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Examination of mycorrhizal associations of *Allanblackia stuhlmannii* – a tree under current domestication

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Department of Forest Mycology and Plant Pathology
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Abstract

Allanblackia is a tree genus native to humid forests in West, Central and East Africa. Its fruits contain large seeds from which edible high-quality fat can be extracted. In order to create a sustainable supply chain of *Allanblackia* seeds, a domestication program has been initiated with the purpose to enable smallholder farmers to grow the tree as an agroforestry component and cash crop. More knowledge is required to understand the tree's biology and possible symbioses involving soil microorganisms with plant growth enhancing potential. This is the first study to report on mycorrhizal associations and the fungal community of *Allanblackia stuhlmannii*, a species indigenous to Tanzania. The aim was to examine whether trees can form arbuscular mycorrhiza (AM), the dominant type of mycorrhiza in tropical ecosystems formed by members of the fungal phyla Glomeromycota. In total 50 samples from seedlings and older trees were included in the study. AM structures, e.g. vesicles and potential arbuscules, were observed using microscopy. A large number of possible AM symbionts were also identified by molecular analysis, as well as fungi belonging to Ascomycota and Basidiomycota. These results suggest that *A. stuhlmannii* is able to form arbuscular mycorrhiza, potentially with a wide range of fungal species. However, it remains to clarify to what extent the growth of *Allanblackia* is benefiting from the association, as well as identify the key fungal species.

Sammanfattning

Allanblackia är ett trädsläkte som växer i Väst-, Central- och Östafrikas regnskogar. Dess frukter innehåller oljerika frön från vilka högkvalitativt, ätligt fett kan utvinnas. I syfte att skapa en hållbar produktion av Allanblackiafrön har ett domesticeringsprogram inletts för att möjliggöra småskalig odling av trädet inom agroforestrysystem. Dock krävs mer kunskap om trädets biologi och eventuella symbiotiska förhållanden med markorganismer. Den här studien av *Allanblackia* är den första i detta sammanhang och undersöker svampsamhället hos *Allanblackia stuhlmannii*, en underart endemisk till Tanzania. Syftet var att undersöka om träden kan bilda arbuskulär mykorrhiza (AM), den dominerande typen av mykorrhiza i tropiska ekosystem vilken bildas av medlemmar i svampfylumet Glomeromycota. Sammanlagt 50 rotprover från unga och äldre träd ingick i studien. Mikroskopering visade potentiella AM strukturer i form av vesikler och arbuskler. Därtill kunde ett stort antal tänkbara AM symbionter identifieras genom molekyläranalys, liksom svampar tillhörande Ascomycota och Basidiomycota. Resultaten tyder på att *A. stuhlmannii* kan utveckla arbuskulär mykorrhiza, eventuellt med ett stort antal svamparter. Det återstår emellertid att utreda i vilken omfattning *Allanblackia* gynnas av dessa förhållanden, samt identifiera viktiga svamparter.

Table of content

1 Background

Sub-Saharan Africa has the fastest growing population in the world and over 60% rely on agriculture for their livelihood (IAASTD, 2009). Most of them are small-holder farmers, largely dependent on the use of locally available natural resources for the subsistence farming system. In order to improve livelihoods and enhance food security and resilience of rural communities, diverse and multifunctional agricultural systems are being promoted (FAO, 2011).

The concept of agroforestry refers to the integration of trees and crops on farm which has both environmental and social benefits (Young, 1989). The biodiversity in agroforestry systems is in general higher compared to conventional agriculture and can provide food, timber, fibers and animal feed, but also improve the soil fertility. The fruits are often better in supplying essential nutrients than staple foods and can additionally be sold at the market to generate extra income to the household. Economic risks are reduced as the system produces multiple products which will increase, diversify and stabilize household income. Moreover, the trees have the potential to be economically profitable through revenues from carbon sequestration. All these strategies play a significant role in poverty alleviation (Syampungani et al., 2010, Sanchez et al., 1997).

For the establishment of economically profitable systems, development of new farming strategies is required. There has long been a lack of investment in domestication of plant species that are specifically suited to African farmers' circumstances, but now there is a growing demand for commercialization and use of local, traditional crops and trees (Akinnifesi et al., 2007). Many indigenous tree species are unknown on the global market, but they may be important locally and contribute to improved rural livelihood and nutrition (Leakey, 2010).

One example is the evergreen tree genus *Allanblackia*, indigenous to humid forests in West, Central and East Africa. Traditionally, *Allanblackia* fruits have been collected from wild stands and high-value edible oil has been extracted from its seeds. The fat has for long been used by local communities for food, medicine and animal feed (Meshack, 2004). *Allanblackia* is a multipurpose tree that is classified as a Cinderella species, i.e. it has long been of importance for local people providing necessities for their everyday life, but has so far been overlooked by science (Leakey et al., 2005). However, during the last decade *Allanblackia* oil has received attention from the industry because of its unique properties which make it a suitable raw material in the production of spreads and soap (EFSA, 2007). This has led to a growing demand for the seeds which cannot be met from wild stands and, as a response, a claim for domestication and commercialization of *Allanblackia* has been raised. By focus on small-scale production, farmers will be able to grow *Allanblackia* on their farms both as an agroforestry tree and a cash crop. Rural communities could therefore improve their livelihoods not only by the benefits of the tree itself, but also through private-public-partnerships (Jamnadass et al., 2010).

To ensure availability and sustainability of *Allanblackia* products, the World Agroforestry Centre (ICRAF, Nairobi, Kenya) launched a research program in 2002 aiming for the domestication of *Allanblackia* tree species on smallholder farms. Up to now, research activities have focused on the selection of highly productive plant materials and propagation methods. To fully optimize the harvest potential of domesticated *Allanblackia*, soil and water requirements of the trees must be met, but very little is known on this today. Work is now carried out on the management of *Allanblackia* seedlings at the nursery and preliminary results show that seedlings do better if grown in soil collected under *Allanblackia* trees. This suggests positive plant-microbial interactions which has been assumed to be mycorrhizal associations (Owusu-Yeboah et al., 2008). Symbiotic relationships between plants and microorganisms are very common and serve important functions in ecosystem composition and productivity, with a fundamental role in shaping the terrestrial landscape (van der Heijden et al., 2008).

In this case of *Allanblackia* domestication it remains to clarify if a symbiotic association occurs, which type of association it is, which organisms are involved in the association, and furthermore how they can be promoted. Knowledge on these relationships will be essential in order to understand the tree's biology and appropriate cultivation practices. Obtained information will be important in the domestication process and could additionally provide the basis for further research and be implemented in ICRAF's outreach material to farmers and nurseries.

2 Objective

This MSc thesis was conducted on the initiative of the "ICRAF Global Research Project 1: Domestication, utilization and conservation of superior Agroforestry germplasm" (GRP1). ICRAF, also known as the World Agroforestry Centre, is an international research centre headquartered in Nairobi, Kenya. They conduct research with focus on improved agroforestry systems in order to mitigate land depletion, forest degradation and thereby obtain food security and alleviating poverty in the tropics. This study was further part of the large research project "Multifunctionality of agroforestry systems; can integration of trees and crops contribute to enhance agricultural productivity, resource utilization and livelihoods for smallholder farmers? A quantitative approach". This project is an ongoing collaboration between the Swedish University of Agricultural Sciences (SLU), Jomo Kenyatta University of Agriculture and Technology (JKUAT), International Centre for Research in Agroforestry (ICRAF), and the Swedish Cooperative Centre-Vi Agroforestry.

The aim of this study was to obtain information about occurrence of potential symbionts with *Allanblackia stuhlmannii*. Since the assumption has been that the tree form mycorrhiza, the objective of the study was to investigate possible presence of mycorrhizal fungi. Field sampling was conducted during September 2012 in the East Usambara Mountains in Tanzania where *A. stuhlmannii* is indigenous.

Literature review

2.1 East Usambara Mountains and Amani Nature Reserve

2.1.1 Location and climate

East Usambara Mountains are located in north-eastern Tanzania (*Figure 1*) and part of the Eastern Arc Mountains that stretch along the East African coast from southern Kenya to southern Tanzania (Frontier Tanzania, 2001). This is considered to be one of the world's 25 biodiversity hotspots with around 4000 plant species described (Myers et al., 2000) of which many are endemic (Lovett, 1989). The forest of East Usambara covers around 45 km^2 (Johansson and Sandy, 1996) ranging between 190 and 1130 m asl. Mainly based on altitude gradient, two forest types can be distinguished: lowland forest and sub-montane forest. The latter in general occurring above 850 m asl (Frontier Tanzania, 2001). East Usambara consists of several forest islands of which Amani Nature Reserve is the largest block, covering 8360 ha. Formed in 1997, Amani is the first nature reserve in Tanzania comprising six former forest reserves, public land and 1068 ha forest donated by East Usambara Tea Company (Frontier Tanzania, 2001).

The mean monthly temperature varies with 5 degrees between the hottest (24.8˚C) and coldest (16.3˚C) months. The climate is monsoonal, receiving mean annual precipitation of about 1900 mm with peaks between March and May and from October to December (Hamilton, 1989a). The bedrock is of late Precambrian period consisting of gneisses with pyroxene, biotites, hornblende and often veins of quartzite. These laterized soils are highly weathered and acidic with low amount of organic matter (FAO, 1983). Precipitation increases with altitude, why soils become more acid and highly leached at higher altitudes, starting from 850-900 m asl (Hamilton, 1989b). Poor soil fertility with regards to low storage capacity of nitrogen and phosphorus could also be resulting from soil disturbance due to gold

mining (Kweyunga and Senzota, 2007). In the lowlands, conditions for agriculture have been described as more favorable (Kessy, 1998).

Figure 1. Location of East Usambara Mountains in Tanzania.

2.1.2 Agriculture and forest conservation

About 84% of the inhabitants in the East Usambaras are farmers (Kessy, 1998). In addition to common food crops, such as maize, beans and vegetables, many of them also grow spice cash crops, for example cardamom, cinnamon and cloves (personal observation). Cardamom agroforestry has been practiced in the area for more than 50 years (Hall et al., 2011) but since the understory vegetation is cleared before planting, this has contributed to the loss of forest (Newmark, 2002). Additionally, many farmers still practice shifting cultivation which puts high pressure on indigenous forests as new land is cleared by burning and old fields left as fallow (Kessy, 1998). Furthermore, bush fires resulting from uncontrolled fire management pose another threat, for forest conservation as well as for the inhabitants (WWF, 2006). Other problems within the area are illegal timber cutting and

animal trapping. However, local communities take part in the forest conservation program and representatives have influence in management issues. To some extent, people have permission to utilize the forest resources, for example by collection of dead wood twice a week (Frontier Tanzania, 2001). In a survey by Vihemäki (2005), most farmers stated that they benefit from forest protection which, besides providing resources, also could have cultural and religious values for local communities (Kessy, 1998). Moreover, forest conservation in the East Usambaras is not only important for biodiversity conservation and the livelihoods of the inhabitants, the forest also serves as the catchment area feeding the Sigi river. Hence, deforestation could result in severe consequences for the water supply of communities in the lowlands (Frontier Tanzania, 2001).

2.2 Allanblackia stuhlmannii

2.2.1 Distribution and biology

The genus *Allanblackia* belongs to the Clusiaceae (Guttiferae) family of flowering plants and is named after the Scottish botanist Allan Black (Anegbeh et al., 2006). This is an evergreen tree native to the tropical rainforests in Africa which comprises nine species, stretching in an equatorial belt from Sierra Leone to Tanzania. In West and Central Africa the species *A. floribunda* Oliv., *A. parviflora* A. Chevalier, *A. marienii* Staner, *A stanerana* Exell & Mendonca and *A. gabonensis* Pellegr. Bamps grow in several countries. *A. kisonghi* Vermoesen and *A. kimbiliensis* Spirl are endemic to the Democratic Republic of the Congo, and *A. ulugurensis* Engl. and *A. stuhlmannii* Engl. are endemic to the Eastern Arc Mountains in Tanzania (Orwa et al., 2009).

Trees are above 20 m in height and grow in humid areas receiving an annual rainfall ranging between 1200-2400 mm at altitudes of 400-1800 m (Munjuga et al., 2008). The bole is straight with dark grey color and smooth or flaking. The simple leaves have opposite position and are oval shaped with a characteristic pointy tip (*Figure 2*). The tree is dioecious, i.e. each individual is male or female, hence the creamy white, red or pink flowers are either male or female (Ruffo et al., 2002). This is not possible to determine before the first flowering, which will take six years from germination (Ofori et al., 2011b). However, female trees seem to occur more frequently than male trees (ratio 3:1) (Peprah et al., 2009). Because the flowers occur on different individuals, *Allanblackia* is thought to be propagated by wind and insect pollination (Mugasha, 1980). Both male and female flowers produce nectar to attract pollinators but male flowers have shown to

Figure 2. Mature *A. stuhlmannii* tree bearing fruit (left), close up of fruit (top right) and leaves of a young seedling (bottom right).

produce significantly more nectar than female flowers. In Tanzania, flowering and flower buds peak in January and February, decline until July, followed by a small peak in August and September. The peak fruit period is January to March with a small peak in October (Mathew et al., 2009). Fruit production seems to occur in cycles, with fruit-booms every fourth year (Munjuga, pers. comm.). The red-brown fruits of *Allanblackia* trees (*Figure 2*) are the largest of any plant in the African rainforests and can weigh between 2.5-5.8 kg (Ruffo et al., 2002). Ripe fruits fall to the ground and it is not possible to tell whether a fruit is ripen or not while still on the tree (Meshack, 2004). The fruits have five sections, each containing 12-38 seeds (Ruffo et al., 2002), however, an average number of 38 seeds per fruit has been reported for *A. stuhlmannii*. There are also indications on increasing seed quantity with tree size (Mathew et al., 2009).

2.2.2 Usages

Allanblackia is a multipurpose tree and besides being used for fuel and construction timber, it also produces valuable non-timber forest products (NTFP) (van Rompaey, 2003). Traditionally the seeds have been harvested from wild stands and the edible fat extracted and used by local communities in cooking, as substitute for butter and cocoa butter or for making candle lights (Meshack, 2004). Different parts of the tree are also used in traditional medicine, e.g. the leaves for

treatment of cough and chest pain. Roots are used to treat impotence and the oil could be smeared on wounds and rashes or to treat rheumatism (Meshack, 2004, Munjuga et al., 2010). Several studies have identified antimicrobial and anti-inflammatory activity in extracts from stem bark (Nguemfo et al., 2007, Azebaze et al., 2008, Azebaze et al., 2006), and there is also evidence of HIV inhibitory substances in extracts from *A. stuhlmannii* (Fuller et al., 1999).

2.2.3 Physicochemical properties

Dehulled, the seed kernels contain almost 70% fat but are low in carbohydrate (17%), protein (4%), ash (2%) and fibre (6%) (Adubofuor et al., 2013). The press cake obtained after processing has a protein content of 14% but the bitter taste and high amount of tannins makes it unsuitable for consumption or as animal feed (Mwaura and Munjuga, 2007). Because of the physicochemical properties of *Allanblackia* fat, it has been referred to as "a food technologist's dream" (Pye-Smith, 2009). The melting point at 34˚C makes it solid in room temperature but melts easily in contact with the body (Buss and Tissari, 2010). As *Allanblackia* fat is almost entirely composed by oleic and stearic triglycerides, the composition of fatty acids distinguish it from other vegetable fats (*Figure 3*), being most similar to Shea butter and Borneo tallow fat.

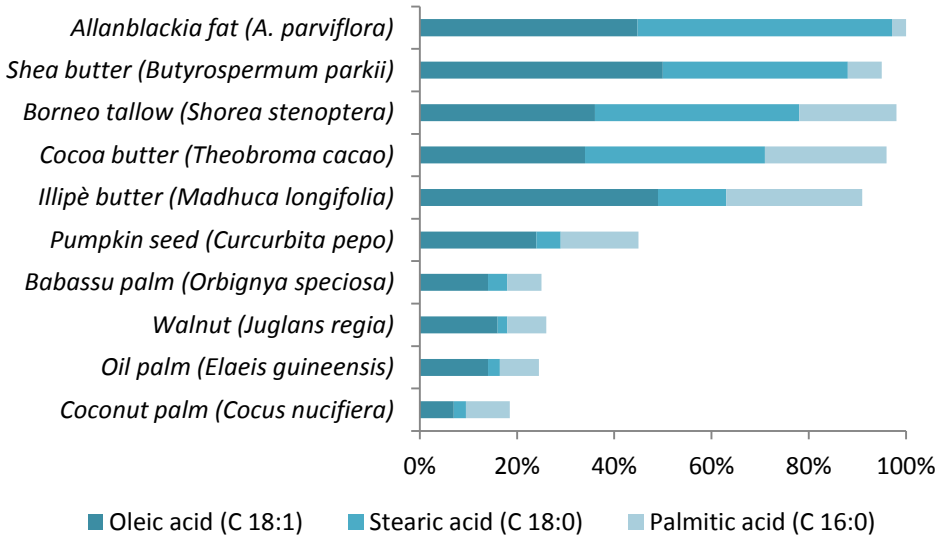

Figure 3. Fatty acid content (weight percentage) of vegetable fats with regards to oleic, stearic and palmitic acid in ten different oil producing tree species. *Allanblackia* fat appears to be most similar to Shea butter and Borneo tallow fat (Belitz et al., 2009, Adubofuor et al., 2013).

The high quality fat of *Allanblackia* makes it a potential substitute for margarine and component in soap-making. It can also be mixed with other fats in order to modify their physical properties (Adubofuor et al., 2013). Unlike, for example, palm-oil it does not require much further modification, and thus the number of steps in the process, as well as the costs of manufacturing, could be reduced (Buss and Tissari, 2010). In 2008, after four years of consideration, *Allanblackia* was authorized by the European Union's Novel Food Regulation (NFR) as safe for the European market (Hermann, 2009). Hence, the tree now has a commercial value.

2.2.4 Commercialization

The Novella Africa Initiative is a private-public-partnership (PPP) initiated by Unilever with the aim to develop an effective, economically viable and environmentally sustainable supply chain of *Allanblackia* oil that is based on small-scale farming systems (Attipoe et al., 2006). The project targets three species in different regions: *A. parviflora* in Ghana, *A. floribunda* in Nigeria and *A. stuhlmannii* in Tanzania (Ofori et al., 2011a). One objective is to involve 200,000 African farmers and plant 25 million trees by 2017. (Unilever, 2008). The initiative brings together a number of different stakeholders including the World Agroforestry Centre (ICRAF), the World Conservation Union (IUCN), local and regional organizations and NGOs, research institutes and governments, and different donors (Jamnadass et al., 2010). Similar partnerships have showed to be successful, for example various fruit and nut species in Cameroon (Asaah et al., 2011). Unilever is the largest investor and buys all the extracted, pre-processed crude oil which is exported for refining in Rotterdam, the Netherlands (Buss and Tissari, 2010).

Cultivation of *Allanblackia* trees is a way for farmers to diversify their production and generate extra income (Jamnadass et al., 2010). A diverse cropping system is important in order to be less dependent on commodity crops and the global market, hence reducing the consequences of falling prices (Leakey and Newton, 1993). Moreover, NTFPs can vary in seasonality and therefore generated income be spread across the year (Leakey et al., 2005). Since *Allanblackia* trees fruit between January and April, the time of seed harvest fall outside the general cropping season, hence providing an income opportunity during the most difficult time of the year (Pye-Smith, 2009). On the local level, formation of community groups are promoted to strengthen their common position in price negotiations as well as to create a platform that will facilitate exchange of local knowledge between farmers (Amanor et al., 2008). Consequently, several community-run Rural Resource Centers (RRC) have been established where training activities in agroforestry techniques are carried out (Ofori et al., 2011a).

ICRAF is coordinating the research program with the main objective to domesticate *Allanblackia* in order to facilitate on farm cultivation of the tree. The Novella Initiative has been suggested to serve as a model for domestication of underutilized tree species. Therefore, in contrast to the domestication of oil palm, the project aims for domestication with focus on small-scale producers. Currently, the main focus is to produce superior genotypes to be able to integrate *Allanblackia* as an agroforestry component on farms (Ofori et al., 2011a). Slow germination rate and long time taken before trees start to produce fruits are some challenges that researchers hope to be able to overcome by developing methods for vegetative propagation (Munjuga et al., 2008).

2.2.5 *Allanblackia* and potential symbionts

Symbiotic relationships between *Allanblackia* and soil microorganisms have so far not received much research attention but one assumption is that mycorrhizal associations are involved. This is partly based on an assessment of growth of superior genotypes of *A. stuhlmannii* which were established in mother blocks in East Usambara. The unpublished results revealed differences in tree performance that were not expected, hence the question whether mycorrhizal symbionts were involved was raised (Mpanda, pers. comm.). However, this hypothesis has not yet been tested. Although mycorrhizal associations are by far the most common form of symbiotic relations involving higher plants, other associations with soil microorganisms could also occur. Plant growth promoting bacteria (PGPB) are widely spread and are beneficial for plants by several mechanisms, for example direct stimulation of plant metabolism through enhanced nutrient acquisition, such as nitrogen fixation and solubilization of phosphate and iron. They can also promote the synthesis of phytohormones and improve the plant's tolerance to stresses. Biocontrol-PGPB promote plant growth indirectly by suppression of e.g. soilborne diseases (Gamalero and Glick, 2011, Bashan et al., 2008). Plants within the *Fabaceae* family can form nitrogen-fixing nodules with *Rhizobium* bacteria (Campbell, 1996). Actinorhizal plants are non-leguminous, from the three orders Cucurbitales, Fagales and Rosales, that are able to form symbiosis with the nitrogen-fixing actinobacteria belonging to genus *Frankia* (Santi et al., 2013). *Allanblackia* does not belong to any of the mentioned groups and therefore nitrogen-fixing symbiosis is not expected. However, this does not exclude that the tree can benefit from other PGPB.

This is the first study to examine potential symbiosis between *Allanblackia* and soil microorganisms. Therefore the focus has only been on providing evidence to support or refute the assumption that *Allanblackia* has mycorrhizal associations.

2.3 Mycorrhiza

The phenomenon of mycorrhiza refers to an intimate symbiotic association between plants and fungi in the soil, through all or part of their life span. This is a mutualistic relationship as both partners are benefitting from a bidirectional nutrient exchange. The fungus receives carbon from the host plant's photosynthesis while facilitating the plant's uptake of nutrients and other minerals (Smith and Read, 2008). Wang and Qiu (2006) estimated that 92% of all terrestrial plant families are able to form mycorrhiza symbiosis and only 5-10% plant species are considered to be non-mycorrhizal (Helgason and Fitter, 2005).

Mycorrhizal symbionts can be grouped into four types based on their fungal associates: ectomycorrhiza, endomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza, of which the former two are most common. Ectomycorrhizas (EM), formed by members of the fungal phyla Ascomycota, Basidiomycota and Zygomycota, are thought to mainly be present in association with woody perennials in temperate zones and in ecosystems with relatively low diversity. However, as stated by Munyanziza et al. (1997), the role of EM in tropical trees have not received much research attention. Features associated with EM are mantles of hyphae enclosing the host root and growing inwards between the epidermal and cortical cells, forming a so called Hartig net. Thus, EM fungi never penetrate to grow inside the host cells. Present in the soil is also an extensive external mycelium (Smith and Read, 2008). Endomycorrhiza, often referred to as arbuscular mycorrhiza (AM), is the dominating type in tropical ecosystem (de Carvalho et al., 2010). Since *A. stuhlmannii* is expected to form AM, this type of association will be further described in the following section.

2.3.1 Arbuscular mycorrhiza

Arbuscular mycorrhiza (AM), formed by members of the fungal phylum Glomeromycota, is the most wide-spread mycorrhizal symbiosis which is found in the majority of families in the plant kingdom and occurs in more than 80% of land plant species (Smith and Read 2008). It has probably played an important role in the evolution and establishment of terrestrial plants (Helgason and Fitter, 2005), but is nowadays believed to be much older, possibly originating more than one billion years ago (Smith and Read, 2008). This symbiosis can be described as a three-component association that involves the host root, as well as both extraradical and intraradical mycel, i.e. the fungus can grow both outside and inside the root cells (Santos-González, 2007). The name arbuscular is derived from the characteristic structures (arbuscules) developed by intraradical hyphae inside the cortical cells of the host's root. These are short-lived organs where the nutrient exchange occurs (Gadkar et al., 2001). Other features characteristic for AM are

hyphal coils formed inside host cells, and vesicles which are storage organs produced by 80% of AM fungal species. Infection structures, so called appressoria, are sometimes formed on the host surface prior to penetration (Smith and Read, 2008). These structures are shown in *Figure 4*.

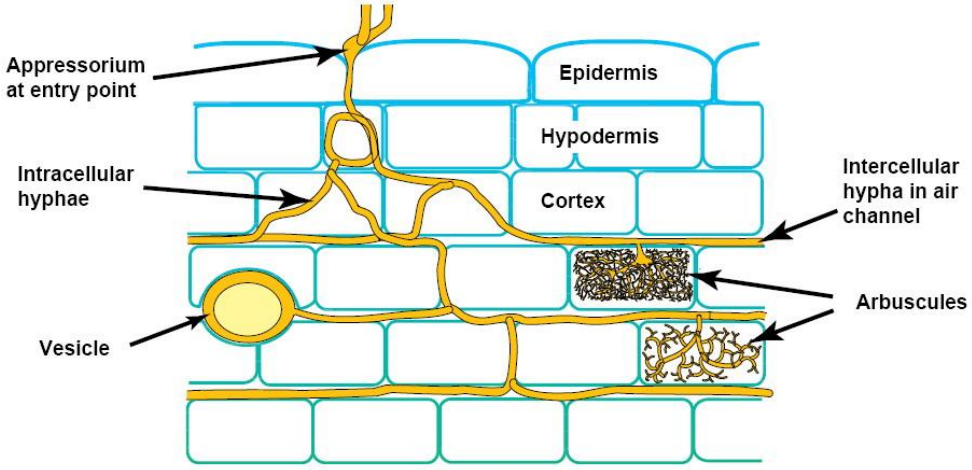

Figure 4. Structures formed by AM fungi inside the host plant root (Brundrett et al., 1996).

AM fungi (AMF) are obligate symbionts and thereby completely dependent on their host for organic carbon, which can correspond up to 20% of total amount fixed by the plant (Smith and Read, 2008). In return, the fungus assist the host plant in acquisition of nutrients in poor environments, in particular phosphorus (Helgason and Fitter, 2005). The extensive fungal mycelium can increase the exploited soil volume and absorb nutrients outside the depletion zone that is created around plant roots as a result of nutrient uptake. Moreover, fungal hyphae are much cheaper to produce compared to roots and therefore a cost-effective way for the plant to access resources (Santos-González, 2007). Besides improving nutrient uptake, other potential benefits include improved water status and increased tolerance to drought (Augé, 2001), as well as resistance to pathogens (Helgason and Fitter, 2005), therefore leading to improved tree growth (Ingleby et al., 2007). It has also been suggested that mycelia networks can connect individual plants, both within and between species (Godbold and Sharrock, 2003).

The hyphae lack septa and are coenocytic, i.e. there are no separate cells with individual nucleus, but the nuclei can move around freely within the mycelium (Helgason and Fitter, 2005). Glomeromycota fungi are assumed to have asexual propagation through spores produced by the mycelium. The spores show high genetic diversity, even from individual fungus, and there is evidence for two underlying explanations. The fungus can either be heterokaryotic with genetically

different nuclei, or homokaryotic with identical nuclei, containing several different copies of the gene (Smith and Read, 2008). Spores are normally dispersed by wind or water and this is one way for the fungi to colonize plants, in addition to infected root fragments and hyphae. Spore production is dependent on physiological factors (Redecker, 2002) as well as plant diversity (Burrows and Pfleger, 2002).

AM associations are characteristic to ecosystems with high biodiversity (Smith and Read, 2008), probably because of the wide range of potential hosts and high root density for colonization (Burrows and Pfleger, 2002). On the other hand, fungal diversity and microbial interactions are major contributing factors to the biodiversity and functioning of ecosystems with influence on its productivity and variability (van der Heijden et al., 1998). AM fungi are thought to have a high degree of adaptability across a wide range of potential host plants (Smith and Read, 2008). However, there is evidence for some extent of host specificity and furthermore different AM species may have certain specific niches, therefore not competing with each other (Burrows and Pfleger, 2002).

Taxonomy of the Glomeromycota has long been based on microscopic observation of spores (Redecker, 2002) but because of their homogenous morphology, only a few species have been described (Helgason and Fitter, 2005). The diversity is however assumed to be very high and probably underestimated (Vandenkoornhuyse et al., 2002). From an assessment of published studies, Kivlin et al. (2011) suggest it to be six times higher than previously thought. According to Helgason and Fitter (2005), there is still no scientific consensus about how AMF should be organized genetically. However, AMF was recently removed from the Zygomycota to establish the new monophyletic phylum Glomeromycota. This is probably a sister group to the phyla Ascomycota and Basidiomycota, sharing the same ancestors (*Figure 5*) (Schüβler et al., 2001).

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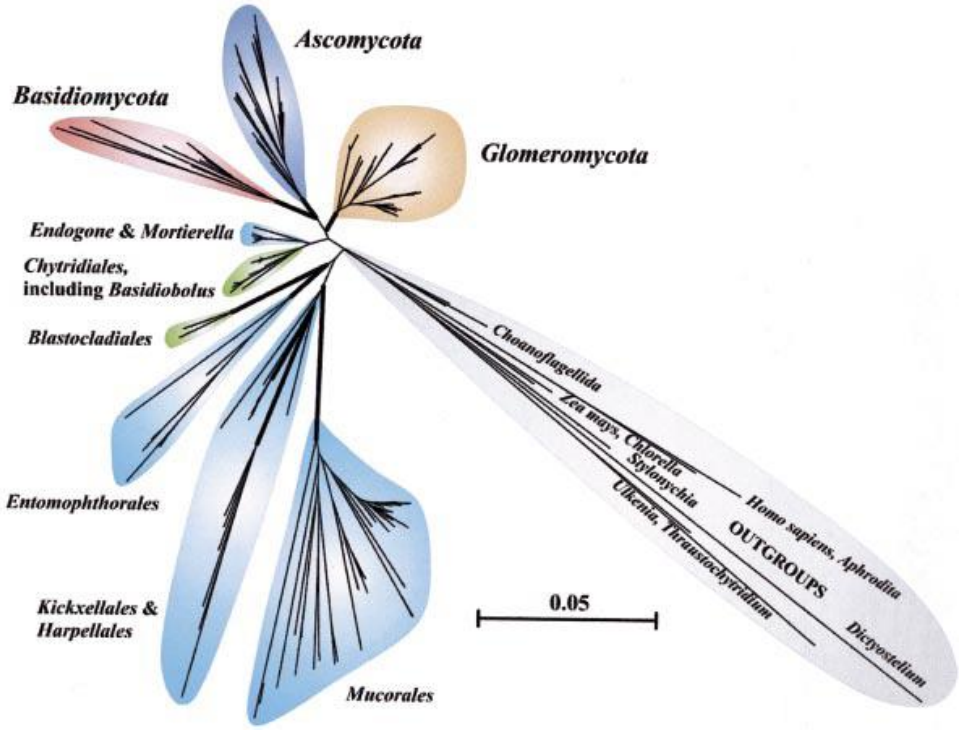

Figure 5. Phylogeny of fungi based on DNA analysis (Schüβler et al., 2001).

The new taxonomy of Glomeromycota, presented in *Figure 6*, is based on DNA analysis of spores and constitutes the four orders Archaeosporales, Diversisporales, Glomerales, Paraglomerales. Around 200 species in ten genera have been described so far (Redecker and Raab, 2006), of which *Glomus* is the most species rich genus (Smith and Read, 2008). It is polyphyletic and has further been divided into three major families *Glomus A*, *B* and *C*. The latter is also known as Diversisporales and contains the Acaulosporaceae, Diversisporaceae, Gerdemanniaceae and Gigasporaceae. *Glomus A* and *B* can also be further diverged into sub-groups (Schüßler and Walker, 2010).

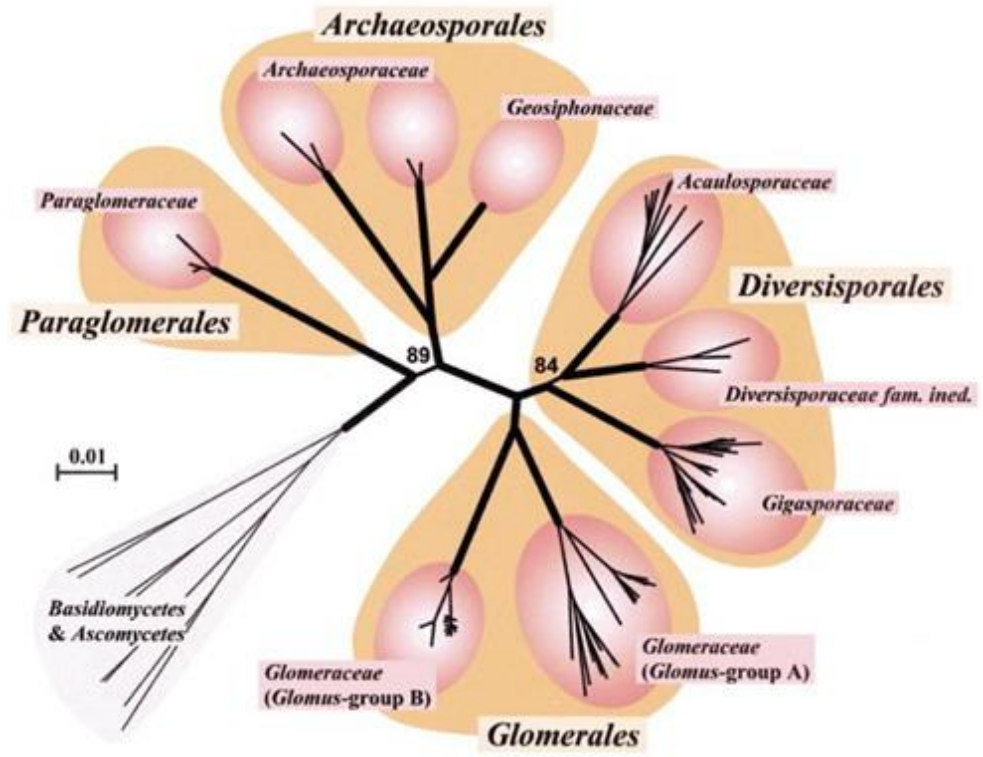

Figure 6. The four orders of Glomeromycota and their families (Schüβler et al., 2001).

2.3.2 AM in tropical ecosystems

Sub-Saharan Africa represents areas with combined environmental stresses, e.g. water and mineral stresses, which are expected in 30% of tropical soils respectively. AM fungi are native to all soils in tropical, terrestrial ecosystems and it is assumed that most plants in stressed conditions are dependent on AM for their maintenance and survival (Sieverding, 1991). Tropical agroforestry systems are designed to mimic natural forest ecosystems and put emphasis on high diversity and interactions between and among species, both above and below ground. AM fungi can therefore be fundamental for the productivity of such systems (de Carvalho et al., 2010). Moreover, environmental conditions appear to influence the prevalence of different AMF groups. Lekberg et al. (2007) found the family Glomeraceae to dominate in clay soils, whereas the family Gigasporaceae occurred more frequently in sandy soils. Also, soil pH is an influencing factor regarding AM colonization (Read et al., 1976). This could be one explanation to the observed differences in AM occurrence and community composition, even within the same location (Kivlin et al., 2011).

3 Materials & Methods

3.1 Investigation site and sampling procedure

Sampling of soil and plant material was carried out in September 2012 within Amani Nature Reserve in the Eastern Usambara Mountains, Muheza District, Tanzania. Prior to the fieldwork, a tree nursery with seedlings of *Allanblackia stuhlmannii* was visited. The purpose was to get familiar with the characteristics of the species, such as shape and color of leaves and roots. In addition, five leaves were collected and dried for later molecular analysis.

Figure 7. *A. stuhlmannii* seedling of the sampled size from the forest (left) and seedlings at Kwamkoro tree nursery, Amani Nature Reserve (right).

Sites with native *A. stuhlmannii* stands were identified with assistance of local expertise and among those sites five were chosen to be part of the study. The GPS coordinates were recorded and transferred to Google Earth in order to determine the elevation at each site (*Table 1*). One criteria was that the sites should be distributed along an altitude transect in order to detect potential differences in soil

properties. For that reason, the sites found at the highest and lowest altitudes were selected first and then three sites in between. The slope at each site was estimated with a plumb-bob and presented in *Table 1*. In addition, the vegetation was observed and tree species identified with assistance of local expertise (Appendix).

Table 1. Coordinates, elevation and slope of sample sites in Amani Nature Reserve

Site	GPS coordinates	Elevation (m asl)	Slope (%)
Derema I	S 05˚05.074', E 038˚38.096'	910	58
Derema II	S 05˚04.929', E 038˚37.723'	988	31
Monga	S 05˚05.496', E 038˚36.077'	1035	19
Mbomole	S 05˚05.769', E 038˚37.744'	944	43
Kibaoni	S 05˚05.432', E 038˚38.451'	816	43

At each site five *A. stuhlmannii* seedlings were identified which were assumed to meet the selection criteria of well-established but still accessible root system. For this reason, seedlings aged 2-3 years were selected, with a height of approx. 40-60 cm. The distance between the seedlings within the site ranged between 10-30 meters. The intact root system was accessed by digging up the seedling and surrounding soil (approx. 40x40 cm) and placed in plastic bags for later processing.

While uprooting, roots suspected to be from older *A. stuhlmannii* trees, due to the characteristic red color and large size (*Figure 8*), were collected from surrounding soil but kept as separate samples from those belonging to the seedling. Additionally, in conjunction with the root sampling, soil was collected adjacent to the *A. stuhlmannii* seedling and taken to Sweden for physical and chemical analyses. The results are presented in an MSc thesis by Alvum-Toll (2013).

Figure 8. Typical root system of *A. stuhlmannii* seedling (left) showing the coarse diameter of roots and root samples stored in ethanol in Falcon tubes (right).

At the Head Office of Amani Nature Reserve, the seedlings were washed in tap water and the root system cleared out. Root fragments of approx. 5-10 cm size were cut off and as many as possible put in 15ml Falcon tubes filled with ethanol (80%). During washing, more of the suspected *A. stuhlmannii* roots (mentioned above) were detected and put in separate test tubes after preparing them in the same way as the seedling roots. The *A. stuhlmannii* roots collected from the soil at the site in the forest were also prepared in the same way and kept as a separate sample. However, at the laboratory in Uppsala, these two root samples of suspected *A. stuhlmannii* roots were pooled together as one sample which hereafter will be referred to as older roots. Hence, from each tree two root samples were subjected for molecular analysis, thus 10 samples per site and in total 50 root samples.

3.2 Microscopic examination

To enable visual examination of potential fungal colonization under light microscope, roots were cleared and stained according to the ink-vinegar method (Vierheilig et al., 1998). Small root fragments of approximately 1-1.5 cm were randomly selected from each sample and boiled in 10% (wt/vol) KOH. Three different times were tested; 5, 10 and 15 min. Roots were then rinsed several times in distilled water. Cleared roots were stained with black ink (A.T. Cross Company, Lincoln, USA) by boiling for 3 min in a 5% ink-vinegar solution (5% acetic acid). De-staining was performed by rinsing 20 min in distilled water acidified with a few drops of 5% acetic acid. Because of the thickness of the roots and dark pigmentation, roots also had to be bleached overnight with H_2O_2 before examination. Two H_2O_2 concentrations (1:1 and 3:2) were tested, but for the lower concentration one additional night in a fresh solution gave better result. The majority of the samples were prepared with 15 min boiling in KOH and bleaching with 3:2 H_2O_2 overnight.

Samples were examined under light microscope (Leica, Wetzlar; Zeiss, Jena, Germany) and the occurrence of mycorrhizal structures, in particular AM (arbuscules, vesicles and coils), were looked for. Initially, AM fungal structures were intended to be quantified using the grid intersect method (Brundrett et al., 1996). However, this proved to be complicated because of the root thickness and the occurrence of many different types of fungi and the patchiness of observed AM features. Still, AM structures were observed, though sporadically (see result), and hence the decision of continuing with molecular analysis was made.

3.3 Molecular analysis

For molecular analysis, both roots from seedlings and older roots were processed. *Figure 9* presents an overview of the analytical procedure. Roots were cut into pieces of approximately 5-10 mm size with a sterile pair of scissors. For each extraction five small fragments were taken randomly, including both root tips and middle parts of the root system. A few extra samples of aggregated hyphae (so called rhizomorphs) found on the root surface were also collected for analysis.

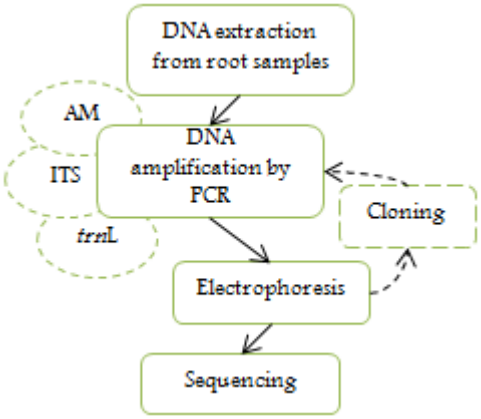

Figure 9. Flowchart describing the different steps in the molecular analysis.

3.3.1 DNA isolation and purification

Each root sample was put into a 2 ml screw cap tube together with two small metal nuts, except for the rhizomorphs where sand and two glass beads were used instead. 1000 µl CTAB (3%) was added and the samples homogenized for 30 seconds using bead beating (5500-1x30-015) in a Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France). The samples were then stored at -20˚C before further processing. Samples were incubated in a heating block at 65˚C for 1 hour and shaken every 10-15 min using a vortex. The mixture was then centrifuged at 10000 rpm for 5 min. Genomic DNA was purified with a single chloroform extraction. Approximately 500 µl of the top mixture was transferred to a new microcentrifuge tube and 500 µl chloroform was added. The tubes were shaken by hand and centrifuged at 10 000 rpm for 7 min. Up to 500 µl of the aqueous phase was transferred to a new centrifuge tube and the DNA was precipitated by adding 1.5 volumes of isopropanol. The mixture was left at room temperature for approx. 30 min. The precipitated DNA was centrifuged at 13 000 rpm for 10 min and the supernatant discarded. The DNA pellet was then washed with 500 µl ethanol (70%) and centrifuged at 13 000 rpm for 5 min. The pellet was left to dry at room temperature for approx. 15 min and re-suspended in 50 µl ddH_2O. The concentration and quality of the isolated DNA were determined using spectroscopy

(NanoDrop, Thermo Scientific, Waltham, USA) at 260 and 280 nm. Before further processing, all samples were diluted with ddH_2O to a concentration of 50 ng/µl. Samples with a total DNA content of <5.0 ng were all diluted 5 times to a final volume of 25 µl.

In order to confirm that the collected roots belonged to *A. stuhlmannii*, DNA was also extracted from dried leaves, two from the nursery and two from native stands in the forest. From each leaf, five small pieces (approx. 1x1 cm) were cut with a sterile pair of scissors, put in screw cap tubes with two nuts and processed the same way as the other samples. However, since no PCR products were obtained DNA from the leaves had to be purified using DNeasy plant mini kit (Qiagen, Hilden, Germany), following the instructions from the manufacturer.

3.3.2 DNA amplification

In order to produce enough starting template for further sequencing, the isolated DNA had to be amplified. Polymerase chain reaction (PCR) is a method which makes it possible to generate multiple copies of a gene by nucleic acid synthesis. In the first step double-stranded DNA is denatured at high temperature and converted into single strands. During the following annealing phase, performed at lower temperatures, specific primers bind to sequences flanking the target DNA and start to synthesize new strands, complementary to the target gene (Klug et al., 2007). This reaction is normally repeated in 20-40 cycles. The PCR mixture, in which the reaction is carried out, contains target specific primers, nucleotides for DNA synthesis, a polymerase to perform extension, i.e. completion of a double-stranded DNA copy, and other co-factors, e.g. $MgCl_2$ for efficient and pure reaction. What part of the genome amplified in the PCR reaction depends upon which region that is targeted by the used primers.

15 µl of PCR mixture (7.5 µl reaction mix + 7.5 µl DNA sample, i.e. 3.75 ng) was loaded to a 96-well microtiter plate or in 0.2 ml reaction tubes in a 2720 Thermal Cycler or a Verti® 96-Well Fast Thermal Cycler (Applied Biosystems, Carlsbad, USA). Depending on the targeted genes, three different sets of primers and programs were used in the PCR.

ITS primers

To examine and describe the general fungal community, the universal primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns, 1993) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990) were used. The primers are designed to exclude plant DNA sequences and target the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). This

method has been suggested as the standard barcode for fungi because of its high identification rate across a broad range of fungi (Schoch et al., 2012), for example Ascomycota, Basidiomycota and Glomeromycota. PCR was performed in a mixture containing 0.4 mM dNTP, 1.5 mM MgCl2, 0.4 µM of each primer, 0.05 U of DreamTaq DNA Polymerase and the supplied reaction buffer (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) using the following program: 5 min initial denaturation at 95˚C, followed by 35 cycles of 30 sec denaturation at 94˚C, 30 sec annealing at 55˚C, 30 sec extension at 72˚C, and a final extension period of 7 min at 72˚C.

AM primers

To exclusively amplify DNA from AM fungi, PCR was carried out with the AM specific primers AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') (Lee et al., 2008). The primers target the small subunit rRNA gene which is less variable than ITS and will enable identification of all subgroups of Glomeromycota but exclude sequences from other taxonomic groups of fungi, such as Ascomycota and Basidiomycota. The reaction mixture contained 0.4 µM of each primer, 0.4 mM dNTP, 1.5 mM MgCl2, 0.05 U of the DreamTaq DNA Polymerase and the supplied reaction buffer (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) and underwent 35 cycles for 1 min at 94˚C with initial denaturation at 94˚C for 1 min, 1 min annealing at 50˚C, 1 min extension at 72˚C followed by final extension at 72˚C for 10 min.

trnL primers

With the purpose to ensure that the root samples were from *A. stuhlmannii*, chloroplast DNA was amplified from extracted DNA from leaves and ten randomly selected root samples. The specific primers trnLc (5'-CGA AAT CGG TAG ACG CTA CG-3') and trnLd (5'-GGG GAT AGA GGG ACT TGA AC-3'), designed to amplify the trnL intron (UAA) of angiosperm and gymnosperm species, were used in a mixture of the following concentrations: 0.12 µM of each primer, 0.4 mM dNTP, 1.5 mM MgCl2, 0.05 U of the DreamTaq DNA Polymerase and the supplied reaction buffer (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). PCR was carried out for 35 cycles of 30 sec at 95˚C, with initial denaturation of 10 min, 30 sec annealing at 50˚C and 2 min extension at 72˚C, followed by final extension at 72˚C for 10 min.

The result from the PCR was checked using electrophoresis. 5 µl of PCR product and 3 µl of ladder were loaded onto a 1% agarose gel stained with gelgreen. The gel was run at 300V and 400A for 30 minutes and visualized under blue light. The

ITS amplifications did, as anticipated, show multiple bands, i.e. DNA sequences from more than one species had been amplified, and the samples therefore had to be separated by cloning before sequencing. The PCR products from the AM amplification were few and weak, i.e. containing little or no DNA and consequently also needed to be cloned to ensure enough template for further sequencing and to isolate the genome from single species. The same was also applied for the chloroplast amplified from root DNA, as the bands too were few and weak.

3.3.3 Purification of PCR products

Before cloning, the PCR products were purified. 10 µl PCR product was mixed with 26 µl of a solution containing 1/10 V NaAc and 2.5 V ethanol (96%). If there was less than 10 µl PCR product left, the sample was diluted with ddH_2O to the required volume. The mixture was incubated for 30 min and then centrifuged at 13 000 rpm for 10 min. The supernatant was discarded, after which 100 µl 70% ethanol was added and the samples centrifuged for 5 min at 13 000 rpm. The ethanol was discarded the same way and samples kept for 30 min at 37˚C to let the ethanol evaporate. Thereafter the pellet was diluted in 1 µl ddH2O.

3.3.4 Cloning

Cloning was performed using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen™, Paisley, UK). In this method, DNA is ligated into a vector and transferred to a host cell where it can replicate during the cell division. 1 µl PCR product was mixed with 0.25 µl salt solution and 0.25 µl of the provided pCR™4-TOPO® TA vector and incubated in room temperature for 30 min. The samples were placed on ice and 25 µl solution containing *Escherichia coli* bacteria added and then incubated for another 30 min. Next, the cells were heat-shocked for 30 sec in a water bath at 42˚C after which the samples were placed on ice for 2 min. 150 µl SOC-medium was added and samples incubated at 37˚C for 1 h while shaking at 200 ppm. However, by mistake the DNA amplified with the ITS primers were only incubated at 25˚C. By a sterile bacterial cell spreader 100 µl of cell suspension was spread on a pre-heated (37˚C) agar plate with ampicillin and Xgal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside). Each plate corresponded to the DNA amplification from one root sample. Plates were incubated upside-down at 37˚C over night.

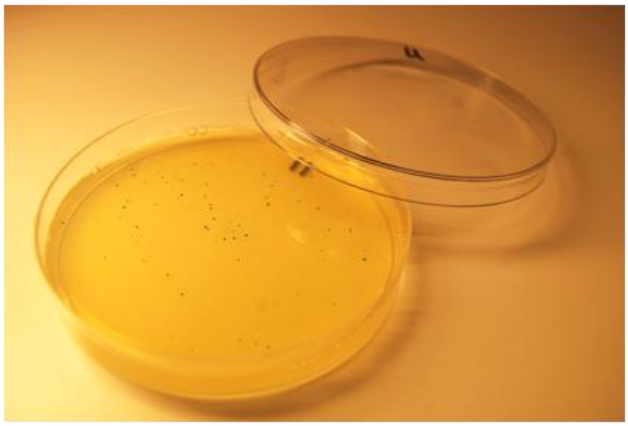

Figure 10. Agar plates after cloning showing *E. coli* colonies. White and light-blue colonies, which contain fungal DNA, were picked for PCR.

Whether cloning of inserted DNA fragments was successful could be identified by the color of the formed bacterial colonies. The vector carries a fragment of the bacterial *lacZ* gene which, if intact, will enable the host cell to metabolize the Xgal, thus producing blue colonies, i.e. the bacteria only contains vector. However, when fungal DNA is ligated into the vector the *lacZ* gene is disrupted and thereby white colonies are formed (Klug et al., 2007).

A total of 16 white and light blue clones from each plate were picked randomly and put in separate wells on a PCR plate filled with 200 µl ddH2O. 25 µl of the suspension was then transferred to another PCR plate with 25 µl reaction mix containing 0.4 mM dNTP, 1.5 mM MgCl2, 0.05 U of the DreamTaq DNA Polymerase and the supplied reaction buffer (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) and x µM of each M13-tailed universal sequencing primers: forward (5'-TGT AAA ACG ACG GCC AGT-3') and reverse (5'-CAG GAA ACA GCT ATG ACC-3'). The PCR was performed with initial denaturation for 5 min at 94˚C, followed by 35 cycles of 30 sec at 94˚C, 30 sec annealing at 55˚C, and 30 sec extension at 72˚C followed by final extension at 72˚C for 7 min.

The PCR products, initially derived from the ITS amplifications, that appeared to be of different size based on the electrophoresis, were selected for sequencing along with 2-3 replicates when possible. From the AM amplifications, all formed PCR products or maximum 11-12 from each sample were selected. PCR products from the amplification of chloroplast DNA were only obtained from four individual root samples and from those a total number of nine clones were sent for sequencing as well as chloroplast DNA from *A. stuhlmannii* leaves from both nursery and native stands.

3.3.5 Purification of PCR product

The PCR products were purified using Agencourt® AMPure Xp® (Beckman Coulter Inc., Brea, USA) following the instructions from the manufacturer. PCR amplicons are bound to magnetic beads and can thereby be separated from contaminants, e.g. excess primers, nucleotides, enzymes and salts. 80 µl of AMPure magnetic bead solution was mixed with the remaining PCR products (40-45 µl) in a 96-well plate and incubated at room temperature for 5 minutes. The samples were then placed on a magnetic plate and incubated for 10 min before the liquid was discarded. The bounded DNA was washed twice by adding 200 µl ethanol (70%) to each well and discarded after 30 sec of incubation. The samples were removed from the magnetic plate and left to dry for 30-60 min at 37˚C. Thereafter, the purified DNA was mixed with 60 µl elution buffer of which 50 µl was transferred to a new 96-well plate and left over night at 37˚C to allow the fluid to evaporate. All dried DNA samples were sent to Macrogen Inc., Seoul, South Korea for sequencing.

3.3.6 Data analyses

The received DNA sequences were analyzed using the SeqMan software (DNASTAR, Madison, USA). Sequences from the three different DNA amplifications (ITS, AML and trnL) were treated as separate projects. First, the sequences were assembled with minimum percentage match of 80%, thus similar sequences are grouped together. Hence, similar sequences were placed in the same group. The sequences were trimmed manually, i.e. vector DNA and bad sequence ends were removed, and poor quality sequences deleted. After re-assembling all remaining sequence groups with minimum percentage match at 98%, they were blasted against the NCBI GenBank. Regarding to the AM project, a large group of sequences (>160) which appeared to have significant differences, where re-assembled at 99% before blasting.

3.3.7 Statistical analyses

A general linear model was used to test for differences in the number of sequence groups between samples from young and old roots and the five sites. The model included the following factors: sample type, site and the interaction term sample type x site. Data were analyzed using the MINITAB 16 software package (Minitab Inc., State College, USA).

4 Results

4.1 Microscopic examination

Microscopy examination of stained roots revealed a large number of fungal hyphae, both with and without septa. Some hyphae were found to form appressoria. Features characteristic to AM were also observed but occurred more sporadically. These included vesicles and potential arbuscules (*Figure 11*). No EM macro structures, e.g. mantles could be observed on the root surface.

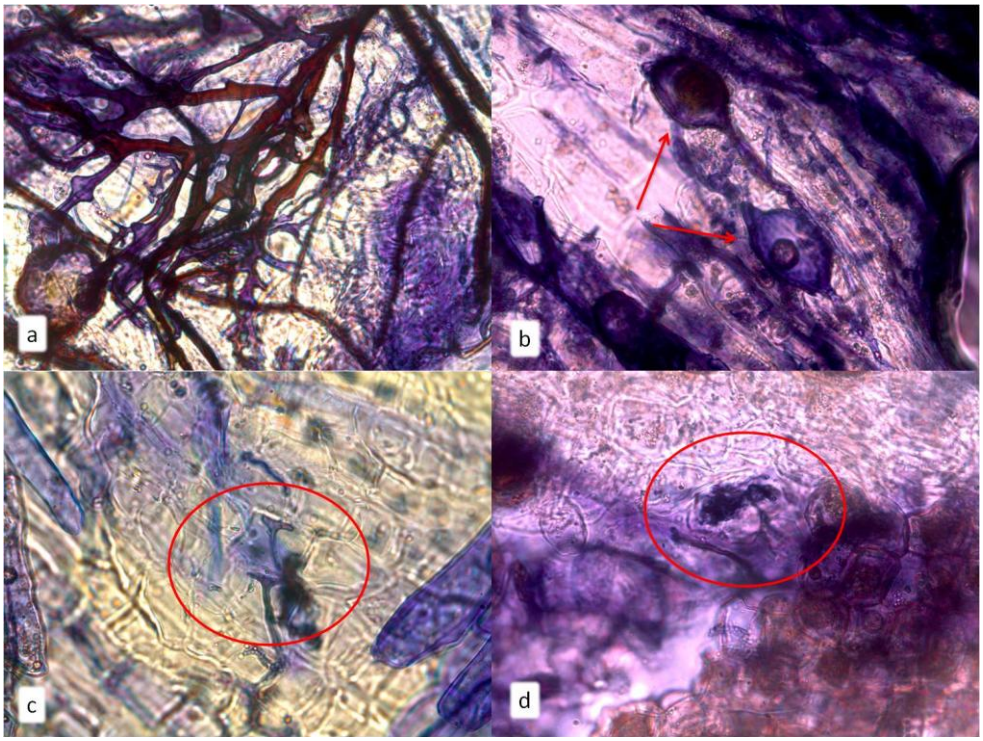

Figure 11. Microscope photographs of *A. stuhlmannii* roots showing: a) fungal hyphae; b) vesicles; c) fungal hyphae forming appressoria; and d) potential degenerated arbuscule. 400 x magnification.

4.2 Molecular analysis

4.2.1 General fungal community

Out of the original 50 DNA extractions, sequences from 42 root samples (84%) were obtained from the ITS amplification. From the total 171 sequences detected, 136 groups were formed and subsequently submitted to the BLAST query in GenBank (*Appendix 2*). Every sequence group comprises sequences with high similarity, likely to be of the same fungal species. The level of identification, i.e. the best match, varied but subphylum, and sometimes class and order, were often possible to assign. *Table 2* summarizes the results from the BLAST query.

Table 2. Results from the ITS amplification, showing the number of analyzed samples at the different sites originally from young and old trees, total number of sequence groups per site and the percentage of sequence groups also found at other sites.

	Derema I	**Derema II**	**Monga**	**Mbomole**	**Kibaoni**
No. of samples analyzed from each site					
Young	3	5	3	5	5
Old	3	3	5	5	5
Total	6	8	8	10	10
Total no. of sequence groups from each site					
Ascomycota	6	17	16	12	9
Basidiomycota	4	5	7	15	6
Glomeromycota	6	12	12	15	13
Total	16	34	25	42	38
Percentage of sequence groups found within each site, which were also detected at one or more other sites					
Ascomycota	50%	41%	38%	42%	32%
Basidiomycota	25%	20%	14%	7%	0%
Glomeromycota	17%	17%	25%	0%	8%

Fungi from the three phyla (Ascomycota, Basidiomycota and Glomeromycota) were found at all five sites, both on young and older roots. In Ascomycota, 46 groups were formed out of the 73 individual sequences. With regard to Basidiomycota and Glomeromycota, 36 of 39 and 54 of 59 sequence groups had been formed respectively. This not only indicates that species of Ascomycota present in more than one root sample occurred more frequently compared to the other phyla, but also that the relative diversity appeared to be higher in the latter two. Furthermore, ten groups of Ascomycota, two of Basidiomycota, and three of Glomeromycota were also observed at more than one site. The percentage of those is indicated in *Table 2*. Many sequence groups, up to nine different (from

Mbomole), could be found within one single sample. Several groups were also observed to be present in both young and old roots. However, the diversity of fungal sequences was significantly higher in samples of older roots. This is the only significant difference from the statistical analyses. *Figure 12* presents the number of detected fungal sequence groups from the analysis, sorted by sample type (young and old roots) and site. Additionally, all sites except Derema I had individual root samples, both young and older, which hosted fungi from all three phyla at the same time.

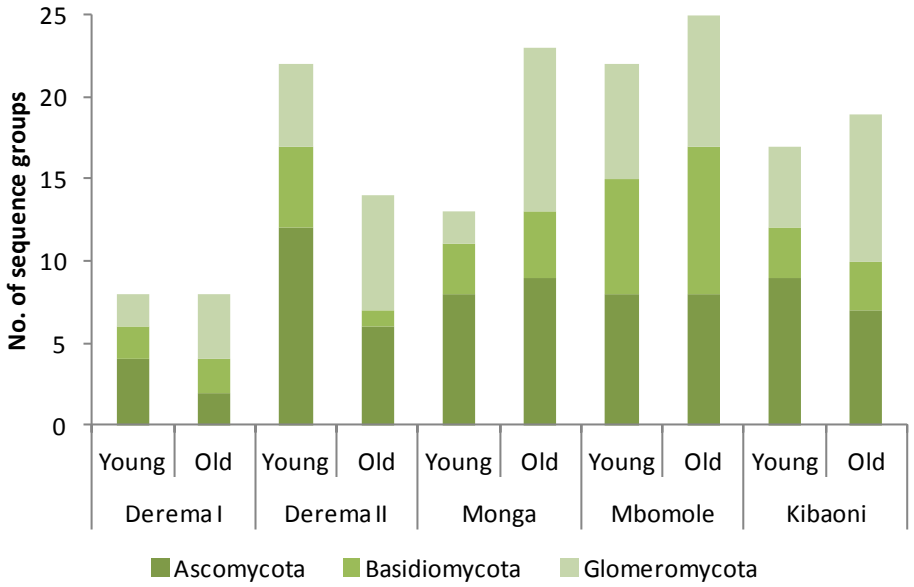

Figure 12. Number of sequence groups of fungal phyla detected in young and old roots of *A. stuhlmannii* and distribution between five sampling sites in Amani Nature Reserve.

The most common subphylum within the Ascomycota appeared to be Pezizomycotina, where the classes Dothideomycetes, Eurotiomycetes, Leotiomycetes, Orbiliomycetes and Sordariomycetes could be distinguished. Saccharomycotina and Taphrinomycotina were other detected subphyla. With exception of two samples of Pucciniomycotina, only sequences of the subphylum Agaricomycotina were obtained among the Basidiomycota, with the classes Agaricomycetes, Phallomycetidae and Tremellomycetes. All samples within the Glomeromycota belonged to the class Glomeromycetes. Except for two samples of the genus *Acoulaspora* within the order Diversisporales, the remaining sequence groups were identified to belong to the order Glomerales.

The result from the analysis of rhizomorphs and one extra root cluster is presented in *Table 3*.

Table 3. Fungi detected from rhizomorphs picked from roots of *A. stuhlmannii* seedlings (tree ID number) and from a root cluster from an unidentified plant, all samples collected from two sites

Monga	**Mbomole**	
Rhizomorph (3)	Rhizomorph (4)	Root cluster (2)
Agaricaceae	*Agaricales*	*Agaricales*
Agaricomycetes		*Chaetosphaeriaceae*
Archaeorhizomcyetes		*Eurotiomycetes*
Cryptococcus		*Glomerales**
Glomerales$_1$		
Glomerales$_2$		

* same sequence also found in old roots of tree 5, Kibaoni.

4.2.2 Arbuscular mycorrhiza

DNA amplification with the AM primers was only successful in 23 root samples. This data set was too small to conduct statistical analysis. All five sites were still represented but regarding Derema I, Monga and Mbomole, fungal sequences could only be obtained from older roots. A total of 96 sequences formed 53 sequence groups that were submitted to the GenBank. The results are presented in *Table 4* and *Appedix 3*. Two genera belonging to the Glomeromycetes were identified: *Gigaspora* (2%) and the *Glomus* groups; *Glomus A* (93%) and *Glomus B* (5%). The distribution of sequence groups between sample types (young and old roots) and the five sites is presented in *Figure 13*. Various sequences of *Glomus A*, which constitute the vast majority, were hosted by both young and older roots. In total, 50 different sequence groups of *Glomus A* could be detected. Only one sequence of *Glomus B* was detected but the species was present at three sites: Derema II, Mbomole and Kibaoni. This occurred in both young and older roots, which also hosted *Glomus A*. Two different sequences of *Gigaspora* were found in older roots sampled at separate sub-locations within the site Monga. Twenty of the sequence groups were found at more than one site, of which 19 were classified as *Glomus A*. Up to eight different sequences could be detected in one single root sample of older roots, which were from Monga. In samples of young roots, i.e. originating from one individual seedling, the maximum number of sequence group found was six.

Table 4. Results from the AM amplification, showing the number of analyzed samples, total number of sequence groups per site and the percentage of groups also found at other sites.

	Derema I	**Derema II**	**Monga**	**Mbomole**	**Kibaoni**
No. of samples analyzed from each site					
Young	-	4	-	-	3
Old	3	4	3	3	3
Total	3	8	3	3	6
Total no. of sequence groups from each site					
Glomus A	11	28	17	13	20
Glomus B	-	3	-	1	1
Gigaspora	-	-	2	-	-
Total	11	31	19	14	21
Percentage of sequence groups found within each site, which were also detected at one or more other sites					
Glomerales	73%	52%	53%	83%	53%

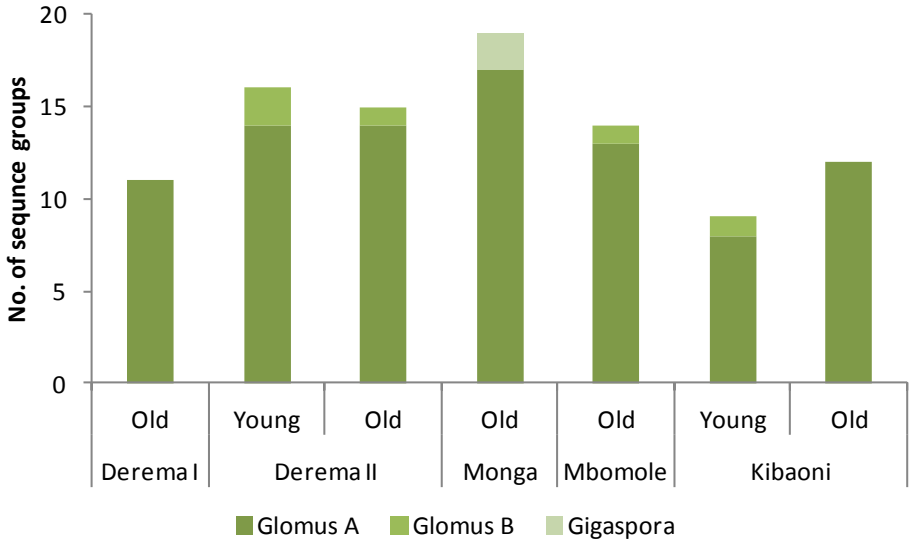

Figure 13. Number of sequence groups of Glomerales fungi, detected in young and old roots of *A. stuhlmannii*, and the distribution between the five sampling sites in Amani Nature Reserve.

4.2.3 Chloroplasts

The DNA amplification of chloroplasts from *Allanblackia* leaves and roots by using the trnL primers was not successful. Only five sequences could be obtained but none of those matched the same plant order as *Allanblackia* (Clusiaceae). This also applies for the chloroplast DNA extracted from the seedlings, i.e. known to be from *Allanblackia*. Consequently, the result cannot be considered reliable. However, in one of the old root samples, *Allanblackia* DNA was amplified by the ITS primers which gave a similar match to a sequence of another *Allanblackia* species in the GenBank.

5 Discussion

This study is the first of its kind to examine the fungal community associated to roots of *Allanblackia stuhlmannii* and the potential occurrence of symbiotic relations. Because the vast majority of tropical trees are considered to form arbuscular mycorrhiza (de Carvalho et al., 2010), the same association was expected in this case. This hypothesis was confirmed, since AM structures were observed during microscopy examination and the prevalence was later verified by molecular analysis.

5.1 Microscopic examination

Due to the large root size, preparing good samples for microscopy examination proved to be difficult. Although, a large number of different fungal hyphae could be studied, their function in the root remains unclear. For example, the observed appressoria (fungal structures to enable penetration of plant cells), could be formed by AM fungi but also be a result of infection by root pathogenic fungi. However, internal structures characteristic for AM fungi were also observed, i.e. arbuscules and vesicles. One reason why so few arbuscules were identified during microscopic examination could, apart from the limited number of samples examined, also be due to their short life span (Gadkar et al., 2001). The root samples in this study had been stored between 2-5 weeks before examination, hence most arbuscules potentially present at the time of sampling were probably already degenerated.

The initial objective was to quantify the colonization of AM fungi using the grid intersect method (Brundrett et al., 1996). This could still be a potential quantification method if using fresh root samples or trying better preparation techniques, such as other staining methods, as described by Vierheilig et al. (2005).

5.2 Molecular analysis

From the 50 root samples, DNA could only be amplified from 23 samples using the AM primers. Samples from all five sites were represented, although in some cases only from older roots. Consequently, this set of data is too limited to statistically determine the quantity of AM. However, the primary aim of the study was not to quantify the rate of colonization but to investigate whether trees of *A. stuhlmannii* are able to form associations with AM fungi. The result from the molecular analysis reveals that *A. stuhlmannii* hosts different species of the fungal phylum Glomeromycota. Since Glomeromycetes are known to be obligate symbionts (Smith and Read, 2008), this finding, in combination with the fungal structures observed during microscopy examination (arbuscules and vesicles), confirms that the tree can form AM associations.

In this study, two genera of Glomeromycota were identified: *Gigaspora* and *Glomus*, of which the latter was distinguished into group A and B. Of the 96 different sequences submitted to GenBank, 53 groups were formed, of which 50 belonged to *Glomus A*. This is consistent with the result from another study by Haug et al. (2010), indicating that *Glomus A* is the dominating type in AM fungal communities in tropical mountain rainforests, also representing the highest species diversity. The diversity of identified fungal taxa in this study may, however, be biased due to the limited range of the AM1 primer which has been reported to exclude the genera *Archaeospora* (Archaeosporaceae) and *Paraglomus* (Paraglomaceae) (Husband et al., 2002, Haug et al., 2010).

The same sequence groups were sometimes found at more than one sampling site or in samples of both young and older roots. Both diversity and distribution of AM fungi are considered to be very high (Kivlin et al., 2011) and even single plant species are able to harbor rich fungal communities (Santos-González, 2007). That was also shown in this study, as many different sequences could be identified in one single root sample. Though samples with older roots are likely to contain root fragments originating from different individual trees of the same *Allanblackia* sp., many sequences were also observed in young root samples originating from one individual seedling. However, the high number of sequence groups identified does not necessarily imply that all are originating from different fungal species. Glomerales are known to have high genetic diversity even within the same species and between individual spores (Vandenkoornhuyse et al., 2001, Ehinger et al., 2012). Species composition of the colonizing fungal community could however change during the tree's lifetime (Husband et al., 2002). The fungal diversity within an ecosystem has also been suggested to change depending on the season (Vandenkoornhuyse et al., 2002).

With the universal ITS primer set, a wide range of different fungi was identified that were associated with the roots of *A. stuhlmannii*. This method can therefore give an indication of the presence of general fungal community, which includes saprotrophs found on the root surface, pathogenic and parasitic fungi, as well as endophytic and symbiotic fungi. In this study it was not possible to determine whether all the identified fungi were able to form mycorrhiza. The finding that *A. stuhlmannii* forms AM does not exclude the potential occurrence of EM, as trees have been observed in other studies to form both associations at the same time (Onguene and Kuyper, 2001, Kottke et al., 2004). However, no EM macroscopic structures were found during examination using stereomicroscope. In several of the analyzed root samples, all three fungal phyla were represented in one single sample. As mentioned, samples of older roots could contain a mixture of root fragments of different individual trees, while young samples were all collected from the same seedling. Therefore it is possible that individual trees (at least during the early lifespan) can host all these three phyla simultaneously. Moreover, the fact that the older root samples could possibly contain several individual trees could be one explanation to why those samples were found to have significantly higher diversity among harbored fungal species than samples of young roots. On the other hand, it could also be due to succession of the fungal community or that older roots have been able to accumulate more symbiotic fungi during a longer period of time.

With regards to the species identification of the sampled roots, the sequences submitted to GenBank were not possible to analyze for leaf and root chloroplast DNA. This could be due to interference from inhibiting substances in the leaf, but DNA amplification using other primer sets, in combination with addition cleaning steps, could potentially be more successful. As a result, this analysis could not ensure that the older root samples belonged to *A. stuhlmannii*. However, due to the characteristic look of the *Allanblackia* roots, other species are unlikely to have been sampled. In addition, from one of the old root samples, *Allanblackia* DNA was amplified by the ITS primers which gave a similar match to a sequence of another *Allanblackia* species in the GenBank. Hence, this finding, in combination with the characteristic look, supports the assumption that also the older roots were of *Allanblackia* origin.

5.3 Outlook

The occurrence of AM fungi in association with *A. stuhlmannii* has been confirmed. The next step before any strategies can be adopted in the domestication program would be to gain more knowledge to understand the complexity and significance of this relationship. Field studies are required to determine whether, and to what extent, establishment and growth of *A. stuhlmannii* are enhanced by AM. Moreover, the beneficial fungal species have to be isolated and identified. If the relationship proves to be favorable for seedling growth, inoculation at the nursery may be advisable. This is also a common practice in commercial cultivation of, for example, oil-palm and black pepper (Phosri et al., 2010, Mala et al., 2009). Inoculation of AM fungi can also have the potential to stimulate plant growth in addition to the effect of the indigenous fungal community (Mohammad et al., 2004) and there is evidence that mycorrhizal fungi can spread to adjacent crops (Ingleby et al., 2007).

The tree's response to inoculation will vary between tree species but also the quality of the inocula (Onguene and Kuyper, 2005). Since AM fungi are obligate symbionts, isolation and cultivation in pure cultures is more difficult than for EM fungi. However, all AM features, e.g. spores, vesicles, mycelia and colonized roots, have the potential to initiate symbiosis (Sieverding, 1991), but in general spores have the greatest infectivity while hyphal fragments the least (Belitz et al., 2009). Ways to isolate and produce AM inocula have been described by several authors (Diop, 2004, Sharma et al., 2000, Fracchia et al., 2001), including on-farm production (Douds Jr et al., 2006, Douds Jr et al., 2010). In addition to native populations of microbial symbionts, inoculation with commercial cultures has also been shown to promote germination and plant growth (Mekonnen et al., 2010). Several inocula are today available in the global market and could be tested on *Allanblackia*.

A successful inoculum must meet several criteria. Besides being cheap and easy to handle, it should be able to adapt to new conditions, for example when the seedling is transplanted, or to changes in the environment, such as leaf litter, soil temperature and shade (Wilson and Coutts, 1985). As observed in other mycorrhizal symbioses, changes in the composition of the fungal community might occur over time and fungi dominant during the seedling phase may later be replaced by other species (Husband et al., 2002). This may be important to take into consideration, as farms in East Usambara are established on previously species-rich forested areas but the abrupt change in land-use may have had impact on the diversity of microorganism community in the soil (Cuenca et al., 1998). The fungal species forming associations with *Allanblackia* may therefore no longer be present in land that

has been converted to agriculture. Understanding the changes of the fungal community over time is important and of great significance for establishment of *Allanblackia* on farms.

This study only examines associations formed between *Allanblackia* and mycorrhizal fungi. In future research, the occurrence of other symbionts with growth enhancing potential, such as PGPB, could be studied. In order to develop strategies in the domestication program for cultivation of *Allanblackia* in nurseries and on-farm, more research within this field is needed.

6 Conclusions

This study shows that *A. stuhlmannii* is able to form arbuscular mycorrhiza. A large number of potential AM symbionts (Glomerales) were found, both on young and older roots. However, it remains to clarify how and to what extent *A. stuhlmannii* is benefiting from the AM associations, and to investigate whether certain fungal species are more important to promote tree growth than others. Therefore, further research will be needed within the domestication program of *A. stuhlmannii* with regards to symbiotic associations with soil microorganisms.

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Appendix

Appendix 1. Trees and shrubs found on the sampling sites in Amani Nature Reserve

	Derema I	Derema II	Monga	Mbomole	Kibaoni
Albizia sp.	x	x		x	
Allanblackia stuhlmannii	x	x	x	x	x
Anthocleista grandiflora		x	x	x	x
Beilschmiedia kweo					x
Cephalosphaera usambarensis	x	x	x	x	x
Clidemia hirta	x			x	x
Cyathea manniana	x			x	
Dracaena sp.		x	x	x	x
Ficus sur			x		
Harungana madagascariensis			x		
Khaya sp.			x		
Landolphia kirkii					x
Macaranga kilimandscharica				x	
Maesopsis eminii	x	x	x	x	x
Myrianthus sp.	x	x	x	x	x
Newtonia buchananii	x	x	x	x	x
Parinari excelsa		x	x	x	x
Phytelephas macrocarpa					x
Polyscias fulva				x	
Psidium sp.		x			
Rauvolfia caffra					x
Sorindeia madagascariensis		x	x	x	
Synsepalum msolo					x
Syzygium sp.					x
Tabernaemontana sp.	x	x	x	x	x

Appendix 2. Fungal sequences obtained from the ITS amplification, sorted by phyla and identified to the best taxonomic match. Subscript numbers refer to separate sequence groups of the same fungal taxa

		Derema I						**Derema II**								**Monga**										**Mbomole**										**Kibaoni**									
Fungal taxa	Tree:	1		2		3		4		5		1		2		3		4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Ascomycota	*Aureobasidium*						x																																		
	Cladosporium								x																																
	Davidiella				x				x																															
	Davidiellaceae									x						x		x														x								
	Pleosporales						x				x																													
	Sydowia									x																														
	Eurotiomycetes									x																														
	Helotiales$_1$						x	x				x																				x							x	
	Helotiales$_2$										x															x														
	Helotiales$_3$																																						x	
	Helotiales$_4$										x																													
	Helotiales$_5$																																		x					
	Helotiales$_6$																												x											
	Leotiomycete$_1$									x										x																	x			
	Leotiomycete$_2$																																						x	
	Leotiomycete$_3$																	x																						
	Orbiliomycetes										x																													
	Chaetosphaeriaceae																										x													

		Derema I						Derema II								Monga								Mbomole										Kibaoni									
Fungal taxa	Tree:	1	2	3	4	5		1	2		3		4	5		1		2	3	4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Ascomycota	*Hypocreales*																	x																									
	Pezizomycotina$_1$																					x				x																	
	Pezizomycotina$_2$																																										
	Pezizomycotina$_3$																						x				x		x						x		x	x	x	x			
	Pezizomycotina$_4$																										x																
	Pezizomycotina$_5$			x																																							
	Pezizomycotina$_6$															x																											
	Pezizomycotina$_7$											x																															
	Pezizomycotina$_8$																												x														
	Pezizomycotina$_9$																								x																	x	
	Pezizomycotina$_{10}$																			x																							
	Pezizomycotina$_{11}$																x																										
	Pezizomycotina$_{12}$															x																											
	Pezizomycotina$_{13}$													x																													
	Galactomyces						x																																				
	Archaeorhizomcyetes																																x										
	Ascomycota$_1$											x										x								x		x											
	Ascomycota$_2$							x																																			
	Ascomycota$_3$																													x	x												
	Ascomycota$_4$																													x		x											

		Derema I						Derema II								Monga								Mbomole										Kibaoni									
Fungal taxa	Tree:	1	2	3	4	5		1	2		3		4	5		1		2	3	4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Ascomycota	*Ascomycota*$_5$															x																		x							x	x	
	Ascomycota$_6$																		x																								
	Ascomycota$_7$													x																													
	Ascomycota$_8$								x																																		
	Ascomycota$_9$																x																										
	Ascomycota$_{10}$																																										
	Ascomycota$_{11}$																x																										
	Ascomycota$_{12}$																				x																						
Basidiomycota	*Agaricales*$_1$																		x																								
	Agaricales$_2$																x																										
	Marasmius																															x											
	Sebacinales																																					x					
	Thelephoraceae$_1$																																										
	Thelephoraceae$_2$																																						x				
	Agaricomycetes$_1$																											x		x													
	Agaricomycetes$_2$																																		x								
	Agaricomycetes$_3$						x																																				
	Agaricomycetes$_4$															x																											
	Agaricomycetes$_5$																			x													x										
	Agaricomycetes$_6$									x																																	

Fungal taxa		Derema I														Derema II														Monga													
	Tree:	1		2		3		4		5						1		2		3		4		5						1		2		3		4		5					
	Root type:	O		O		Y		Y		Y		O				Y		Y		O		Y		O		Y		Y		O		O		O		Y		O		Y		O	
Basidiomycota	*Agaricomycetes*$_{7}$																																											
	Agaricomycetes$_{8}$																																											
	Agaricomycetes$_{9}$																																											
	Agaricomycetes$_{10}$																							x																				
	Agaricomycetes$_{11}$																																					x						
	Agaricomycetes$_{12}$																																					x						
	Agaricomycetes$_{13}$																																												
	Agaricomycetes$_{14}$																																												
	Agaricomycetes$_{15}$																																												
	Agaricomycetes$_{16}$																															x												
	Geastrum																																											
	Cryptococcus$_{1}$																	x						x																				
	Cryptococcus$_{2}$																																												
	Cryptococcus$_{3}$																	x																										
	Cystofilobasidiales$_{1}$																																												
	Cystofilobasidiales$_{2}$																																												
	Cystofilobasidium																																												
	Dioszegia																																												
	Filobasidiales																																												
	Tremellomycetes																																											

Fungal taxa		Monga														Mbomole														Kibaoni													
	Tree:	1		2		3		4		5						1		2		3		4		5						1		2		3		4		5					
	Root type:	Y		O		O		O		Y		O				Y		O		Y		O		Y		O		Y		O		Y		O		Y		O		Y		O	
Basidiomycota	*Agaricomycetes*$_{7}$																			x																								
	Agaricomycetes$_{8}$																																											
	Agaricomycetes$_{9}$																																	x										
	Agaricomycetes$_{10}$																																												
	Agaricomycetes$_{11}$																																												
	Agaricomycetes$_{12}$																																												
	Agaricomycetes$_{13}$																											x																
	Agaricomycetes$_{14}$																											x																
	Agaricomycetes$_{15}$																									x																		
	Agaricomycetes$_{16}$																																												
	Geastrum																																												
	Cryptococcus$_{1}$																																												
	Cryptococcus$_{2}$																									x																		
	Cryptococcus$_{3}$																																												
	Cystofilobasidiales$_{1}$																x																												
	Cystofilobasidiales$_{2}$																x																												
	Cystofilobasidium															x																													
	Dioszegia																			x																										
	Filobasidiales															x																													
	Tremellomycetes																		x																										

Fungal taxa		Derema I										Derema II										Monga										Mbomole										Kibaoni									
	Tree:	1		2		3		4		5		1		2		3		4		5		1		2		3		4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Basidiomycota	*Agaricomycotina*$_1$			x																																													
	Agaricomycotina$_2$	x																																															
	Agaricostilbomycetes																																	x															
	Sporobolomyces								x					x																																				
Glomeromycota	*Acaulospora*$_1$																																																
	Acaulospora$_2$																													x																			
	Glomerales$_1$														x									x																								
	Glomerales$_2$																																	x															
	Glomerales$_3$	x															x													x																		
	Glomerales$_4$																																																x
	Glomerales$_5$												x																																				
	Glomerales$_6$																							x																								
	Glomerales$_7$																																															x	
	Glomerales$_8$															x																																
	Glomerales$_9$																																									x							
	Glomerales$_{10}$																																									x						
	Glomerales$_{11}$																																															x	
	Glomerales$_{12}$																															x																	
	Glomerales$_{13}$																																	x															
	Glomerales$_{14}$												x																																				

		Derema I						Derema II								Monga								Mbomole										Kibaoni									
Fungal taxa	Tree:	1	2	3	4	5		1	2		3		4	5		1		2	3	4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Glomeromycota	*Glomerales*$_{15}$																																								x		
	Glomerales$_{16}$																																							x			
	Glomerales$_{17}$																														x												
	Glomerales$_{18}$											x																															
	Glomerales$_{19}$																															x											
	Glomerales$_{20}$											x																															
	Glomerales$_{21}$															x																							x		x		
	Glomerales$_{22}$																x																										
	Glomerales$_{23}$											x																															
	Glomerales$_{24}$							x																																			
	Glomerales$_{25}$	x																																									
	Glomerales$_{26}$							x																																			
	Glomerales$_{27}$							x																																			
	Glomerales$_{28}$																								x																		
	Glomerales$_{29}$																											x															
	Glomerales$_{30}$																																										x
	Glomerales$_{31}$																																			x							
	Glomerales$_{32}$																																								x		
	Glomerales$_{33}$																															x											
	Glomerales$_{34}$																				x																						

Fungal taxa		Derema I										Derema II										Monga										Mbomole										Kibaoni									
	Tree:	1		2		3		4		5		1		2		3		4		5		1		2		3		4		5		1		2		3		4		5		1		2		3		4		5	
	Root type:	O	O	Y	Y	Y	O	Y	Y	O	Y	O	Y	Y	O	Y	O	O	O	Y	O	Y	O	O	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Glomeromycota	*Glomerales*$_{35}$																																																	
	Glomerales$_{36}$																																																	
	Glomerales$_{37}$		x																																															
	Glomerales$_{38}$	x																																																
	Glomerales$_{39}$																																																	
	Glomerales$_{40}$																																																	
	Glomerales$_{41}$																	x																																
	Glomerales$_{42}$																																																	
	Glomerales$_{43}$																																																	
	Glomerales$_{44}$																									x																								
	Glomerales$_{45}$																																																	
	Glomerales$_{46}$			x																																														
	Glomerales$_{47}$																											x																								
	Glomerales$_{48}$																									x																								
	Glomerales$_{49}$																									x																								
	Glomerales$_{50}$																					x																												
	Glomerales$_{51}$							x																																										
	Glomerales$_{52}$																																			x															

Appendix 3. Sequences of AM fungi, sorted by genus and group. Subscript numbers refer to separate sequence groups of the same fungal taxa

	Derema I			**Derema II**								**Monga**			**Mbomole**			**Kibaoni**					
Tree:	1	3	4	1		2		3		4		1	4	5	2	4	5	1	2	3	4	5	
Root type:	O	O	O	Y	O	Y	O	Y	O	Y	O	O	O	O	O	O	O	O	Y	Y	O	Y	O
Gigaspora$_1$												x											
Gigaspora$_2$													x										
Glomus A$_1$	x			x										x									
Glomus A$_2$										x													
Glomus A$_3$													x										
Glomus A$_4$																			x				
Glomus A$_5$	x																						
Glomus A$_6$				x				x							x		x						
Glomus A$_7$						x								x	x				x				
Glomus A$_8$																						x	
Glomus A$_9$				x																			
Glomus A$_{10}$		x		x				x						x									
Glomus A$_{11}$													x										
Glomus A$_{12}$																					x		
Glomus A$_{13}$															x								
Glomus A$_{14}$							x			x													
Glomus A$_{15}$	x														x		x	x	x		x		
Glomus A$_{16}$												x											
Glomus A$_{17}$				x												x							
Glomus A$_{18}$				x																			
Glomus A$_{19}$								x		x		x		x									
Glomus A$_{20}$										x							x						
Glomus A$_{21}$		x										x	x							x	x		
Glomus A$_{22}$										x													
Glomus A$_{23}$														x	x				x				x
Glomus A$_{24}$	x																						
Glomus A$_{25}$											x												
Glomus A$_{26}$	x						x									x							
Glomus A$_{27}$							x						x										
Glomus A$_{28}$		x											x										
Glomus A$_{29}$	x																						
Glomus A$_{30}$					x																		
Glomus A$_{31}$														x									
Glomus A$_{32}$							x							x				x					

	Derema I			**Derema II**								**Monga**			**Mbomole**			**Kibaoni**					
Tree:	1	3	4	1		2		3		4		1	4	5	2	4	5	1	2	3	4	5	
Root type:	O	O	O	Y	O	Y	O	Y	O	Y	O	O	O	O	O	O	O	O	Y	Y	O	Y	O
Glomus A_{33}																x					x		
Glomus A_{34}													x										
Glomus A_{35}														x									
Glomus A_{36}				x																			
Glomus A_{37}		x																x					
Glomus A_{38}						x										x							
Glomus A_{39}																			x				
Glomus A_{40}																						x	
Glomus A_{41}						x																	
Glomus A_{42}			x															x					
Glomus A_{43}								x															
Glomus A_{44}																		x					
Glomus A_{45}																x							
Glomus A_{46}								x															
Glomus A_{47}				x																			
Glomus A_{48}																					x		
Glomus A_{49}																					x		
Glomus A_{50}						x																	
Glomus B					x		x				x					x				x			