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Fusarium spp. associated with ambrosia beetles on *Acacia crassicarpa* in Indonesia

By

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DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

I, **Kira Mary Theresa Lynn** declare that the dissertation, which I hereby submit for the degree **Magister Scientiae** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any

other tertiary institution.

SIGNATURE:  .....

DATE: .....10th February 2020.....

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Preface

Ambrosia beetles are an ecological guild of plant associated insects that consume symbiotic fungi as a primary source of nutrition. In the ecosystems of their tropical and sub-tropical native ranges, most ambrosia beetle species live in decaying wood and generally remain economically inconsequential. However, changes in their ecological interactions, particularly their anthropogenic introduction into non-native regions, have resulted in a few ambrosia beetle species becoming some of the most important pests on various economically significant plant hosts. Despite this, the diversity of the fungal species associated with these emerging ambrosia beetle pests is understudied and requires further investigation. This dissertation consists of three chapters; a literature review and two research chapters.

The **first chapter** of this dissertation provides an overview of the general biology and ecology of ambrosia beetles and introduces examples of some of the most economically important ambrosia beetle pests affecting agricultural and forestry ecosystems globally. The potential factors that have led to the emergence of ambrosia beetles as pests is examined, and the three known distinct modes of ambrosia beetle damage are discussed. Finally, the need for basic research regarding the biodiversity and pathogenic potential of certain ambrosia beetles, and their fungal associates in their native environments, is discussed.

Most of the research on ambrosia beetle pests has been conducted in non-native environments where they have been accidently introduced and have caused significant damage to local forestry and agricultural sectors. An understanding of the ecology of ambrosia beetle pests in their native environment can provide a basis for better management strategies, that can be implemented to mitigate their impact if they are accidentally introduced into non-native regions. The **second chapter** aimed to better understand the ambrosia beetle and its corresponding fungal associate that

has emerged as a pest on the globally important forestry species *Acacia crassicarpa*, in its native environment of Indonesia. This chapter has been published in *Antonie van Leeuwenhoek* (DOI: 10.1007/s10482-020-01392-8).

The severe economic impacts caused by some of the most prolific invasive ambrosia beetles is largely attributed to the interaction of their fungal associates with naïve plants hosts that lack coevolved adaptation. Therefore, identifying the fungal associates of these emerging ambrosia beetle pests, and determining their diversity and pathogenic potential, is pivotal to understanding their disease biology. The **third chapter** aimed to investigate the diversity of the *Fusarium* fungi associated with two *Euwallacea* species affecting *A. crassicarpa* plantations in Riau, Indonesia.

The results of this research show the importance of understanding the genetic diversity and pathogenic potential of ambrosia beetles and their fungal associates in their native environment.

Chapter 1: Literature review

Ambrosia beetles and their fungal associates causing economic losses in forestry, agricultural and urban environments

Introduction

Ambrosia beetles (see Box 1 for definition) belong to the subfamilies Scolytinae and Platypodinae in the Curculionidae (Kirkendall et al. 2015). They are wood boring insects that are defined by a shared ecological strategy known as fungus farming, as all ambrosia beetles have a nutritional symbiosis with ambrosia fungi (see Box 1 for definition) (Farrell et al. 2001; Six 2012). There are approximately 3400 known species of ambrosia beetles, residing in 11 tribes. They are distributed around the globe and are associated with almost all terrestrial plant groups (Farrell et al. 2001; Hulcr et al. 2015). Most ambrosia beetles infest and reproduce in dead or compromised plant hosts where they perform important ecological roles (Batra 1967; Kirkendall et al. 2015; Hulcr and Stelinski 2017; Skelton et al. 2019). However, under certain circumstances, their activity can be detrimental to otherwise healthy plant hosts, causing substantial economic losses, particularly when they infest agricultural crops or forestry plantations (Ploetz et al. 2013; Raffa et al. 2015).

Diseases associated with ambrosia beetles have been recognised in urban landscapes, and in forestry and agricultural ecosystems, in geographically diverse locations (Ploetz et al. 2013; Smith and Hulcr 2015). The incidence and impact of diseases associated with ambrosia beetles is becoming more prevalent as a consequence of the homogenization of the world's biota via anthropogenic activities (Ploetz et al. 2013; Hulcr and Stelinski 2017). When ambrosia beetle infestations have an economic impact, a variety of interactions are evident. In this review, the general biology and ecology of ambrosia beetles is briefly summarized. Using selected examples of some of the most economically important ambrosia and closely related bark beetle pests, the biological characteristics and ecological circumstances that have led to the emergence of these pests, is considered. By examining these trends, it might be possible to predict, and thus prevent and/or mitigate future outbreaks.

Primary features of ambrosia beetles

Most bark and ambrosia beetles are in some way associated with fungi (Raffa et al. 2015). Ambrosia beetles are a group of unrelated weevil clades in the Curculionidae that fall at the end of a spectrum whereby xylomycetophagy, or strict mycophagy, are the ecological feeding strategies implemented (Beaver 1989; Kirkendall et al. 2015 and see Box 1 for definitions). Thus, rather than directly consuming plant host tissues, ambrosia beetles and their larvae predominantly

feed on symbiotic fungi as their main source of food (Beaver 1989; Farrell et al. 2001; Hulcr et al. 2015; Kirkendall et al. 2015).

After locating a suitable host, usually dead or stressed trees, ambrosia beetles' tunnel into the host xylem. During this process, they simultaneously release spores of their associated ascomycete fungi along the gallery walls (Batra 1963). The fungi then penetrate the xylem tissue of the infested plant, extracting nutrients, and concentrating these nutrients on and/or near the beetle gallery surface, where they are subsequently consumed by the beetle and larvae as their primary food source (Batra 1963). The strategy of farming and consuming fungi has been so successful that ambrosia beetles have independently evolved approximately 15 times from their bark beetle ancestors (Six 2012). Thus, the term 'ambrosia beetles' does not refer to a phylogenetic group or clade, because they do not share a common ancestor. The term rather refers to an ecological guild of beetles that exploit the same feeding strategy (Farrell et al. 2001; Hulcr et al. 2015).

Box 1 Terminology of specialised terms used within the review	
Ambrosia beetles	Ambrosia beetles include all Platypodinae and several Scolytinae, whose larvae and adults feed predominately on coevolved symbiotic ambrosia fungi, that are grown in fungal gardens cultivated by adults in tunnel systems in woody tissues, mainly the xylem. Wood may (xylomycetophagy) or may not (mycophagy) be consumed in the process.
Ambrosia fungi	Ambrosia fungi are a diverse group of fungal species associated with ambrosia beetles. Most confirmed fungal associates act as nutritional mutualists of ambrosia beetles and include species from various genera such as; *Ambrosiella, Raffaelea, Ambrosiozyma, Dryadomyces* and *Fusarium.*
Bark beetles	Members of Scolytinae that breed in the inner bark and whose larvae feed predominantly on the woody tissues, mainly the phloem (phloeophagous).
Frass	The dust that is pushed out during the boring process and gallery formation of tunnelling insect larvae or adults. The dust is a mixture of faeces and wood bits (digested or not).
Mycangium (plural mycangia)	Structure/s of an organism that are specialized to carry symbiotic fungi (usually in spore form).
Mycophagy	The feeding on fungal hyphae and conidia exclusively as a primary source of nutrition.
Non-reproductive hosts	Non-reproductive hosts are trees where primary beetles probe the host but find it unfavourable for brood establishment and either leave or

	die without reproducing. However, while probing, the beetle can inoculate the potential plant host with its associated fungus. The fungus can eventually kill or damage the host, making it more favourable for subsequent infestation by other primary and/or secondary ambrosia beetles.
Primary ambrosia beetles (Primary attackers)	In the context of this study, primary ambrosia beetles are the first to colonize and successfully establish a brood gallery in a host plant. Additionally, primary ambrosia beetles can colonize seemingly healthy, living plant tissue.
Reproductive hosts	Reproductive hosts are plant hosts in which infesting beetles complete their life cycle. These beetles colonize and establish a successful brood gallery in which fungal gardens are cultivated. These fungal gardens feed the developing larvae and young adults.
Secondary ambrosia beetles	In the context of this study, secondary ambrosia beetles attack dead or dying plant hosts, that have already been colonized by at least one aggressive primary ambrosia/bark beetle.
Xylomycetophagy	Ambrosia beetles whose adults and larvae consume cultivated, associated fungi but may also consume some wood while feeding on mycelia.
Xylophagy	Scolytinae taxa that establish brood galleries in sapwood and don't cultivate fungi.

The adults of most wood-boring insects leave their natal galleries to find food, mating partners and oviposition sites and only tunnel into wood as larvae (Kirkendall et al. 2015). In contrast, ambrosia beetles complete almost their entire life cycle within dead or compromised plant tissues, predominantly in the host's xylem (Hulcr and Stelinski 2017). This behaviour most likely evolved as an adaptive strategy due to the harshness of foraging in open landscapes (Kirkendall et al. 2015), where species are exposed to parasitoids and predators, and where they must compete for resources with other diverse insect communities.

The selective advantage to complete the ambrosia beetle lifecycle within dead/compromised plant tissues provides a protective environment for the beetle (Kirkendall et al. 2015). By removing the need to search for food and allowing the beetles to remain protected in the galleries throughout most of their life cycle, the evolution of xylomycetophagy has enabled ambrosia beetles to colonize an otherwise nutritionally poor environment and thrive in a niche that is exploited by few other insects (Batra 1966; Beaver 1989; Raffa et al. 2015; Kasson et al. 2016).

For most ambrosia beetle species, the associated fungi are stored and transported in a highly diverse and specialised array of structures, pockets or sacs, termed mycangia (Batra 1963 and see Box 1 for definition). One of the key factors in host selection is determined by the ability of the host to support the growth of the associated fungal gardens – allowing some ambrosia beetles to have a broad host spectrum (Gibson and Hunter 2010; Hulcr and Dunn 2011; You et al. 2015). For example, species in the *Euwallacea fornicatus sensu lato* species complex have been reported to successfully infest at least 412 plant species in 75 families across their native and introduced ranges (Gomez et al. 2019).

Despite their potentially large host range, most ambrosia beetles and their fungal associates remain ecologically restricted to dead host plants, as these have fewer defence mechanisms that must be overcome (Hulcr and Dunn 2011; Ranger et al. 2015; Hulcr et al. 2017). However, several ambrosia beetles, such as the red bay ambrosia beetle (*Xyleborus glabratus*) (Fraedrich et al. 2008; Hanuu et al. 2008), and the polyphagous shot hole borer (PSHB; *E. fornicatus*) (Eskalen et al. 2013), can colonize living plant tissues (Hulcr et al. 2017). To do this, the beetles carry necrotrophic or mild pathogens that possess some virulence factors that are able to suppress the defence mechanisms of living host plant tissue (Six and Wingfield 2011). Thus the ability to colonize living plant tissues is a normal part of the ecology of some ambrosia beetle species (Hulcr et al. 2017). However, it should be noted that the role of environmental and pre-existing conditions of the trees within the disease paradigm also needs to be considered, as these factors appear to influence the plant host selection of the beetle (Ranger et al. 2010, 2016; Hulcr and Stelinski 2017).

With the preferences of any particular ambrosia beetle species to colonize a plant host xylem being fairly dynamic, so too are the fungal symbionts associated with the beetle hosts (Six 2012). Ambrosia beetles are not only associated with nutritional fungal symbionts, but also harbour a diverse and promiscuous microbiota of organisms (Baker and Norris 1968; Beaver 1989; Kolařík et al. 2008; Gibson and Hunter 2010; Kostovcik et al. 2015; Lynch et al. 2016). Dominant, coevolved nutritional symbionts form the core community, but the beetle mycangia also carry many interchangeable fungi from the environment, some of which can be transient species and may include plant pathogens and endophytes (Beaver 1989; Hulcr and Dunn 2011). Many of these other associates have unknown functions (Kolařík and Hulcr 2009; Gibson and Hunter 2010; Kostovcik et al. 2015). However, some have been suggested to aid with detoxification of the host

plant tissues, thereby reducing the competition of secondary contaminants and enabling successful colonization of the nutritional symbionts (Lynch et al. 2016).

The effect and abundance of a certain fungal taxon on a plant host, and stored within the beetle mycangia, may vary with the type of beetle mycangia and with ecological context. For example, this can relate to the freshness of the host, the stage of gallery development, or the presence of other organisms (Kajimura and Hijii 1992; Klepzig et al. 2009). In the case of *E. fornicatus* (PSHB), *Graphium euwallaceae* is the most abundant fungal symbiont of the immature beetles, suggesting that it is the main food source (Lynch et al. 2016). As the beetles mature, the ratio of fungi within the mycangia changes and *Fusarium euwallaceae* becomes the most abundant fungal symbiont, suggesting this is the main food source of mature beetles (Lynch et al. 2016). Alternatively, depending on whether the beetle inoculates the fungi into living or dead trees, some symbiotic *Fusarium* or *Raffaelea* species appear to act as both a nutritional symbiont and an agent for disarming host defences (Norris and Baker 1968; Bumrungsri et al. 2008; Fraedrich et al. 2008; Hulcr and Dunn 2011). This ecological flexibility of the symbioses can become a threat for plants in non-native environments, where novel encounters can result in unpredictable consequences, including unprecedented death of the new plant hosts (Freeman et al. 2013; Hughes et al. 2017; Hulcr and Stelinski 2017).

Damage caused by ambrosia beetles

Ambrosia beetle infestations can cause serious damage to trees in three main ways. Firstly, they can cause structural damage and staining to the host trees via mass coordinated attacks, regardless of their microbial associates (Krokene 2015). The second mode of damage is through a combination of mass repeated infestations by the beetle and by the disease that is caused by their corresponding, moderately pathogenic microbial associates, on stressed hosts. Lastly, the most severe mode of ambrosia beetle damage involves microbial associates that after one infection, can result in host mortality or serious damage (Hulcr and Stelinski 2017).

The tree diseases that are caused by ambrosia beetles and their associated fungi are unusual given the biology of the beetles. Firstly, the fungi involved in ambrosia beetle-associated diseases did not evolve to be pathogenic, and don’t usually play a phytopathogenic role in their association

with their co-evolved beetle hosts. Rather they act as benign nutritional symbionts (Kühnholz et al. 2001; Hulcr and Dunn 2011; Ploetz et al. 2013). Secondly, ambrosia beetles typically infest dead or immunocompromised plant hosts in which the defence mechanisms utilized by healthy trees are weakened, allowing for successful colonization to occur (Batra 1967). When accidently introduced to naïve hosts, particularly in non-native regions, most species remain ecologically constrained to these stressed or dead hosts. However, some species undergo an apparent shift in host selection and predominantly colonize living plant tissue, sometimes leading to serious damage or disease emergence on infested hosts (Kühnholz et al. 2001; Ranger et al. 2010).

Shifts in the virulence of microbial associates from benign nutritional symbionts to phytopathogens and changes in plant host selection mechanisms of the beetle vectors from dead/dying to living plants hosts, is only beginning to be understood (Hulcr and Dunn 2011). What is clear is that such shifts in the genetic and environmental context in which ambrosia beetles dynamically interact with their plant hosts and associated microbiota, underlie the emergence of ambrosia beetle associated diseases (Hulcr and Dunn 2011; Ploetz et al. 2013). Some of the factors related to the development of the most significant ambrosia beetle–associated tree diseases are summarised in Table 1.

Table 1 Summary of some of the general factors that are related to the development of some of the most significant ambrosia beetle–associated tree diseases		
Factor	Brief summary of influencing factor	Examples
1. Manipulation of native habitat	Human activities that manipulate the ambrosia beetles' landscape in a way that reduces the diversity of their habitats or inhibits the success or movement of their natural predators.	The native ambrosia beetle *Euwallacea perbrevis* (TSHBa), infests naïve exotic monoculture agricultural and forestry crops in its native range of south East Asia, but has not been reported to cause substantial damage in the surrounding native forests with which it coevolved (Walgama 2012).
2. Climatic changes, specifically elevated temperatures	(1) Warmer, shorter winters, decrease overwintering mortality, accelerate beetle maturation and can increase evapotranspiration stress of plant hosts. (2) Dryer seasons can result in severe drought, that compromises tree defences. (3) Climatic changes can result both in the increase in the frequency and size of outbreaks within historical habitats and result in a migration of beetle species into new areas where plant hosts lack coevolved adaptation.	The migration of the ambrosia beetle *Platypus quercivorus* northward into the once climatically unsuitable cooler forested areas of Japan, where it has encountered and subsequently become a pest of the naïve host *Quercus crispula* (Kamata et al. 2002).
3. The accidental anthropogenic movement of beetles and their symbionts to non-native environments	The accidental movement and successful establishment of ambrosia beetles outside of their native range in which favourable native trees lack effective coevolved defences.	The ambrosia beetle *Xyleborus glabratus*, and its fungal symbiont *Raffaelea lauricola*, is a major pest on species in the Lauraceae plant family in parts of the United States, where it is invasive (Harrington et al. 2011). No such reports have been made on any members of the Lauraceae plant family that are endemic to Asia, and is hypothesized to be due to the presence of coevolved resistance to the disease (Ploetz et al. 2017a).
4. Acquired pathogenicity due to	(1) The horizontal transmission of fungal symbionts between different ambrosia beetle species co-inhabiting a single host.	(1) The horizontal transfer of *R. lauricola* to nine other ambrosia beetle species has contributed to the spread and impact of laurel wilt in avocados (Harrington et al. 2010;

anthropogenic movements	(2) Novel associations of the beetle vector with fungi.	Harrington and Fraedrich 2010; Ploetz et al. 2013; Carrillo et al. 2014; Ploetz et al. 2017a, b). Although *R. lauricola* does not play a nutritional role to these other beetle vectors, it increases the overall stress of the plant host making it a more favourable reproductive host for later infestation (Ranger et al. 2015). (2) The new association of the plant pathogen *Ceratocystis ficicola*, hypothesised to be native to Japan (Kajitani and Masuya 2011), with the invasive ambrosia beetle *Euwallacea interjectus*, resulted in the wilt symptoms and death of fig trees in Japan (Morita et al. 2012; Kajii et al. 2013, Kajitani et al.1996).
5. Ambrosia beetles that act as secondary vectors of plant pathogens	Through gallery construction, ambrosia beetles can create entry wounds for secondary plant pathogens to enter and infect a host. The entry wounds can either be visited by other insects that may vector plant pathogens or be an entry point for wind or water dispersed inoculum (Montoya and Wingfield 2006; Hayslett et al. 2008).	The rapid dispersal of Rapid ʻŌhiʻa Death, caused by both *Ceratocystis lukuohia* and *C. huliohia* in Hawaii is aided by the non-native ambrosia beetle *Xyleborus ferrugineus* (Roy et al. 2018). As the adult beetles construct their galleries, frass inoculated with fungal propagules of the infecting pathogen can be expelled. This inoculated frass can be dispersed by wind or rain splash and contribute to air- and soil- borne inoculum of the pathogen.

1. *Manipulation of native habitats*

Natural forested areas globally and where different ambrosia beetle species are native, are often partially converted into production forests due to their suitable environmental conditions. These production forests are commonly comprised of exotic monoculture species, many of which are naïve hosts for the native ambrosia beetle fauna (Wingfield 1999; Kirkendall et al. 2015). The reduction of heterogenicity in these landscapes and the inherent stressful settings of non-natural environments, can provide favourable naïve uniform reproductive hosts for many native and invasive ambrosia beetle species.

An example of such anthropogenic manipulation of native landscapes is found in south East Asia, were the native pioneering ambrosia species *E. perbrevis*, more frequently referred to as the Tea shot hole borer clade A (TSHBa), has been reported as a pest on many naïve agricultural and forestry crops. These include for example exotic crops such as tea (*Camelia sinensis*) in India and Sri Lanka (Walgama 2012), and *Acacia* spp. in Indonesia and Vietnam (Nuhamara, 1993 in Stouthamer et al. 2017). No such reports of infestation and damage have been made in the native surrounding forests where the beetles occur naturally and where they have coevolved with their tree hosts.

In environments subject to high levels of anthropogenic modification, ambrosia beetle infestations on plant hosts are often preceded by environmental stresses. Many of these such as poor drainage, graft incompatibility, poor soil or site conditions, or excessive or improperly timed nutrient addition and irrigation may not be apparent to the grower (Ranger et al. 2016). In the absence of aggressive ambrosia beetles, most trees would be sufficiently resilient and able to recover. However, mass mechanical damage on an already compromised host can result in tree death. Fragmentation of the natural landscape can also limit the dispersal and success of many natural predators, aiding in the expansion of beetle populations that have adapted to colonize new habitats (Six et al. 2011). A boom in the beetle population can accelerate the spread of an infestation through homogenous environments, resulting in even greater economic losses (Six et al. 2011; Raffa et al. 2015).

Compromised plant hosts emit semiochemical cues that can be detected by many ambrosia beetle species (Ranger et al. 2010; Kendra et al. 2014; Smith et al. 2019a). For example, species of *Xylosandrus* and *Euwallacea* are highly sensitive to stress-related volatiles, predominantly ethanol (Ranger et al. 2016), and quercivorol (Carrillo et al. 2015), respectively. After a suitable host has been located and successfully colonized by a small subset of pioneering ambrosia beetles and their ambrosia fungi, a mass accumulation of secondary ambrosia beetles can occur (Hulcr and Stelinski 2017 and see Box 1 for definitions). Mass accumulation of secondary ambrosia beetles on trees is synchronized by the use of pheromones secreted by ambrosia beetles that have already successfully established in the infested plant host, and can occur over a period of months (Shoda-Kagaya et al. 2010; La Spina et al. 2013).

Secondary ambrosia beetles, although not able to attack living plant tissues, can also have severe economic impacts (Lindgren and Fraser 1994). This is particularly true for harvested timber, which can be quickly colonized by masses of ambrosia beetle species and their corresponding fungi, causing structural damage and unwanted staining respectively (Hulcr and Stelinski 2017). For example, *X. affinis* has spread from its native tropical and subtropical regions of America, to most tropical and subtropical areas in the world (Rabaglia et al. 2009). Its natural attraction to recently felled wood has made it an important structural pest of moist, untreated timber, in both its native and introduced areas (Rabaglia et al. 2009).

2. Impact of changing environmental conditions

Environmental conditions, particularly increasing environmental temperatures, can favour the reproduction of ambrosia beetles, contributing to the expansion of ambrosia beetle populations in both native and invasive areas (Carroll et al. 2003; Hicke et al. 2006; Régnière and Bentz 2007; Cudmore et al. 2010; Six et al. 2011; Dysthe et al. 2015; Kirkendall et al. 2015). This can occur in four main ways; (1) warmer, shorter winters can increase the beetle population (Bentz et al. 1991; Evans et al. 2011) and (2) expand their infestation range into previously climatically unsuitable areas where trees lack appropriate coevolved adaptation (Carroll et al. 2003; Régnière et al. 2012), (3) warmer, dryer seasons resulting in drought stress, can increase overall vulnerability of plant hosts, making them more suitable for infestation (Bentz and Jönsson 2015) and (3) longer warm seasons benefit the growth of fungal associates of ambrosia beetles, which can improve successful host colonization (Addison et al. 2013).

Warmer and shorter winter seasons decrease winter mortality of ambrosia beetles and increase the rate at which they develop (Evans et al. 2011; Bentz and Jönsson 2015). In response to the subsequent population growth, beetles can coordinate mass attacks on more trees. This can include infestation of healthy trees to meet the demand for food, resulting in mass infestation events (Raffa et al. 2015). In combination with this, warmer seasons may also expand the number of hosts available for beetle colonization, either via the increase in stressed trees during droughts (Hart et al. 2014; Raffa et al. 2015), or by allowing the migration of beetles into areas that previously had an unsuitable climate (Carroll et al. 2003; Hicke et al. 2006; Régnière et al. 2012).

An example of how warmer seasons can favour brood development and host availability, is the widespread impact of the mountain pine beetle *Dendroctonus ponderosae.* In this situation, warmer climatic conditions have partially resulted in the increase in the size and number of outbreaks (Hicke et al. 2006; Régnière and Bentz 2007; Negrón and Cain 2019). The bark beetle, *Dendroctonus ponderosae*, is native to parts of western North America and plays an important ecological role by infesting old, large diameter or weakened trees, and aiding the development of a younger forest (Negrón and Cain 2019). It does this via a combined attack of larval feeding and the inoculation of the blue stain fungi *Leptographium longiclavatum Grosmannia clavigera* and *Ophiostoma montium*, which girdle and eventually kill the infested tree (Hicke et al. 2006). These fungi although not nutritional associates (Negrón 2018), immobilize the host's defences, preventing the trees from repelling and killing the attacking beetles, and blocking the flow of nutrients and water within the trees, resulting in eventual mortality (Abdel-Kerim Farahat et al. 2006; Negrón 2018). Thus, the natural ecology of *D. ponderosae* assists in maintaining the natural regeneration of forests, by targeting weakened, old or large diameter host and allowing for younger ones to establish.

In British Colombia, a combination of factors has resulted in the growth of *D. ponderosae* beetle populations beyond their natural threshold and made the widely occurring *Pinus contorta* more vulnerable to attack. These factors include; (1) unusually mild winters with temperatures averaging above freezing point, and warm, dry summers during the last decade (http://www.env.gov.bc.ca/soe/indicators/climate-change/temp.html), (2) monocultural forests filled with mature large diameter *P. contorta*, and (3) approximately a century of forest fire suppression (Taylor and Carroll 2003; Abdel-Kerim Farahat et al. 2006; Negrón 2018). This climate-related increase in the *D. ponderosae* beetle population, and subsequent migration to healthier hosts, has caused the death of nearly all *P. contorta* of favourable host size class in many

plots, resulting in an unprecedented epidemic, devastating forests in 19 Western U.S.A States and Canada, and destroying approximately 88 million acres (approx. 36 million ha) of timber (Carroll et al. 2003; Shore et al. 2004; Raffa et al. 2015).

Expansion of host range due to more favourable climatic conditions can also lead to new interactions between ambrosia beetle species and naïve plant hosts (Cudmore et al. 2010; Six et al. 2011). Although contested, it is hypothesized that the oak dieback in Japan, caused by the native ambrosia beetle *Platypus quercivorus,* and it fungal associate *Raffaelea quercivora,* is the result of rising temperatures over the past century (https://www.nippon.com/en/features/h00067/hot-and-getting-hotter.html) (Kamata et al. 2002). *Platypus quercivorus* was first noticed as a native beetle in Japan in the 1920s, having no serious effect on living trees until the 1980s when a mass dieback of oaks occurred (Hijii et al. 1948; Hamaguchi and Goto 2010). This sudden outbreak has been hypothesized as partly due to the warmer climate altering the distribution of the ambrosia beetle and enabling it to migrate northward into the cooler forested areas of Japan, where it has encountered, and subsequently become a pest of *Quercus crispula* (Kamata et al. 2002). *Platypus quercivorus* can realize a greater reproductive success on *Q. crispula* than on other hosts, due in part to the lack of co-evolution between *Q. crispula* and the beetle-fungal symbiosis. This has resulted in an explosion in *P. quercivorus* populations and their subsequent rapid spread and infestation of susceptible host across Japan, destroying approximately 1000 ha of oak forest each year since the 1980s (Hijii et al. 1948; Kamata et al. 2002; Kubono and Ito 2002; Murata et al. 2009) .

The mechanisms underlying the oak die-back caused by *R. quercivora* are believed to mimic those of some invasive pests, in that the resulting disease is caused by an exaggerated response of naïve plant tissues to a new threat (Murata et al. 2009). The resulting mortality is thus caused by tree “suicide” rather than by any virulence factors produced by the fungus (Murata et al. 2009; Kendra et al. 2010; Hulcr and Dunn 2011). Once colonized by the fungus, the oak trees react by plugging the xylem with gum and tyloses as a defence mechanism, in an attempt to block the fungus spreading (Kuroda and Yamada 1996). As this immune response has not been refined via the co-evolution between the fungi and host, an exaggerated response is initiated, resulting in partial blockage of the vascular system (Hulcr and Dunn 2011). This blockage, in combination with the structural damage caused by gallery formation to the vascular system, and the spread of the associated fungi throughout the xylem, results in host mortality (Kuroda 2001; Murata et al. 2009).

The symbioses between the ambrosia beetles and their fungal associates can also be influenced by climatic changes. The sporulation and growth rates of fungi are closely tied to temperature, therefore, temperature changes can influence the presence and abundance of mycangial microorganism present within and between populations of their beetle hosts (Rice et al. 2008). Temperature dependent symbioses have been demonstrated for several beetle species of *Dendroctonus* (Six and Bentz 2007; Addison et al. 2013; Dysthe et al. 2015). For example, the relative prevalence of two fungi associated with *D. ponderosae* have been shown to fluctuate with temperature - one symbiont is better adapted to cooler conditions (*Grosmannia clavigera*), and the other to warmer ones (*Ophiostoma montium*) (Six and Bentz 2007; Addison et al. 2013; Dysthe et al. 2015). This mechanism is believed to aid with the maintenance of a simultaneous stable community of symbiotic fungi within a single beetle host (Six and Bentz 2007; Rice et al. 2008).

The ability to accommodate a community of microbial associates potentially enables symbiont beetles to adapt and survive under different or varying environmental conditions (Batra 1963; Kajimura and Hijii 1992; Klepzig et al. 2009; Hulcr et al. 2012; Kostovcik et al. 2015; You et al. 2015). In turn, the structure of these microbial communities can be altered by changes in climate, such that the prevalence of associates can vary between host populations in climatically different geographical locations, or in single locations with varying climatic conditions over time (Six and Bentz 2007; Rice et al. 2008). The temperature influenced fluctuations in the prevalence of associates, as well as in their growth rates, can influence the rate of nutrient acquisition, which in turn can have strong effects on beetle fitness (Six and Bentz 2007). This can subsequently impact the rate of brood development, adult dispersal and secondary host colonization; all of which can influence the level of impact of the host beetles (Six and Bentz 2007; Addison et al. 2013; Dysthe et al. 2015).

3. Invasive ambrosia beetles

Bark and ambrosia beetles, are being introduced into non-native regions, faster than any other group of invasive forest pests (Aukema et al. 2010; Hulcr and Dunn 2011). The adaptive ability of ambrosia beetles has enabled them to adapt and thrive in many conditions, resulting in their repeated successful establishment outside of their native range (Hulcr and Dunn 2011; Raffa et al. 2015). By shifting from infesting dying or dead trees to colonizing living trees that lack coevolved resistance, a subset of these introduced fungus–insect associates have become some of the most economically devastating pests to numerous agricultural and forestry crops (Harrington 2005;

Eskalen et al. 2016; Hughes et al. 2017). This is particularly true for symbioses involving beetles in the Tribe Xyleborini, that have had the highest invasive establishment success of any ambrosia beetle (Hulcr and Dunn 2011). The successful establishment of these beetles after an invasion is in part due to their haplo-diploid mating system, where unmated diploid females produce haploid dwarf males whom that they can later mate with (Jordal et al. 2001; Eskalen et al. 2013). Thus, if a suitable plant host can be located, and environmental conditions are favourable, the introduction of only a small number of mated ambrosia beetle females is needed to establish an active population (Hulcr and Dunn 2011).

The severely negative economic impacts of invasive ambrosia beetles on urban landscapes and agricultural industries is demonstrated by *E. fornicatus* (PSHB). This invasive ambrosia beetle and its complex of fungal associates is responsible for the current outbreak of Fusarium Dieback (FD) in parts of America, Israel, Mexico and South Africa (Eskalen et al. 2013; Garcia-Avila et al. 2016; Ploetz et al. 2017a; Paap et al. 2018). The female beetle carries three fungal species: *G. euwallaceae*, *Paracremonium pembeum* and the most economically important *F. euwallaceae* (Freeman et al. 2013; Lynch et al. 2016). In its native environment of Southeast Asia, the beetle and fungi have not been reported to cause serious damage. This is likely because the beetle populations are controlled by a suite of natural predators and because the native tree species have evolved resistance to the beetles' fungal associates. However, in areas where the beetle and fungus have been accidently introduced, over 200 tree species have been found susceptible to Fusarium Dieback (Eskalen et al. 2013).

The true extent of economic damage of *E. fornicatus* and its fungal associates on native ecosystems and agricultural settings in their introduced ranges, has yet to be established. But the estimated economic and ecological impacts of these invasive pests on urban areas are significant. For example, in Southern California an estimated 23.2 million trees are believed to be susceptible to the PSHB-FD complex (McPherson et al. 2017). If half of these susceptible trees die, the estimated cost for removing and replacing them is U$15.9 billion. These costs could be even greater if the impact on local economies, biodiversity, wildlife and human health are also considered (McPherson et al. 2017).

A second example of the economic impact that invasive ambrosia beetle-fungal symbioses can have is that of Laurel wilt, caused by the invasive beetle *Xyleborus glabratus* and its fungal associate *R. lauricola* (Norris and Baker 1968; Dreaden et al. 2014). The beetle and pathogen are

both believed to be native to Asia, and are causing mortality on species within the Lauraceae family in parts of south-eastern United States, where they were accidently introduced (Harrington et al. 2011). Potential movement of this symbiosis to commercial avocado orchids in the USA and Mexico, or to the west coast where laurel species are densely planted, could have devastating economic impacts of up to U$356 million of predicted losses (Evans et al. 2010). No such reports have been made on any members of the Lauraceae that are endemic to Asia, and this is believed to be due to a coevolved resistance to the *R. lauricola* (Ploetz et al. 2017a).

Despite the dramatic impacts of invasive epidemics caused by ambrosia beetle fungal symbioses, most insect–fungus consortia that are introduced into non-native environments don't cause economically significant damage. Rather, they remain restricted to dead hosts and go unnoticed. Colonization of living plant tissues is uncommon for ambrosia beetles under natural circumstances (Nair 2000). Likewise, fungal symbionts e.g. *Raffaelea, Ambrosiella, Fusarium* etc., seldom act as phytopathogens (Hulcr and Dunn 2011). Thus it appears that shifts in the behaviour of insect–fungus symbioses in non-native environments and the presence of naive favourable plant host is a pivotal component when predicting the emergence of economically impactful outbreaks (Hulcr and Dunn 2011).

4. Acquired pathogenicity

The disease epidemiology of Laurel wilt highlights and important mechanism for the emergence of new ambrosia beetle associated disease outbreaks. This is of acquired pathogenicity. The dominant fungal symbionts of ambrosia beetles aren't passed directly from the mother to the offspring during vertical transmission. Rather these fungal symbionts grow independently along the surfaces of the brood galleries, where the larvae obtain them during feeding (Beaver 1989; Klepzig et al. 2009). Because several ambrosia beetle species can simultaneously inhabit in a single plant host, multiple beetle species can interact with each other's brood galleries and associated fungi, allowing horizontal transmission of these fungal symbionts to occur (Six 2012; Carrillo et al. 2014). In addition, lateral transfer of genes coding for virulence factors between closely related species, could potentially increase the pathogenicity of some necrotrophic pathogens and contribute to the observed increase in host mortality (Ma et al. 2010).

The highly specialized types of mycangia possessed by different ambrosia beetle species supports the assembly of a distinct microbial community allowing for the persistence and preservation of the core community (Kajimura and Hijii 1992; Hulcr and Cognato 2010; Kostovcik et al. 2015). These features enable different ambrosia species to carry over-lapping, but unique sets of microbial associates and contaminants, each with their own degree of fidelity and community structure (Carrillo et al. 2014). These new associations can involve microorganism that are more aggressive than the original symbionts, resulting in new or more severe disease outbreaks. For Laurel wilt, the horizontal transfer of *R. lauricola* to nine other ambrosia beetle species has aided in the spread and impact of Laurel wilt in avocados (Harrington et al. 2010; Harrington and Fraedrich 2010; Ploetz et al. 2013; Carrillo et al. 2014; Ploetz et al. 2017a, b).

The microbial associates acquired by ambrosia beetles may not play a nutritional role to ambrosia beetles, but rather, they increase the overall stress on the plant host, making it a more favourable reproductive host for subsequent infestation (Ranger et al. 2015). Xyleborine ambrosia beetles commonly utilise semiochemicals and other cues to locate host trees (Niogret et al. 2011; Inch and Ploetz 2012; Kendra et al. 2012). This mechanism can sometimes result in the 'probing' of hosts, on which beetles will land and attempt to penetrate, but then recognise a lack of suitability for colonization and leave to locate another host (Gomez et al. 2019). This 'probing' process can be sufficient for the inoculation of the attacking beetle's fungal symbiont, which can be a weak pathogen, or create an entry wound for other pathogens to infect (Ploetz et al. 2017a; Paap et al. 2018; Gomez et al. 2019).

Ambrosial beetle 'probing" was reported to occur during the initial stages of the Laurel wilt outbreak, where primary attacks by *X. glabratus* on healthy trees were aborted (Fraedrich et al. 2008). Although successful colonization did not occur, aborted attacks resulted in the inoculation of "probed" trees with *R. lauricola* (Fraedrich et al. 2008). Brood development of *X. glabratus* was observed after disease development in inoculated hosts (Fraedrich et al. 2008, 2015; Ploetz et al. 2017a). This 'probing' mechanism may reflect the original host selection biology of ambrosia beetles, in which immunocompromised hosts are favoured for colonisation (Raffa et al. 2015; Hulcr and Stelinski 2017).

Acquired pathogenicity can also occur via non-mycangial fungi that can be acquired from the environment and be transported either directly on the beetles exoskeleton or by mycophagous

mites carried by the beetle (Klepzig et al. 2001; Moser et al. 2010; Six 2012). The new associations of the insect vector with fungi can arise either by the joining of a native or introduced pathogen to a pre-existing or novel beetle, and/or plant host relationship. Examples include (1) the loose association of the plant pathogen, *Ceratocystis manginecans*, with the native mango bark beetle, *Hypocephalus mangiferae* (previously *Hypocryphalus mangiferae*), resulting in a new mass infection of mango crops across Asia (Al Adawi et al. 2013; Fourie et al. 2016), and by (2) the novel association of the plant pathogen *C. ficicola*, hypothesised to be native to Japan (Kajitani and Masuya 2011), to the invasive ambrosia beetle *E. interjectus*, resulting in the wilt symptoms and death of fig trees in Japan (Morita et al. 2012; Kajii et al. 2013, Kajitani et al. 1996).

5. Secondary vectors

Ambrosia beetles can act as secondary vectors of plant pathogens. Ambrosia beetles create entry wounds through gallery construction, which can enable secondary plant pathogens to enter and infect a host. The entry wounds may either be visited by other insects that can vector plant pathogens, or be an entry point for wind or water dispersed inoculum (Montoya and Wingfield 2006; Hayslett et al. 2008). This can occur in both reproductive and non-reproductive hosts (see Box 1 for definitions), if sufficient entry wounds are created (Gomez et al. 2019).

Ambrosia beetle gallery construction into already infected hosts can aid in the dispersal of the infecting pathogen via the excretion of inoculated frass during gallery formation (Harrington 2000; Ocasio-Morales et al. 2007; Harrington and Appel 2016; Roy et al. 2018 and see Box 1 for definitions). For example, *C. cacaofunesta* and *C. platani* can be dispersed in frass when ambrosia beetles infest trees already colonized by these pathogens (Harrington 2000; Ocasio-Morales et al. 2007). In Hawaii, native ʻōhiʻa lehua trees (*Metrosideros polymorpha*) are threatened by a disease known as Rapid ʻŌhiʻa Death (ROD), caused by both *C. huliohia* and *C. lukuohia* (Barnes et al. 2018). The rapid dispersal of ROD throughout Hawai'i is believed to be aided by the non-native ambrosia beetle *X. ferrugineu* (Roy et al. 2018). As the adult *X. ferrugineu* beetles construct their galleries, frass inoculated with fungal propagules of *C. huliohia* and *C. lukuohia* can be expelled. This inoculated frass can be dispersed by wind or rain splash and contribute to air- and soil- borne inoculum of the pathogen (Roy et al. 2018).

Conclusions

Ambrosia beetle–associated damage to trees appears to be on the rise, manifesting in diverse and geographically varied environments with significant negative impacts (Kubono and Ito 2002; Fraedrich et al. 2008; Kim et al. 2009; Mendel et al. 2012; Eskalen et al. 2014; Garcia-Avila et al. 2016; Paap et al. 2018). Those ambrosia beetles that emerge as economically important pests can cause damage in three ways; either via structural damage, mass accumulation on stressed trees or through their associations with virulent tree pathogens (Hulcr and Stelinski 2017). However, the rise in damage by ambrosia beetles and their associated fungi, regardless of mode, is strongly associated with shifts in their genetic and environmental interactions. In these cases, the beetles interact dynamically with their plant hosts and associated microbiota in a way that can favour the reproductive success of these beetles.

Most populations of ambrosia beetles in their native environments are dynamic, changing in size depending on resource availability and climate. These populations tend to shrink with resource depletion and grow with resource availability driven by greater population sizes. In conjunction with these environmental factors, cooperative behaviour of mass coordinated attack, and diverse, density-dependent plant host selection strategies, also drive the flux in these native beetle populations. However, factors such as warmer temperature, drought, wide-spread landscape manipulation or anthropogenic introduction can favour the growth of populations to exceed a threshold and result in severe outbreaks as discussed above.

As shown in this review, the factors that cause the shifts in the interactions of the beetles are not mutually exclusive. Most of the interactions are characterised by a lack of coevolved adaptation between the naïve plant host and the ‘novel’ beetle and/or their associated microorganisms (Ploetz et al. 2013). A few key characteristics of some ambrosia beetles ecology, such as their ability to colonize living plant tissues, can influence the potential degree of impact they can have (Ranger et al. 2010, 2016; Hulcr and Stelinski 2017). Thus, although the ecology and biology of ambrosia beetles did not evolve to make ambrosia beetles economically significant pests, it has enabled them to adapt and thrive when shifts in their environmental circumstances and microbial associates occur.

There is a lack of understanding of the ecological characteristics that influence the degree of impact of emerging ambrosia beetle pests. This is partly because most ambrosia beetle related damage is studied when the beetles emerge as pests outside their native environment. Consequently, only a small fraction of their original ecological interactions are present and can be studied. This provides a significant challenge in determining how the beetles have evolved and what economic impacts they will have when shifts in their environment occur.

A much more comprehensive understanding is needed regarding the diversity of microbial associates cultivated by ambrosia beetles. Similarly, how the prevalence of these communities can change depending upon host selection and geographical distribution. This is particularly true for ambrosia beetle–fungus symbioses that can colonize living plant tissues. Thus, investigating ambrosia beetles in their native habitats, delving more deeply into their diversity, and understanding how the different components of these emerging pathosystems interact, will aid in identifying future tree-killing invasive pests (Cognato et al. 2015).

Movement of flora and fauna around the world via anthropogenic activities will continue and likely grow in the future. As a result, damage caused by ambrosia beetles and their fungal associates is expected to rise. Countless examples of established economically impactful beetle outbreaks have already emerged and some of these, such as the outbreak of an *Euwallacea* sp. in commercially important *Acacia* plantations in Indonesia have been presented in this review. In order to better prepare for and manage these emerging outbreaks, simple screening of ambrosia beetle–fungal symbioses that colonize living plant tissues in their native habitats, could help to differentiate future tree-killing invasive pests from those that are harmless. In addition, application of many new and emerging technologies to study and understand both components of the ambrosial symbiosis; such as advances in the genetics tools to correctly identify cryptic beetle species (Smith et al. 2019b) and artificial diet experiments to understand the stringency of fungal associates, have been made (Carrillo et al. 2020). Continuing this trend of research, further evaluating the biological variation and interaction among emerging ambrosia beetle pests in their native environments, may reveal significant ecological traits that can mitigate their impact, particularly if they are accidentally introduced into non-native regions.

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Chapter 2

Euwallacea perbrevis (Coleoptera: Curculionidae: Scolytinae), a confirmed pest on *Acacia crassicarpa* in Riau, Indonesia, and a new fungal symbiont; *Fusarium rekanum* sp. nov.

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Abstract

Non-native *Acacia* plantations in Indonesia were first reported to be infested by a native ambrosia beetle species, identified as *Euwallacea fornicatus* in 1993. Recently the level of infestation in these plantations by ambrosia beetles has steadily increased. The recent redefinition of the taxonomic parameters of the *Euwallacea fornicatus* species complex has resulted in the identity of the ambrosia beetle species in these plantations becoming unclear. This is also true for their obligate fungal associates. Therefore, the aim of this study was to identify the ambrosia beetle species, as well as its corresponding fungal associate/s, infesting *Acacia crassicarpa* plantations in Riau, Indonesia. Morphological identification and phylogenetic analysis of the mitochondrial cytochrome oxidase c subunit I (COI) gene, revealed that the beetles are *E. perbrevis*, previously a synonym of *E. fornicatus* and commonly referred to as the Tea Shot Hole Borer A (TSHBa). Multi-locus phylogenetic analyses of the fungal associate of *E. perbrevis* revealed a *Fusarium* sp. that is among members of the Ambrosia Fusarium Clade (AFC), but that is genetically distinct from other previously identified *Fusarium* symbionts of *Euwallacea* species. This novel fungal species is described here as *Fusarium rekanum* sp. nov.

Introduction

Ambrosia beetles are wood-boring mycetophagous insects belonging to the subfamilies Scolytinae and Platypodinae in the larger Curculionidae (Jordal et al. 2011; Hulcr et al. 2015). They are defined by a shared ecological strategy known as fungus farming, with the beetles having obligate nutritional symbioses with fungi (Farrell et al. 2001; Kirkendall et al. 2015). In most ambrosia beetle species, the insects store and transport their fungal associates to and from their natal galleries, in specialised structures known as mycangia (Batra 1963). Once released from the mycangia, the fungi penetrate the xylem tissues of typically dying or occasionally living plant hosts, and grow in the galleries to provide nutrition for the beetles and their offspring (Batra 1963; Mueller et al. 2005).

Ambrosia beetles perform a key role in the ecosystems of their tropical and sub-tropical native ranges by introducing a diverse consortium of fungi into the woody tissues they infest, influencing their rate of decay (Skelton et al. 2019). Many ambrosia beetles and their associated fungi are

ecologically constrained to dying trees and usually remain harmless even after establishment in non-native regions. Therefore, they are generally economically inconsequential (Hulcr and Dunn 2011; Ranger et al. 2015; Hulcr et al. 2017). However, several ambrosia beetle species are emerging as prominent pests on trees, particularly in regions where they have been accidently introduced (Hughes et al. 2017; Stouthamer et al. 2017; Gomez et al. 2018).

Ambrosia beetles in the genus *Euwallacea* Hopkins 1915 within the tribe Xyleborini, are particularly successful invaders (Cognato et al. 2015; Stouthamer et al. 2017). This is in part due to their haplodiploid mating system, wide host range and their primary association with *Fusarium* Link species, which can act as both nutritional symbionts and weak phytopathogens (Kasson et al. 2013; O'Donnell et al. 2015). The fusaria associated with *Euwallacea* form a monophyletic group within Clade 3 of the *F. solani* species complex (FSSC), known as the Ambrosia *Fusarium* Clade (AFC, Kasson et al. 2013). Recently, however, Sandoval-Denis et al. (2019) proposed that species in the FSSC, including the AFC species, be placed in the genus *Neocosmospora*.

The AFC comprises of two strongly supported subclades. Clade A includes species that produce curved fusiform septate macroconidia, typical of *Fusarium*. In contrast, most of the species in Clade B produce clavate macroconidia (Kasson et al. 2013). To date, only seven of the 18 species in the AFC have been formally described and include *F. ambrosium* Agnihothr. & Nirenberg (AF-1) (Gadd and Loos 1947), *F. euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell (AF-2) (Freeman et al. 2013), *F. oligoseptatum* T. Aoki, M.T. Kasson, S. Freeman, D.M. Geiser & K. O'Donnell (AF-4) (Aoki et al. 2018), *F. kuroshium* F. Na, J.D. Carrillo & Eskalen (AF-12) (Na et al. 2018), *F. floridanum* T. Aoki, Kasson, S. Freeman, Geiser & O'Donnell (AF-3), *F. tuaranense* T. Aoki, Kasson, S. Freeman, Geiser & O'Donnell (AF-5) and *F. obliquiseptatum* T. Aoki, Kasson, S. Freeman, Geiser & O'Donnell (AF-7) (Aoki et al. 2019). Three of these are associated with species in the redefined *E. fornicatus* species complex (Gomez et al. 2018; Smith et al. 2019). The four pests in this redefined complex include *E. perbrevis* Schedl 1951 (Tea Shot Hole Borer Clade a) associated with *F. ambrosium* (AF-1), *E. fornicatior* Eggers 1923 (Tea Shot Hole Borer Clade b) associated with a still unknown symbiont, *E. fornicatus* Schedl 1951 (Polyphagous Shot Hole Borer) associated with *F. euwallaceae* (AF-2), and *E. kuroshio* Gomez & Hulcr 2018 (Kuroshio Shot Hole Borer) associated with *F. kuroshium* (AF-12) (Gomez et al. 2018; Smith et al. 2019).

Species in the *E. fornicatus* complex, along with their AFC associates, are responsible for economic losses to forestry and agricultural industries in various parts of the world (Eskalen et al. 2013; O'Donnell et al. 2016; Na et al. 2018). However, due to previous ambiguous morphological species boundaries, all four species were treated as *E. fornicatus,* despite differences in fungal symbionts, geographic range, and host preference (Gadd and Loos 1947; Freeman et al. 2013; Gomez et al. 2018; Na et al. 2018). This ambiguous level of identification and the revision of the *E. fornicatus* complex, has resulted in confusion regarding the economic importance of these four species.

In Indonesia, *E. fornicatus* has been reported as a pest on *Acacia* spp. where large areas of these exotic trees are planted (Nuhamara, 1993 in Stouthamer et al. 2017). In recent years, there has been a gradual increase in ambrosia beetle infestations within these *Acacia* plantations. This has resulted in significant economic losses to the forestry industry in the region due to tree mortality, a decrease in yield and wood property, and the potential of these insects to vector new aggressive plant pathogens (Roy et al. 2018; Boland and Woodward 2019).

The taxonomic revision of the *E. fornicatus* complex has led to a situation where the identity of the most common ambrosia beetle infesting *Acacia* spp. in Riau, Indonesia is not known. Likewise, the identity of its fungal symbiont/s has not been determined. The aim of this study was therefore to, (1) identify the ambrosia beetles infesting *A. crassicarpa* plantations in Riau, Indonesia, (2) identify the fungal associate/s of the most common beetle species, and (3) determine whether these fungi are pathogens of *A. crassicarpa*.

Materials and methods

Collections of beetle specimens and brood galleries

Plantations of *A. crassicarpa* in Riau, Indonesia, showing heavy ambrosia beetle infestation were identified, and four geographically separated compartments were chosen as collection sites. Based on the infestation scale of Eskalen et al. (2013), three moderately infested living trees [10–30 entry holes] were selected from each site, felled at 50 cm above ground level, and cut into 50 cm logs.

These logs were transported to the laboratory where they were dissected, and beetles, along with their galleries, were extracted for morphological and molecular identification.

Fungal isolations

Fungal isolates were either obtained by direct isolation from the mandibular mycangia of mature female beetles (Lynch et al. 2016), or by culturing from the surfaces of the beetle galleries, using methods described by Eskalen et al. (2013). Isolations were performed on Petri dishes containing either potato dextrose agar (PDA; PDA 20g/l, BD Difco^{TM}) or *Fusarium* selective medium (FSM, Leslie and Summerell 2006) amended with 100 µg streptomycin sulphate (Sigma, Steinheim, Germany) to limit the growth of contaminating bacteria and fungi.

For the mycangial extractions, beetles were surface disinfected by submerging individual specimens into Eppendorf tubes containing 70% (v/v) ethanol. Tubes were agitated with a vortex mixer for 10 s before the beetle specimens were removed, rinsed with sterilized distilled water, and air-dried. The beetle heads were separated from the thoracic segments, macerated or crushed, and were either smeared directly onto the surface of agar or suspended in 200 µl of sterile water in 1.5 ml Eppendorf tubes, shaken for 10 s, and individually pipetted (50 µl) onto the agar and spread using sterile glass rods. The Petri dishes were incubated at 25 °C for 3–5 days to allow for fungal growth. The remaining thoracic and abdominal segments of the beetles were individually stored in 200 µl of 70% (v/v) ethanol for further DNA extractions and identification.

Fungi growing in the beetle galleries were obtained using three different methods as follows: (1) sterile needles were scraped along the gallery surfaces and washed in 200 µl of sterile water. Fifty µl of this suspension was pipetted onto growth medium (FSM and PDA) and spread using sterile glass rods (Kajimura and Hijii 1992); (2) mycelial strands growing inside the beetle galleries, observed using a dissection microscope, were lifted using sterile needles and plated onto FSM and PDA; (3) small pieces of discoloured wood were cut from the edges of the galleries, surface disinfested with 70% (v/v) ethanol and placed on FSM and PDA growth media.

Primary isolations were incubated at 25 °C for 3–5 days to allow for fungal growth. Fungal colonies were sub-cultured onto 2% malt extract agar (MEA: 20 g/l malt extract, 20 g/l agar, Biolab, Midrand, South Africa), amended with 100 µg streptomycin. Five days post sub-culturing, single hyphal tip cultures were made onto MEA, to obtain pure colonies for further identification.

All cultures obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 1, Supplementary Table 1). Representative isolates of the novel species were deposited in the living collection (PPRI) of the South African National Collection of Fungi (NCF), Roodeplaat, South Africa. Dried specimens of sporulating cultures were deposited in the herbarium collection (PREM) of the South African National Collection of Fungi, Roodeplaat, South Africa.

DNA extraction and PCR

Beetle specimens

Specimens were initially characterised based on morphology and grouped utilising similarities in body form and declivital sculpturing. A subset of 30 individual specimens was selected for DNA extraction, including a representative from each morphological group. DNA was extracted from the beetle wings and legs using the prepGEM™ Universal DNA extraction kit (Zygem, Biocom Africa (Pty) Ltd.), following the manufacturers protocol, except that the final product was not diluted. The extracted DNA was subjected to PCR amplification of the mitochondrial cytochrome oxidase c subunit I gene (COI), using the primer pair LCO1490 and HCO2198 (Folmer et al. 1994). Each PCR reaction mixture consisted of 0.2 µl FastStart™ Taq DNA Polymerase (Roche), 10.8 µl sterile DNase-free water, 1 µl of 10 mM both forward and reverse primer, 3 µl of 25mM $MgCl_2$, 2.5 µl 10 × PCR Buffer and 4 µl of DNA template, for a 25 µl total reaction mixture. PCR amplification was carried out on a Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA,USA), programmed for an initial denaturing step of 95 °C for 7 min; followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; ending with a final extension of 7 min at 72 °C. Amplification was confirmed by staining 2 µl PCR product with 2 µl GelRed™ Nucleic Acid Gel

stain (Biotium, Hayward, CA, USA), and separating them on a 2% agarose gel, followed by visualization under UV light.

Fungal isolates

Three-day-old cultures of all isolates resembling *Fusarium* spp., were used for DNA extraction using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. PCR amplification was performed for: the ribosomal internal transcribed spacer region and the domains D1 and D2, at the 5' end of the nuclear large subunit (ITS 1-5.8S-ITS2 + LSU rDNA), using the primers ITS4/ITS5 (White et al. 1990) and NL1/NL4 (Kurtzman and Robnett 1997), respectively; the translation elongation factor 1-α (TEF1-α) using the primers EF1/EF2 (O'Donnell et al. 1998) and the RNA polymerase subunit II (RPB2), using the primer pairs 5F2/7CR (RPB2-1) and 7CF/11AR (RPB2-2) (O'Donnell et al. 2007). PCR amplifications were performed in 13 µl reactions containing 2.5 µl 5× MyTaq buffer (Bioline, London, UK), 0.25 µl MyTaq DNA polymerases (Bioline), 1 µl DNA template, 0.5 µl of each primer (10 mM), and 8.25 µl of sterile deionized water. PCR was performed for each primer pair using published cycling parameters (White et al. 1990; O'Donnell and Cigelnik 1997; Jacobs et al. 2004; O'Donnell et al. 2007). Amplification of products was confirmed with gel electrophoresis as stated above. PCR amplification of the RNA polymerase subunit I (RPB1), using the primer pairs F5/R8 (RPB1-1) and F7/G2R (RPB1-2) (O'Donnell et al. 2010), was unsuccessful across all isolates.

DNA Sequencing

PCR products were purified using 6% Sephadex G-50 columns following the manufacturer's protocol (Sigma, Aldrich Germany). Products were sequenced in both directions in 12 µl reactions, using the same primers used for PCR amplification. The reaction mixture contained 1 µl BigDye® Terminator v. 3.1 ready reaction mixture (Perkin-Elmer, Warrington, UK), 1 µl sequencing buffer, 1 µl of either the forward or reverse primer (10 mM) for each gene region and 1.5 µl cleaned PCR product. The thermal cycling conditions included 25 cycles of 10 s at 96 °C, 5 s at 55 °C and 4 min at 60 °C. Sequencing products were cleaned using Sephadex G-50 columns and dried in an Eppendorf 5301 vacuum concentrator at 60 °C for 5 min. Sequencing was performed at the

sequencing facility of the University of Pretoria, on an ABI PRISM^{TM} 3500xl Auto-sequencer (Applied Biosystems, Foster City, California, USA). The forward and reverse sequencing reads were assembled into contigs using CLC Bio Main Workbench v. 6 (CLC Bio, www.clcbio.com), and from which consensus sequences were extracted and exported for phylogenetic analysis.

Phylogenetic analyses

Beetles specimens

A preliminary identity for the beetle specimens was obtained by performing a nucleotide BLAST of the COI sequences against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Based on the results, sequences of closely related taxa were downloaded to compile datasets for phylogenetic analyses. Additionally, sequences available from previous studies (Gomez et al. 2018; Na et al. 2018), were also incorporated into the datasets. Sequences were aligned in MEGA v. 7 (Kumar et al. 2016), using the inbuilt MUSCLE alignment software (Edgar 2004), and manually inspected and adjusted. Genealogical relationships among individual beetles were reconstructed using maximum likelihood (ML) analyses, which were performed on the online CIPRES Science Gateway v 3.3 platform (Miller et al. 2010), using the online RAxML-HPC BlackBox v. 8.2.10 tool (Stamatakis 2014). Maximum likelihood analyses were carried out using the GTRGAMMA + I substitution model with all other parameters set to default. A non-parametric analysis of the sequence data with 1000 bootstrap replicates provided statistical support for the branches of the generated ML trees. *Ambrosiophilus sexdentatus* (HM06405) was used to root the ML analysis as suggested by Gomez et al. (2018).

The DNA for the representative specimens sequenced in this study are stored in the Entomological database of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. All additional specimens collected in this study are stored in 70% (v/v) ethanol at 5 °C at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Fusarium isolates

Initial BLAST searches of the resulting translation elongation factor 1-α (TEF1-α) sequences, against the NCBI GenBank database, were performed to putatively identify fungal isolates. As with the beetles, sequences of closely related taxa were then downloaded, along with those used in previous studies (Kasson et al. 2013; O'Donnell et al. 2015; Carrillo et al. 2019) (Table 1), to construct datasets for phylogenetic analyses. DNA sequences for the ITS, TEF1-α and RPB2 gene regions (Table 1), from 14 selected isolates of a *Fusarium* sp. obtained in this study, were individually aligned with each respective gene dataset in MEGA v. 7, following the same procedure as stated above. The individual datasets were subsequently combined using FASconCAT-G (Kück and Longo 2014).

Using the concatenated dataset, a maximum likelihood (ML) multigene phylogeny was constructed. jModelTest2 (Darriba et al. 2015) was used on the combined dataset to determine the best substitution model. Maximum likelihood tree construction was performed using PhyML v. 3.1 (Guindon et al. 2010), with the following criteria: proportion of non-variable sites 0.484, gamma shape 0.311 and the number of substitution sites set to 6. The starting tree was obtained using a BioNJ approach and the branch swopping strategy was set to select the best NNI algorithm. Statistical support for the branches in the ML trees was obtained by performing 1000 bootstrap replicates. *Fusarium neocosmosporiellum* (NRRL 22468) and *Fusarium lichenicola* (NRRL 32434) were used as the outgroups based on previous studies (Kasson et al. 2013; O'Donnell et al. 2015).

Morphological characterization of Fusarium *spp.*

To investigate the morphological characters of the fungus, three representative isolates (CMW 52862, CMW 53688 and CMW 53691) were selected for the study. Fourteen-day-old cultures grown on PDA and synthetic low-nutrient agar (SNA: 0.2 g glucose, 0.2 g sucrose, 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$, 7 H_2O, 0.5 g KCl, 20 g Difco agar per litre; Leslie and Summerell 2006) in darkness, under continuous UV light or under an ambient daylight photoperiod, at ambient room temperature (23–25 °C), were used to evaluate the microscopic characters considered by Aoki et al. (2005) and Freeman et al. (2013). Microscope slides were prepared for each isolate with structures mounted in water and 25–50 measurements were recorded for each characteristic, using a Nikon H550L microscope (Nikon, Yokohama, Japan). Means, standard deviation, standard error, max, and min values of the microscopic characters were

calculated and presented as minimum–(mean minus standard deviation)–mean–(mean plus standard deviation)–maximum.

To determine optimum growth conditions, colony growth rate was determined for each representative isolate of the *Fusarium* sp. Three replicates of each isolate were assessed at temperatures ranging from 10–35 °C at 5 °C intervals grown in the dark. Agar plugs (5 mm) were cut from the edges of 1-week-old cultures and placed at the centres of 90 mm Petri dishes containing PDA. Two measurements of colony diameter perpendicular to each other were made, every day for 10 days, after which averages were determined. After 14 days, the cultures grown at 25 °C in the dark were used to characterize colony colour (surface and reverse), odour and colony morphology. Colony colours (surface and reverse) were described using the colour charts of Rayner (1970).

Pathogenicity assay

To determine the pathogenicity of the *Fusarium* sp. on *A. crassicarpa*, two isolates (Treatment 1 = CMW 52862 and Treatment 2 = CMW 51764) were selected and each inoculated into the stems of 16, seven-to-eight-month-old, *A. crassicarpa* trees. The fungal cultures were grown on PDA for 7 days at 25 °C, and 6 mm agar plugs were cut from the leading edges of the cultures using a sterilized cork borer. The same size cork borer was flame-sterilized and used to make wounds in the stems of the trees, removing the phloem tissues to expose the cambium. The agar plugs were then placed in the wounds, mycelium-side facing the cambium and the inoculation sites were covered with Parafilm® (Bemis NA) and sealed with adhesive tape. Sixteen trees were inoculated in the same manner with sterile PDA to serve as controls.

Inoculations were inspected after 30 days, by removing the bark to assess lesion development. Lesion lengths were measured from the edges of visible discolouration in the wood. A Shapiro–Wilk test and Levene's test were performed in R (v. 3.2.3), to assess whether the data was normally distributed and homogeneous, respectively. The data were subsequently raised to the power of -0.5 in R to obtain a normal distribution. To test the difference between lesion length of the different treatments and the controls, an analysis of variance (ANOVA) using the agricolae package in R was performed (de Mendiburu 2019). *Ad hoc* Tukey's bootstrap test was performed with the log-

transformed data at α= 0.05, to determine whether there were significant differences between the individual isolates compared to the control and between the isolates themselves. All plots were made using ggplot in the package R tidyverse (Wickham 2017).

To test Koch's postulates, xylem samples from the margins of the lesions were taken to attempt re-isolation of the inoculated fungi. Small pieces of the xylem tissue, including those from the control plants, were plated onto PDA and FSM amended with 100 µg streptomycin, and incubated at 25 °C for five days. The resulting isolates were identified using DNA sequences for the translation elongation factor 1-α (TEF1-α) as described above. Resulting sequences were aligned to the original isolate sequence data in CLC Bio Main Workbench v. 6 (CLC Bio, www.clcbio.com), to confirm the presence of the inoculated fungi.

Results

PCR amplification and sequencing

Beetle specimens

In total, 51 beetle galleries were extracted from infested trees (Fig. 1a), and 61 beetle specimens were collected. Based on preliminary morphological characterisation, five different beetle taxa were identified. Successful PCR amplification of the COI gene was carried out for all beetle specimens, with a resulting amplicon length of approximately 800 bp.

Morphological characters, such as body size (Fig. 1b), and ML phylogenetic analysis of the DNA sequence data (Fig. 2), showed that *Euwallacea perbrevis* (TSHBa) was the most abundantly collected beetle species, representing 49% of the samples. The 15 individual TSHBa beetles sequenced represented two distinct COI haplotypes (Fig. 2), one of which is novel. These haplotypes differed from each other by 6 bp, but both grouped into the same clade with other specimens collected from Indonesia (Gomez et al. 2018), with a 100% bootstrap support. The novel haplotype, represented by seven individuals, differed by 4bp to its closest lineage (MH276909), isolated from East Java, Indonesia. The second haplotype was represented by eight

individuals and grouped with a previously identified specimen from East Java, Indonesia (MH276910) with a bootstrap support of 89% (Gomez et al. 2018). The aligned dataset for COI (94 taxa, 569 characters) was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S25628) and all sequences obtained for the TSHBa beetle specimens in this study were deposited in GenBank (Table 2).

Morphological identification using the online, Southeast Asia ambrosia beetles identification resource (http://idtools.org/id/wbb/sea-ambrosia/index.php), and BLAST results of COI sequences of representative individuals, identified the remaining three beetle taxa as: *Xyleborinus exiguus* Wood & Bright 1992 (syn. *X. perexiguus*; 97% identity to HM064109), *Xylosandrus crassiusculus* Motschulsky 1866 (99% identity to KX035196) and *E. similis* Ferrari 1867 (94% identity to KU727036). The remaining beetle was identified as *Hypothenemus eruditus* Westwood 1836 (99% identity to KY800193), using the BLAST results of COI sequences and the online site 'Featured Creatures' (http://entnemdept.ufl.edu/creatures/). With the exception of *H. eruditus*, these are all ambrosia beetles that reside in the tribe Xyleborini. *Hypothenemus eruditus* is a true weevil in the Cryphalini.

Fungal isolates

Nighty-nine fungal isolates were obtained from the beetle mycangia and brood galleries. These included 18 different fungal genera (Supplementary Table 1), of which *Fusarium* spp. were most commonly isolated across all isolation methods. Due to the fact that beetles in the *E. fornicatus* complex commonly have an association with *Fusarium* spp., (Kasson et al. 2013), the *Fusarium* isolates obtained from the selected sub-population of *E. perbrevis* became the focus of this study.

The second most abundant fungi isolated were Hypocrealean fungi. In addition, a single isolate of a *Graphium* sp. and *Paracremonium* sp. were collected, both of which have previously been identified as community members associated with other species in the *E. fornicatus* complex (Lynch et al. 2016; Na et al. 2018). A single isolate was also collected that is genetically very similar to *Ambrosiella roeperi* T.C. Harr. & McNew (99% identity to NR154684), the mycangial symbiont of *X. crassiusculus* (Harrington et al. 2014).

A collection of 14 isolates of a *Fusarium* sp., originating from both mycangial isolations and isolations from the beetle galleries, was selected for further phylogenetic analysis using the ITS-LSU, TEF1-α and RPB 2 datasets. The amplicon lengths for the ITS-LSU, TEF1-α and RPB 2 gene regions were approximately 1100 bp, 700 bp and 1400 bp, respectively. All sequences obtained for the 14 *Fusarium* sp. isolates in this study were deposited in GenBank (Table 1). The aligned sequences for the ITS (89 taxa, 393 characters), RPB 2 (89 taxa, 1145 characters), TEF1-α (89 taxa, 278 characters), and the combined ITS, RPB2 and TEF1-α (89 taxa, 1815 characters) datasets, were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S25627). The best model of evolution for the combined dataset was identified as TIM+I+G.

Maximum likelihood phylogenetic analyses of the individual TEF1-α (Supplementary Fig. 1) and RPB2 (Supplementary Fig. 2) datasets, as well as the combined ITS, RPB 2 and TEF1-α (Fig. 3) dataset, consistently showed that the isolates from both the *E. perbrevis* mycangia and brood galleries, represented a distinct and previously undescribed species. This new taxon, which had high bootstrap support in all analyses, formed a new AFC lineage in subclade B, identified here as AF-19.

RPB 2 was the most informative gene region, providing high bootstrap support for eight of the 19 AFC species, including the new lineage (AF-19) emerging from this study (Supplementary Fig 2). In contrast, bootstrap analysis of the ITS rDNA provided the weakest signal and resolved only three of the 19 AFC species (Supplementary Fig 3). As has been found previously, the combined analysis (ITS, RPB2 and TEF1-α), provided the strongest support for the evolutionary relationships within members of the AFC, with high bootstrap support for 11 of the 19 AFC species. In the combined analysis, a total of 13 out of 19 AFC members were resolved with the exceptions of AF-12, AF-13, AF-14, AF-15, AF-17 and AF-18 (Fig. 3). Thus, phylogenetic analyses across two of the three gene regions consistently supported the presence of a previously unknown *Fusarium* lineage (AF-19), distinct from all other previously identified clades for *Fusarium* spp. associated with ambrosia beetles.

The topology of the ML phylogram presented in this study based on the combined data set, differed slightly from that in a previous study by Carrillo et al. (2019). Unlike their analyses, we

excluded the RPB 1 gene region as we were unable to successfully amplify this gene region. Major groupings within both analyses were, however, similar. Bootstrap support for the two AFC clades (Clade A and B of Kasson et al. 2013) was well supported (Fig. 3). However, support for the backbone of the AFC Clade B, in which our isolates from *E. perbrevis* grouped, was weak with 11 nodes having a bootstrap value below 50%.

Taxonomy

Based on morphological and molecular evidence, the *Fusarium* isolates from *E. perbrevis* mycangia and brood galleries collected from infested *A. crassicarpa,* represent a novel associate of the TSHBa beetle. It is consequently described as follows:

Fusarium rekanum sp. nov. Lynn & Marinc. (Figs. 3, 4)

MycoBank (MB 831926)

Etymology The name *rekanum* is derived from the Bahasa Indonesia word "rekan" meaning associate and reflects the relationship between the fungus and its beetle vector, *E. perbrevis*.

Culture characteristics Colony colour on PDA at 25 ºC for 14 d in dark white to buff (pale yellow). Colony margin smooth and colony elevation raised to umbonate. Reverse pigmentation pale luteous to luteous. Odour of yeast or brewing beer. Exudates absent. Colonies on PDA in dark radial mycelial growth rates of an average of 2.49 mm/d at 15 ºC, 4.94 mm/d at 20 ºC, 7.66 mm/d at 25 ºC, 7.52 mm/d at 30 ºC and 0.9 mm/d at 35 ºC. *Conidial pustules*, produced on sporodochia, formed on older cultures grown on PDA, pale luteous to saffron, luteous to sienna, and ochreous. *Aerial mycelium* sparse with occasional pionnotal colony appearance, or developed abundantly, loose to floccose, white. *Chlamydospores* formed abundantly in hyphae, rarely in conidia, mostly round to oval, intercalary or terminal, single, paired, rarely clustered, or often in chains, hyaline, smooth, rarely rough-walled, 6–(7)–9.5–(11.5)–14.5 × 4–(7)–9–(11)–14 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, retarded on PDA. *Sporodochia* formed sparsely on SNA, abundantly on PDA. *Aerial conidiophores* formed abundantly on SNA and PDA,

erect, tall, thin walled and narrow with no tapering towards apex, mostly unbranched, 24–(49.5)–77.5–(105.5)–139.5 × 2–(4.5)–6–(7.5)–10 µm, forming monophialides integrated in apices. *Aerial phialides* simple, sub-cylindrical with slight bulging at midsection, with discreet collarette. *Aerial conidia* (1) mostly oval, two-celled oval to obovoid with truncated base, 0–1(–2)-septa, 7.5–(10)–13–(16)–18 × 3–(4.5)–5–(6)–7 µm, (2) falcate to clavate, slightly curved cylindrical, (0–)2–5(–6)-septate conidia, morphologically similar to the falcate to clavate sporodochial conidia. *Sporodochial conidiophores* thick, short and typically undulate, mostly unbranched or irregularly branched, forming apical monophialides. *Sporodochial phialides* simple, curved or crooked tube shaped, with discreet collarette. *Sporodochial conidia* (1) hyaline, mostly curved clavate, swollen in upper parts, tapering toward the base, often with a round, blunt or occasionally slightly papillate apical cell, with a barley notched distinct foot–like basal cell, (0–)2–6 (–6)-septate, formed on PDA and SNA, 33–(36)–40–(44)–48 × 6.5–(7)–8.5–(9.5)–11.5 µm, (2) obovoid or short-clavate to pyriform, curved or straight conidia, with a rounded apex and truncate base, (0–)1(–2)-septate, formed sometimes together with multi-septate conidia from sporodochial conidiophores, particularly on PDA.

Habitat Acacia crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia

Distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation, isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa), April 2017, K. M. T. Lynn, HOLOTYPE PREM 62333 (dried culture of CMW 52862), living culture ex-type CMW 52862 = PPRI 27163.

Additional specimens Indonesia, Riau, Pelalawan, *A. crassicarpa* plantation, isolated from a mycangium of *E. perbrevis* (TSHBa). November 2018, K. M. T. Lynn, PREM 62334 (dried culture of CMW 53688), living culture CMW 53688 = PPRI 27165; PREM 62335 (dried culture of CMW 53691), living culture CMW 53691 = PPRI 27164.

Notes: Fusarium rekanum resembles *Fusarium* spp. residing in the clade that accommodates the associates of *Euwallacea* spp., particularly *F. euwallaceae*, *F. kuroshium*, and *F. ambrosium*,

in various morphological features. It differs from *F. euwallaceae, F. kuroshium* and *F. ambrosium* in producing very few rough-walled chlamydospores formed on conidia. *Fusarium rekanum* also differs from both *F. euwallaceae* and *F. kuroshium* in the pigmentation of the conidial pustules, which are lighter in colour on PDA, and the lack of pigmented sporodochial conidia. *Fusarium kuroshium* has darker colonies after 14 days when compared to *F. rekanum*.

Pathogenicity assay

The *A. crassicarpa* trees inoculated with the two isolates of *F. rekanum* sp. nov., showed no external signs of disease after 30 days. However, red-coloured lesions, that were absent in the control inoculation, were observed around the inoculation sites, underneath the bark (Fig. 5). *Acacia crassicarpa* inoculated with isolate CMW 52862 (Treatment 1) produced lesions with a mean length of 8.2 ± 4.8 cm and *A. crassicarpa* inoculated with isolate CMW 51764 (Treatment 2) produced lesions with a mean length of 13.9 ± 7.6 cm. Trees treated with sterile agar plugs as controls produced wound response lesions having a mean length of 5.2 ± 1.6 cm. Mean lesion lengths associated with isolate CMW 51764 (Treatment 2) were found to be significantly larger than the controls ($P < 0.05$). Isolate CMW 52862 (Treatment 1) did not produce significantly longer lesions when compared to the negative control ($P > 0.05$) (Fig. 6)

The TEF1-α sequences generated for the cultures re-isolated from the lesions, aligned perfectly to the TEF1-α sequence data for the isolates (CMW 52862 and CMW 51764) used for the inoculations. Isolations from the stained margins of the lesions (wound response) associated with the control inoculations yielded only secondary contaminant fungi.

Discussion

Based on morphological and molecular characteristics, the results of this study show that *E. perbrevis* (the Tea Shot Hole Borer A) is the most commonly encountered beetle infesting healthy *A. crassicarpa* trees in the sampled areas of Riau, Indonesia. This beetle was identified following the recent taxonomic revision of Smith et al. (2019), that included the same species collected in East Java, Indonesia. The dominant fungal associate of this beetle was identified as a new AFC *Fusarium* species, described here as *F. rekanum* sp. nov. A preliminary inoculation trial with *F.*

rekanum sp. nov. showed that the fungus is able to cause lesions but that it is likely not an aggressive pathogen on *A. crassicarpa*.

Specimens collected across multiple geographically separated compartments, at varying time points and on trees of different ages, show that *E. perbrevis* is a common pest on the sampled, non-native *A. crassicarpa* in Riau, Indonesia. This is the same species that has previously been treated in the aggregate species *E. fornicatus* (Smith et al. 2019), and that is believed to be native to the area. The common occurrence of this beetle over a relatively wide distribution suggests that they have migrated from the natural forests into the *A. crassicarpa* plantations.

Fusarium rekanum could be distinguished as a novel lineage, separate from the 18 other previously identified *Fusarium* spp. (AFC species) associated with *Euwallacea* spp., (Carrillo et al. 2019) based on multilocus phylogenetic inference. The invasive haplotype (H8) of *E. perbrevis* (TSHBa), has recently been shown to vector five members (AF-1, AF-13, AF-14, AF-17, AF-18) of the AFC (Carrillo et al. 2019). Thus far, only *F. ambrosium* (AF-1) has been formally described (Gadd and Loos 1947; Kasson et al. 2013), while the remaining four taxa (AF-13, 14, 17, 18), originating from Taiwan, have yet to be named (Carrillo et al. 2019). None of these AFC members were isolated from *E. perbrevis* mycangia or corresponding brood galleries such as those analysed in the present study. Our phylogenetic analysis suggests that *F. rekanum* is relatively distantly related to these other AFC members (Fig. 3). Because *F. rekanum* is the only species thus far described from Indonesia, this could reflect the geographic origins, plant hosts, and different haplotypes of the *Euwallacea* beetles from which the fungi were isolated.

The two isolates of *F. rekanum* tested for pathogenicity in this study resulted in lesions, but these were relatively small, and similar to those produced in the control inoculated *A. crassicarpa*. This implies that the fungus has a low level of aggressiveness, although this was a preliminary study including only a small number of trees. Future studies including a greater number of isolates would be required to better understand whether the fungus might play a role in tree decline. Therefore, it is possible that this species is purely involved in providing nutrition for its beetle symbiont (Beaver 1989; Farrell et al. 2001).

Currently, the damage observed on *A. crassicarpa* appears to be the combination of the mechanical damage caused by mass attack of beetles, and the repeated inoculation of the weakly pathogenic *Fusarium* sp. The contribution, if any, of the other fungi isolated in this study (Supplementary Table 1), in the observed tree decline would also need to be further investigated. This would especially be true for the *Graphium* sp. and *Paracremonium* sp. as they have previously been shown to play an important role in the development and establishment of their beetle hosts (Lynch et al. 2016).

Mass accumulation of a variety of ambrosia beetles in a host can occur over a period of months, with multiple species coexisting in the same habitat (Carrillo et al. 2014). In the present study, five different beetle species were found in moderately infested tree hosts. However, as South East Asia is believed to be the native range of many species of the Xyleborini (Hulcr et al. 2015), it is possible that many more species of ambrosia beetle and their associated fungi could infest *A. crassicarpa*. This overlap in gallery occupation by different ambrosia beetle species, may give rise to lateral transmission of the associated fungi between different beetle species (Ploetz et al. 2017). How specific these beetle and fungal associations are, and whether *F. rekanum* is obligately required for the survival and development of *E. perbrevis,* remains to be determined.

The extensive native range of species in the *E. fornicatus* species complex and the results of phylogenetic analyses presented here, and in previous studies on these insects, suggests that there is substantial biological variation among native populations of *E. perbrevis* and their *Fusarium* spp. associates (Gomez et al. 2018; Smith et al. 2019). Consistent with a recent study by Carrillo et al. (2019), we hypothesise that a promiscuous relationship exists between members of the *E. fornicatus* complex and *Fusarium* spp., in areas where these organisms are native. This is in contrast to the strict symbiosis observed in non-native areas (O'Donnell et al. 2015; Stouthamer et al. 2017).

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Tables

Table 1 Isolates and sequence data used in the phylogenetic analysis of this study

Isolate	Species	Host	Location	GenBank numbers[a]		
				ITS	TEF1- α	RPB2[b]
CMW52862[c,d,e,f]	*Fusarium* sp.	*Euwallacea perbrevis* (TSHBa)	PKU, Indonesia	MN249094	MN249151	MN249137, MN249108
CMW53702[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249095	MN249152	MN249138, MN249109
CMW53701[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249096	MN249153	MN249139, MN249110
CMW53691[ef]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249097	MN249154	MN249140, MN249111
CMW53690[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249098	MN249155	MN249141, MN249112
CMW53689[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249099	MN249156	MN249142, MN249113
CMW53688[ef]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249100	MN249157	MN249143, MN249114
CMW51756[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249101	MN249158	MN249144, MN249115
CMW51757[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249102	MN249159	MN249145, MN249116
CMW51760[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249103	MN249160	MN249146, MN249117
CMW51764[de]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249104	MN249161	MN249147, MN249118
CMW51765[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249105	MN249162	MN249148, MN249119
CMW51767[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249106	MN249163	MN249149, MN249120

CMW51769[e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249107	MN249164	MN249150, MN249121
UCR3641[c]	*F. kuroshium* [AF-12]	*Platanus racemosa*	El Cajon, CA	KX262196	KX262216	KX262256
UCR3644	*F. kuroshium* [AF-12]	*P. racemosa*	El Cajon, CA	KX262197	KX262217	KX262257
UCR3651	*F. kuroshium* [AF-12]	*Euwallacea* sp.	Fallbrook, CA	KX262198	KX262218	KX262258
UCR3653	*F. kuroshium* [AF-12]	*Persea americana*	Bonsall, CA	KX262200	KX262220	KX262260
UCR3654	*F. kuroshium* [AF-12]	*P. americana*	Bonsall, CA	KX262201	KX262221	KX262261
UCR3652	*F. kuroshium* [AF-12]	Unknown	Unknown	KX262199	KX262219	KX262259
UCR4672	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taichung, Taiwan	KX262206	KX262226	KX262266
UCR4673	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taichung, Taiwan	KX262207	KX262227	KX262267
UCR4674	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taichung, Taiwan	KX262208	KX262228	KX262268
UCR4675	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taichung, Taiwan	KX262209	KX262229	KX262269
UCR4676	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Taichung, Taiwan	KX262210	KX262230	KX262270
UCR4677	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taichung, Taiwan	KX262211	KX262231	KX262271
UCR4678	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taichung, Taiwan	KX262212	KX262232	KX262272
UCR4679	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Taichung, Taiwan	KX262213	KX262233	KX262273
UCR4680	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Taichung, Taiwan	KX262214	KX262234	KX262274

UCR4681	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taichung, Taiwan	KX262215	KX262235	KX262275
NRRL20438	*F. ambrosium* [AF-1]	*E. perbrevis* (TSHBa)	India	AF178397	AF178332	JX171584
NRRL22231	*F. tuaranense* [AF-5]	*Hevea brasiliensis*	Malaysia	KC691570	KC691542	KC691631, KC691660
NRRL22346	*F. ambrosium* [AF-1]	*E. perbrevis* (TSHBa)	India	EU329669	FJ240350	EU329503
NRRL22468	*F. neocosmosporiellum*	*Arachis hypogaea*	Guinea	DQ094318	AF178349	EU329512
NRRL22643	*Fusarium* sp. [AF-9]	*Xyleborus ferrugineus*	Costa Rica	KC691583	DQ247628	KC691644, KC691673
NRRL32434	*F. lichenicola*	*Homo sapiens*	Germany	DQ094444	DQ246977	EF470161
NRRL46518	*F. tuaranense* [AF-5]	*H. brasiliensis*	Malaysia	KC691571	KC691543	KC691632, KC691661
NRRL54722	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038014	JQ038007	JQ038028
NRRL54723	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038015	JQ038008	JQ038029
NRRL54724	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038016	JQ038009	JQ038030
NRRL54725	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038017	JQ038010	JQ038031
NRRL54726	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038018	JQ038011	JQ038032
NRRL54727	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038019	JQ038012	JQ038033
NRRL62578	*F. oligoseptatum* [AF-4]	*E. validus*	PA (U.S.A.)	KC691565	KC691537	KC691626, KC691655
NRRL62579[c]	*F. oligoseptatum* [AF-4]	*E. validus*	PA (U.S.A.)	KC691566	KC691538	KC691627, KC691656

NRRL62584	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691582	KC691554	KC691643, KC691672
NRRL62585	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691577	KC691554	KC691638, KC691667
NRRL62590	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691574	KC691546	KC691635, KC691664
NRRL62591	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691573	KC691545	KC691634, KC691663
NRRL62606	*F. floridanum* [AF-3]	*Euwallacea* sp.	FL (U.S.A.)	KC691561	KC691533	KC691622, KC691651
NRRL62610	*F. obliquiseptatum* [AF-7]	*Euwallacea* sp.	Australia	KC691575	KC691547	KC691636, KC691665
NRRL62611	*F. obliquiseptatum* [AF-7]	*Euwallacea* sp.	Australia	KC691576	KC691548	KC691637, KC691666
NRRL62629	*F. floridanum* [AF-3]	*E. interjectus*	FL (U.S.A.)	KC691564	KC691536	KC691625, KC691654
NRRL62941	*Fusarium* sp. [AF-10]	Unknown	Singapore	KM406633	KM406626	KM406647
NRRL62942	*F. ambrosium* [AF-1]	*Camellia sinensis*	Sri Lanka	KM406631	KM406624	KM406645
NRRL62944	*Fusarium* sp. [AF-11]	*C. sinensis*	Sri Lanka	KM406634	KM406627	KM406648
NRRL66088	*Fusarium* sp. [AF-9]	*Delonix regia*	FL (U.S.A.)	KM406632	KM406632	KM406646
TW1	*Fusarium* sp. [AF-18]	*P. americana*	Danei District, Taiwan	MK432860	MK435437	MK435521
TW2	*Fusarium* sp. [AF-14]	*P. americana*	Danei District, Taiwan	MK432862	MK435439	MK435523
TW4	*Fusarium* sp. [AF-16]	*P. americana*	Danei District, Taiwan	MK432866	MK435443	MK435527
TW15	*Fusarium* sp. [AF-15]	*P. americana*	Danei District, Taiwan	MK432861	MK435438	MK435522

TW25	*Fusarium* sp. [AF-16]	*P. americana*	Danei District, Taiwan	MK432863	MK435440	MK435524
TW34	*Fusarium* sp. [AF-16]	*P. americana*	Danei District, Taiwan	MK432864	MK435441	MK435525
TW37	*Fusarium* sp. [AF-16]	*P. americana*	Danei District, Taiwan	MK432865	MK435442	MK435526
TW40	*Fusarium* sp. [AF-17]	*P. americana*	Danei District, Taiwan	MK432867	MK435444	MK435528
TW43	*F. kuroshium* [AF-12]	*P. americana*	Danei District, Taiwan	MK432868	MK435445	MK435529
TW44	*Fusarium* sp. [AF-18]	*P. americana*	Danei District, Taiwan	MK432869	MK435446	MK435530
TW45	*Fusarium* sp. [AF-15]	*P. americana*	Danei District, Taiwan	MK432870	MK435447	MK435531
TW55	*Fusarium* sp. [AF-18]	*P. americana*	Danei District, Taiwan	MK432871	MK435448	MK435532
TW56	*Fusarium* sp. [AF-14]	*P. americana*	Danei District, Taiwan	MK432872	MK435449	MK435533
UCR5499	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Danei District, Taiwan	MK432873	MK435450	MK435534
UCR5508	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Danei District, Taiwan	MK432874	MK435451	MK435535
UCR5509	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Danei District, Taiwan	MK432875	MK435452	MK435536
UCR5513	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Danei District, Taiwan	MK432876	MK435453	MK435537
UCR5545	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Danei District, Taiwan	MK432877	MK435454	MK435538
UCR5546	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Danei District, Taiwan	MK432878	MK435455	MK435539
UCR5557	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Danei District, Taiwan	MK432879	MK435456	MK435540

UCR5584	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Danei District, Taiwan	MK432880	MK435457	MK435541
UCR6394	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Danei District, Taiwan	MK432881	MK435458	MK435542
UCR6395	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Danei District, Taiwan	MK432882	MK435459	MK435543
UCR6403	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Danei District, Taiwan	MK432883	MK435460	MK435544
UCR6405	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Danei District, Taiwan	MK432884	MK435461	MK435545
UCR6408	*F. kuroshium* [AF-12]	*Euwallacea* sp.	Danei District, Taiwan	MK432885	MK435462	MK435546
UCR6409	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Danei District, Taiwan	MK432886	MK435463	MK435547
UCR6411	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Danei District, Taiwan	MK432887	MK435464	MK435548
UCR6414	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Danei District, Taiwan	MK432888	MK435465	MK435549
UCR6417	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Danei District, Taiwan	MK432889	MK435466	MK435550
UCR6432	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Danei District, Taiwan	MK432890	MK435467	MK435551
UCR6436	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Danei District, Taiwan	MK432891	MK435468	MK435552

[a] ITS = internal transcribed spacer region; TEF1- α = translation elongation factor 1- α; RPB2 = DNA-directed RNA polymerase II subunit.

[b] Two accession numbers correspond to unjoined RPB2-1 and RPB2-2 sequences.

[c] Type-species

[d] Isolates used in the pathogenicity test.

[e] Indonesian isolates collected and sequenced in this study

[f] Isolates used in morphological descriptions and growth studies

PKU = Pekanbaru; CA = California; PA = Pennsylvania; FL = Florida

Table 2 Descriptions and sequences of specimens collected in this study and used for phylogenetic analysis

Specimen	Genetic lineage	Country	Locality	GenBank accession COI gene region
EB471	*Euwallacea perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249122
EB472	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249123
EB473	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249124
EB474	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249125
EB475	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249126
EB476	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249127
EB477	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249128
EB478	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249129
EB479	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249130
EB480	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249131
EB481	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249132
EB482	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249133
EB483	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249134
EB484	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249135
EB485	*E. perbrevis* (TSHBa)	Indonesia	Pekanbaru, Riau	MN249136

Figures

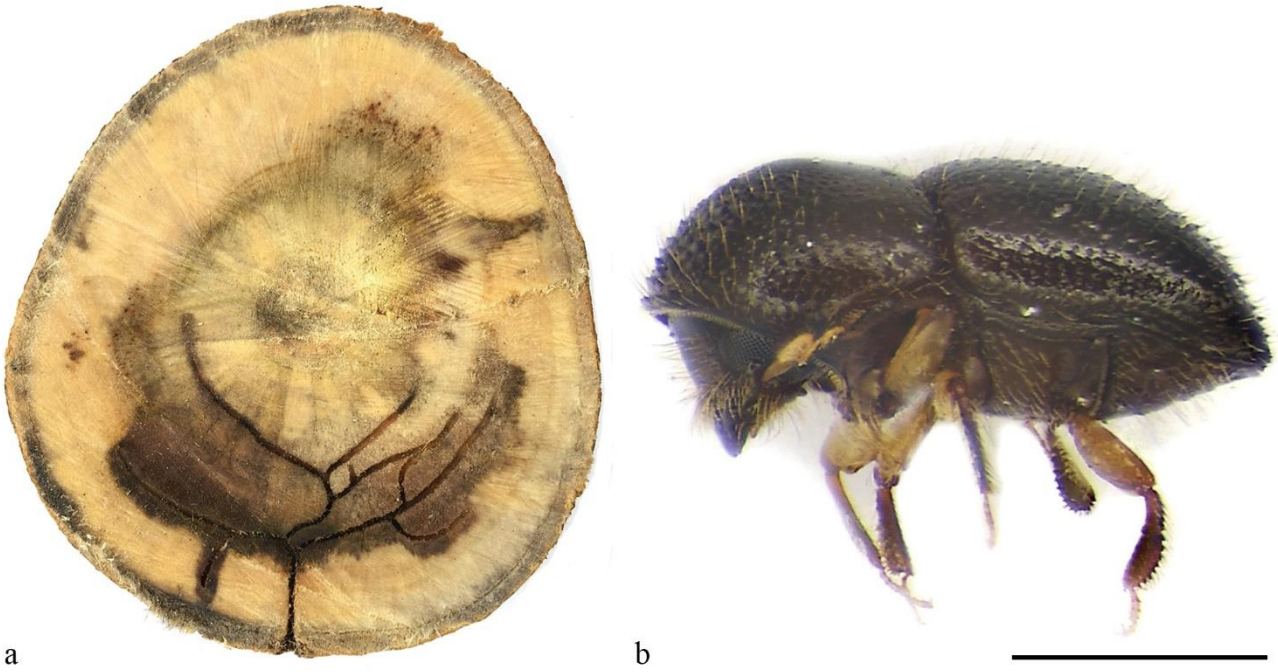


Fig. 1 *Euwallacea perbrevis* and its brood gallery. **a.** Brood gallery of *Euwallacea perbrevis* (TSHBa) in two-year-old *A. crassicarpa*. **b.** Mature female *Euwallacea perbrevis* (TSHBa) beetle collected from infested *Acacia crassicarpa* in Indonesia. Scale bar = 1.0 mm

Fig. 2 Phylogenetic tree based on maximum likelihood (ML) analysis of COI gene sequences for various ambrosia beetle species in the *Euwallacea fornicatus* sensu lato complex (Gomez et al. 2018). Specimens in bold were sequenced in this study and represented two distinct haplotypes. Both haplotypes grouped with the other specimens collected from Indonesia with 100% bootstrap support. One novel haplotype represented by seven individuals was supported with a bootstrap of 95%, whereas the second haplotype grouped with other previously identified specimen from Indonesia (MH276910) and contained eight individuals with a bootstrap support of 89%. *Ambrosiophilus sexdentatus* (HM064051) represents the outgroup.

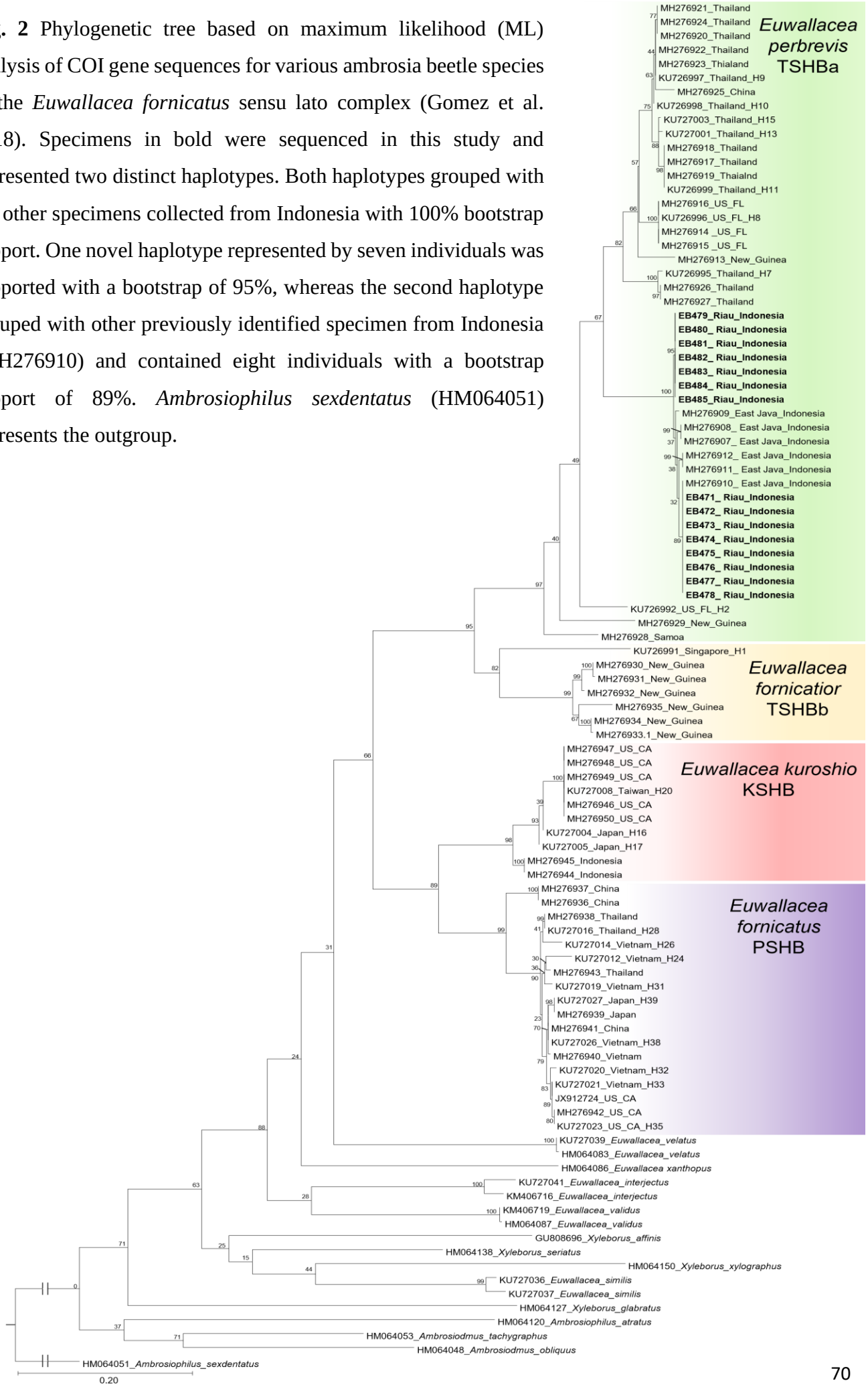
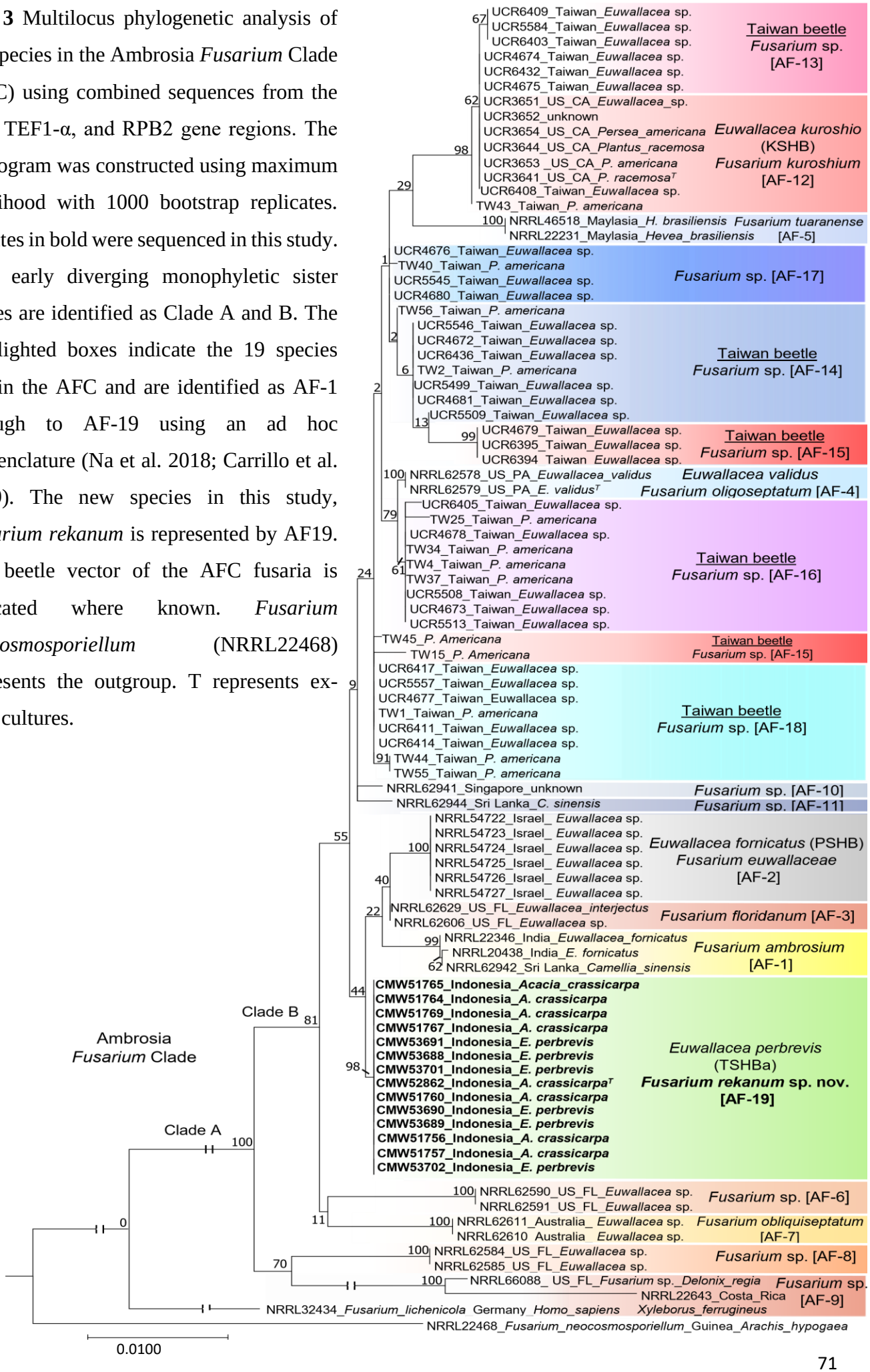


Fig. 3 Multilocus phylogenetic analysis of the species in the Ambrosia *Fusarium* Clade (AFC) using combined sequences from the ITS, TEF1-α, and RPB2 gene regions. The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. Two early diverging monophyletic sister clades are identified as Clade A and B. The highlighted boxes indicate the 19 species within the AFC and are identified as AF-1 through to AF-19 using an ad hoc nomenclature (Na et al. 2018; Carrillo et al. 2019). The new species in this study, *Fusarium rekanum* is represented by AF19. The beetle vector of the AFC fusaria is indicated where known. *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type cultures.



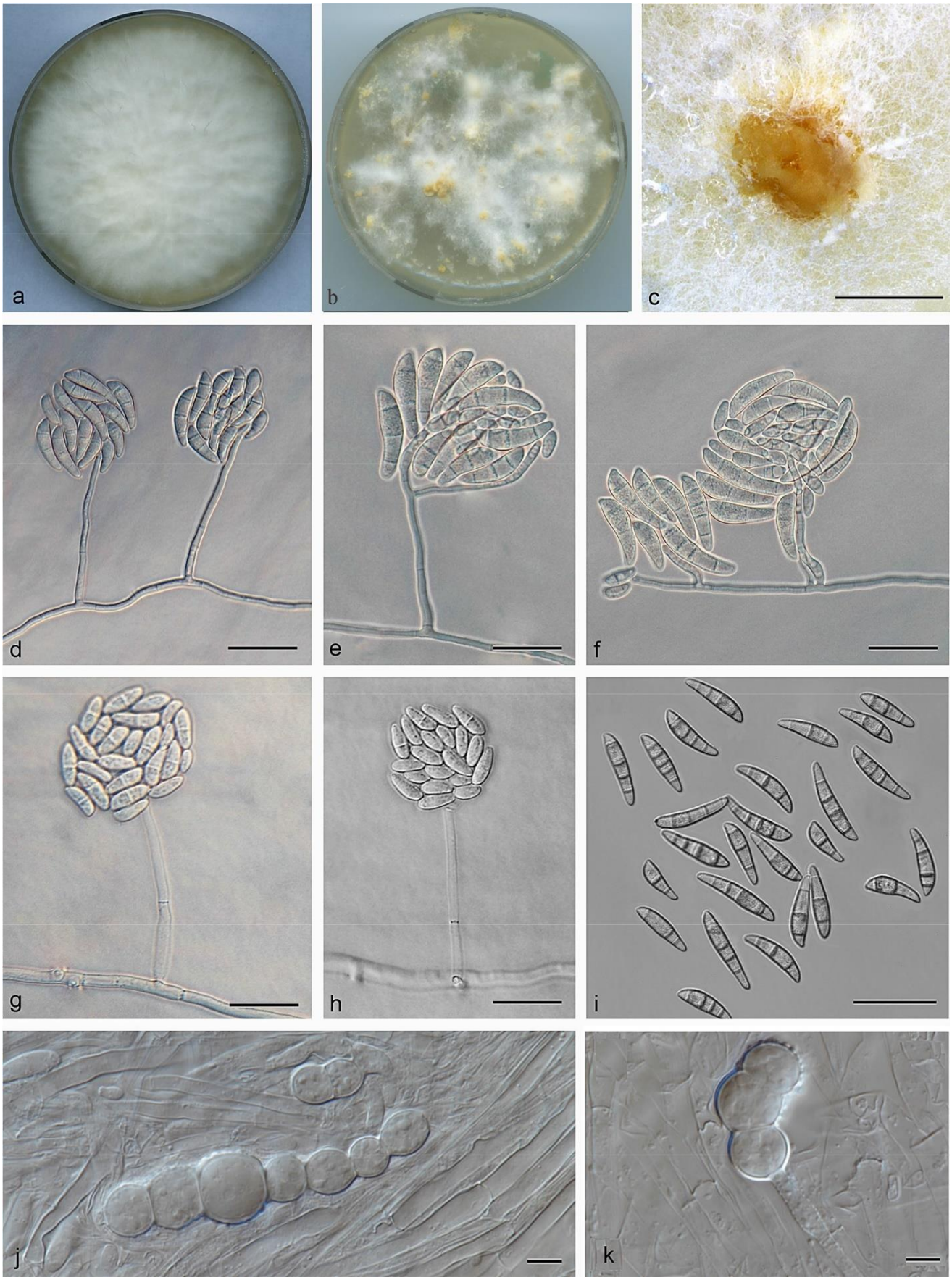


Fig. 4 Morphological characteristics of *Fusarium rekanum* (ex-type CMW 52862 = PPRI 27163) on PDA and SNA. **a–c, i.** Morphology of *F. rekanum* cultures grown on potato dextrose agar (PDA), **d–h, j–k.** morphology of 14-day-old *F. rekanum* cultures grown on synthetic low-nutrient

agar (SNA). **a.** Colony morphology on PDA at 2 weeks. **b, c.** White-buff conidial masses formed in culture on PDA after 1 month. **d–h.** Simple to branched aerial conidiophores forming 0-multiseptate conidia, often swollen apically with 1–5 septa or oval to short clavate with 0–1 septa. **i.** Multiseptate sporodochial conidia with a round, blunt apical cell, and a barley notched distinct foot-like basal cell with 4–5 septa. **j–k.** Round to oval chlamydospores, in chains, formed intercalary or terminally in hyphae. Scale bars: **c** = 2 mm, **d–i** = 30 µm, **j–k** = 5 µm.

Fig. 5 Lesions formed on seven to eight-month-old *Acacia crassicarpa* trees inoculated with a control and with *Fusarium rekanum* after a 30-day incubation period. **a.** Wound response of *A. crassicarpa* inoculated with the control. **b.** Red lesions on *A. crassicarpa* inoculated with CMW 52862 (Treatment 1).

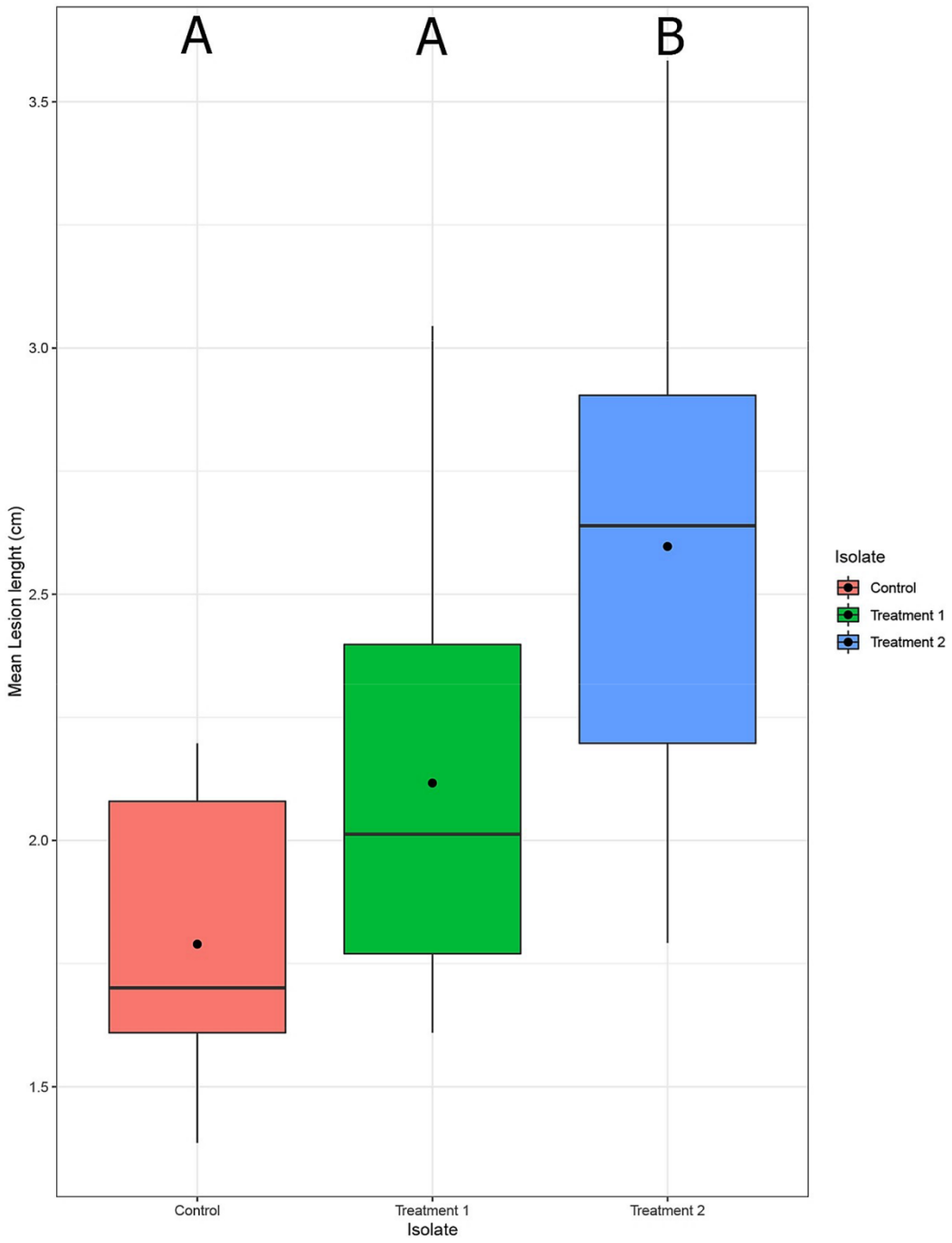


Fig. 6 Statistical variance and mean of log transformed lesion lengths (cm) on inoculated *A. crassicarpa*, represented in a boxplot. Based on the ad hoc Tukey's bootstrap test, Treatment 1 (CMW 52862) is not significantly different from the control as denoted by (A). Treatment 2 (CMW 51764) is the only treatment that is significantly different, both from the control and from treatment 2 (B).

Additional text

Supplementary Table 1 List of all fungi isolated from the beetle mycangium and brood galleries of ambrosia beetles infesting *A. crassicarpa*.

CMW Culture number Isolate	Host	Country	Date collected	Preliminary identification (Closest hit on NCBI)
51753	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51754	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51755	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51756	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51757	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51758	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51759	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51760	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51761	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	Unidentified
51762	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.

51763	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	Unidentified
51764	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51765	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51766	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51767	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51768	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
51769	Infested *Acacia crassicarpa*	Riau, Indonesia	October 2017	*Fusarium* sp.
52862	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52864	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52865	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52866	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52867	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52868	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.
52869	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	*Fusarium* sp.

-	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Phaeoacremonium Fusariumcum*
52848	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	100% *Gliomastix polychroma*
52849	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Xylariales* sp.
52850	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Phialemonium curvatum*
52851	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	Unidentified
52852	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	98% *Ascomycota* sp.
52853	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	98% *Paracremonium contagium*
52854	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	100% *Chaetomium* sp.
52855	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Xenoacremonium recifei*
52856	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Gliomastix polychrom*
52857	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	100% *Cosmospora butyri*
52858	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Xylariales* sp.
52859	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Ambrosiella roeperi*
52860	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	Unidentified

52861	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	Unidentified
52863	Beetle gallery in *A. crassicarpa*	Riau, Indonesia	April 2018	99% *Phialemonium* sp.
53687	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53688	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53689	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53690	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53691	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53692	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53693	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	95% *Actinomucor elegans* var. *meitauzae* strain
53694	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53695	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53696	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53697	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified
53698	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified

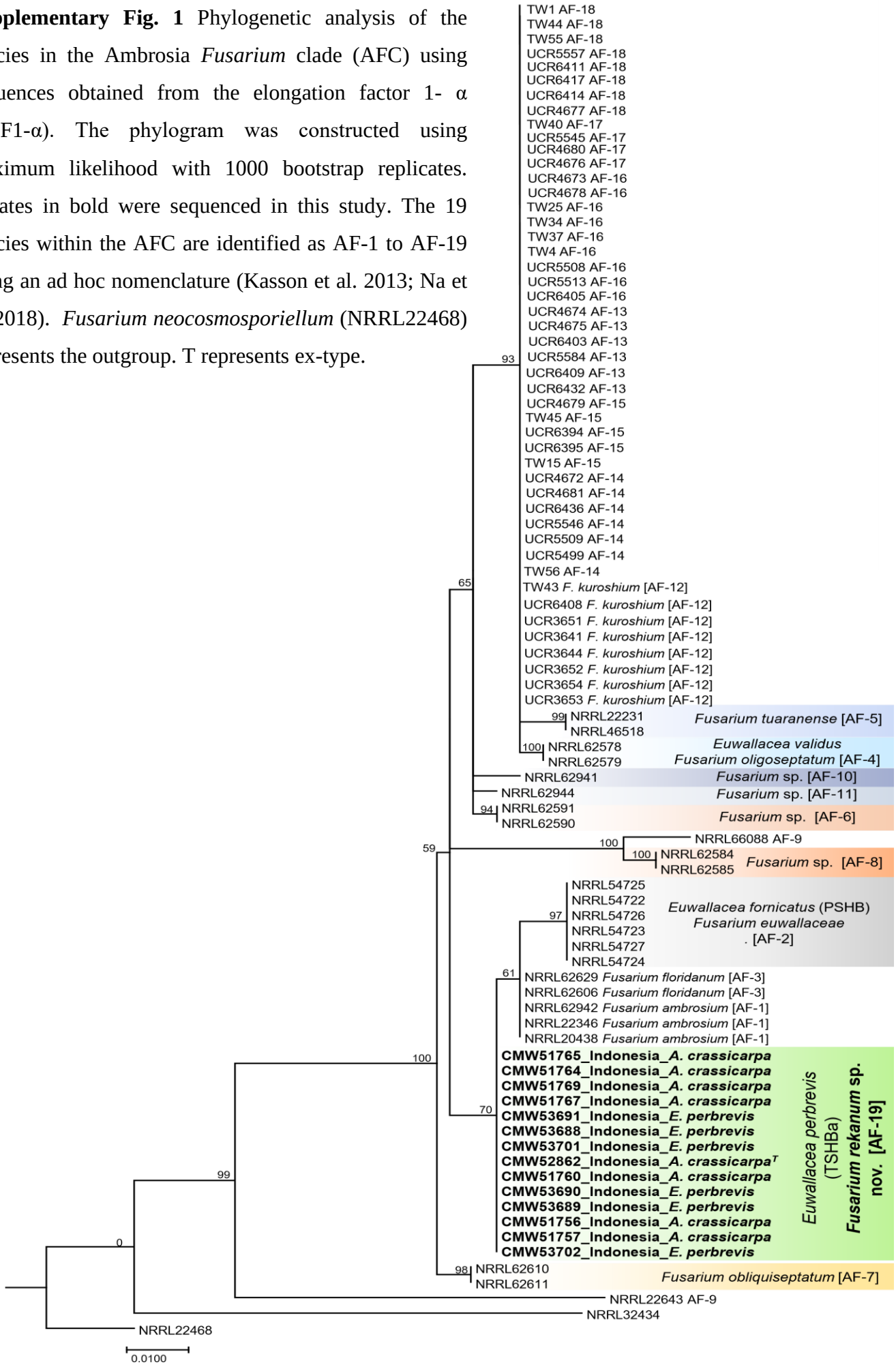
53699	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53700	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53701	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53702	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53703	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53704	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	97% *Hypocreales* sp.
53705	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53706	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53707	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53708	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53709	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	96.7% *Phialemoniopsis curvata*
53710	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	96.7% *Phialemoniopsis curvata*
53711	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53712	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified

53713	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53714	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53715	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53716	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53717	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53718	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53719	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53720	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53721	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53722	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53723	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53724	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53725	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99.5% *Graphium euwallaceae*
53726	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	94.2% *Rhizomucor variabilis*

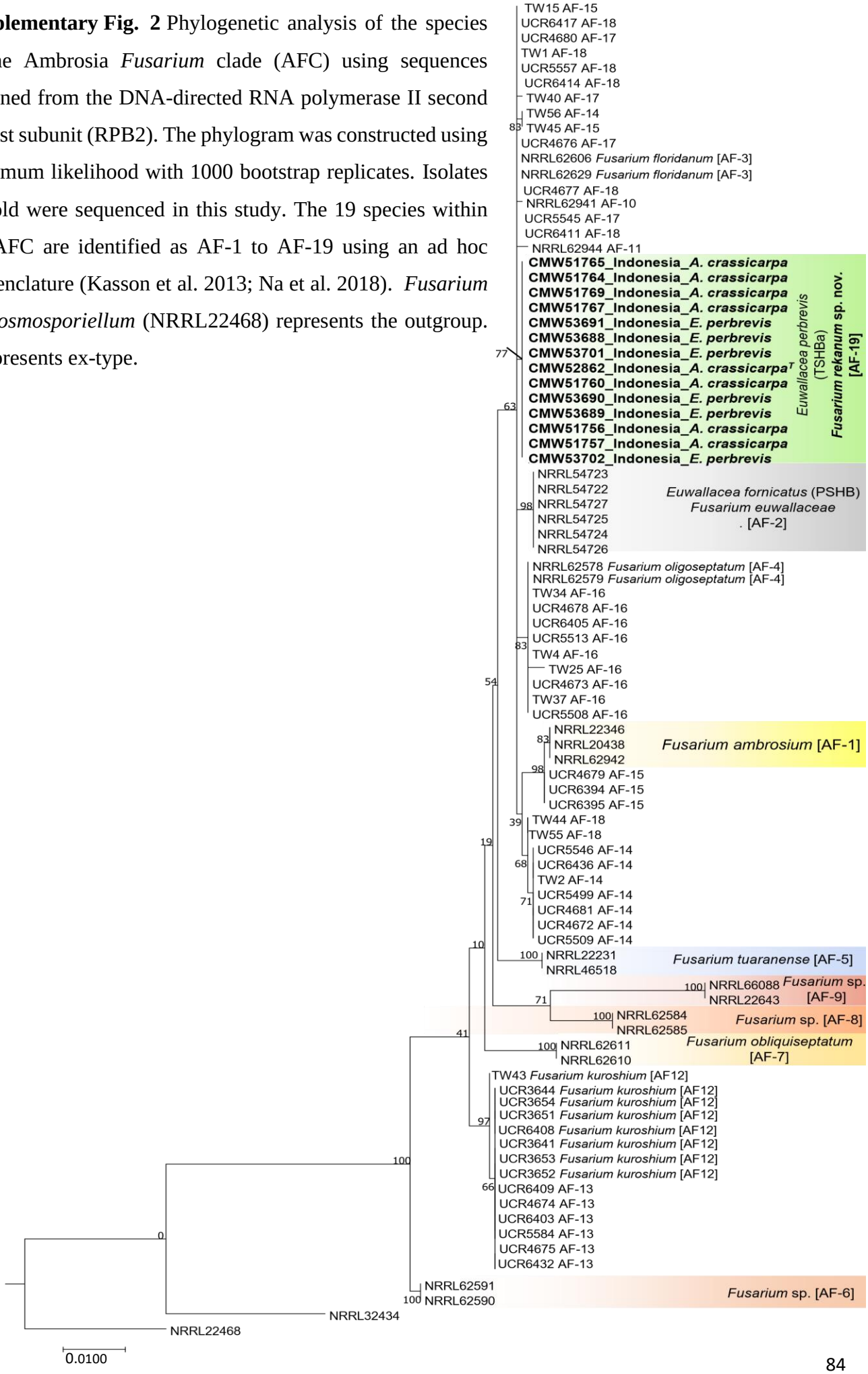
53727	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99.7% *Geotrichum ghanense*
53728	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53729	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
"-	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53730	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53731	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53732	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified
53733	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	97.4% *Hypocreales* sp.
53808	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified
53734	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53735	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	*Fusarium* sp.
53736	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	97% *Hypocreales* sp.
53737	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	98% *Hypocreales* sp.
53738	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99% *Hypocreales* sp.

53739	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unidentified
53740	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99% *Hypocreales* sp.
53741	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99% *Hypocreales* sp.
53742	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	Unknown
53743	Xyleborini beetle mycangium	Riau, Indonesia	September 2018	99% *Hypocreales* sp.

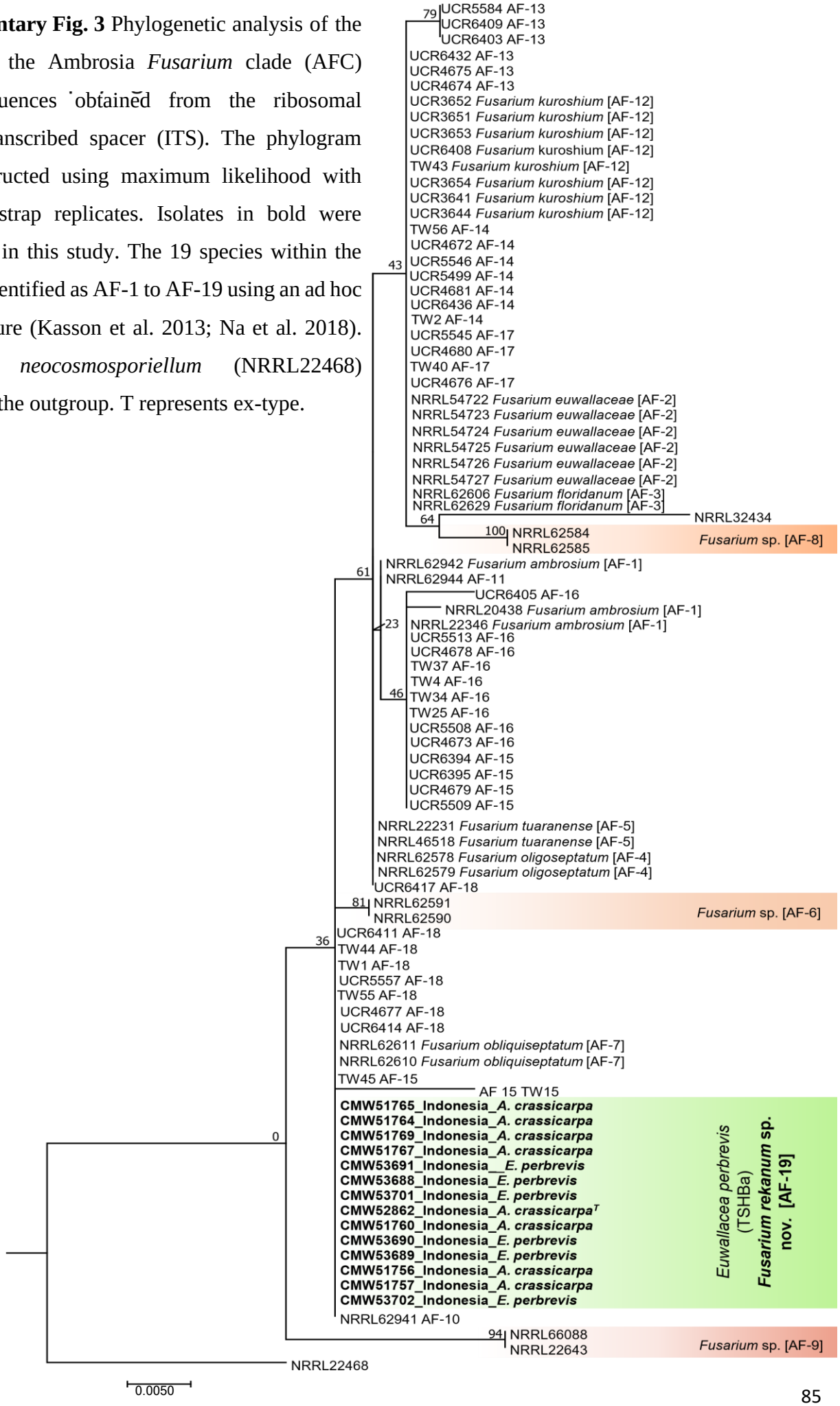
Supplementary Fig. 1 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the elongation factor 1- α (TEF1-α). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 19 species within the AFC are identified as AF-1 to AF-19 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Supplementary Fig. 2 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the DNA-directed RNA polymerase II second largest subunit (RPB2). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 19 species within the AFC are identified as AF-1 to AF-19 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Supplementary Fig. 3 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the ribosomal internal transcribed spacer (ITS). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 19 species within the AFC are identified as AF-1 to AF-19 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Chapter 3

Two *Euwallacea* spp. infesting *Acacia crassicarpa* in Indonesia and their promiscuous novel *Fusarium* associates

Abstract

Several species in the *Euwallacea fornicatus* complex have emerged as important pests on woody plants globally, particularly in habitats where they are invasive. These beetles live in obligate symbioses with fungi in the genus *Fusarium.* The aim of our study was to identify *Euwallacea* spp. and their associated fungal associates that have emerged as pests on planted *Acacia crassicarpa* in Riau, Indonesia. Morphological identification and phylogenetic analyses of the mitochondrial cytochrome oxidase c subunit I (COI) gene, confirmed that *E. similis* and *E. perbrevis* are the most abundant beetles infesting these trees. Multi-locus phylogenetic analyses of their fungal associates revealed their non-specific association with six *Fusarium* spp., including *F. rekanum* and five novel members of the Ambrosia *Fusarium* Clade (AFC). These five novel *Fusarium* spp. as are provided with the names *F. akasia* sp. nov., *F. awan* sp. nov., *F. mekan* sp. nov., *F. variasi* sp. nov. and *F. warna* sp. nov.

Introduction

Ambrosia beetles are plant-associated insects that have a primary nutritional association with fungal symbionts (Hulcr and Stelinski 2017). Interest in ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) along with their co-evolved fungal associates has increased substantially in recent years, due to their potential to cause disease in various urban and agricultural environments, across geographically diverse locations (Ploetz et al. 2013). Although ambrosia beetles do not normally result in significant economic impact, several species such as those in *Euwallacea* and *Xylosandrus*, have become significant pests, particularly in invaded areas (Hughes et al. 2017; Hulcr and Stelinski 2017).

Ambrosia beetles rely on fungal symbionts as an obligate food source, storing and transporting these microbial associates within specialized organs known as mycangia (Batra 1963). By successfully cultivating these symbiotic associates, ambrosia beetles are able to survive and breed in the nutritionally poor xylem tissue of their infested plant hosts (Beaver 1989). This requires the fungal symbiont/s to overcome various plant host defences with the aid of several virulence factors, to successfully establish within the infested host (Raffa et al. 2015).

Despite the presence of some virulence factors, the fungi associated with ambrosia beetles are typically not plant pathogens and rather act as benign nutritional symbionts in their association with their co-evolved beetle hosts (Kühnholz et al. 2001; Hulcr and Dunn 2011; Ploetz et al. 2013). However, in some cases, they can become aggressive tree pathogens (Norris and Baker 1968; Bumrungsri et al. 2008; Fraedrich et al. 2008; Hulcr and Dunn 2011). For example, the invasive ambrosia beetles *Euwallacea fornicatus* and *Euwallacea kuroshio* are responsible for spreading the disease known as Fusarium Dieback (FD), in parts of California and Florida. This disease poses a serious threat to the local avocado industry, as well as to the urban landscapes (Eatough Jones and Paine 2017; Stouthamer et al. 2017). The disease is caused by the complex of fungi associated with these two beetle species, mainly *Fusarium euwallaceae* and *Fusarium kuroshium* (Freeman et al. 2013; Na et al. 2018). Neither of these two ambrosia beetles have been reported as pests in their native environments, presumably due the coevolution between their fungal associates and the surrounding native flora (Stouthamer et al. 2017; Paap et al. 2018). Similarly, *Euwallacea perbrevis* and its identified symbionts, *Fusarium ambrosium* and *Fusarium rekanum*, have recently been reported as a pest in its native range, but only on non-native trees such as tea (*Camellia sinensis*) in India and Sri Lanka (Danthanarayana 1968), and on *Acacia crassicarpa* in Indonesia (Lynn et al. 2020). *Euwallacea perbrevis* has also become an invasive pest on Avocado trees in Florida (USA) after their accidental introduction (Carrillo et al. 2016).

The shift in the virulence of the microbial associates of ambrosia beetles, from benign nutritional symbionts to phytopathogens, is incompletely understood (Hulcr and Dunn 2011). What is clear is that an increase in the number of these ambrosia beetle-related diseases is usually associated with activities that facilitate naïve encounters between the beetle-fungus symbiosis and naïve plant hosts (Ploetz et al. 2013). More specifically, the emergence of these diseases is largely attributed to the interaction of the fungi associated with the beetles and naïve plants hosts that lack coevolved adaptation (Hulcr and Dunn 2011).

Several of the most prolific ambrosia beetle-related diseases have been examined only once they have become invasive (Cognato et al. 2015; Hughes et al. 2017; Paap et al. 2018). The level of diversity of these invasive species and their fungal associates, is therefore most likely underrepresented. This is because invasive pests likely undergo bottleneck events after introduction and thus represent only a small portion of the diversity present in their natural populations (Stouthamer et al. 2017). This is exemplified by three of the four cryptic species in

the *Euwallacea fornicatus* species complex and their primary *Fusarium* fungal associates that reside in the Ambrosia *Fusarium* Clade (AFC) (Smith et al. 2019).

Members of the AFC are hypothesised to be the primary symbionts and main food sources of *Euwallacea* spp. (Kasson et al. 2013; O'Donnell et al. 2015). To date, 19 AFC species have been identified, several of which were collected in countries outside their native range, particularly in the United States (Na et al. 2018; Aoki et al. 2019; Lynn et al. 2020). Of these, only eight AFC taxa have been formally described, of which only three were collected from their native environments (Lynn et al. 2020). Thus knowledge regarding the fungal symbionts transported within the mandibular mycangia of *E. fornicatus* complex species, is largely restricted to invasive populations and that likely only represent a very small segment of the natural diversity (Mendel et al. 2012; Kasson et al. 2013). This has resulted in the incorrect notion that species in the *E. fornicatus* complex have a strict association with their dominant *Fusarium* spp. associates (Kostovcik et al. 2015; Carrillo et al. 2019).

Recent research on members of the *E. fornicatus* complex in their native range of Taiwan, showed that the relationship between the species in the *E. fornicatus* complex and the ambrosia fungi they cultivate, is likely more diverse and promiscuous than previously described in invaded areas (Carrillo et al. 2019). A recent study by Lynn et al. (2020) on the native ambrosia beetle *E. perbrevis* infesting *A. crassicarpa* in Indonesian plantations, suggested that a similar pattern of promiscuous symbiosis might occur. In this study 18 different fungal genera were isolated from various ambrosia beetles and their brood galleries, of which several *Fusarium* spp. were most commonly isolated. Because members of the *E. fornicatus* complex have a primary association with *Fusarium* spp. (Kasson et al. 2013), we hypothesised that *E. perbrevis* is the vector of a more diverse array of *Fusarium* spp. than previously believed. The aim of this study was thus to further investigate the ambrosia beetles previously reported as pests on *A. crassicarpa* in their native environment of Indonesia and to broaden available knowledge of their symbionts.

Materials and methods

Sample collection and isolation

Field surveys of five geographically separated compartments of *A. crassicarpa* plantations in Riau, Indonesia, showing ambrosia beetle infestation, were conducted during 2018 and 2019. Several moderately [10–30 entry holes] and several highly [>31 entry holes]) infested, two to three-year-old living trees, were selected at each site, felled 50 cm above ground level, and cut into 50 cm billets. These billets were immediately transported to the laboratory where they were split with sterilized equipment, and living female beetles were extracted and placed in conical screw cap tubes containing sterile filter paper. Each tube contained five beetles that were grouped based on morphology and stored for later identification and mycangial fungus isolation. Data regarding the compartments where collections were done, are summarized in Table 1.

Fungal isolates used in this study were either obtained from the heads of female beetles using methods similar to those described by Lynch et al. (2016), or by culturing them from the surfaces of the beetle galleries using methods described by Eskalen et al. (2013). For the mycangial extractions, beetles were surface disinfected by submerging individual specimens in Eppendorf tubes containing 70% (v/v) ethanol. Tubes were agitated with a vortex mixer for 20 s, rinsed with sterile de-ionized water, and allowed to air-dry on sterile filter paper.

Beetle heads were separated from the thoracic and abdominal segments under a dissection microscope, and then individually macerated in sterile 1.5 mL microcentrifuge tubes with sterile surgical tweezers. To select for *Fusarium* spp., the macerated heads were processed in one of two ways: either by smearing them directly onto the surface of *Fusarium* selective medium agar (FSM, Leslie and Summerell 2006) amended with 100 µg streptomycin sulphate (Sigma, Steinheim, Germany). Alternatively, they were suspended in 200 µl of sterile water in 1.5 ml Eppendorf tubes, shaken for 10 s, and pipetted (50 µl) and spread onto FSM agar plates amended with 100 µg streptomycin sulphate (Sigma, Steinheim, Germany), using sterile glass rods. The Petri dishes were incubated at 25 °C for 3–5 days to allow for fungal growth.

Fungal colonies having unique morphologies were sub-cultured onto 2% malt extract agar (MEA: 20 g/l malt extract, 20 g/l agar, Biolab, Midrand, South Africa), amended with 100 µg streptomycin. To obtain pure colonies for further downstream identification, single hyphal tip cultures were made onto MEA, five days post sub-culturing. The remaining thoracic and abdominal segments of the dissected beetles were individually stored in 200 µl of 70% (v/v)

ethanol for downstream beetle identification. Care was taken to store the beetles accurately to link the fungi to the beetles from which they were isolated.

All cultures obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 2).

DNA extraction, PCR, and phylogenetic analyses

Beetle specimens

DNA was extracted from both wings and two legs of stored beetle specimens using the prepGEM™ Universal DNA extraction kit (Zygem, Biocom Africa (Pty) Ltd.), following the manufacturers protocol, with the exception that the final product was not diluted. The extracted DNA was used for PCR amplification of the mitochondrial cytochrome oxidase c subunit I gene (COI), using the primer pair LCOI490 and HCO2198 (Folmer et al. 1994). Each PCR reaction mixture consisted of 0.2 µl FastStart™ Taq DNA Polymerase (Roche), 10.8 µl sterile DNase-free water, 1 µl of 10 mM both forward and reverse primer, 3 µl of 25mM $MgCl_2$, 2.5 µl 10 × PCR Buffer and 4 µl of DNA template, for a 25 µl total reaction mixture. PCR amplification protocols were the same as those described by Lynn et al. (2020), for all the beetle specimens. Successful amplification was confirmed by staining 2 µl PCR product with 2 µl GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, CA, USA), and separating them on a 2% agarose gel, followed by visualization under UV light.

PCR products were purified using 6% Sephadex G-50 columns following the manufacturer's protocols (Sigma, Aldrich Germany). Products were sequenced in both directions in 12 µl reactions, using the same primers used for PCR amplification. The reaction mixture contained 1 µl BigDye® Terminator v. 3.1 ready reaction mixture (Perkin-Elmer, Warrington, UK), 1 µl sequencing buffer, 1 µl of either the forward or reverse primer (10 mM) for the COI gene region, and 1.5 µl cleaned PCR product. The thermal cycling conditions included 25 cycles of 10 s at 96 °C, 5 s at 55 °C and 4 min at 60 °C. Sequencing products were cleaned using Sephadex G-50 columns and dried in an Eppendorf 5301 vacuum concentrator at 60 °C for 5 min. Sequencing was

performed at the sequencing facility of the University of Pretoria, on an ABI PRISM™ 3500xl Auto-sequencer (Applied Biosystems, Foster City, California, USA). The forward and reverse sequencing reads were assembled into contigs using CLC Bio Main Workbench v. 6 (CLC Bio, www.clcbio.com), and the consensus sequences were extracted and exported for phylogenetic analyses.

A preliminary identity for the beetle specimens was obtained by performing a nucleotide BLAST of the COI sequences against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Based on the results, sequences of closely related taxa were downloaded and incorporated into datasets provided by Lynn et al. (2020) and used for phylogenetic analyses. Sequence alignments for the gene region were generated using MAFFT v. 7 (Katoh and Standley 2013) and manually checked and corrected where necessary using MEGA v. 7 (Kumar et al. 2016). Genealogical relationships among individual beetles were reconstructed using maximum likelihood (ML) analyses which were executed on the online CIPRES Science Gateway v 3.3 platform (Miller et al. 2010), using the online RAxML-HPC BlackBox v. 8.2.10 tool (Stamatakis 2014), with default parameters. A non-parametric analysis of the sequence data with 1000 bootstrap replicates provided statistical support for the branches of the generated ML trees. *Ambrosiophilus sexdentatus* (HM06405) was used to root the ML analysis as suggested by Gomez et al. (2018).

The DNA for the representative specimens sequenced in this study are stored in the Entomological DNA collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). All additional specimens collected in this study are stored in 70% (v/v) ethanol in the Scolytine beetle collection at FABI, University of Pretoria, South Africa.

Fungal isolates

Genomic DNA of three-day-old cultures was extracted from all isolates resembling *Fusarium* spp. obtained from the mandibular mycangia of female beetles and their corresponding brood galleries. For this purpose, 50 µl of the Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used, following the manufacturers protocols. The resulting

extracted suspension was diluted with 200 µl of sterile deionized water. PCR amplification was performed for; (1) the ribosomal internal transcribed spacer region and the domains D1 and D2, at the 5' end of the nuclear large subunit (ITS 1-5.8S-ITS2 + LSU rDNA), using the primers ITS4/ITS5 (White et al. 1990) and NL1/NL4 (Kurtzman and Robnett 1997), respectively, for (2) the translation elongation factor 1-α (TEF1-α) using the primers EF1/EF2 (O'Donnell et al. 1998), for (3) the RNA polymerase subunit I (RPB1) using primers pairs F5/R8 (RPB1-1) (O'Donnell et al. 2010) and F7/G2R (RPB1- 2) (O'Donnell et al. 2010), and for (4) the RNA polymerase subunit II (RPB2), using the primer pairs 5F2/7CR (RPB2-1) and 7CF/11AR (RPB2-2) (O'Donnell et al. 2007). PCR amplifications were performed in 13 µl reactions containing 2.5 µl 5× MyTaq buffer (Bioline, London, UK), 0.25 µl MyTaq DNA polymerases (Bioline), 1 µl DNA template, 0.5 µl of each primer (10 mM), and 8.25 µl of sterile deionized water. PCR was performed for each primer pair using published cycling parameters (White et al. 1990; O'Donnell and Cigelnik 1997; Jacobs et al. 2004; O'Donnell et al. 2007). Amplification of products was confirmed with gel electrophoresis as described above.

PCR products were purified using 6% Sephadex G-50 columns following the manufacturer's protocols (Sigma, Aldrich Germany) and sequenced and assembled using the same protocols described above. The extracted consensus sequences were exported for phylogenetic analyses. Initial BLAST searches against the NCBI GenBank database of the resulting translation elongation factor 1-α (TEF1-α) sequences were performed to confirm that the isolates were those of *Fusarium* spp. The confirmed sequences were then combined with the datasets generated by Lynn et al. (2020) (Table 2), to construct datasets for phylogenetic analyses. DNA sequences for the ITS, TEF1-α and RPB2 gene regions obtained in this study (Table 2), were individually compiled with each respective gene dataset, aligned and inspected following the same procedures described above for the beetles. The individual datasets were combined using FASconCAT-G (Kück and Longo 2014).

Phylogenetic analyses of *Fusarium* spp. were conducted using concatenated DNA sequences of the ITS + LSU, TEF1-α, and RPB2 gene regions from 40 isolates of *Fusarium* spp. obtained in this study, together with those obtained from GenBank for 88 isolates previously used in AFC phylogenetic analyses (Kasson et al. 2013; Carrillo et al. 2019; Sandoval-Denis et al. 2019; Lynn et al. 2020) (Table 2). Maximum likelihood (ML) analyses were performed using the online CIPRES Science Gateway v 3.3 platform (Miller et al. 2010) and with the online RAxML-HPC

BlackBox v. 8.2.10 tool (Stamatakis 2014). Maximum likelihood analyses were carried out using the GTRGAMMA + I substitution model with all other parameters set to default. Statistical support for the branches in the ML trees was obtained by performing 1000 bootstrap replicates. *Fusarium neocosmosporiellum* (NRRL 22468) and *Fusarium lichenicola* (NRRL 32434) were used as the outgroups based on previous studies (Kasson et al. 2013; O'Donnell et al. 2015).

Morphological characterization of Fusarium *spp.*

For morphological characterisation, isolates of *Fusarium* spp. were grown on potato dextrose agar (PDA; PDA 20g/l, BD Difco™, UK) and synthetic low-nutrient agar (SNA: 0.2 g glucose, 0.2 g sucrose, 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$, 7 H_2O, 0.5 g KCl, 20 g Difco agar per litre; Leslie and Summerell 2006) in darkness, under continuous UV light and under an ambient daylight photoperiod. Cultures were grown at ambient room temperature (23–25 °C) for fourteen days and used to evaluate the considered characteristic for these fungi (Aoki et al. 2005; Freeman et al. 2013). Microscope slides were prepared for each isolate with structures mounted in water and 25–50 measurements were recorded for each defining characteristic using a Nikon H550L microscope (Nikon, Yokohama, Japan). Conidia and conidiophores produced on SNA under continuous black light were either mounted in water or directly observed on the agar plates to which a cover slip had been added and then examined and photographed. Means, standard deviations, standard error, maximum and minimum values for the microscopic characters were calculated and presented as minimum–(mean minus standard deviation)–mean–(mean plus standard deviation)–maximum.

To determine optimum growth conditions for growth in culture, colony growth rates of each representative isolate were determined. Three replicates of each of these isolates were assessed at six temperatures ranging from 10–35 °C at 5 °C intervals grown in the dark. Agar plugs (5 mm diam.) were cut from the edges of 1-week-old cultures and placed at the centres of 90 mm Petri dishes containing PDA. Two measurements of colony diameter perpendicular to each other were made every day for 10 days, after which averages were determined. Cultures incubated for 14 days and for 1 month at 25 °C in the dark and in ambient daylight on PDA in 90 mm Petri dishes were used to characterize colony colour (surface and reverse), odour and colony morphology. Colony colours (surface and reverse) were described using the colour charts of Rayner (1970).

Results

Beetle specimen collection and fungal isolations

A total of 114 living adult *Euwallacea* spp. female beetles were extracted from 30 infested logs collected from the five sampled compartments. Morphological identification using the online, Southeast Asia ambrosia beetle's identification resource (http://idtools.org/id/wbb/sea-ambrosia/index.php), identified *Euwallacea perbrevis* (TSHBa; Fig. 2a) and *E. similis* (Fig. 2b) as the most abundant beetle species present. Isolations from the mycangia of these beetles and their corresponding brood galleries, yielded 157 fungal isolates that had morphological characteristics typical of *Fusarium* spp.

PCR amplification and sequencing

Beetle specimens

Maximum likelihood phylogenetic analysis of the beetle COI sequence data (146 taxa, 569 characters) (Fig. 1), confirmed that *Euwallacea perbrevis* (TSHBa; Fig. 2a) and *E. similis* (Fig. 2b) were the most abundant beetle species present in the sampled trees. Three distinct COI haplotypes (Fig. 1) of *E. perbrevis* were obtained, one of which had not previously been described. All three of these haplotypes grouped into the same clade with other specimens collected from Indonesia (Gomez et al. 2018, Lynn et al. 2020), with 100% bootstrap support. The *E. perbrevis* specimens sequenced and identified as haplotype A in this study, were the most commonly collected *E. perbrevis* haplotype and had the widest distribution, as they were collected from four of the five sampled compartments (Table 1). In contrast, haplotype C and the novel haplotype B, were less abundant and were collected only in two of the five sampled compartments (Table 1).

Among the *E. similis* specimens sequenced in this study, four novel COI haplotypes were identified (Fig. 1). Novel haplotypes D and F were the least abundant haplotypes, each with only one representative specimen collected from a single, but different compartment each (Table 1). Novel haplotype E was collected from three compartments and was the most widely distributed *E. similis* haplotype. Haplotype G was the most commonly collected *E. similis* haplotype and was

collected in two different compartments (Table 1). All sequences obtained for the beetle specimens sequenced in this study were deposited in GenBank (Table 1).

Fungal isolates

PCR amplification of the RNA polymerase subunit I (RPB1), using the primer pairs F5/R8 (RPB1-1) and F7/G2R (RPB1-2) (O'Donnell et al. 2010), was not uniformly successful for all isolates. The amplicon lengths for the ITS-LSU, TEF1-α and RPB 2 gene regions that successfully amplified, were approximately 1100 bp, 700 bp and 1400 bp, respectively. The combined multilocus dataset included 128 taxa and 1 824 characters (tef1= 278, ITS+LSU = 401 & rpb2 =1 145), including alignment gaps. Of the 1824 characters, 204 were phylogenetically informative. Separate trees for each partition are presented as Supplementary Fig. 1-3.

Multi-locus phylogenetic analyses based on three loci (ITS-LSU, TEF1-α, and RPB2) showed that the *Fusarium* spp. recovered from *Euwallacea* spp. represented six AFC species, including *F. rekanum* and five novel AFC species ([AF-20]-[AF- 24]) (Fig. 3). Three of these novel AFC members reside in AFC species clade B (AF-20, AF-22 and AF-24), one in clade A (AF-23), and one species (AF-21) fell outside clade A and B, forming a sister clade with *F. lichenicola* (NRRL 32434) (Kasson et al., 2013; O'Donnell et al., 2015) (Fig. 3).

RPB 2 was the most informative gene region in the phylogenetic analyses, providing high bootstrap support for 13 of the 24 AFC species, including four of the five novel lineages (AF-20, 21, 23 and 24) (Supplementary Fig. 1). In contrast, bootstrap analysis of the ITS rDNA provided the weakest signal and resolved only five of the 24 AFC species, including the novel lineages AF-21 and AF-23 (Supplementary Fig. 2). The combined analyses of ITS, RPB2 and TEF1-α, provided the strongest support for the evolutionary relationships within members of the AFC, with high bootstrap support for 16 of the 24 AFC species.

The topology of the ML phylogram based on the combined data set, differed from that in a previous study by Carrillo et al. (2019). Unlike their analyses, we excluded the RPB 1 gene region because we were unable to successfully amplify this gene region for almost all isolates and rather

included the LSU gene region. Major groupings within both analyses were however, similar. Bootstrap support for the two AFC clades (Clade A and B of Kasson et al. 2013) was robust (Fig. 3). However, ML bootstrapping failed to support most of the nodes along the backbone of the phylogeny, but 16 of the species lineages represented by two or more isolates were strongly supported as genealogically exclusive (Fig. 2). The only exceptions were for AF-10 - AF-15, and AF-17 - AF-18 (Fig. 3) but including the AFC species described herein.

Representative isolates of the novel species were deposited in the living collection (PPRI) of the South African National Collection of Fungi (NCF), Roodeplaat, South Africa. Dried specimens of sporulating cultures were deposited in the herbarium collection (PREM) of the South African National Collection of Fungi, Roodeplaat, South Africa.

Taxonomy

Based on multilocus phylogenetic analyses and a detailed comparison of phenotypic/morphological characters, five novel AFC species in the *Fusarium solani* species complex (FSSC) are described as follows:

Fusarium akasia sp. nov. Lynn (Fig. 4)

MycoBank (MB 834436)

Etymology The name *akasia* is derived from the Bahasa Indonesia word ‘akasia’ meaning *Acacia.* It reflects the plant host infested and damaged by the beetle vectors, *E. perbrevis* and *E. similis*.

Culture characteristics Colony colour on PDA white, buff (pale yellow) to saffron (light orange yellow) or fulvous in dark, buff to honey darkening to red, blood red after 1 month in ambient daylight. Colony margin on PDA at 14 days entire, occasionally lobate or curled/concentric, colony elevation convex to umbonate. Reverse pigmentation yellowish white to buff darkening to isabelline or cinnamon dark, saffron to orange darkening to rust and blood red after 1 month in

ambient daylight. Odour absent. Occasional apricot to red exudates. Colonies on PDA radial mycelial growth rates of an average of 2.59 mm/d at 15ºC, 4.83 mm/d at 20ºC, 7.34 mm/d at 25ºC, 7.48 mm/d at 30ºC and 1.28 mm/d at 35ºC in dark. *Conidial pustules*, produced on sporodochia, form on older cultures grown on PDA and are luteous to ochreous. *Aerial mycelium* sparse with pionnotal colony appearance, or abundant, loose to floccose, white to buff. *Chlamydospores* formed sparsely in hyphae, often in mature conidia, mostly round to oval, intercalary, often single or paired, ordinary hyaline, smooth, often rough-walled, 2–(5)–8–(11)–13 x 2–(6)–8–(10)–10.5 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, slow on PDA. *Sporodochia* formed sparsely on SNA, abundantly on PDA. *Aerial conidiophores* formed abundantly on SNA and PDA, often arched, varying in length, thick with slight tapering towards the apex and base, often branched, 31–(36)–87–(138)–219 µm x 3–(5)–6–(7)–8 µm, forming monophialides integrated in apices. *Aerial phialides* simple, subcylindrical, with discreet collarette. *Aerial conidia* [1] mostly oval, two-celled oval to obovoid, slightly curved, short clavate with truncated bases, 0–1(–2) septa, 10.5–(13)–16–(19)–22 x 4–(5)–6–(7)–10 µm, [2] clavate, sometimes slightly curved cylindrical, (0-)1–4(–5)-septate conidia, morphologically similar to the clavate sporodochial conidia. *Sporodochial conidiophores* thick with slight bulging at midsection, often branched, forming apical monophialides. *Sporodochial phialides* simple, subcylindrical with crooked tube shape and discreet collarette. *Sporodochial conidia* ordinarily hyaline, mostly slightly curved clavate, ridged appearance, swollen in upper parts, tapering toward the base, often with a round, blunt apical cell, and a barley notched foot–like basal cell, (0-)1–4(5)-septate, formed on PDA and SNA, 22–(28)–35–(42)–49 µm x 6–(8)–10–(12)–13 µm. Short-clavate to obovate, straight or curved conidia, with rounded apex and truncate base, 0–1-septate, sometimes formed together with multiseptate conidia borne on thick sporodochial conidiophores.

Habitat Acacia crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia.

Known distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia, March 2019, K. M. T. Lynn, HOLOTYPE PREM 62607, culture ex-type CMW 54735 = PPRI (Waiting for numbers to be assigned).

Additional specimens Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia. March 2019, K. M. T. Lynn, PARATYPE PREM 62608, culture ex-type CMW 54741 = PPRI (Waiting for numbers to be assigned); Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia. March 2019, K. M. T. Lynn PARATYPE PREM 62609, culture ex-type CMW 54752 = PPRI (Waiting for numbers to be assigned).

Diagnosis: The species is easily distinguished by its clavate sporodochial conidia that have a ridged appearance. The abundance of the arched thick aerial conidiophores that taper slightly at the base is also a diagnostic feature.

Fusarium awan sp. nov. Lynn (Fig. 5)

MycoBank (MB 834437)

Etymology The name *awan* is derived from the Bahasa Indonesia word 'awan' meaning cloud and reflects the white mycelial growth of the fungus on the yellowish agar. Thus, the fungi in culture appear as sun shining through large white cumulus clouds.

Culture characteristics Colony colour on PDA white in dark, white darkening to honey after 1 month in ambient daylight. Colony margin on PDA after 14 days entire, occasionally curled, particularly under ambient daylight, colony flat, often irregularly umbonate. Reverse pigmentation yellowish white to buff in dark, buff darkening to ochreous after 1 month in ambient daylight. Odour absent. Exudates absent. Colonies on PDA radial mycelial growth rates of average 1.44 mm/d at 15°C, 4.54 mm/d at 20°C, 6.12 mm/d at 25°C, 6.57 mm/d at 30°C, and 3.26 mm/d at 35°C in dark. *Conidial pustules*, luteous to ochreous, produced on sporodochia that form in older cultures on PDA. *Aerial mycelium* sparse with pionnotal colony appearance, or abundant, loose to floccose, white to buff. *Chlamydospores* abundant in hyphae and in mature conidia, mostly oval to round ellipsoidal, intercalary or terminal, often single or paired, occasionally in chains, ordinary hyaline, smooth, often rough-walled, 4–(6)–7–(8)–10 x 5–(6)–7–(8)–10 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, retarded on PDA. *Sporodochia* sparse

on SNA, abundant on PDA. *Aerial conidiophores* abundant on SNA and PDA, often with multiple branching, varying in length, with slight tapering towards apex, 47–(69)–120–(171)–245 µm x 2–(3)–4–(5)–6 µm, forming monophialides integrated in apices. *Aerial phialides* simple, subcylindrical, with discreet collarette. *Aerial conidia* of two forms [1] mostly oval, two-celled and three-celled oval, 0-1(2) septa, 8–(11)–13–(15)–20 x 3–(3)–4–(5)–6 µm, [2] long, barely curved, flûte shaped with discreet tapering toward the base, 1-3 septate conidia, 12–(20)–24–(28)–31 µm x 3–(4)–5–(6)–8 µm. *Sporodochial conidiophores* thick, often branched, forming apical monophialides. *Sporodochial phialides* simple, subcylindrical, tube shaped, with discreet collarette. *Sporodochial conidia* hyaline, varying in shape and size, [1] curved, narrow, cylindrical, or slightly clavate, often swollen in upper parts with narrow papillate apical cells, tapering abruptly toward base, with barley notched foot–like basal, or simply rounded at base, often abundantly producing chlamydospores, 2-4 septa, 19.5–(27)–35–(43)–36 µm x 3.5–(4.5)–5.5–(6.5)–8 µm; [2] ordinarily hyaline, barely curved, flûte shaped, no or slight tapering toward the base, often with round, blunt apical cell, and barley notched foot–like basal cell, 0–2-3(–4) septate, 14–(21)–25–(29)–33 µm x 3–(3.5)–4.5–(5.5)–7 µm. Short-clavate to oval, straight or slightly curved conidia, with rounded apex, occasionally truncate base, 0–1-septate, often formed together with multiseptate conidia borne on thick sporodochial conidiophores.

Habitat A. crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia.

Known distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea similis* mycangia. March 2019, K. M. T. Lynn, HOLOTYPE PREM 62602, culture ex-type CMW 54719 = PPRI (Waiting for numbers to be assigned).

Additional specimens Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa) and *E. similis*. November 2018, K. M. T. Lynn, PARATYPE PREM 62594, culture ex-type CMW 53705 = PPRI (Waiting for numbers to be assigned); Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *E. similis* mycangia. March 2019, K. M. T. Lynn PARATYPE PREM 62604, culture ex-type CMW 54722 = PPRI (Waiting for numbers to be assigned).

Diagnosis: The species has abundant chlamydospores both in the hyphae and within mature conidia. It can also be recognised by the formation of multi-septate aerial conidia having an elongated oval shape and by the production of very narrow sporodochial conidia compared to other species in the AFC.

Fusarium mekan sp. nov. Lynn (Fig. 6)

MycoBank (MB 834438)

Etymology The name *mekan* is derived from the Bahasa Indonesia word 'mekan' meaning eat or food. It reflects the relationship this fungus has with its beetle vector, *E. similis*, after a beetle was observed to be eating a culture of this fungi under a microscope.

Culture characteristics Colony colour on PDA white, greyish flax blue to greyish violet in dark, white to pale mouse grey, darkening to purple state and rust after 1 month in ambient daylight, occasionally with white, albino segments or streaks. Colony margin on PDA undulate, colony umbonate, occasionally raised. Reverse pigmentation yellowish white to fawn in dark, bay darkening to chestnut and blood red after 1 month in ambient daylight. Odour absent. Rose exudates occasionally present. Colonies on PDA radial mycelial growth rates of average 2.46 mm/d at 15ºC, 4.84 mm/d at 20ºC, 5.89 mm/d at 25ºC, 6.69 mm/d at 30ºC, and 0.75 mm/d at 35ºC in dark. *Conidial pustules* luteous to ochreous, produced on sporodochia on older cultures grown on PDA. *Aerial mycelium* sparse, or developed abundantly, loose to floccose, white to greyish/brown white. *Chlamydospores* formed abundantly in hyphae and mature conidia, mostly round to oval, intercalary or terminal, often single or paired, rarely clustered, ordinary hyaline, smooth, often rough-walled, 5–(6)–8–(10)–13 x 6–(7)–8–(9)–11 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, retarded on PDA. Sporodochia formed sparsely on SNA, abundantly on PDA. *Aerial conidiophores* formed abundantly on SNA and PDA, often branching, erect or occasionally slightly curved, varying in length, thin walled, tapering towards base and slightly towards apex, 63–(83)–133–(183)–232 µm x 2–(5)–5–(6)–8 µm, forming monophialides integrated in apices. *Aerial phialides* simple, subcylindrical, with discreet collarette. *Aerial conidia* [1] mostly oval, two-celled oval to obovoid with truncated base, occasionally pyriform, 0-1(-2) septa, 8–(9)–12–(15)–29 x 3–(3)–4–(5)–5.5 µm, [2] thick, curved

with slight tapering toward the base, often with a round, blunt apical cell, and rounded basal cell, 0–3(-4) septate, morphologically similar to long oval shaped sporodochial conidia. *Sporodochial conidiophores* thick, cylindrical, varying in length, occasionally branched, forming apical monophialides. *Sporodochial phialides* simple, subcylindrical, tube shaped, with discreet collarette. *Sporodochial conidia* ordinarily hyaline, occasionally pigmented, thick, straight or occasionally slightly curved with discrete tapering toward base, often with round, blunt apical cell, and rounded or barley notched foot–like basal cell, 0–5(-6) septate, formed on PDA and SNA, 20.5–(24)–31.5–(38.5)–45 µm x 4–(5)–6–(7)–9 µm. Obovoid to short-clavate or reniform, truncated base, slightly curved, with swollen rounded apex, 0–1 septate, often formed together with multiseptate conidia on thick sporodochial conidiophores.

Habitat Acacia crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia.

Known distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *E. similis* mycangia, March 2019, K. M. T. Lynn, HOLOTYPE PREM 62600, culture ex-type CMW 54714 = PPRI (Waiting for numbers to be assigned).

Additional specimens Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa) and *E. similis*. November 2018, K. M. T. Lynn, PARATYPE PREM 62601, culture ex-type CMW 53696 = PPRI (Waiting for numbers to be assigned); Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *E. similis* mycangia. November 2018, K. M. T. Lynn PARATYPE PREM 62602, culture ex-type CMW 54717 = PPRI (Waiting for numbers to be assigned).

Diagnosis: A distinguishing characteristic of this species is its multi-septate conidia that are slightly curved, elongate and oval, and in which the septa appear consistently spaced from each other. The production of chlamydospores at both the apex and base of single mature conidia is also a diagnostic feature.

Fusarium variasi sp. nov. Lynn (Fig. 7)

MycoBank (MB 834439)

Etymology The name *variasi* is derived from the Bahasa Indonesia word 'variasi' meaning variation. It refers to the observed variation in the size and shape of the conidia.

Culture characteristics Colony colour on PDA white or livid purple to fawn in dark, white with livid purple to bay segments, darkening to dark brick or violate slate or black after 1 month in ambient daylight. Colony margin on PDA entire, rarely filamentous, raised. Reverse pigmentation yellowish white to fawn in dark, white with rust to umber segments, occasionally entirely darkening to umber or black after 1 month in ambient daylight. Odour absent. Occasional greenish yellow or red exudates present. Colonies on PDA radial mycelial growth rates of average 2.47 mm/d at 15ºC, 3.49 mm/d at 20ºC, 5.02 mm/d at 25ºC, 6.79 mm/d at 30ºC, and 0.36 mm/d at 35ºC in dark. *Conidial pustules* produced on sporodochia, form on older cultures grown on PDA and SNA, luteous to ochreous or dull green to dark violet, particularly under UV light. *Aerial mycelium* sparse, or abundant, loose to floccose, white. *Chlamydospores* abundant in hyphae, mostly round to oval, intercalary or terminal, single, paired or in chains, often clustered, hyaline, smooth, often rough-walled, 6–(7)–8–(9)–11 x 6–(7)–8–(9)–11 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, retarded on PDA. *Sporodochia* formed on SNA, abundant on PDA. *Aerial conidiophores* abundant on SNA and PDA, often branching, erect, varying in length, thin walled and narrow, tapering towards apex, 19–(40)–65–(90)–121 µm x 2–(3)–4–(5)–6 µm, forming monophialides integrated in apices. *Aerial phialides* simple, subcylindrical, with discreet collarette. *Aerial conidia* [1] mostly oval, obovoid with truncated base or globose, 0 septa, 4–(5)–6–(7)–9 x 2–(3)–4–(5)–5 µm, [2] mostly two celled oval, short clavate, curved, 0-1(2) septa, 13–(14)–17–(20)–25 x 4–(5)–6–(7)–9 µm, [3] falcate to clavate, slightly curved, 3-5(-6) septate, morphologically almost indistinguishable from falcate to clavate sporodochial conidia. *Sporodochial conidiophores* thick, cylindrical, often branched, forming apical monophialides. *Sporodochial phialides* simple, subcylindrical, tube shaped, with discreet collarette. *Sporodochial conidia* ordinarily hyaline, falcate to clavate, thick, curved with tapering toward the base, with papillate, occasionally rounded apical cell, and barley notched or distinct foot–like basal cell, 3-6(-7) septate, formed on PDA and SNA, 44–(47)–52–(57)–64 µm x 8–(10)–12–(14)–15 µm.

Short-clavate to oval, straight conidia, with rounded apex and occasionally truncate base, 0–1-septate, often formed together with multiseptate conidia borne on thick sporodochial conidiophores.

Habitat Acacia crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia.

Known distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa), November 2018, K. M. T. Lynn, HOLOTYPE PREM 62595, culture ex-type CMW 53734 = PPRI (Waiting for numbers to be assigned).

Additional specimens Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa). November 2018, K. M. T. Lynn, PARATYPE PREM 62596, culture ex-type CMW 53735 = PPRI (Waiting for numbers to be assigned); Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *A. crassicarpa* infested with *E. perbrevis* (TSHBa). November 2018, K. M. T. Lynn PARATYPE PREM 62597, culture ex-type CMW 54696 = PPRI (Waiting for numbers to be assigned).

Diagnosis: This species is characterised by the highly variable size and shape of the aerial conidia and the clustering of these varied conidia on a single conidiophore. The conidia are small, obovoid, and clavate, multiseptate, and swollen, with papillate apical end or are shorter, clavate shaped, septate conidia that vary in shape. This species also produces abundant chlamydospore in clusters unlike other species in the AFC.

Note: This species might represent more than one taxon, based on the variation in the morphological characteristics and the strong bootstrap support for two branches in the phylogenetic trees. However, we have chosen not to describe separate species before additional isolates can be collected to resolve the species boundaries more rigorously.

Fusarium warna sp. nov. Lynn (Fig. 8)

MycoBank (MB 834440)

Etymology The name *warna* is derived from the Bahasa Indonesia word 'warna' meaning colour. It reflects the different colours of the fungi in culture, including the range of colours of the mycelium, the exudates produced and the pigmented conidia.

Culture characteristics Colony colour on PDA white to livid purple to vinaceous purple, with white segments, to fawn on outer edges of culture in dark, lavender to violet or livid violet with white segments, darkening to livid vinaceous or dark vinaceous to dark purple with sepia edges after 1 month in ambient daylight. Colony margin on PDA entire, flat to umbonate. Reverse pigmentation yellowish white to fawn in dark, pale vinaceous grey white with rust to umber darkening to dark brick after 1 month in ambient daylight. Odour absent. Red to purple exudates often present. Colonies on PDA radial mycelial growth rates of an average of 2.17 mm/d at 15^{o}C, 4.69 mm/d at 20^{o}C, 6.83 mm/d at 25^{o}C, 5.28 mm/d at 30^{o}C and 2.33 mm/d at 35^{o}C in dark. *Conidial pustules* produced on sporodochia, form on older cultures grown on PDA, occasionally on SNA, are luteous to ochreous, or dull green to sepia. *Aerial mycelium* sparse, or abundant, loose to floccose, white to light purple. *Chlamydospores* sparse in hyphae and mature conidia, mostly round to oval, intercalary or terminal, single, paired, often clustered, ordinary hyaline, smooth, often rough-walled, 3.5–(4)–5–(6)–8 x 3–(4)–5–(6)–6.5 µm. *Sclerotia* absent. Sporulation on SNA abundant, particularly under UV light, retarded on PDA. *Sporodochia* formed on SNA, abundantly on PDA. *Aerial conidiophores* formed abundantly on SNA and PDA, often branching, slightly curved, varying in length, thick with no tapering towards the apex, 40–(57)–78–(99)–144 µm x 3–(3)–4–(5)–7 µm, forming monophialides integrated in apices. *Aerial phialides* simple, subcylindrical, with discreet collarette. *Aerial conidia* [1] mostly obovoid with truncated base, oval, or short clavate, rarely curved, 0-3(-4) septa, 12–(14)–17–(20)–25 x 4–(6)–7–(8)–10 µm, [2] clavate, straight, with bulging apical end and prominent tapering towards base, 1-5(-7) septate, morphologically almost indistinguishable from short clavate sporodochial conidia. *Sporodochial conidiophores* thick, cylindrical, often branched, forming apical monophialides. *Sporodochial phialides* simple, subcylindrical, tube shaped, with discreet collarette. *Sporodochial conidia* ordinarily hyaline or pigmented, thick, short clavate, papillate or rounded swollen apical cell,

tapering toward the base, indistinct foot–like or rounded basal cell, 1-4(-6) septate, formed on PDA and SNA, 27.5–(30)–32–(34)–37.5 µm x 10–(12)–13–(14)–15 µm. Short-clavate to oval, straight conidia, with rounded apex and occasionally truncate base, non-septated, often formed together with multiseptate conidia borne on thick sporodochial conidiophores.

Habitat Acacia crassicarpa plantations infested with ambrosia beetles in Riau, Indonesia.

Known distribution Riau, Indonesia.

Specimen examined Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia. March 2019, K. M. T. Lynn, HOLOTYPE PREM 62603, culture ex-type CMW 54720 = CBS (Waiting for numbers to be assigned).

Additional specimens Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia. March 2019, K. M. T. Lynn, PARATYPE PREM 62605, culture ex-type CMW 54724 = PPRI (Waiting for numbers to be assigned); Indonesia, Riau, Pelalawan, *Acacia crassicarpa* plantation. Isolated from *Euwallacea perbrevis* (TSHBa) mycangia. March 2019, K. M. T. Lynn PARATYPE PREM 62606, culture ex-type CMW 54726 = PPRI (Waiting for numbers to be assigned).

Diagnosis: This species is characterised by multiseptate, thick, short, papillated, spindle-shaped sporodochial conidia that taper towards the base. The production of smaller chlamydospores compared with other species in the AFC is an additional diagnostic feature.

Discussion

This study confirmed the observations of Lynn et al. (2020), in that the ambrosia beetle species infesting *A. crassicarpa* in Riau, Indonesia represent *Euwallacea similis* and *Euwallacea perbrevis* and are the most abundant beetles infesting these trees. Six *Fusarium* spp. residing in the AFC were found associated with these beetles, with five of the species shown to be novel taxa. These

five novel *Fusarium* spp. were described as *F. akasia* sp. nov. [AF-20], *F. awan* sp. nov. [AF-21], *F. mekan* sp. nov. [AF-22], *F. variasi* sp. nov. [AF-23] and *F. warna* sp. nov. [AF-24]. An important outcome of the study was that the *Fusarium* spp. had a non-specific association with their beetle symbionts.

Both *E. perbrevis* and *E. similis* were commonly found infesting the same host and were occasionally also found co-inhabiting a single brood gallery. However, *E. similis* was never found infesting a host where *E. perbrevis* was not also present. Thus, *E. similis* appears to be a secondary invader requiring the tree to be weakened or already infested before colonization can occur. This supports the findings of Lynn at al. (2020) and Balasundaran and Sankaran (1991) where *E. similis* was also reported to usually infest only stressed or dying trees.

Phylogenetic analyses of the COI gene region showed a high level of intraspecific genetic diversity in both of the identified *Euwallacea* spp. The COI gene region is known to have high levels of intraspecific variation when distinguishing species in Xyleborini (Jordal and Kambestad 2014). Consequently, it is not possible to determine whether the COI data presented in this study, showing distinct divergence between the four novel *E. similis* haplotypes, provides indication of cryptic speciation. Alternatively, this might simply reflect genetic diversity within the species due to the intraspecific COI variation (Jordal and Kambestad 2014).

The five new *Fusarium* spp. residing in the AFC clade (Kasson et al. 2013), were identified based on phenotypic and morphological characters (Gadd and Loos 1947; Freeman et al. 2013; Aoki et al. 2018, 2019; Na et al. 2018). *Fusarium akasia* sp. nov. [AF-20], *F. mekan* sp. nov. [AF-22], and *F. warna* sp. nov. [AF-24], reside in Clade B of the AFC. *Fusarium variasi* sp. nov. [AF-23] grouped with AF-8 and AF-9 in group Clade A of the AFC and *F. awan* sp. nov. [AF-21] formed a sister clade with *F. lichenicola* (NRRL 32434). Interestingly, the species that have been formally described and that reside in clade B of the AFC, have been found to almost exclusively produce clavate multiseptate sporodochial conidia, or a combination of fusiform and clavate multiseptate sporodochial conidia. This is in contrast to the predominately fusiform conidia found by *Fusarium* species in Clade A (Kasson et al. 2013; Aoki et al. 2018). The shift from producing predominately fusiform to apically swollen clavate sporodochial conidia is believed to be an evolutionary adaptation of these fungi to their symbiotic *Euwallacea* spp. (Kasson et al. 2013).

However, two of the described species in this study do not follow this pattern. *Fusarium mekan* sp. nov. [AF-22] in Clade B, in addition to AF-6, produce discrete fusiform conidia rather than clavate shape conidia. Similarly, *F. variasi* sp. nov. [AF-23] in Clade A produces masses of clavate shaped conidia rather than fusiform conidia.

Results of this study might suggest that the five new *Fusarium* spp. are cultivated by the two *Euwallacea* ambrosia beetles from which they were isolated (Table 3). *Fusarium akasia* sp. nov. was isolated from the mycangia of *E. similis* and *E. perbrevis* specimens; *F. awan* sp. nov. [AF-21], *F. mekan* sp. nov. [AF-22] and *F. warna* sp. nov. [AF-24] were recovered from the mycangia of *E. similis* specimens; *F. variasi* sp. nov. [AF-23] was recovered from a gallery harboring either *E. similis* or *E. perbrevis* larvae; and *F. warna* sp. nov. [AF-24] was isolated from the mycangia of *E. perbrevis* specimens. Additionally, *F. rekanum,* a previously identified associate of *E. perbrevis* (Lynn et al. 2020), was also obtained from mycangia of *E. similis* specimens in this study. Thus, investigation into the association of two *Euwallacea* spp. with their symbiotic fungi in Riau, Indonesia, revealed further evidence for a non-specific association of these beetle species with AFC members and is consistent with the findings of Carrillo et al. (2019).

A previous study by Balasundaran and Sankaran (1991) suggested that *F. solani* is associated with *E. similis* and that the fungus results in a canker and die-back disease of teak (*Tectona grandis*) in Kerala, India. However, *F. solani* was never isolated from the mycangia of *E. similis* in this study. Rather, five novel *Fusarium* spp. were associated with the beetle, all of which appeared to be nutritional associates.

Euwallacea perbrevis has now been reported to be associated with eight members of the AFC, two of which are novel species described in the present study. Five of these associates were isolated from countries other than Indonesia. These are AF-1 from India/ Sri Lanka, and AF-13, AF-14, AF-17, AF-18 from Taiwan. All were isolated from tree species other than *A. crassicarpa* (Carrillo et al. 2019). None of these five AFC species were isolated from the mycangia of *E. similis* or *E. perbrevis,* or from their corresponding brood galleries such as those analysed in the present study. The absence of these other AFC associates from the beetle specimens analyzed in the present study could reflect the geographic origins, plant hosts, and different haplotypes of the beetle vectors.

A non-exclusive relationship appears to exist between members of the *E. fornicatus* species complex and their *Fusarium* associates. This includes the two *Euwallacea* spp. investigated in the present study, and their closely related AFC *Fusarium* associates (Table 3). However, the prevalence of these AFC members can clearly differ. *Fusarium rekanum* was the most abundant AFC member isolated from the mycangia of *E. perbrevis* specimens, followed by *F. akasia* and then *F. warna.* Similarly, *F. awan* was the most abundant AFC member isolated from the mycangia of *E. similis* specimens, followed by *F. mekan, F. rekanum,* and *F. akasia.* This could be a consequence of the sampling strategy used, or that these two *Fusarium* spp. are better suited to enable the beetle to colonize *A. crassicarpa* compared to other AFC members (Kajimura and Hijii 1992; Klepzig et al. 2009).

It is possible that the association of the *Fusarium* spp. with their *Euwallacea* beetle vectors is influenced by prevailing temperatures where the beetles are found. For example, Riau, Indonesia, where the present study was conducted, has a relatively high average annual temperature which appears to be consistent with the optimal temperatures for growth of *F. rekanum* and *F. awan.* This follows the findings of Lynn et al. (2020) who showed that that optimum growth rate of *F. rekanum* in culture was 25 ^{o}C.

Observations made in this study lead us to hypothesise that the five novel AFC members identified are nutritional symbionts of their *Euwallacea* vectors. This will, however, need to be tested with diet experiments similar to those conducted by Freeman et al. (2013). Future studies should also consider the survival of these beetle species on all the various AFC members as suggested (or done) by Carrillo et al. (2020). It is apparent that some ambrosia beetles have promiscuous relationships with their fungal associates (Hulcr and Cognato 2010; Carrillo et al. 2014; Kostovcik et al. 2015), while others appear to be more specific (Beaver 1989). Although most fungus-farming *Euwallacea* spp. currently have no significant economic consequences (Hulcr and Stelinski 2017), they have potential to cultivate the symbionts of other closely related beetle species that do (O'Donnell et al. 2015). The potential to switch symbionts can result in the emergence of more destructive and economically impactful *Euwallacea-Fusarium* associations. These emerging issues regarding the biology and importance of *Euwallacea* spp. are gaining increasing importance and will likely need to be studied more thoroughly in the future.

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Tables

Table 1 Descriptions and sequences of *Euwallacea* spp. specimens collected in this study and used for phylogenetic analysis

Specimen	Genetic lineage	Haplotype	Location	Compartment	Corresponding AFC member	GenBank No. (COI)
A2	*Euwallacea similis*	F	PKU, Indonesia	I091	*Fusarium awan* [AF-21]	MT026195
A4	*E. similis*	G	PKU, Indonesia	I091	N/A	MT026196
A6	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026197
A10	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026198
A12	*E. similis*	E	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026199
A14	*E. similis*	G	PKU, Indonesia	I091	N/A	MT026200
A17	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026201
A18	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026202
A19	*E. similis*	G	PKU, Indonesia	I091	N/A	MT026203
A32	*E. similis*	G	PKU, Indonesia	I091	*Fusarium rekanum* [AF-19]	MT026204
A36	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026205
A45	*E. similis*	E	PKU, Indonesia	I091	*Fusarium mekan* [AF-22]	MT026206
A46	*E. similis*	G	PKU, Indonesia	I091	*F. rekanum* [AF-19]	MT026207
A48	*E. similis*	G	PKU, Indonesia	I091	*F. mekan* [AF-22]	MT026208

A49	*E. similis*	G	PKU, Indonesia	I091	*F. awan* [AF-21]	MT026209
A54	*Euwallacea perbrevis* (TSHBa)	C	PKU, Indonesia	B012	*Fusarium warna* [AF 24]	MT026210
A57	*E. similis*	G	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026211
A58	*E. perbrevis* (TSHBa)	C	PKU, Indonesia	B012	*F. warna* [AF 24]	MT026212
A64	*E. perbrevis* (TSHBa)	C	PKU, Indonesia	B012	*F. warna* [AF 24]	MT026213
A66	*E. perbrevis* (TSHBa)	C	PKU, Indonesia	B012	*F. warna* [AF 24]	MT026214
A67	*E. similis*	G	PKU, Indonesia	B012	*F. awan* [AF-21]	MT026215
A68	*E. similis*	G	PKU, Indonesia	B012	*F. awan* [AF-21]	MT026216
A69	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026217
A70	*E. similis*	G	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026218
A72	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026219
A75	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026220
A78	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*Fusarium akasia* [AF-20]	MT026221
A80	*E. similis*	G	PKU, Indonesia	B012	*F. awan* [AF-21]	MT026222
A81	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*F. akasia* [AF-20]	MT026223

A83	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B012	*F. akasia* [AF-20]	MT026224
A85	*E. similis*	G	PKU, Indonesia	B012	*F. awan* [AF-21]	MT026225
A88	*E. similis*	D	PKU, Indonesia	B012	*F. rekanum* [AF-19]	MT026226
A89	*E. similis*	G	PKU, Indonesia	B012	*F. awan* [AF-21]	MT026227
A91	*E. similis*	E	PKU, Indonesia	C012	*F. awan* [AF-21]	MT026228
A92	*E. perbrevis* (TSHBa)	B	PKU, Indonesia	C012	*F. akasia* [AF-20]	MT026229
A93	*E. perbrevis* (TSHBa)	B	PKU, Indonesia	C012	*F. akasia* [AF-20]	MT026230
A98	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	C012	*F. rekanum* [AF-19]	MT026231
A99	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	C012	*F. rekanum* [AF-19]	MT026232
A100	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	B023	*F. rekanum* [AF-19]	MT026233
A106	*E. similis*	E	PKU, Indonesia	B023	*F. awan* [AF-21]	MT026234
A107	*E. similis*	E	PKU, Indonesia	B023	*F. rekanum* [AF-19]	MT026235
A108	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	K045	*F. akasia* [AF-20]	MT026236
A109	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	K045	*F. akasia* [AF-20]	MT026237
A111	*E. perbrevis* (TSHBa)	A	PKU, Indonesia	K045	*F. rekanum* [AF-19]	MT026238

Table 2 Isolates and sequence data of *Fusarium* spp. used in the phylogenetic analysis of this study

Isolate	Species	Host	Location	GenBank numbers[a]		
				ITS	TEF1- α	RPB2[b]
CMW52862[c]	*Fusarium rekanum* [AF-19]	*Euwallacea perbrevis* (TSHBa)	PKU, Indonesia	MN249094	MN249151	MN249137, MN249108
CMW53691	*F. rekanum* [AF-19]	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249097	MN249154	MN249140, MN249111
CMW53690	*F. rekanum* [AF-19]	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249098	MN249155	MN249141, MN249112
CMW53688	*F. rekanum* [AF-19]	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249100	MN249157	MN249143, MN249114
CMW51764	*F. rekanum* [AF-19]	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249104	MN249161	MN249147, MN249118
CMW51765	*F. rekanum* [AF-19]	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN249105	MN249162	MN249148, MN249119
CMW54723[d]	*F. rekanum* [AF-19]	*E. similis*	PKU, Indonesia	MN954349	MT009963	MT009923, MT010003
CMW54730[d]	*F. rekanum* [AF-19]	*E. similis*	PKU, Indonesia	MN954353	MT009967	MT009927, MT010007
CMW54737[d]	*F. rekanum* [AF-19]	*E. similis*	PKU, Indonesia	MN954359	MT009973	MT009933, MT010013
CMW51753[d]	*Fusarium* sp.	*Acacia crassicarpa*	PKU, Indonesia	MN954324	MT009938	MT009898, MT009978
CMW51754[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954325	MT009939	MT009899, MT009979
CMW51755[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954326	MT009940	MT009900, MT009980
CMW51758[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954327	MT009941	MT009901, MT009981
CMW52864[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954329	MT009943	MT009903, MT009983
CMW52865[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954330	MT009944	MT009904, MT009984

CMW52866[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954331	MT009945	MT009905, MT009985
CMW52867[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954332	MT009946	MT009906, MT009986
CMW52868[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954333	MT009947	MT009907, MT009987
CMW52869[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954334	MT009948	MT009908, MT009988
CMW54732[d]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954354	MT009968	MT009928, MT010008
CMW54733[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954355	MT009969	MT009929, MT010009
CMW54734[d,c,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954356	MT009970	MT009930, MT010010
CMW54735[d,c,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954357	MT009971	MT009931, MT010011
CMW54741[d,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954360	MT009974	MT009934, MT010014
CMW54751[d]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954362	MT009976	MT009936, MT010016
CMW54752[d,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954363	MT009977	MT009937, MT010017
CMW51766[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954328	MT009942	MT009902, MT009982
CMW53705[d,e]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954337	MT009951	MT009911, MT009991
CMW53731[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954338	MT009952	MT009912, MT009992
CMW54719[d,c,e]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954345	MT009959	MT009919, MT009999
CMW54722[d,e]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954348	MT009962	MT009922, MT010002

CMW54728[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954352	MT009966	MT009926, MT010006
CMW54736[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954358	MT009972	MT009932, MT010012
CMW54748[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954361	MT009975	MT009935, MT010015
CMW53696[d,e]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954335	MT009949	MT009909, MT009989
CMW53699[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954336	MT009950	MT009910, MT009990
CMW54714[d,c,e]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954342	MT009956	MT009916, MT009996
CMW54717[d,e]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954343	MT009957	MT009917, MT009997
CMW54718[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954344	MT009958	MT009918, MT009998
CMW53734[d,c]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954356	MT009953	MT009913, MT009993
CMW53735[d]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954357	MT009954	MT009914, MT009994
CMW54696[d,e]	*Fusarium* sp.	*A. crassicarpa*	PKU, Indonesia	MN954341	MT009955	MT009915, MT009995
CMW54720[d,c,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954346	MT009960	MT009920, MT010000
CMW54721[d]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954347	MT009961	MT009921, MT010001
CMW54724[d,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954350	MT009964	MT009924, MT010004
CMW54726[d,e]	*Fusarium* sp.	*E. perbrevis* (TSHBa)	PKU, Indonesia	MN954351	MT009965	MT009925, MT010005
CMW54748[d]	*Fusarium* sp.	*E. similis*	PKU, Indonesia	MN954361	MT009975	MT009935, MT010015

UCR3641[c]	*F. kuroshium* [AF-12]	*Platanus racemosa*	El Cajon, CA	KX262196	KX262216	KX262256
UCR3644	*F. kuroshium* [AF-12]	*P. racemosa*	El Cajon, CA	KX262197	KX262217	KX262257
UCR3651	*F. kuroshium* [AF-12]	*Euwallacea* sp.	Fallbrook, CA	KX262198	KX262218	KX262258
UCR3653	*F. kuroshium* [AF-12]	*Persea americana*	Bonsall, CA	KX262200	KX262220	KX262260
UCR3654	*F. kuroshium* [AF-12]	*P. americana*	Bonsall, CA	KX262201	KX262221	KX262261
UCR3652	*F. kuroshium* [AF-12]	Unknown	Unknown	KX262199	KX262219	KX262259
UCR4672	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taichung, Taiwan	KX262206	KX262226	KX262266
UCR4673	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taichung, Taiwan	KX262207	KX262227	KX262267
UCR4674	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taichung, Taiwan	KX262208	KX262228	KX262268
UCR4675	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taichung, Taiwan	KX262209	KX262229	KX262269
UCR4676	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Taichung, Taiwan	KX262210	KX262230	KX262270
UCR4677	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taichung, Taiwan	KX262211	KX262231	KX262271
UCR4678	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taichung, Taiwan	KX262212	KX262232	KX262272
UCR4679	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Taichung, Taiwan	KX262213	KX262233	KX262273
UCR4680	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Taichung, Taiwan	KX262214	KX262234	KX262274
UCR4681	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taichung, Taiwan	KX262215	KX262235	KX262275

NRRL20438	*F. ambrosium* [AF-1]	*E. perbrevis* (TSHBa)	India	AF178397	AF178332	JX171584
NRRL22231	*F. tuaranense* [AF-5]	*Hevea brasiliensis*	Malaysia	KC691570	KC691542	KC691631, KC691660
NRRL22346	*F. ambrosium* [AF-1]	*E. perbrevis* (TSHBa)	India	EU329669	FJ240350	EU329503
NRRL22468	*F. neocosmosporiellum*	*Arachis hypogaea*	Guinea	DQ094318	AF178349	EU329512
NRRL22643	*Fusarium* sp. [AF-9]	*Xyleborus ferrugineus*	Costa Rica	KC691583	DQ247628	KC691644, KC691673
NRRL32434	*F. lichenicola*	*Homo sapiens*	Germany	DQ094444	DQ246977	EF470161
CBS279.34[c]	*F. lichenicola*	*Homo sapiens*	Somalia	LR583725	LR583615	LR583840
CBS279.59[c]	*F. lichenicola*	Soil	Tahiti	LR583726	LR583616	LR583841
CBS483.96	*F. lichenicola*	*Homo sapiens*	Japan	LR583727	LR583617	LR583842
CBS509.63[c]	*F. lichenicola*	Air	Brazil	LR583728	LR583618	LR583843
CBS522.63[c]	*F. lichenicola*	Air	Brazil	LR583729	LR583619	LR583844
CBS623.92	*F. lichenicola*	*Homo sapiens*	Germany	LR583730	LR583620	LR583845
NRRL34123	*F. lichenicola*	*Homo sapiens*	India	DQ094645	DQ247192	EU329635
NRRL46518	*F. tuaranense* [AF-5]	*H. brasiliensis*	Malaysia	KC691571	KC691543	KC691632, KC691661
NRRL54722	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038014	JQ038007	JQ038028
NRRL54723	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038015	JQ038008	JQ038029

NRRL54724	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038016	JQ038009	JQ038030
NRRL54725	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038017	JQ038010	JQ038031
NRRL54726	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038018	JQ038011	JQ038032
NRRL54727	*F. euwallaceae* [AF-2]	*Euwallacea* sp.	Israel	JQ038019	JQ038012	JQ038033
NRRL62578	*F. oligoseptatum* [AF-4]	*E. validus*	PA (U.S.A.)	KC691565	KC691537	KC691626, KC691655
NRRL62579[c]	*F. oligoseptatum* [AF-4]	*E. validus*	PA (U.S.A.)	KC691566	KC691538	KC691627, KC691656
NRRL62584	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691582	KC691554	KC691643, KC691672
NRRL62585	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691577	KC691554	KC691638, KC691667
NRRL62590	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691574	KC691546	KC691635, KC691664
NRRL62591	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691573	KC691545	KC691634, KC691663
NRRL62606	*F. floridanum* [AF-3]	*Euwallacea* sp.	FL (U.S.A.)	KC691561	KC691533	KC691622, KC691651
NRRL62610	*F. obliquiseptatum* [AF-7]	*Euwallacea* sp.	Australia	KC691575	KC691547	KC691636, KC691665
NRRL62611	*F. obliquiseptatum* [AF-7]	*Euwallacea* sp.	Australia	KC691576	KC691548	KC691637, KC691666
NRRL62629	*F. floridanum* [AF-3]	*E. interjectus*	FL (U.S.A.)	KC691564	KC691536	KC691625, KC691654
NRRL62941	*Fusarium* sp. [AF-10]	Unknown	Singapore	KM406633	KM406626	KM406647
NRRL62942	*F. ambrosium* [AF-1]	*Camellia sinensis*	Sri Lanka	KM406631	KM406624	KM406645

NRRL62944	*Fusarium* sp. [AF-11]	*C. sinensis*	Sri Lanka	KM406634	KM406627	KM406648
NRRL66088	*Fusarium* sp. [AF-9]	*Delonix regia*	FL (U.S.A.)	KM406632	KM406632	KM406646
TW1	*Fusarium* sp. [AF-18]	*P. americana*	Taiwan	MK432860	MK435437	MK435521
TW2	*Fusarium* sp. [AF-14]	*P. americana*	Taiwan	MK432862	MK435439	MK435523
TW4	*Fusarium* sp. [AF-16]	*P. americana*	Taiwan	MK432866	MK435443	MK435527
TW15	*Fusarium* sp. [AF-15]	*P. americana*	Taiwan	MK432861	MK435438	MK435522
TW25	*Fusarium* sp. [AF-16]	*P. americana*	Taiwan	MK432863	MK435440	MK435524
TW34	*Fusarium* sp. [AF-16]	*P. americana*	Taiwan	MK432864	MK435441	MK435525
TW37	*Fusarium* sp. [AF-16]	*P. americana*	Taiwan	MK432865	MK435442	MK435526
TW40	*Fusarium* sp. [AF-17]	*P. americana*	Taiwan	MK432867	MK435444	MK435528
TW43	*F. kuroshium* [AF-12]	*P. americana*	Taiwan	MK432868	MK435445	MK435529
TW44	*Fusarium* sp. [AF-18]	*P. americana*	Taiwan	MK432869	MK435446	MK435530
TW45	*Fusarium* sp. [AF-15]	*P. americana*	Taiwan	MK432870	MK435447	MK435531
TW55	*Fusarium* sp. [AF-18]	*P. americana*	Taiwan	MK432871	MK435448	MK435532
TW56	*Fusarium* sp. [AF-14]	*P. americana*	Taiwan	MK432872	MK435449	MK435533
UCR5499	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taiwan	MK432873	MK435450	MK435534

UCR5508	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taiwan	MK432874	MK435451	MK435535
UCR5509	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taiwan	MK432875	MK435452	MK435536
UCR5513	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taiwan	MK432876	MK435453	MK435537
UCR5545	*Fusarium* sp. [AF-17]	*Euwallacea* sp.	Taiwan	MK432877	MK435454	MK435538
UCR5546	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taiwan	MK432878	MK435455	MK435539
UCR5557	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taiwan	MK432879	MK435456	MK435540
UCR5584	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taiwan	MK432880	MK435457	MK435541
UCR6394	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Taiwan	MK432881	MK435458	MK435542
UCR6395	*Fusarium* sp. [AF-15]	*Euwallacea* sp.	Taiwan	MK432882	MK435459	MK435543
UCR6403	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taiwan	MK432883	MK435460	MK435544
UCR6405	*Fusarium* sp. [AF-16]	*Euwallacea* sp.	Taiwan	MK432884	MK435461	MK435545
UCR6408	*F. kuroshium* [AF-12]	*Euwallacea* sp.	Taiwan	MK432885	MK435462	MK435546
UCR6409	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taiwan	MK432886	MK435463	MK435547
UCR6411	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taiwan	MK432887	MK435464	MK435548
UCR6414	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taiwan	MK432888	MK435465	MK435549
UCR6417	*Fusarium* sp. [AF-18]	*Euwallacea* sp.	Taiwan	MK432889	MK435466	MK435550

UCR6432	*Fusarium* sp. [AF-13]	*Euwallacea* sp.	Taiwan	MK432890	MK435467	MK435551
UCR6436	*Fusarium* sp. [AF-14]	*Euwallacea* sp.	Taiwan	MK432891	MK435468	MK435552

[a] ITS = internal transcribed spacer region; TEF1- α = translation elongation factor 1- α; RPB2 = DNA-directed RNA polymerase II subunit.
[b] Two accession numbers correspond to unjoined RPB2-1 and RPB2-2 sequences.
[c] Type-species
[d] Isolates collected in Indonesia and sequenced in this study
[e] Isolates used in morphological descriptions and growth studies
[AF-] indicates the Ambrosia fusaria species within the AFC
PKU = Pekanbaru; CA = California; PA = Pennsylvania; FL = Florida

Table 3 Summarized data of *Fusarium* spp. and the beetle vectors they are associated with in this study

Fusarium spp. based on combined analysis	Number of isolates	Beetle vectors based on morphology	Confirmed *Euwallacea* spp. vectors based on COI sequence data
F. rekanum [AF-19]	51	*Euwallacea perbrevis*	30
		Euwallacea similis	11
F. akasia sp. nov. [AF-20]	21	*E. perbrevis*	10
		E. similis	1
F. awan sp. nov. [AF-21]	34	*E. similis*	23
F. mekan sp. nov. [AF-22]	5	*E. similis*	2
F. variasi sp. nov. [AF-23]	3	Unknown	N/a
F. warna sp. nov. [AF-24]	4	*E. perbrevis*	4

*Although 114 *Euwallacea* beetles were collected, the COI gene region did not successfully amplify in all 114 specimens. Therefore, only selected fungal isolates that could be correctly correlated to a beetle vector that had COI sequence data, were used in the phylogenetic analysis presented in this study.

Figures

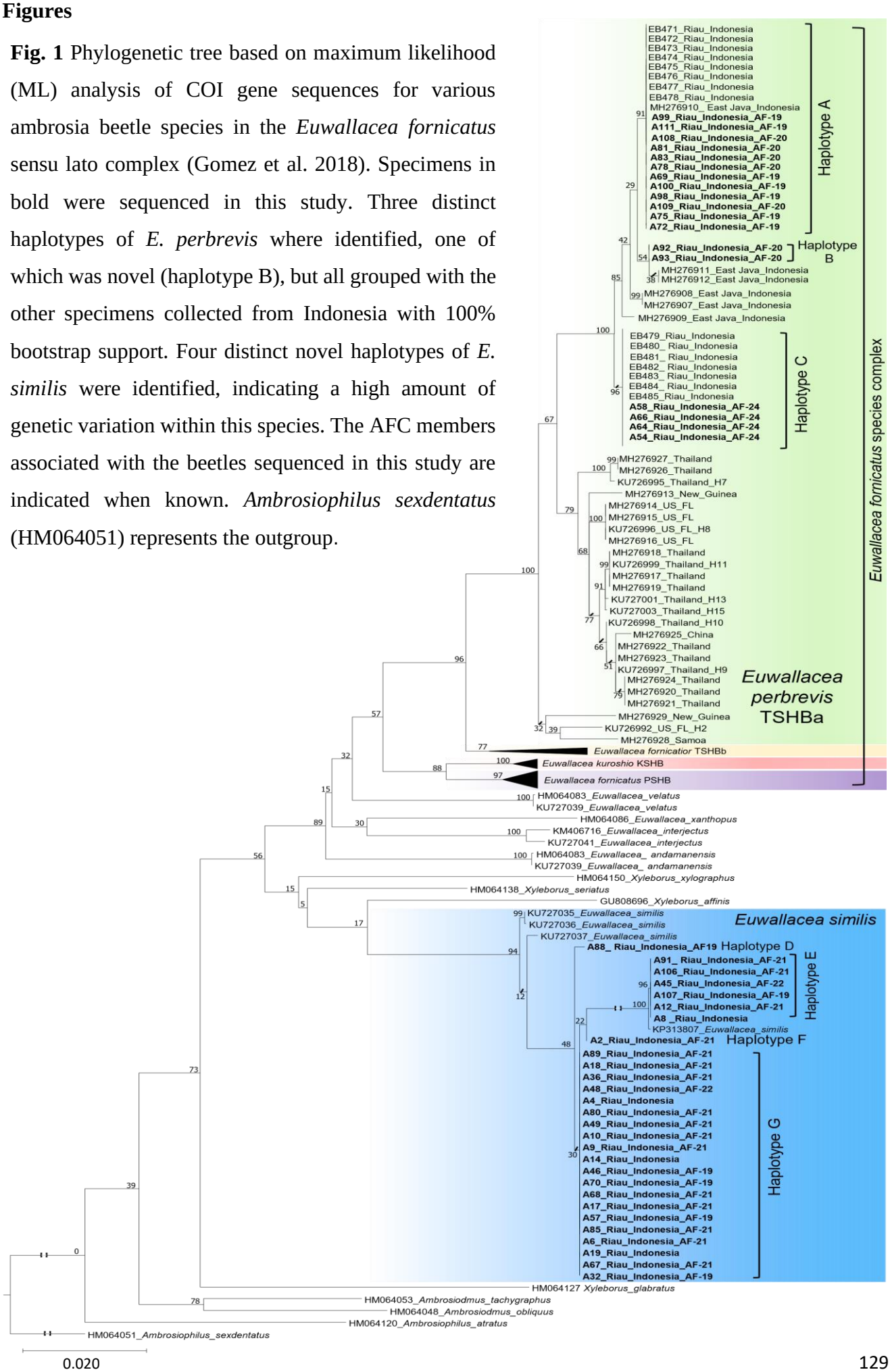


Fig. 1 Phylogenetic tree based on maximum likelihood (ML) analysis of COI gene sequences for various ambrosia beetle species in the *Euwallacea fornicatus* sensu lato complex (Gomez et al. 2018). Specimens in bold were sequenced in this study. Three distinct haplotypes of *E. perbrevis* where identified, one of which was novel (haplotype B), but all grouped with the other specimens collected from Indonesia with 100% bootstrap support. Four distinct novel haplotypes of *E. similis* were identified, indicating a high amount of genetic variation within this species. The AFC members associated with the beetles sequenced in this study are indicated when known. *Ambrosiophilus sexdentatus* (HM064051) represents the outgroup.

a

b

Fig. 2 *Euwallacea perbrevis* and *Euwallacea similis* **a.** Mature female *Euwallacea perbrevis* (TSHBa) beetle collected from infested *Acacia crassicarpa* in Indonesia. **b.** Mature female *Euwallacea similis* beetle collected from infested *Acacia crassicarpa* in Indonesia. Bar corresponds to 1.0 mm. We thank Andrew J Johnson and You Li from the UF Forest Entomology Laboratory, who kindly provided the photos for Fig. 1a and 1b.

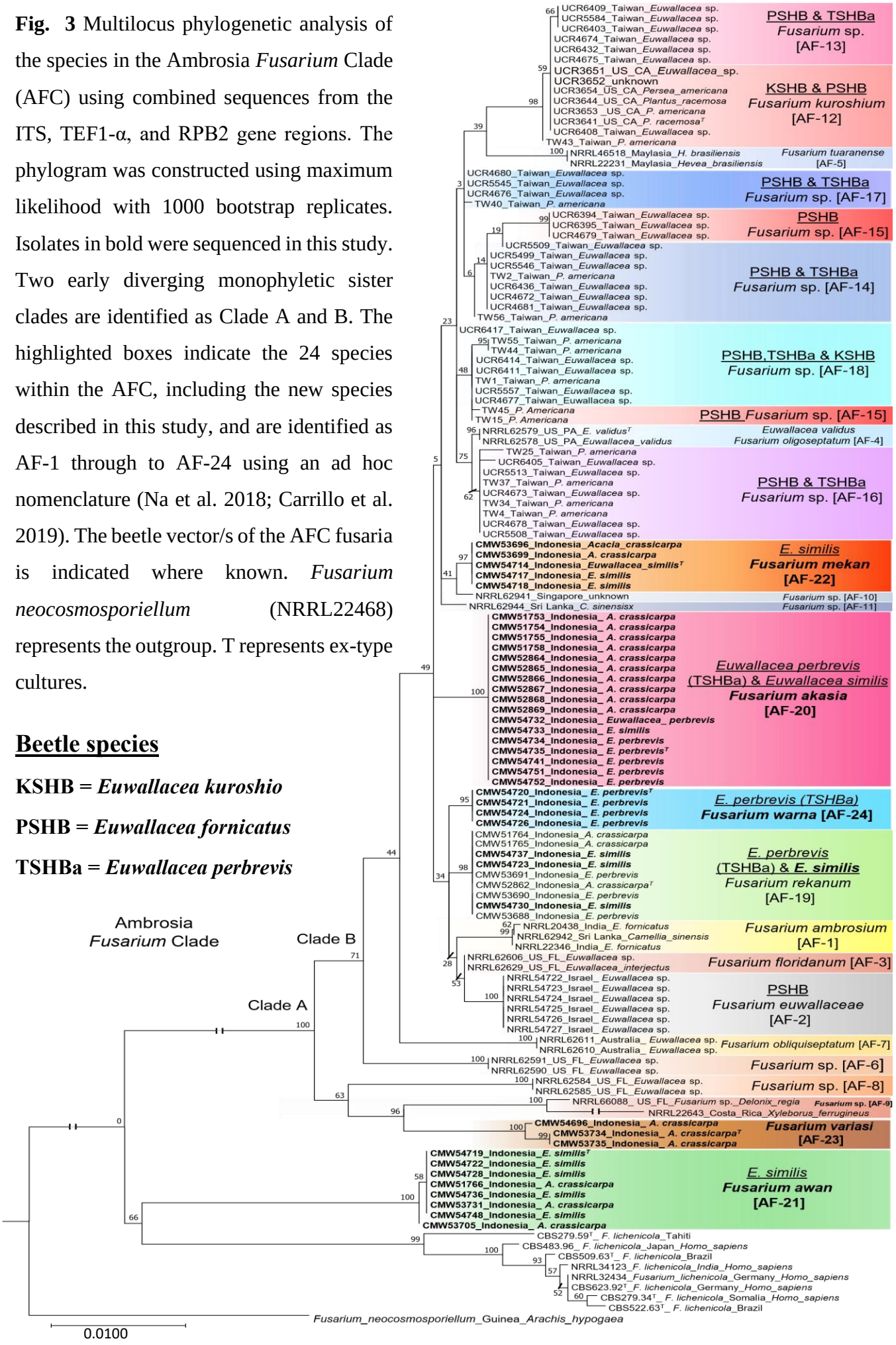
Fig. 3 Multilocus phylogenetic analysis of the species in the Ambrosia *Fusarium* Clade (AFC) using combined sequences from the ITS, TEF1-α, and RPB2 gene regions. The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. Two early diverging monophyletic sister clades are identified as Clade A and B. The highlighted boxes indicate the 24 species within the AFC, including the new species described in this study, and are identified as AF-1 through to AF-24 using an ad hoc nomenclature (Na et al. 2018; Carrillo et al. 2019). The beetle vector/s of the AFC fusaria is indicated where known. *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type cultures.

Beetle species

KSHB = *Euwallacea kuroshio*

PSHB = *Euwallacea fornicatus*

TSHBa = *Euwallacea perbrevis*



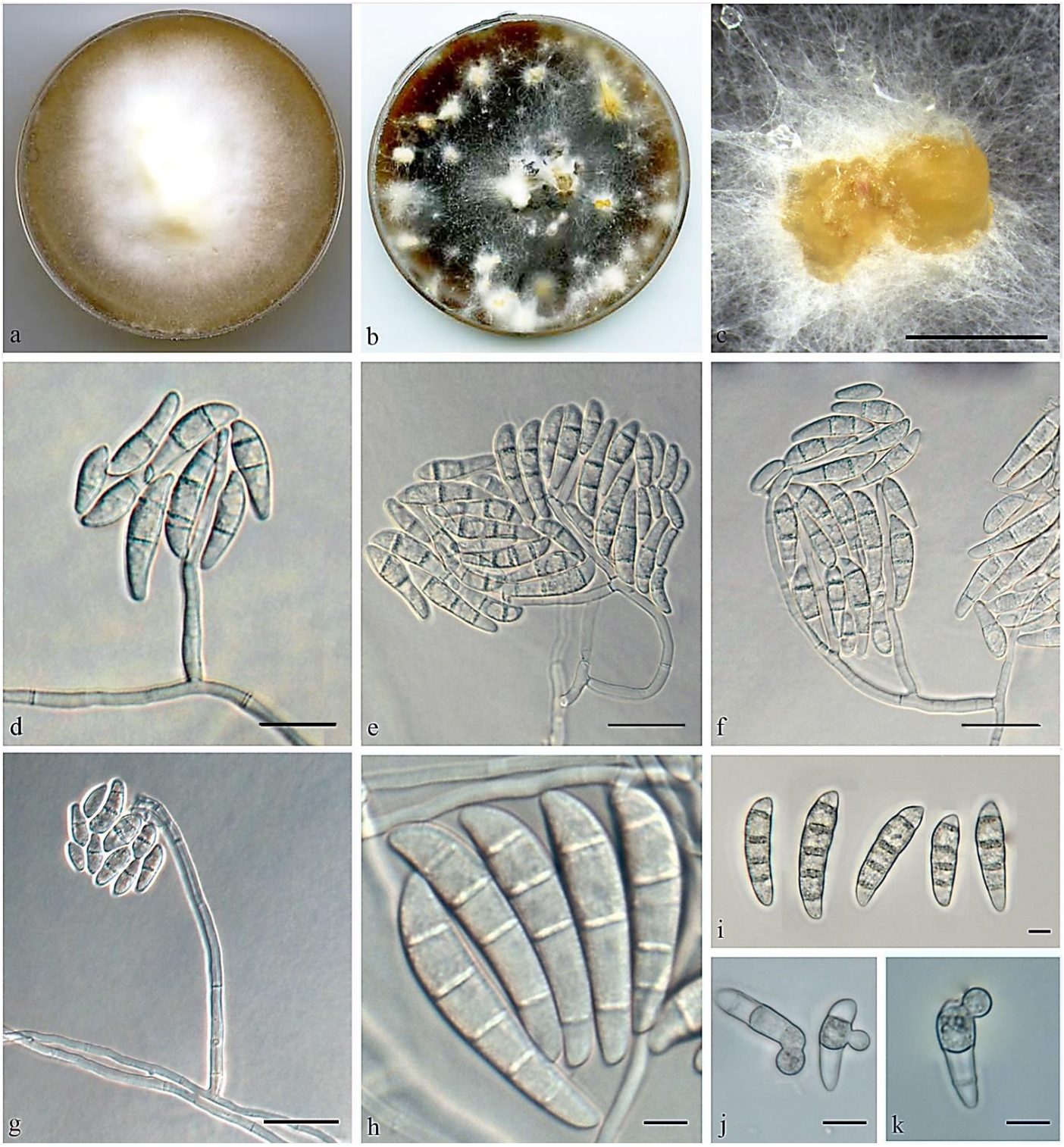


Fig. 4 Morphological characteristics of *Fusarium akasia* on PDA and SNA (CMW 54735, CMW 54741 and CMW 54752). **a-c, i-k.** Morphology of *F. akasia* cultures grown on potato dextrose agar (PDA) and **d**–**h.** morphology of 14-day-old *F. akasia* cultures grown on synthetic low-nutrient agar (SNA) **a.** Colony morphology on PDA at 2 weeks. **b-c.** Luteous-ochreous conidial masses formed in culture on PDA after 1 month. **d-g.** Simple to branched aerial conidiophores forming non-septated conidia, often swollen apically with 1-5 septa or oval to short clavate with 0-2 septa. **h.** Curved clavate multiseptated aerial conidia with 3-5 septa, that are morphologically indistinguishable from sporodochial conidia. **i.** Multiseptate sporodochial conidia with a round,

blunt apical cell, and a barley notched distinct foot-like basal cell with 4-5 septa. **j-k.** Multiseptate conidia with round to oval, rough-walled, ridged appearance, chlamydospores formation. **c** = 1 mm; **d-g** = 30 µm; **h** = 5 µm; **I** = 5 µm; **j-k** = 10 µm.

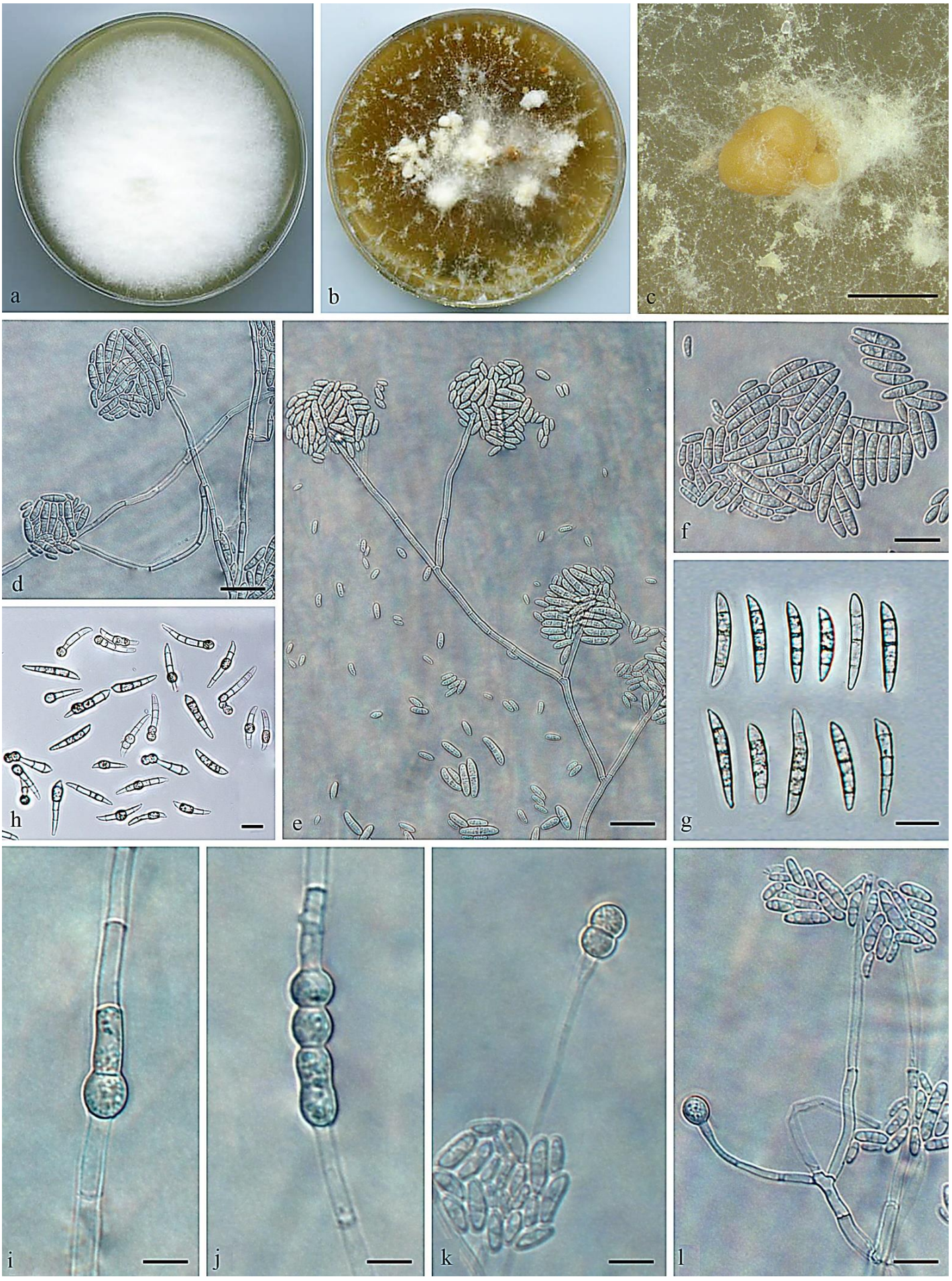

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Fig. 5 Morphological characteristics of *Fusarium awan* on PDA and SNA (CMW 54719, CMW 53705 and CMW 54722). **a–c, g-h**. Morphology of *F. awan* cultures grown on potato dextrose agar (PDA) and **d–f, i-l.** morphology of 14-day-old *F. awan* cultures grown on synthetic low-nutrient agar (SNA) **a.** Colony morphology on PDA at 2 weeks. **b, c.** Luteous-ochreous conidial masses formed in culture on PDA after 1 month. **d-e**. Mature multiseptated conidia with 0-3 septa, formed on branched aerial conidiophores. **f.** Long, barely curved, flûte shaped multiseptated aerial conidia with 0-3 septa, that are morphologically similar to sporodochial conidia. **g.** Multiseptate narrow sporodochial conidia with a round or papillate apical cell, and a barley notched foot-like basal cell with 3-4 septa. **h-l.** Round to oval chlamydospores, single, paired or in chains, formed intercalary or terminally in hyphae or in mature narrow conidia **c** = 2 mm; **d-g** = 20 µm; **h-I** = 10 µm.

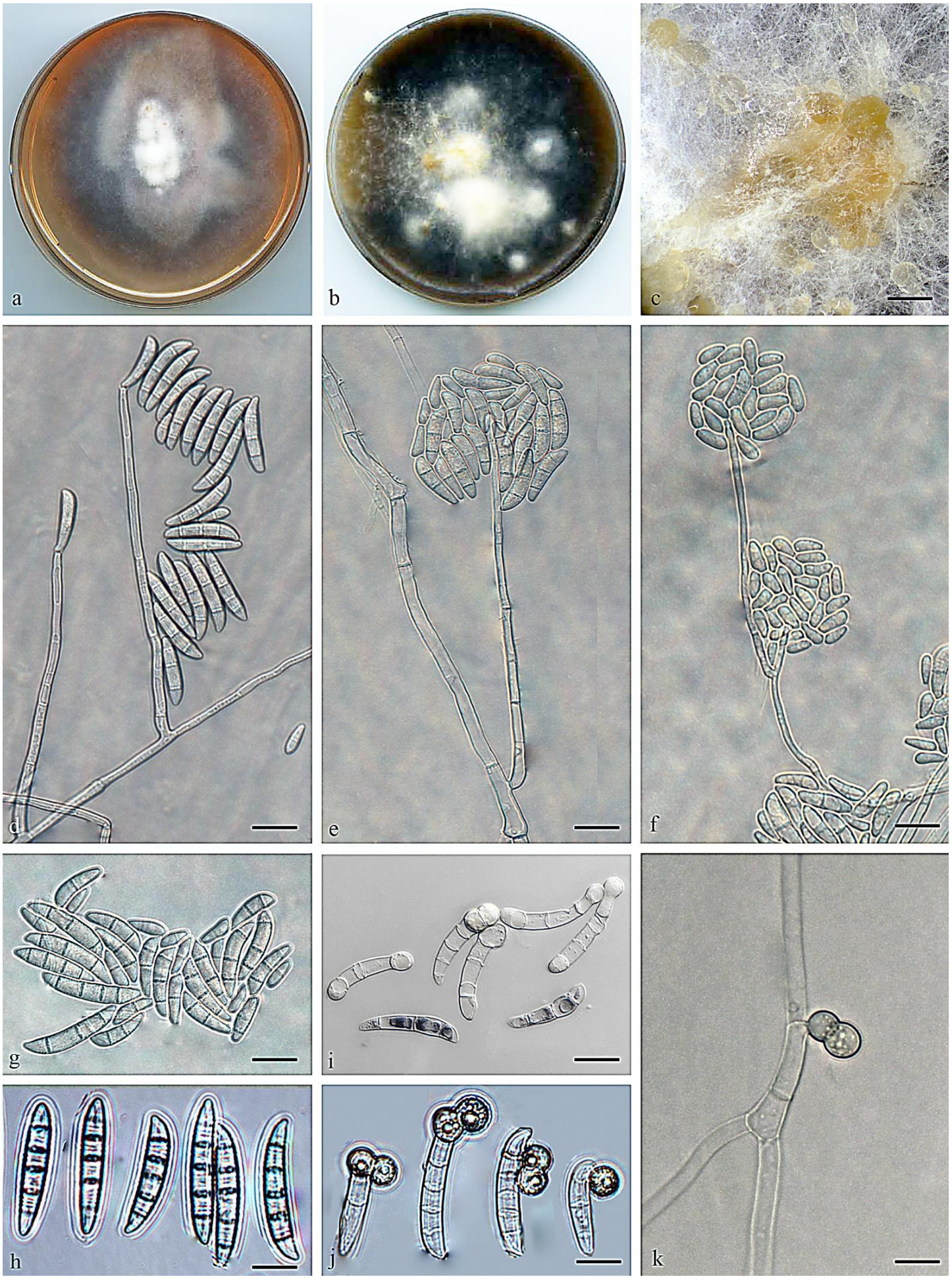
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Fig. 6 Morphological characteristics of *Fusarium mekan* on PDA and SNA (CMW 54714, CMW 53696 and CMW 54717). **a**–**c, h-j.** Morphology of *F. mekan* cultures grown on potato dextrose agar (PDA) and **d**–**g, k.** morphology of 14-day-old *F. mekan* cultures grown on synthetic low-nutrient agar (SNA) **a.** Colony morphology on PDA at 2 weeks. **b, c.** Luteous-ochreous conidial masses formed in culture on PDA after 1 month. **d-e.** Simple to branched aerial conidiophores forming non-septated conidia, often slightly swollen apically with 1-5 septa or oval to short clavate with 0-1 septa. **g.** Slightly curved multiseptated aerial conidia with 2-4 septa, that are morphologically indistinguishable from sporodochial conidia. **h.** Multiseptate sporodochial conidia with a round, blunt apical cell, and a rounded or barley notched distinct foot-like basal cell with 5 approximately equidistant septa. **i.** Pigmented multiseptate sporodochial conidia. **i-j.** Multiseptate mature conidia with round to oval, rough-walled chlamydospores formation. **k.** Paired round to oval chlamydospores formed in hyphae. **c** = 1 mm; **d-g** = 20 µm; **h-j** = 15 µm; **k** = 10 µm.

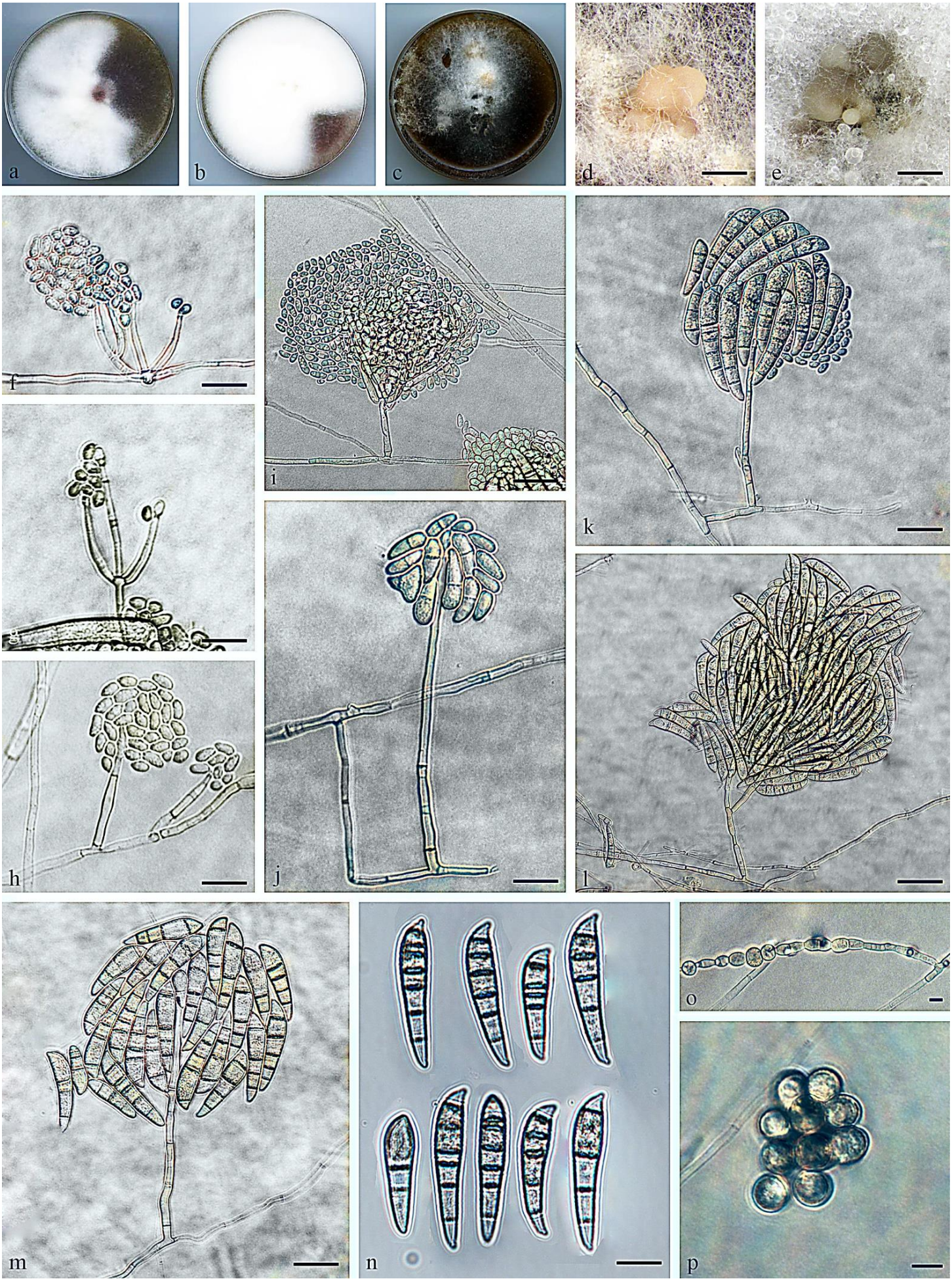
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Fig. 7 Morphological characteristics of *Fusarium variasi* on PDA and SNA (CMW 53734, CMW 53735 and CMW 54696). **a**–**e, n.** Morphology of *F. variasi* cultures grown on potato dextrose agar (PDA) and f-m, o-p, morphology of 14-day-old *F. variasi* cultures grown on synthetic low-nutrient agar (SNA). **a-b.** Colony morphology on PDA at 2 weeks of CMW 53734 and CMW 54696 respectively. **c-e**. Luteous-ochreous conidial masses formed in culture on PDA after 1 month. **f-m.** Simple to branched aerial conidiophores varying in length forming either, [1] oval, obovoid or globose, non-septated conidia, [2] two celled oval, short clavate, curved, 0-1(2) septa conidia or [3] clavate, slightly curved, apically papillated, 3-6 septa conidia. These conidia can form together on the same aerial conidiophore or separately. **n.** Multiseptate clavate shaped sporodochial conidia with narrow papillate or occasionally rounded apical cell, and barely notched or distinct foot-like basal cell with 2-7 septa. **o-p.** Round to oval chlamydospores in chains or clustered formed in hyphae. **d-e** = 1 mm; **f-m** = 20 µm; **n** = 15 µm; **o-p** = 10 µm.

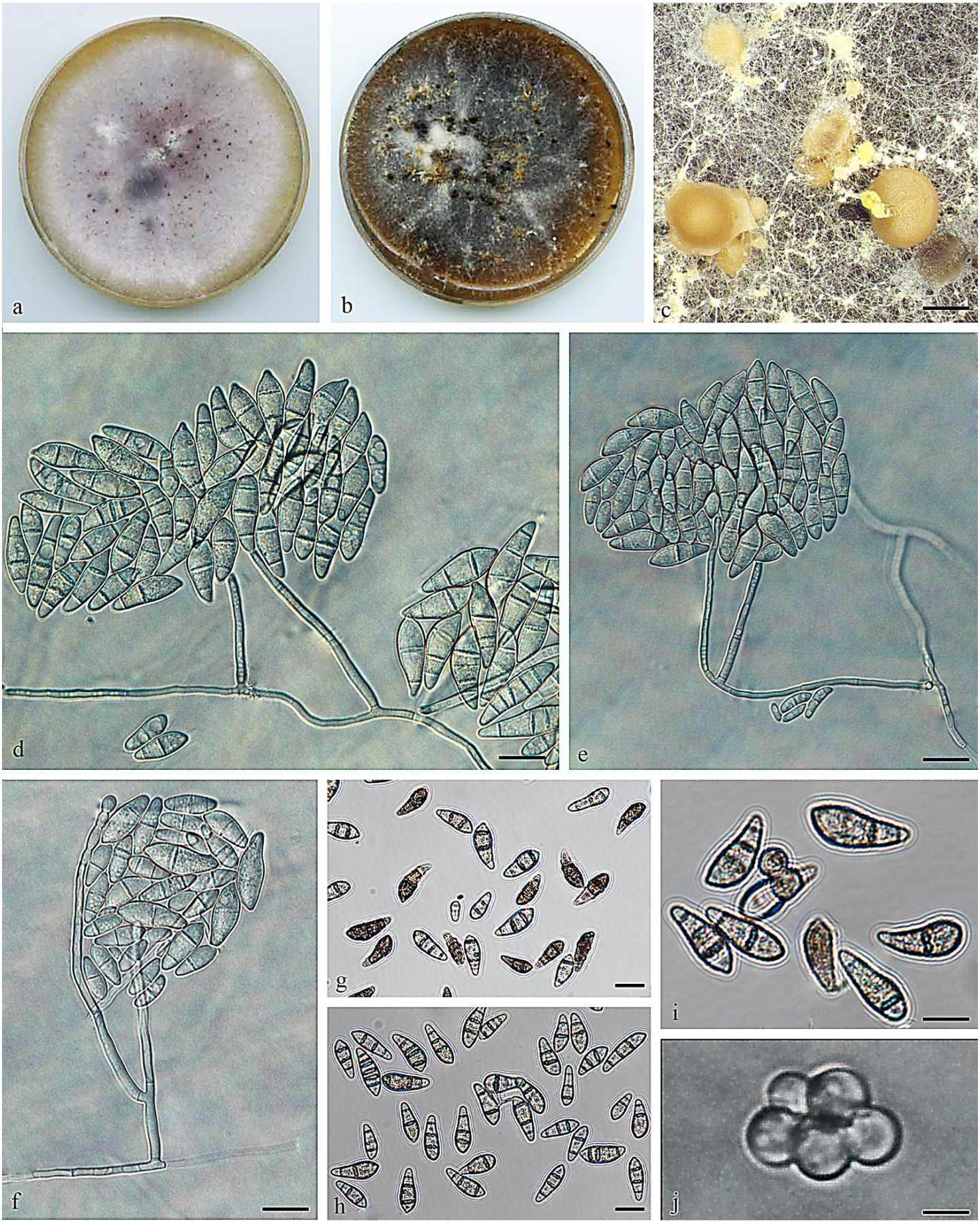
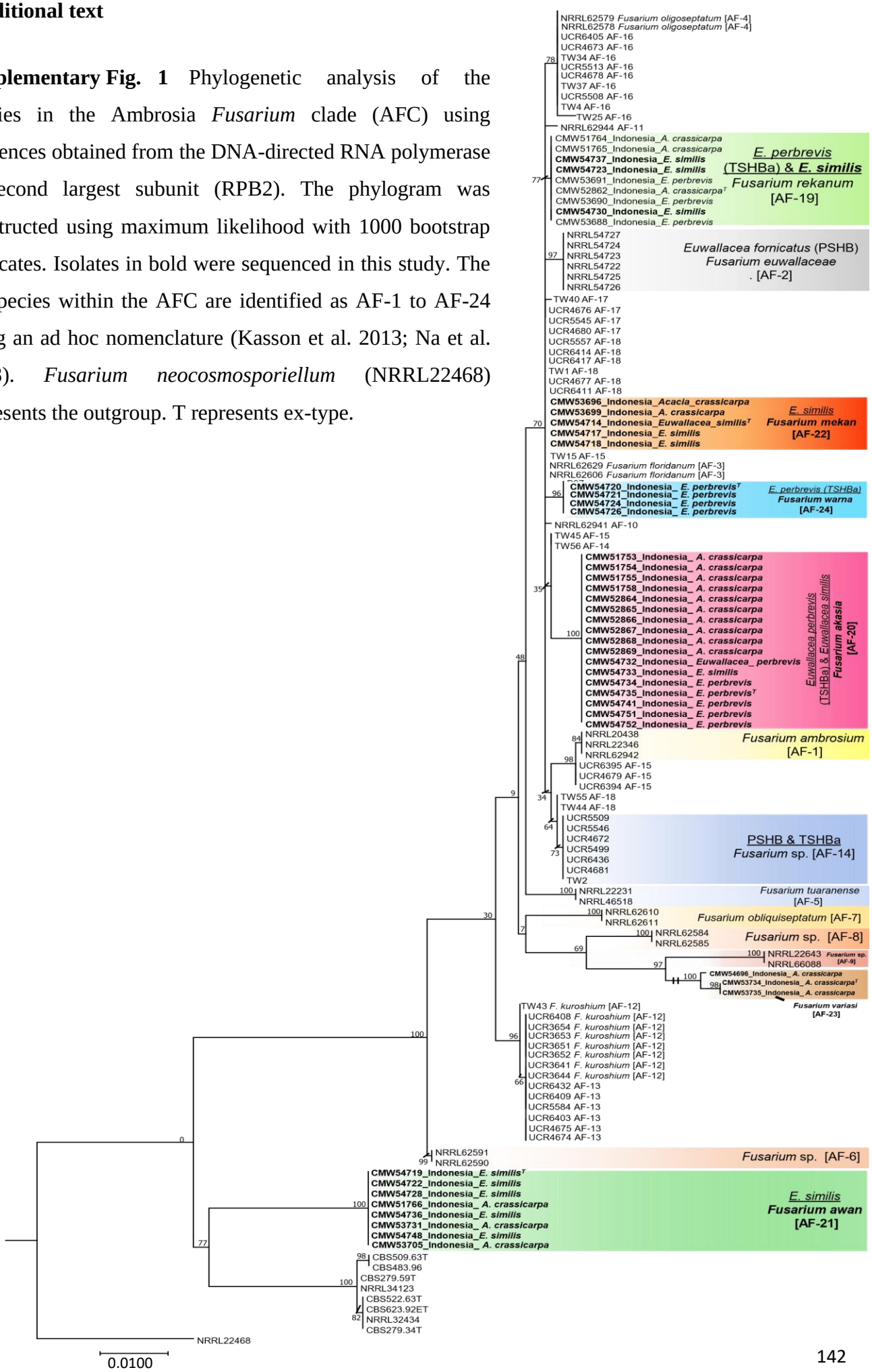


Fig. 8 Morphological characteristics of *Fusarium warna* on PDA and SNA (CMW 54720, CMW 54724 and CMW 54726). **a**–**c, g-i.** Morphology of *F. warna* cultures grown on potato dextrose agar (PDA). **d-f, j.** morphology of 14-day-old *F. warna* cultures grown on synthetic low-nutrient

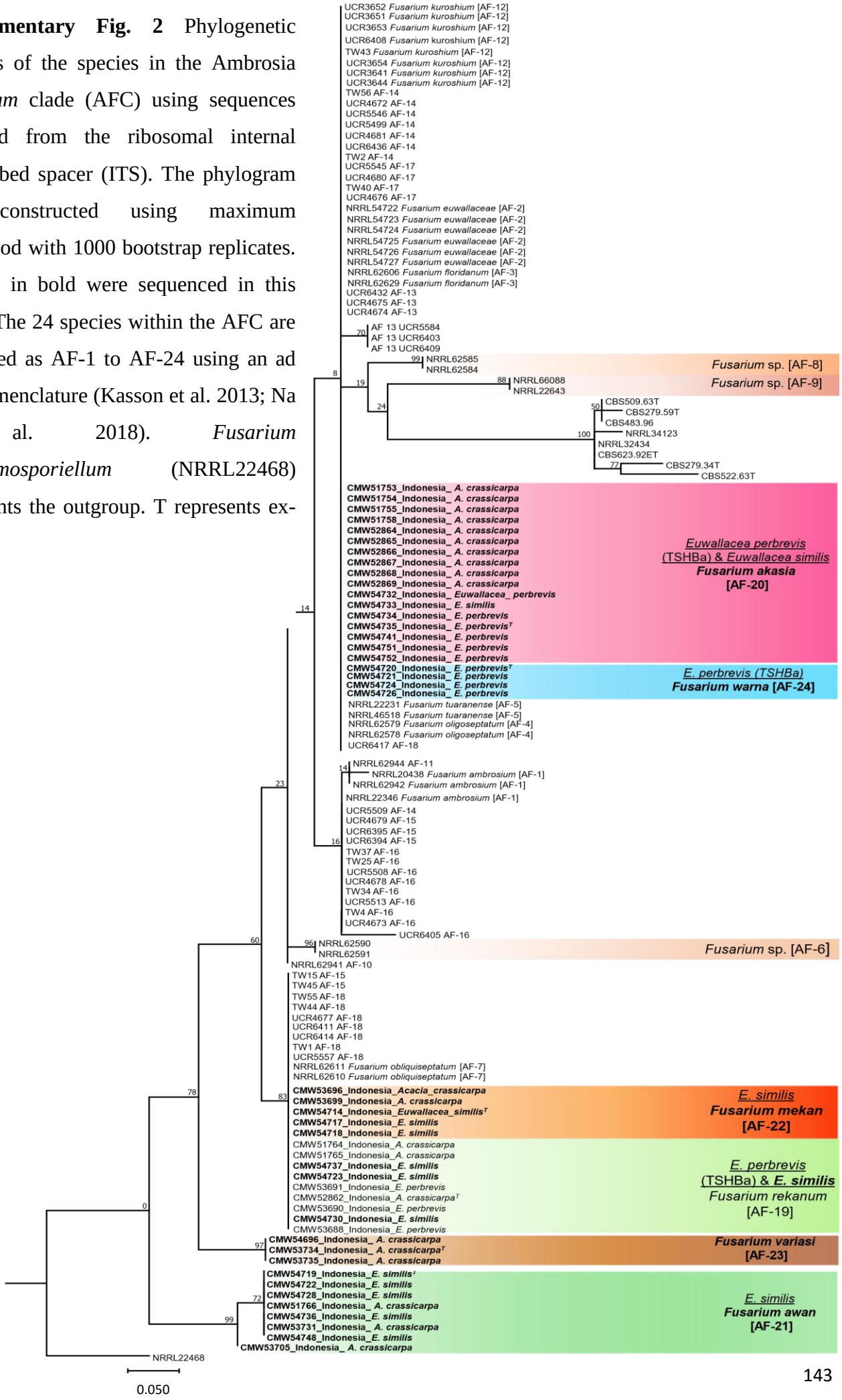
agar (SNA). **a-b.** Colony morphology on PDA at 2 weeks. **c.** Luteous-ochreous to sepia conidial masses formed in culture on PDA after 1 month. **d-e.** Simple to branched aerial conidiophores varying in length forming short obovoid conidia with truncated base with 0-3(-4) septa. **g-h.** Pigmented multiseptated short, clavate shaped sporodochial conidia. **i.** Multiseptate conidia with round to oval, rough-walled chlamydospores. **j.** Round to oval chlamydospores, clustered, formed in hyphae. **c** = 1 mm; **d-m** = 15 µm; **g-j** = 10 µm.

Additional text

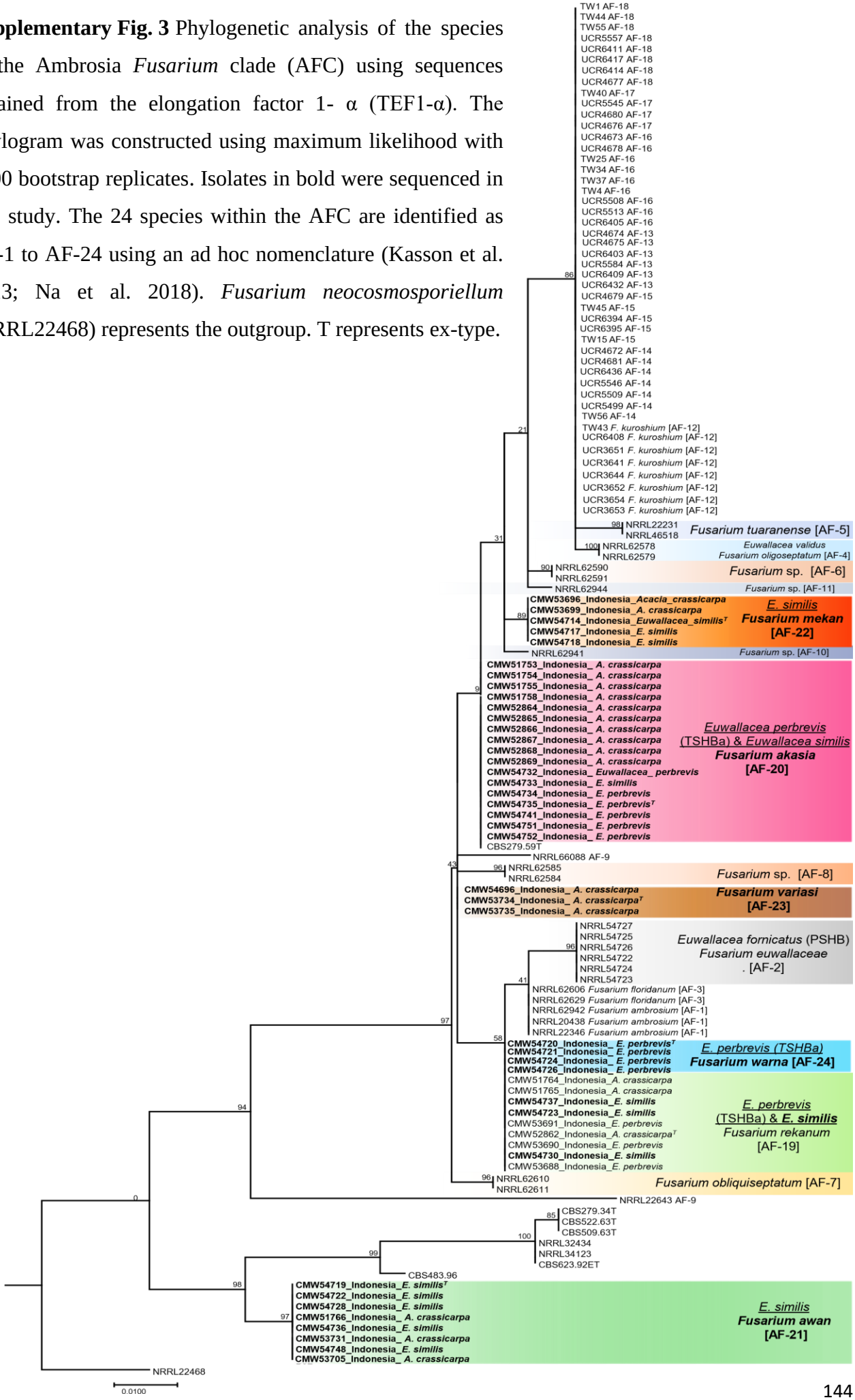
Supplementary Fig. 1 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the DNA-directed RNA polymerase II second largest subunit (RPB2). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 24 species within the AFC are identified as AF-1 to AF-24 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Supplementary Fig. 2 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the ribosomal internal transcribed spacer (ITS). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 24 species within the AFC are identified as AF-1 to AF-24 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Supplementary Fig. 3 Phylogenetic analysis of the species in the Ambrosia *Fusarium* clade (AFC) using sequences obtained from the elongation factor 1- α (TEF1-α). The phylogram was constructed using maximum likelihood with 1000 bootstrap replicates. Isolates in bold were sequenced in this study. The 24 species within the AFC are identified as AF-1 to AF-24 using an ad hoc nomenclature (Kasson et al. 2013; Na et al. 2018). *Fusarium neocosmosporiellum* (NRRL22468) represents the outgroup. T represents ex-type.



Summary

This dissertation investigated ambrosia beetles and their fungal associates that have emerged as pests in their native environment of Indonesia, on the globally important exotic forestry species, *Acacia crassicarpa,* with the aim to broaden knowledge regarding the biodiversity, ecology and pathogenic potential of this beetle-fungus symbiosis. This study identified four ambrosia beetles infesting *A. crassicarpa* plantations in the peatlands of Riau, Indonesia, however only two of these were consistently isolated from infested hosts. This study found that *Euwallacea similis* and *Euwallacea perbrevis* were the most abundant beetles infesting *A. crassicarpa.* However, *E. similis* appears to act as a secondary borer during the infestation process, as it was rarely found infesting a host independent of *E. perbrevis.* Furthermore, three novel haplotypes of *E. perbrevis* and four novel haplotypes of *E. similis* were identified in this study.

Three novel *Fusarium* spp. residing in the Ambrosia *Fusarium* Clade (AFC) were found to be associated with *E. perbrevis*, increasing the number of AFC members associated with *E. perbrevis* to eight. Of these eight identified AFC associates, only four have been formally described, three of which were done so in this study. These three novel fusaria species were described as *F. rekanum* sp. nov. [AF-19] (Chapter 2), *F. akasia* sp. nov. [AF-20] and *F. warna* sp. nov. [AF-24] (Chapter 3). Similarly, four novel *Fusarium* spp. residing in the AFC were found to be associated with *E. similis.* Until now, very little was known about the fungal associates harboured by *E. similis*. Here we confirm that *F. rekanum* sp. nov. [AF-19], *F. akasia* sp. nov. [AF-20], *F. awan* sp. nov. [AF-21], and *F. mekan* sp. nov. [AF-22] (Chapter 3) are all associates of *E. similis*. Finally, a sixth AFC member was isolated from a brood gallery of either *E. perbrevis* or *E. similis*, and formally described as *F. variasi* sp. nov. [AF-23].

This study not only gave insight into the diversity of the two *Euwallacea* spp. infesting *A. crassicarpa* plantations in Riau, Indonesia, but also on the diversity of AFC members they harbour and how this beetle-fungi symbiosis appears to be promiscuous in nature. Investigating these exotic *Fusarium*-farming *Euwallacea* spp. that have emerged as pests in their native environment, can give insight into their pathogenic potential and inform future quarantine strategies.