB	QLD	**18**
E. ?siderophloia or ?fibrosa	Symphyomyrtus	Adnataria	Siderophloiae			Stem and leaf PG	?	**18**
E. sideroxylon	Symphyomyrtus	Adnataria	Melliodorae			LB	ACT, VIC, NSW	**4, 33**
E. sideroxylon	Symphyomyrtus	Adnataria	Melliodorae			Axial U-PG	ACT	**33**
E. sideroxylon	Symphyomyrtus	Adnataria	Melliodorae			M-PG	ACT	MF Purcell unpub. data
E. sieberi	Eucalyptus	Cineraceae	Psathyroxylon			M-SBG	NSW	**33**
E. smithii	Symphyomyrtus	Maidenaria	Compactae			M-SBG	NSW	**33**
E. stellulata	Eucalyptus	Longitudinales	..	*Fn. herbaservus*		M-SBG	NSW	**25, 34**
E. stellulata	Eucalyptus	Longitudinales	..			M-FBG	ACT, NSW	**34**
E. stellulata	Eucalyptus	Longitudinales	..			LB	NSW	MF Purcell unpub. data
E. stricta	Eucalyptus	Eucalyptus	Strictae			M-FBG	NSW	MF Purcell unpub. data
E. stricta	Eucalyptus	Eucalyptus	Strictae	*Fn. omlandi*		M-SBG	NSW	**33**
E. tenuiramis	Eucalyptus	Aromatica	Insulanae			?	TAS	**14**
E. tereticornis	Symphyomyrtus	Exsertaria	Erythroxylon	*Fn. tillyardi*	*Fb. curriei*	M-FBG	VIC (21), QLD	**4, 11, 34**
E. tereticornis	Symphyomyrtus	Exsertaria	Erythroxylon		*Fb. minimus*	M-SBG	NSW, QLD	**18, 28**
E. tereticornis	Symphyomyrtus	Exsertaria	Erythroxylon			Pea gall		**18**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales	*Fn. manchesteri*		M-SBG	NSW	**25**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
E. viminalis	Symphyomyrtus	Maidenaria	Viminales		*Fb. viminalisae*	FL	NSW, ACT	**18, 30**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales			LB	SA, NSW	**34**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales			Axial U-PG	NSW	**33**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales			Stem U-PG sessile	NSW	**33**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales			U-SBG	NSW	**33, 35**
E. viminalis	Symphyomyrtus	Maidenaria	Viminales			LB	NSW	MF Purcell unpub. data
E. yalatensis	Symphyomyrtus	Bisectae	Subulatae			?	?	**32**
E. zopheraphloia	Symphyomyrtus	Bisectae	Accedentes			?	?	**32**
Leptospermum								
L. laevigatum					*Fb. leptospermum*	U-SBG	NSW	**36**
L. madidum				*Fn. madidum*		M-SBG	QLD	**36**
Melaleuca								
M. argentea						M-SBG	NT	**18, 34**
M. armillaris					*Fb. armillarisae*	U-PG	NSW	**13, 18, 27, 34**
M. cajuputi				*Fn. purcelli*	*Fb. cajuputiae*	M-SBG	QLD	**10, 13, 18, 34**
M. dealbata				*Fn. makinsoni*	*Fb. dealbatae*	M-SBG	QLD	**10, 13, 18, 34**
M. decora					*Fb. decorae*	U-PG	QLD	**18, 34**
M. fluviatilis				*Fn. turneri*	*Fb. quinquenerviae*	M-SBG	QLD	**10, 12, 13, 18, 34**
M. fluviatilis						M-SBG	QLD	**34**
M. leucadendra				*Fn. centeri*	*Fb. leucadendrae*	M-SBG	QLD	**10, 13, 18, 34**
M. leucadendra						M-SBG	WA	**13**
M. linariifolia					*Fb. linariifoliae*	U-PG	NSW	**18, 34**
M. nervosa				*Fn. goolsbyi*		Basal rosette	QLD	**10, 11, 13, 18, 34**

Host plant Species	Host Subgenus	Host Section	Host Series	*Fergusonina*	*Fergusobia*	Gall type	Location	References
M. nervosa				*Fn. schefferae*	*Fb. nervosae*	M-SBG	QLD	**10, 11, 13, 18, 34**
M. nodosa						U-PG	NSW	**18, 34**
M. quinquenervia				*Fn. turneri*	*Fb. quinquenerviae*	Rosette, M-SBG or M-FBG	QLD	**9, 10, 12, 13, 18**
M. quinquenervia					*Fb. rosettae*	Rosette shoot bud	QLD	**26**
M. stenostachya						M-SBG	QLD	**10, 11, 13, 18, 34**
M. viridiflora				*Fn. burrowsi*	*Fb. viridiflorae*	M-SBG	QLD	**10, 13, 18, 34**
M. viridiflora						M-SBG	QLD	**13**
M. viridiflora						M-SBG	NT	**13**
Metrosideros								
Metrosideros excelsa				*Fn. metrosiderosi*	*Fb. pohutukawa*	U-PG	NZ	**18, 15, 34**
Syzygium								
S. cumini				*Fn. syzygii*	*Fb. jambophila*	M-SBG	India	**19, 37**
S. luehmannii					*Fb. tolgaensis*	U-PG	QLD	**18, 23, 26**
Unknown								
Host unknown					*Fb. indica*			**6, 39**
Host unknown				*Fn. atricornis*				**2, 3, 4**
Host unknown				*Fn. microcera*				**1, 2, 3, 4**
Host unknown				*Fn. scutellata*				**2, 3, 4**
Host unknown				*Fn. flavicornis*			QLD	**2, 3, 4, 17, 38**
Angophora nr *woodsiana*					*Fb. pimpamensis*	M-SBG	QLD	**18, 28**
Corymbia sp.					*Fb. rileyi*	LB	NSW	**18, 23**
Eucalyptus sp.						LB	ACT	**18**

Host plant Species	**Host Subgenus**	**Host Section**	**Host Series**	***Fergusonina***	***Fergusobia***	**Gall type**	**Location**	**References**
Eucalyptus sp.						Axial U-PG	QLD	**18**
Eucalyptus sp.						LB	NSW	**18**
Eucalyptus sp.						M-PG		**18**
Eucalyptus nr E. acmenioides						M-SBG		**18**
Eucalyptus near E. tereticornis								**18**
Eucalyptus sp.				*Fn. davidsoni*				**3, 4**

1. Malloch 1924; **2.** Malloch 1932; **3.** Tonnoir 1937; **4.** Currie 1937; **5.** Fisher & Nickle 1968; **6.** Siddiqi 1986; **7.** Davies & Lloyd 1996; **8.** Siddiqi 1994; **9.** Giblin-Davis et al. 2001; **10.** Davies & Giblin-Davis 2004; **11.** Giblin-Davis *et al*. 2004; **12.** Scheffer *et al*. 2004; **13.** Taylor 2004; **14.** Taylor *et al*. 2005; **15.** Taylor *et al*. 2007; **16.** Taylor & Davies 2008; **17.** Taylor & Davies 2010; **18.** Davies *et al*. 2010a; **19.** Davies *et al*. 2010b; **20.** Nelson *et al*. 2011a; **21.** Nelson *et al*. 2011b; **22.** Nelson *et al*. 2012; **23.** Davies *et al*. 2012a; **24**. Davies *et al*. 2012b; **25.** Purcell *et al*. 2013; 26. Davies *et al*. 2014a; 27. Davies *et al*. 2013a; 28. Davies *et al*. 2013b; 29. Davies *et al*. 2014b; **30.** Davies *et al*. 2014c; **31.** Davies *et al*. 2014d; **32.** Nelson *et al*. 2014; **33.** Purcell *et al*. 2016; **34.** Scheffer *et al*. 2017; **35.** Purcell *et al*. 2017; **36.** Davies *et al*. 2017; **37.** Harris 1982; **38.** Taylor *et al*. 1996; **39.** Jairajpuri 1962.

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APPENDIX II

Descriptions of *Fergusonina microcera* taken from Malloch (1924; 1925; 1932) and Tonnoir (1937).

Genus FERGUSONINA, novum.

Generic characters.—Antennae inserted close to lower margin of eye in profile; head as in Figure 4, the antennae small and in distinct pits. Thorax as in *Agromyza,* the mesopleura and sternopleura bristled. Female with a chitinous tube-like ovipositor. Fore femur with rather long postero-ventral bristles. Costal vein ending a little beyond apex of second vein, third vein distinct, ending in apex of wing, fourth and fifth veins less distinct on apical portions than third, outer cross-vein evanescent or absent.

Genotype, the following species.

FERGUSONINA MICROCERA, n.sp.

Female.—Yellow, narrow rings surrounding ocelli, and the arista black. Dorsum of basal four visible abdominal tergites fuscous, ovipositor glossy black. Wings hyaline.

Ocellar and post-vertical bristles equal; each orbit with one bristle; arista subnude; mouth-parts small. Mesonotum with two pairs of dorsocentrals and one pair of acrostichals on hind margin; scutellum with four bristles, basal pair short. Legs strong, tarsi stout. Inner cross-vein below apex of first vein and nearly two-thirds from base of discal cell, last section of fourth vein over four times as long as preceding section. Length, 1.5 mm.

Type, North Harbour, 30 March, 1923.

Named in honour of Dr. E. W. Ferguson.

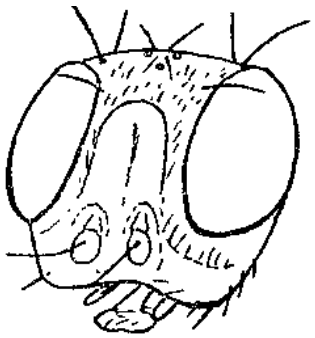

Fig. 4.—*Fergusonina microcera.* Head from the side and in front.

(Malloch 1924)

Genus Fergusonina Malloch.

In a lot of Diptera recently received from Dr. Ferguson I find four specimens of this genus, three of them males, which sex I did not have before me when I described the genus. I note that the venation of some of the specimens shows a departure from that of the genotype in having the outer cross-vein distinct. There are, however, no important changes necessary in the generic definition previously given, the remarkable flattened head, with the much enlarged frontal lunule, being characteristic of all.

I give below a key for the identification of the species now on hand.

Key to Species.

1. Head and thorax yellow in both sexes, only a blackish tinge round ocelli; venation of wing as in Figure 6 ..... *microcera* Malloch.

   Head with at least the ocellar spot, and thorax with at least partial vittae, black .. 2

2. Third antennal segment deep black; venation of wing as in Figure 7 .. *atricornis*, n. sp.
   Antennae entirely clear yellow 3

3. Disc of thorax with six black vittae, median pair extending from anterior margin to beyond suture, lateral pairs not extending to anterior margin, but reaching beyond hind margins of median pair; scutellum entirely yellow; venation of wing as in Figure 8 ..... *flavicornis*, n. sp.

   Disc of thorax black, lateral margins yellow; scutellum black at base; venation of wing as in Figure 9 ..... *scutellata*, n. sp

Fergusonina microcera Malloch.

The male of this species is all yellow, except dark rings round the ocelli. The abdominal and femoral bristles are mostly luteous.

Fifth visible abdominal tergite about as long as the two preceding tergites combined; hypopygium with base subglobose, the forceps long, slender, heavily chitinized, directed forward below venter. Hind femur stout, with a long preapical bristle and one or two shorter bristles basad of it on anteroventral surface.

Length, 1.5-1.75 mm.

Male, Sydney, N.S.W., 29.10.24.

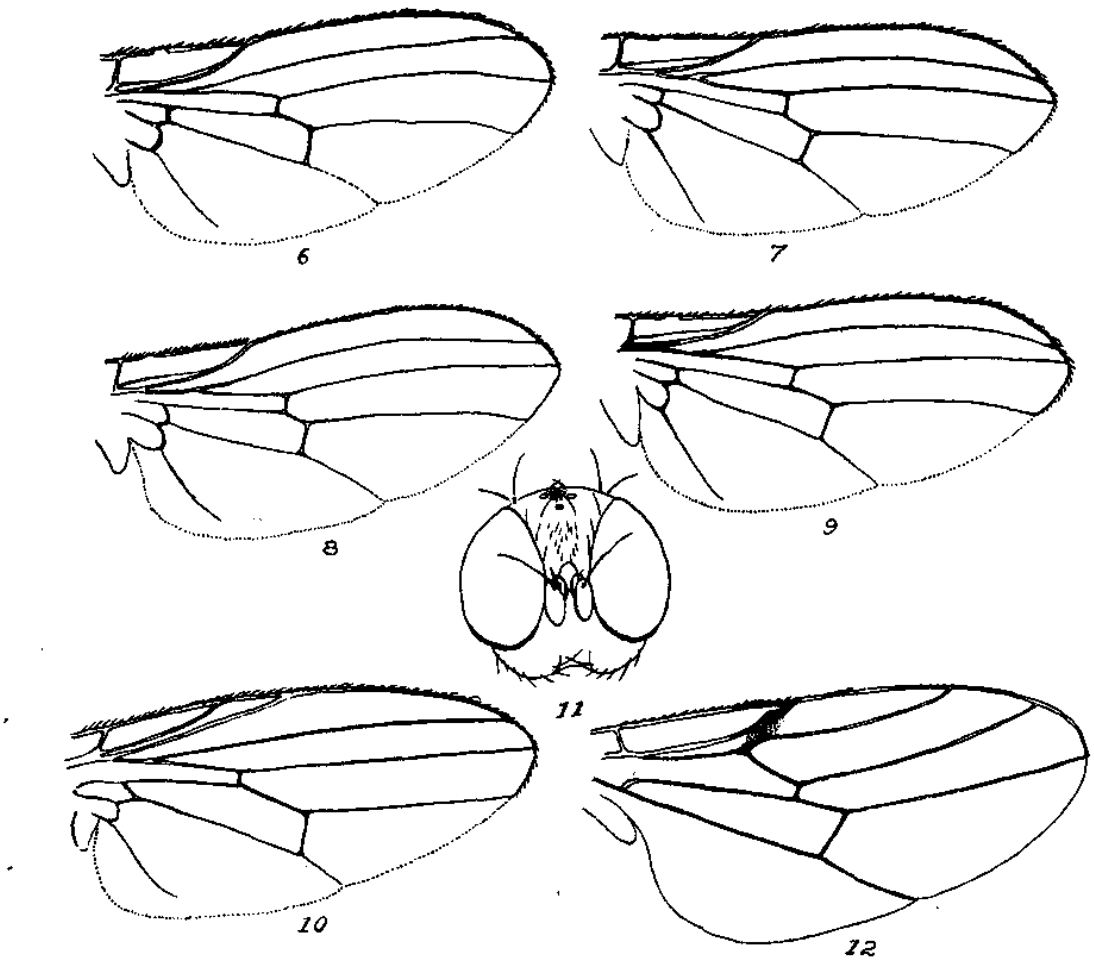

Fig. 6. *Fergusonina microcera*, wing.
Fig. 7. *Fergusonina atricornis*, wing.
Fig. 8. *Fergusonina flavicornis*, wing.
Fig. 9. *Fergusonina scutellata*, wing.
Fig. 10. *Pseudoleucopis magnicornis*, wing.
Fig. 11. *Aphaniosoma nigridorsum*, head from front.
Fig. 12. *Euhippelates pallidiseta*, wing.

(Malloch 1925)

Family Agromyzidae.

Genus Fergusonina Malloch.

Proc. Linn. Soc. N.S.W., 49, pt. 3, 1924, 337; *op. cit.*, 50, pt. 2, 1925, 90.

When I originally described this genus I had but one rather poorly preserved specimen before me. Subsequently I received another and better specimen of the genotype, and single specimens of three additional species, from which I described the species as indicated below. Until recently nothing was known of the larval habits, but in a recent shipment of reared material from Mr. W. B. Gurney there are examples of two species of the genus obtained from bud galls on seeds of *Eucalyptus maculatus*. It is very gratifying to me to learn that the genus named after the gentleman who was instrumental in having me work on the Australian Diptera is so clearly identified with an Australian plant and in all probability exclusively Australian.

Because of the condition of the type and the lack of material for comparison I erred in a few details of the original description and omitted some which did not at the time appear to me to be of consequence. These defects I correct herein and hope that the present paper will enable students to identify not only the genus, but all the species that have been described up to this time.

The postvertical bristles are placed close behind the ocellar triangle and are invariably divergent as shown in my original figure of the genotype; the ocellars are small, proclinate, and divergent, the frons is covered with short stiff hairs except on the large lunate or subtriangular central portion, which extends upward from the antennal bases to or above middle; the antennae are inserted close to or below lower margin of eyes, distant from these, and separated by a more or less well developed carina; the fronto-orbitals vary from two pairs to none, and the verticals are generally four in number, with the inner one on each side the smaller; antennae small, the arista almost bare; vibrissae developed or undeveloped; parafacials haired; proboscis small and stout; palpi of moderate length. Mesopleura and sternopleura setulose; mesonotum with two, rarely three, posterior pairs of postsutural dorsocentral bristles; scutellum with four bristles. Ovipositor of female in the form of a long slender chitinized cone, hypopygium of male rather inconspicuous. Costal vein weak beyond apex of third vein, sometimes practically indistinguishable on its apical section.

Genotype, *Fergusonina microcera* Malloch.

Fergusonina microcera Malloch.

Proc. Linn. Soc. N.S.W., 49, 1924, 338; *op. cit.*, 50, 1925, 92.

The genotype was described from a female, and subsequently I described the male.

Localities.—North Harbour, and Sydney, N.S.W.

Key to the Species.

1. Third antennal segment black or fuscous, much darker than the basal two; costal vein quite well developed between apices of second and third veins and distinct under a transmitted light to apex of fourth vein; frontal orbits with the usual upper outwardly-directed bristles very weak or indistinguishable 2

   Antennae entirely yellow 3

2. Parafacials with but one series of short black hairs from about middle of frontal lunule to lower margin of eye; hind femur of male with some strong black setulae at apex on posterior side, stronger below; mesonotum with four conspicuous dull black vittae behind the suture ..... *atricornis* Malloch

   Parafacials with two or more series of short black hairs on entire extent; hind femur in neither sex with strong black hairs at apex on posterior side; mesonotum without distinct postsutural black vittae ..... *eucalypti*, n. sp.

3. Mesonotum entirely fulvous-yellow; cell between second and third wing veins not narrowed at apex 4

   Mesonotum with black marks on disc; cell between second and third wing veins generally distinctly narrowed at apex 5

4. Each frontal orbit with but one well developed outwardly-curved bristle on upper third ..... *microcera* Malloch

   Each frontal orbit with two rather shorter outwardly-curved bristles on upper third ..... *biseta*, n. sp.

5. Mesonotum with the disc broadly black, the usual vittae so closely contiguous that the yellow ground colour is obliterated; scutellum yellow, the base dark; all sclerites of the pleura largely blackened; all the frontal bristles, including the ocellar, two pairs of orbitals, and the inner vertical well developed, the latter about half as long as the outer verticals ..... *scutellata* Malloch

   Disc of mesonotum with well defined blackish vittae, scutellum not black at base, the pleura less extensively blackened 6

6. Scutellum entirely yellow; mesonotum with six black vittae, the central pair much shortened posteriorly, the two on each side of them extending farther back, but not to posterior margin, all more or less broken at the suture; mesopleura with a slight dark mark near upper anterior angle, otherwise yellow .... *flavicornis* Malloch

   Scutellum blackened on each side at base; mesonotum with four black vittae, the outer one consisting of the two present in the above species, which are so closely contiguous that the yellow ground colour is completely obliterated, the central pair faint posteriorly but traceable to hind margin; mesopleura with an oblique black stripe from middle of anterior margin to lower posterior angle ..... *gurneyi*, n. sp.

(Malloch 1932)

REVISION OF THE GENUS *FERGUSONINA* MALL. (DIPTERA, AGROMYZIDAE).

By A. L. Tonnoir, Division of Economic Entomology, C.S.I.R., Canberra.

(*Communicated by Dr. G. A. Currie.*)

(Sixteen Text-figures.)

[Read 30th June, 1937.]

Although the genus *Fergusonina* has been erected within recent years by Malloch (1924), a revision of it has already become necessary on account of the many species brought to light by the work of Dr. G. A. Currie on the early stages of these interesting gall makers. No less than 19 species are now known to us, many of which would not have been easily detected but for the evident specific characters exhibited in the larval stages and also, in many instances, by the shape of the gall. There is no doubt that a large number of other species are awaiting discovery, since each species of *Eucalyptus* seems to have its corresponding species of *Fergusonina*, and even in some instances several of them, according to the part of the tree on which the galls are found.

As many of the newly-discovered species could not readily be distinguished from the seven known ones by means of the few characters, mainly of coloration, used by Malloch in his descriptions, a more complete study, including the genitalia of all the species, and especially of the known ones, became imperative. This revision was greatly facilitated through Mr. Malloch's kindness in making his types available to the writer; they will eventually be returned to the institutions from which he had received them for study.

The seven species described by Malloch are: *F. microcera*, genotype (♀ 1924, ♂ 1926; gall unknown), *F. atricornis* (♂ 1925; gall unknown), *F. flavicornis* (♀ 1925; gall unknown), *F. scutellata* (♂ 1925; gall unknown), *F. biseta* (♂ 1932, from galls of *E. maculata*), *F. gurneyi* (♂, ♀ 1932, from galls of *E. maculata*), *F. eucalypti* (♂, ♀ 1932, from galls of *E. maculata*).

All except the last two were described from single specimens; both sexes of *F. eucalypti* and *F. gurneyi* were obtained through breeding from a certain type of gall, but the two sexes of *F. microcera* have been collected in the field, in different localities, near Sydney. It is therefore doubtful whether they actually belong to the same species since the multiplicity of forms is so great. The flies of both sexes given as belonging to the same new species described in this paper have always been obtained from the same type of galls on the same host; it is, therefore, very likely that the correlation is correct. All errors are, however, not completely excluded by this method, since two species, such as *F. eucalypti* and *F. gurneyi*, may sometimes breed in very similar galls on the same part of the tree.

It is remarkable that none of the twelve new species bred recently from known galls can be referred to any of the seven described species, yet the localities of some of them are not so very far apart; this shows that the number of species must be very large indeed.

...

Key to Species.

1. Antennae partly black or brownish 2
   Antennae completely yellowish 4
2. Third antennal segment black or brown, orbital bristles very small 3
   Second antennal segment blackish-brown, sometimes rather faintly; four complete dark vittae on the mesonotum and longitudinal blackish streaks on pleurae; orbital bristles well developed ..... *F. carteri*, n. sp.
3. Mesonotum shining without dark vittae or markings; parafacials wide, with two rows of setulae; wing-length 3 mm. ..... *F. eucalypti* Mall.
   Mesonotum dull, with dark markings on the side past the suture; wing-length 2·2 mm. ..... *F. atricornis* Mall.
4. Posterior cross-vein missing or represented only by a very small stump on the median vein* 5
   Posterior cross-vein complete 6
5. Genitalia as in fig. 4 ..... *F. evansi*, n. sp.
   Genitalia as in fig. 5 ..... *F. davidsoni*, n. sp.
6. Legs extensively dark or with a few small dark markings 7
   Legs completely yellow 9
7. Mesonotum black with exception of the side margins, dark markings on the femora only ..... *F. scutellata* Mall.
   Mesonotum mostly yellowish-orange or else the four dark vittae are not fused together and the area in front of the scutellum is yellow 8
8. Legs with small dark markings on the tibiae only ..... *F. brimblecombi*, n. sp.
   Legs with extensive black markings on the femora and tibiae, hypopygium blackish ..... *F. morgani*, n. sp.
9. Mesonotum extensively dark or with four almost complete dark vittae 10
   Mesonotum without dark markings or at most with a few faint ones past the suture 15
10. Mesonotum extensively dark, the dark vittae being fused or almost fused and extending to the scutellum 11
    The dark vittae well separated, or if fused the area in front of the scutellum is yellowish 12
11. The mesonotal vittae completely fused ..... *F. scutellata* Mall.
    The vittae distinctly separated by very thin yellow streaks ..... *F. gurneyi* Mall.
12. No dark markings on pleurae or alae, or else they are small and faint 13
    Dark markings on pleurae extensive 14
13. Four complete dark vittae on the mesonotum, the lateral ones not split longitudinally past the suture and not extending on the alar callus ..... *F. pescotti*, n. sp.
    The four dark vittae somewhat interrupted before the suture, the lateral ones split past the suture so that there appear to be six vittae across the middle of the notum ..... *F. flavicornis* Mall.
14. Median vittae of mesonotum interrupted in their middle, area in front of scutellum and sides of the latter infuscated; mesopleurae almost completely dark; 8th abdominal tergite present in male and as big as the 9th .... *F. newmani*, n. sp.

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* *F. microcera*, whose type is the only known specimen, has the posterior cross-vein obsolete on one wing only; it is not placed in this section.

Median vittae complete and fused with the lateral ones; area in front of scutellum yellow; mesopleurae dark on their upper and lower margins; 8th abdominal segment apparently missing in the male ..... *F. lockharti*, n. sp.

15. Thorax entirely yellowish or orange without trace of darker vittae on the mesonotum past the suture or dark markings on the pleurae 16
    Mesonotum with a few dark markings past the suture* 19
16. Orbital and ocellar bristles very small, distance between the two cross-veins shorter than the anterior cross-vein ..... *F. frenchi*, n. sp.
    These bristles of normal length, distance between the two cross-veins sub-equal to the length of the posterior cross-vein 17
17. Only one orbital bristle ..... *F. microcera* Mall.

...

15. FERGUSONINA MICROCERA Mall.

PROC. LINN. SOC. N.S.W., xlix, 1924, p. 338; l, 1925, p. 91, fig. 6.

♀. *Head* yellow, a narrow black ring round each ocellus, the rest of the ocellar triangle yellow; antennae yellow; arista slightly rufous at base and distinctly pubescent. One large orbital only, behind which there is a small coarse hair directed outwards instead of forwards like all the other hairs of the vertex, it is also a little thicker than these and may represent the posterior orbital bristle. Vibrissae ochraceous at base, hair of face with a rufous tinge. *Thorax* completely rufous-yellow, contrasting with the bright yellow of the head, the postnotum somewhat infuscated. A row of five dorso-central bristles, the two anterior ones very small but yet distinct from the coarse hairs of mesonotum; prescutellar acrosticals longer than usual, somewhat more than half as long as the largest dorso-central. *Legs* yellow, the longer bristles of the femora rufous at base, hind femora not more incrassate than in other species. *Wing*: Costa extending only a little way over R_{2+3}, and the two branches of the radial sector distinctly divergent. Posterior cross-vein obsolete on one wing, nearly complete on the other; distance between the two cross-veins equal to length of the posterior one. *Abdomen*: Dorsum of the first five abdominal tergites fuscous; posterior border of the fifth and the sixth completely dull orange; the seventh glossy-black, except on basal half on the sides where it is luteous. Sixth segment with six dorsal submarginal bristles, the two outside ones being larger than the median ones; there are also numerous small bristles on the dorsal and ventral surfaces of these segments. The seventh segment carries many small bristles on all its surface proximal to the pairs of long subapical bristles. Wing-length 2 mm.

Holotype: North Harbour, Sydney, 30th March, 1923, E. Ferguson. In coll. N.S.W. Dept. of Health, Sydney.

I have not seen the male which was described by Malloch (loc. cit., p. 92), who may have retained this specimen. This male was collected by Dr. Ferguson at Sydney on 2nd October, 1924. As it was not bred from a gall with the female, it is somewhat doubtful, in view of the numerous species of this genus, whether it belongs to the same species as the female redescribed above. The only characters which may allow their being linked together are those of coloration: all yellow, the ocelli ringed with black. The hypopygium of this specimen, as described by Malloch, is quite different from that of the other members of the genus since "its forceps are long, slender, heavily chitinized and directed forward below the venter" (1921).

(Tonnoir 1937)

Malloch JR. 1924. Notes on Australian Diptera III. *Proceedings of the Linnean Society of New South Wales* **49**, 329-338.

Malloch JR. 1925. Notes on Australian Diptera VI. *Proceedings of the Linnean Society of New South Wales* **50**, 80-97.

Malloch JR. 1932 Notes on Australian Diptera XXXII. *Proceedings of the Linnean Society of New South Wales* **57**, 213-217.

Tonnoir A. 1937. Revision of the genus *Fergusonina* Mall. (Diptera, Agromyzidae). *The Proceedings of the Linnean Society of New South Wales* **62**, 126-146.