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Full Length Research Paper

Activation of TLRs/NF-κB signaling pathway and production of diverse cytokines in the incidence and development of invasive pulmonary aspergillosis

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Aspergillus fumigatus **is a common saprophytic fungus in the air. It can cause a severe infectious disease called invasive pulmonary aspergillosis (IPA). To establish the activation of TLRs/NF-κB signaling pathway in invasive pulmonary aspergillosis (IPA) model of wild type mouse, three groups of mouse were used in the study; the normal mice; mice infected with *A. fumigatus* conidia; IPA mouse infected with *A. fumigatus* conidia. *A. fumigatus* burden in lung tissue and lung pathology were detected and evaluated in IPA mouse. The levels of TLR2, TLR4 mRNA, and some inflammatory cytokines were investigated. (1) Severe inflammatory responses were found in IPA mice after nose inhalation of *A. fumigatus* conidia 72 h; In addition, *A. fumigatus* burden in IPA group was higher than that of normal mice with infection in all time. (2) Compared with normal mice with infection, IPA group displayed a low level of TLR2 mRNA in the early stage of infection, whereas it was strongly expressed in the late stage (120 h and 144 h), the expression level of TLR4 mRNA was always low during *A. fumigatus* infection; interestingly, the expression level of NF-κB p65 protein was quickly increased in the early stage (24 h) of infection, and then continuously declined. (3) Normal mice with *A. fumigatus* inoculation expressed high levels of pro-inflammatory cytokines (TNF-α, IL-1β) in the early stage of infection. The highest expression levels appeared at 48 or 72 h, and then declined to normal level. Simultaneously, anti-inflammatory cytokine IL-10 protein was elevated in the late stage of infection. However, IPA mice had a lot secretion of anti-inflammatory cytokine IL-10 protein the early stage of infection, and then displayed a significant decrease in the late stage, whereas the pro-inflammatory cytokines (TNF-α, IL-1β) was slowly secreted in low levels. The abnormal activation of TLRs/NF-κB signaling pathway induced the loss of balance between pro-inflammatory and anti-inflammatory cytokines, and eventually leads to the incidence and development of IPA.**

Key words: *Aspergillus fumigatus,* IPA, TLRs/NF-κB, cytokine, pathogenesis.

INTRODUCTION

Aspergillus fumigatus is a common saprophytic fungus in the air. It has a small diameter and can be passively inhaled into the respiratory tract. *A. fumigatus* conidia will not result in disease inhaled by healthy people, for it can be eliminated by innate immune system in the lungs. However, in hosts with impaired immunity, it can cause a

severe infectious disease called invasive pulmonary aspergillosis (IPA). The mortality rate of IPA ranged from 60 to 94% (Jeurissen et al., 2012; Slavin et al., 2015; Bassetti et al., 2018; Botero et al., 2018). Hitherto, the pathogenesis of IPA is not well illuminated. Toll-like receptors (TLRs) is one major pattern-recognition receptors (PRRs) involved in the early host defence against pathogen invasions (Slavin et al., 2015). By recognizing pathogen-associated molecules, TLRs can trigger corresponding signal transduction, activate host immune system, and remove invaded pathogens.

Wang et al. (2001) firstly reported the function of TLRs in response to *A. fumigatus* (Wang et al., 2001). After that, a lot of *in vitro* studies showed TLRs, mainly TLR2 and TLR4, played roles in immune responses in *A. fumigatus* infection. However, contradicted results still existed in current studies. Meier et al. (2003) indicated both TLR2 and TLR4 involved in response to *A. fumigatus* stimulation. Similarly, *in vivo* studies also showed inconsistent even opposite results. By using $TLR2^{-/-}$ and $TLR4^{-/-}$ mice immunosuppressed with cyclophosphamide, Bellocchio et al. (2004) elucidated that it was TLR4 rather than TLR2 involved in host immune response to *A. fumigatus.* On the contrary, Balloy et al. (2005) showed the key role of TLR2 in immune responses stimulated by *A. fumigatus* using $TLR2^{-/-}$ mice with reduced neutrophils caused by vincristine treatment. Although $MyD88^{-/-}$ mice were more sensitive to *A. fumigatus* infection compared with control or single TLR knockout mice, Dubourdeau et al. (2006) argued that TLR2, TLR4 and MyD88 were not necessary for the elimination of *A. fumigatus*. By use of gene knockout mice and *in vitro* transfection, current studies usually focused on the investigation of TLR functions in a single time point during *A. fumigatus* infection (Wu et al., 2015; Rizzetto et al., 2013; Carvalho et al., 2012).

We established IPA model of wild type mouse. With dynamic investigation of the expression levels of TLR2 and TLR4 mRNA, the protein levels of NF-κB p65, and the levels of inflammatory cytokines including TNF-α, IL–1β and IL-10 protein in pulmonary tissues, together with evaluation of the *A. fumigatus* dosage, and the lung pathology, we elucidated the functions of both TLRs/NF-κB signaling and its multiple downstream cytokines in the development of IPA. This study will provide an insight into the pathogenesis of IPA.

MATERIALS AND METHODS

Experimental animals and grouping

BALB/c SPF mice (Certificate of Conformity: SCXK 2003-0002, male, 6 to 8 weeks old, 20~25 g) were provided by Shanghai SINO-BRITISH SIPPR / BKLAB animal center. Mice were divided into three groups randomly, 25 rats in each group: (1) Normal Group (normal mice); (2) Normal mice with infection (N + *A. fumigatus*); (3) IPA Model Group (normal mice were given with Immunosuppressant and inoculated with *A. fumigatus*).

Strain and culture medium

A. fumigatus (clinical isolates, Separate No. 3910): was purchased from the Fungal Culture Collection of Chinese Medicine Centre (Nanjing). Cells were cultured in Czapek's medium at 26°C.

Main reagents

Cyclophosphamide (CY, NO.: 06060521) was purchased from Jiangsu Hengrui Medicine Co., Ltd.; Trizol reagent was from invitrogen company; TaKaRa RNA PCR Kit 3.0 (AMV) Kit was purchased from Dalian TaKaRa Biotechnology Co., Ltd.; antibodies (Rabbit anti-NF-κB p65, Rabbit anti-IL-1β, Goat anti-rabbit HRP secondary antibodies) were purchased from Santa Cruz Biotechnology (Beijing, China). Polymerase chain reaction (PCR) primers were from Shanghai Biological Engineering company; ultrapure water (UPW, NO.: 07020201) was from U.S. MIUIOORE Inc..

IPA model of mice

According to the literature, the method was given as follows: BALB/c mice were injected intraperitoneally with 100 $mg \cdot kg^{-1} \cdot d^{-1}$ of CY within 2 days. Whereafter, mice were administered intranasally with 50 μL (Concentration: 10^7/mL) spore suspension of *A. fumigatus*. In order to maintain the effect of immunosuppression, mice were given additional CY (100 $mg \cdot kg^{-1} \cdot d^{-1}$) when inoculated with *A. fumigatus* at 96 h (Tang et al., 1993).

Collection and processing of specimen

Mice with nose inhalation of *A. fumigatus* conidia were sacrificed at different time points of 24, 48, 72, 120 and 144 h (5 mice at each time point), then lung tissue were isolated in sterile manner, and conserved in -80°C refrigerator.

A. fumigatus colony counting of lung tissue

100 mg of lung tissue were taken and made into 10% homogenate, then 0.1 ml of it was inoculated on Czapek's medium after diluting 100 times, counting colony after 5 days.

Lung tissue pathology

Observing histological injury and spore germination after all the mice produced paraffin sections of lung tissue and conventional HE staining.

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Table 1. Primer sequences and polymerase chain reaction (PCR) reactive conditions.

Gene	Name	Sequence	temperature (°C)	product (bp)
TLR2	Upstream: downstream:	5'-ACCTCCCTTGACATCAGC-3' 5'-TCGTACTTGCACCACTCG-3'	59	902
TLR4	Upstream: downstream:	5'-ATCTGGTGGCTGTGGAGAC-3' 5'-TTCCCTGAAAGGCTTGGTC-3'	59	288
β-tublin	Upstream: downstream:	5'-AAGGGTCACTACACCGAG-3' 5'-GCGAATCCTGGCATGAAGAAGT-3	59	506

Table 2. Mice lungs burdern of *A. fumigatus* ($\bar{x} \pm s$, n = 5, Unit: CFU/mL).

	Time (h)				
Group	**24**	**48**	**72**	**120**	**144**
Normal mice with infection	1605 ± 298.8	1738 ± 254.0	210 ± 43.2	78 ± 11.3	36 ± 5.2
IPA Group	25800 ± 5533*	28100 ± 6129*	15750 ± 3096*	17500 ± 2543*	9500 ± 1011*

*p<0.05, vs normal mice with infection.

Detection of the target genes expression of lung tissue by RT-PCR

The expression of mRNA was determined using reverse transcription polymerase chain reaction (RT-PCR) analysis. After conidia stimulation, cells were homogenized with 1 ml Trizol reagents, and 1 µg of total RNA was reverse transcribed with a RT-PCR kit (TaKaRa Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions. PCR was performed in tubes using PTC-200 DNA Engine Cycler. After extraction of total RNA and construction of cDNA, PCR amplification using EC3 Chemi HR410 Imaging System, and PCR products were determined using Bandscan image analysis software. The expression of the corresponding target gene was standardized against β-actin. Specific primers for PCR analysis were synthesized using ProTaq DNA polymerase (TaKaRa Biotechnology Co., Ltd.) (Table 1). Values were normalized to β-actin gene expression and are expressed relative to the control group. Primers and PCR reaction conditions are listed in Table 1.

Detection of the target proteins expression of lung tissue by Western blot

Firstly, nuclear protein and total protein from 100 mg lung tissue was extracted; secondly, SDS-PAGE electrophoresis was carried out and transferred to semi-dry membrane; and again, incubated with the corresponding primary antibody (1:250) and secondary antibodies (1:8000) at 37°C for 1 h and finally, the film were exposed to X-ray after colouring with ECL.

Statistical methods

The values of optical density scanning of target band which was measured in the agarose gel and X-ray film by image analysis software Bandscan were read. Afterwards, the expression of its corresponding target gene and protein were respectively standardized by scanning values in each group of β-tublin and β-actin bands. Here, each experiment was repeated three times, and results were indicated as $\bar{x} \pm s$. Statistical software SPSS 10.0 was used to conduct t-test analysis and significant difference was determined at P<0.05.

RESULTS

Morphological analysis of lung pathology

Seventy-two hours after inoculation with *A. fumigatus,* compared with normal mice, the alveolus space in normal mice with infection enlarged, accompanied with inflammatory responses including inflammatory cell infiltration and hemorrhage injury (Figure 1A and B); as a comparison, IPA group had a lung abscess and severe hemorrhage (Figure 1C); in addition, airway epithelial desquamation and mycelium formation were also observed in the IPA group (Figure 1C).

Assessment of *A. fumigatus* burden in pulmonary tissue

The CFU assay indicated that pulmonary tissue from the IPA Group had heavy *A. fumigatus* load (p<0.05) (Table 2) compared with normal mice with infection. As a contrast, normal mice without *A. fumigatus* inoculation showed negative signal in this assay.

Investigating the expression levels of TLR2 and TLR4 mRNA in mice pulmonary tissue

The mRNA levels of TLR2 and TLR4 with RT-PCR were

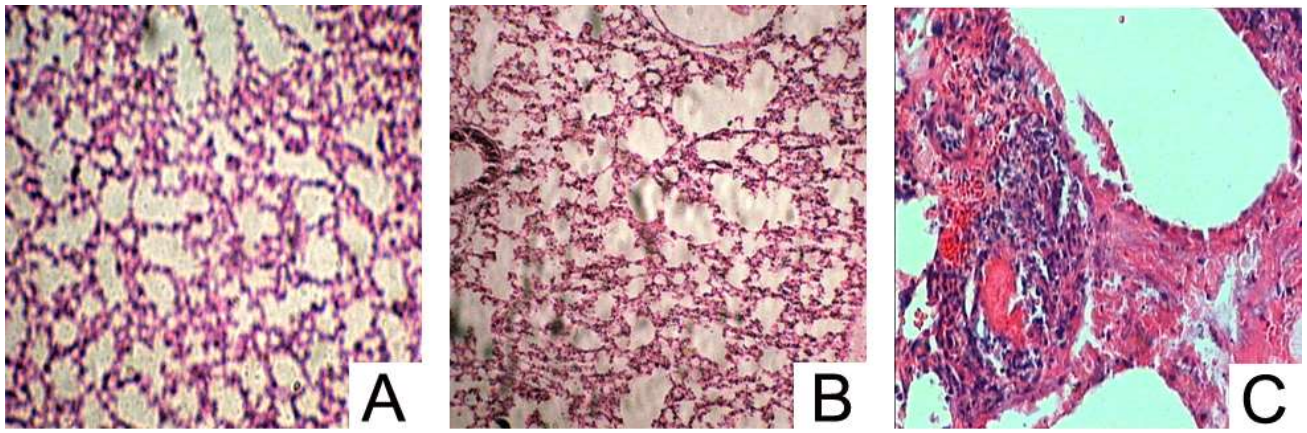

Figure 1. HE staining of the lungs at 72 h after inoculation with *A. fumigatus* (× 200). A, Normal group; B, normal mice with infection; C, IPA Group.

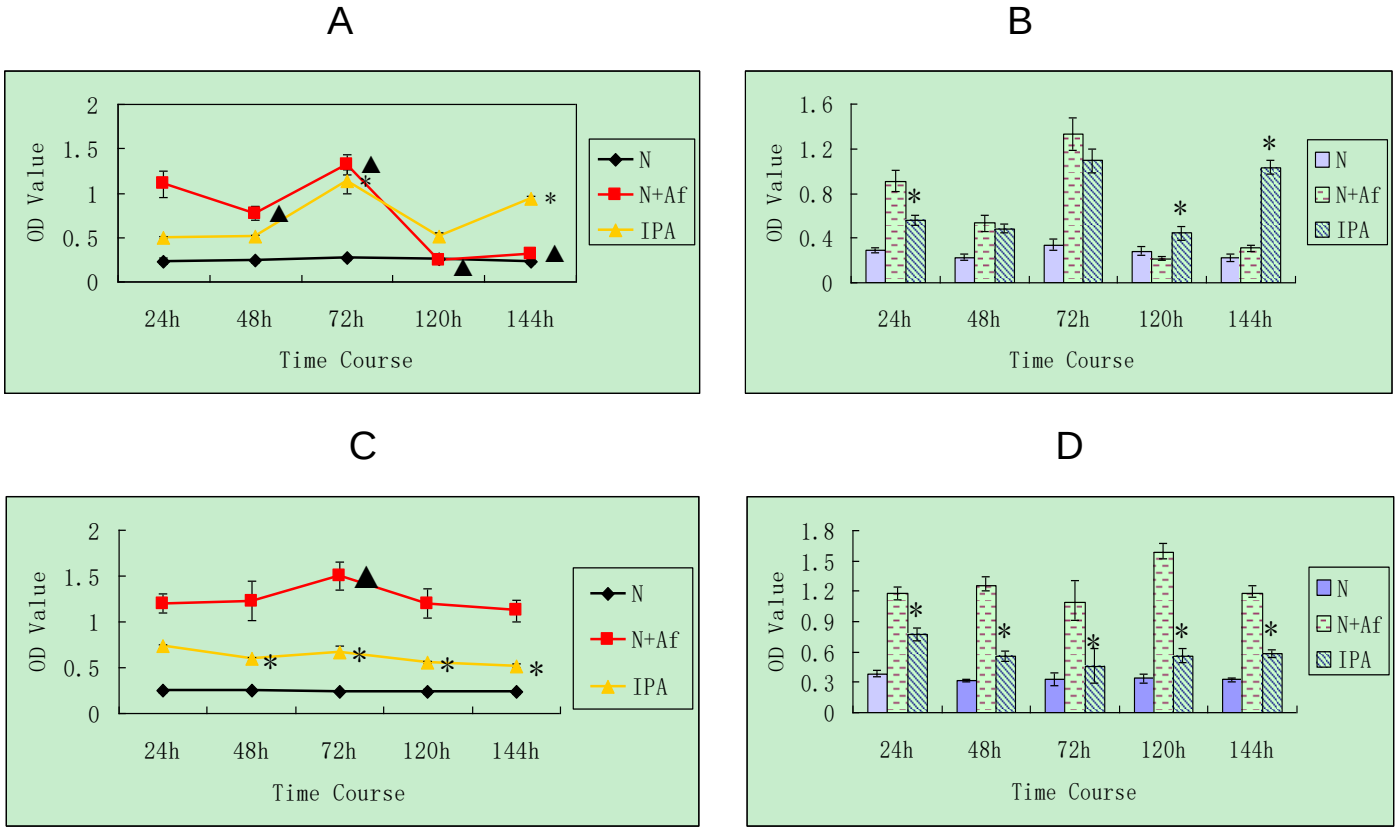

Figure 2. The expression levels of TLR2 and TLR4 mRNA in mouse pulmonary tissue. N, Normal Group; N+ *A. fumigatus;* normal mice with infection; IPA, IPA Group. **A.** The expression of TLR2 mRNA at different time. ▲ $p<0.05$, vs at 24 h in N+ *A. fumigatus* Group; *$p<0.05$, vs at 24 h in IPA Group. **B.** The expression of TLR2 mRNA at the same time point of each group. *$p<0.05$, vs N+ *A. fumigatus*. **C.** The expression of TLR4 mRNA at different time. ▲$p<0.05$, vs at 24 h in N+ *A. fumigatus* Group; * $p<0.05$, vs at 24 h in IPA Group. **D.** The expression of TLR4 mRNA at the same time point of each group. Note:* $p<0.05$, vs N+ *A. fumigatus*.

tested by time course (Figure 2). Compared with normal group, TLR2 mRNA in normal mice with infection was highly expressed before 72 h and then decreased. Until 120 h later, the expression of TLR2 mRNA recovered to a normal level. However, IPA group constantly presented high expression ($p<0.05$); in contrast with normal mice

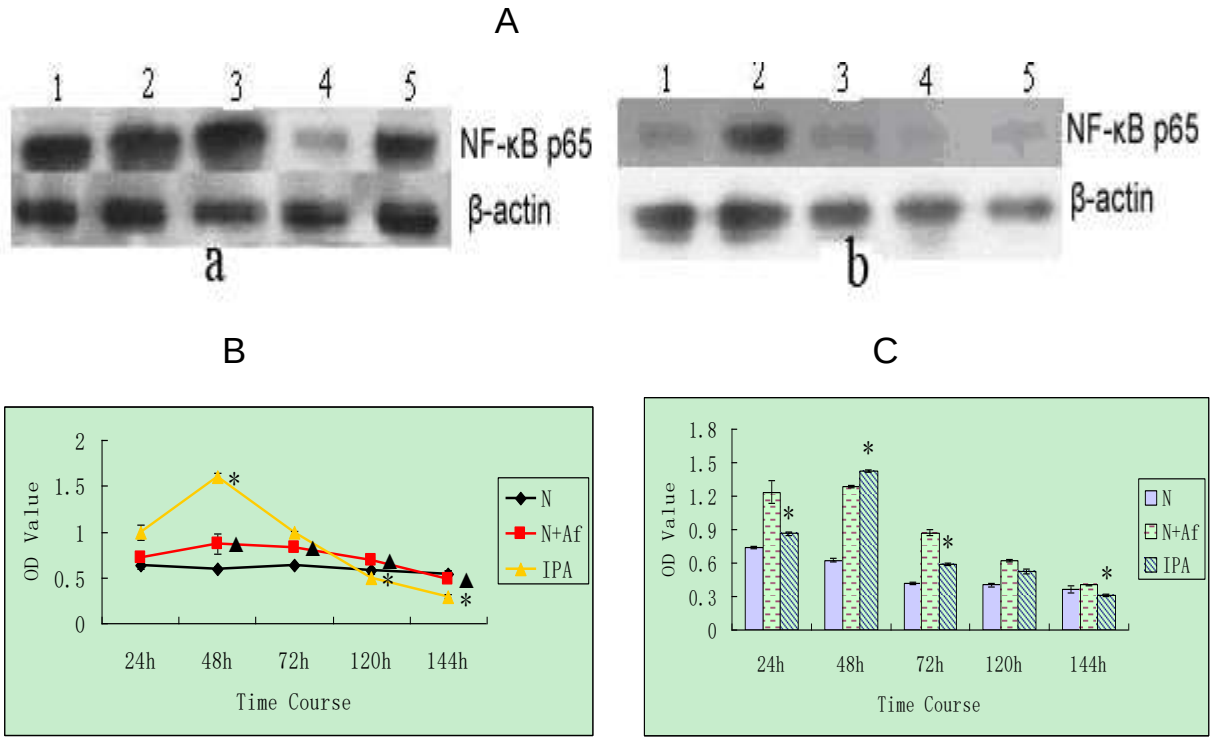

Figure 3. The expression of NF-κB p65 protein. **A.** Western--Blot bands of NF-κB p65 at different time. a. N+ *A. fumigatus*; b. IPA Group. 1: 24 h; 2: 48 h; 3: 72 h; 4: 120 h; 5: 144 h. **B.** The expression of NF-κB p65 at different time. ▲$p<0.05$ vs at 24 h in N+ *A. fumigatus* Group; * $p<0.05$ vs at 24 h in IPA Group. **C.** The expression of NF-κB p65 at the same time point of each group .Note： * $p<0.05$, vs N+ *A. fumigatus*.

with infection, IPA group had a low expression level of TLR2 mRNA in the early stage of infection (24 h), whereas its expression reached a high level in the later stage (120 and 144 h) (Figure 2).

As shown in Figure 2C and D, during the infection of *A. fumigatus,* the expression of TLR4 mRNA in normal group and IPA group showed an increasing status; in comparison with normal mice with infection, the expression of IPA group displayed a continual lower level. By analysis of the altered expression levels of TLR2 and TLR4 mRNA in different mouse group, the results showed that TLR2 mRNA in IPA group was lowly expressed in the early stage of *A. fumigatus* infection (24 h) but strongly expressed in the late stage (120 and 144 h), while the expression levels of TLR4 mRNA remained in a low level during infection.

Analysis of the expression of NF-κB p65 protein

Time course experiments were conducted to evaluate the expression of NF-κB p65 protein in mouse pulmonary tissues from different treatment groups. As shown in Figure 3, NF-κB p65 protein levels were measured by western blot. Twenty-four hours after inoculation with *A. fumigatus,* the expression of NF-κB p65 protein of normal group gradually increased, and then decreased to normal levels after 72 h. On the other hand, IPA mice had a sharp elevation of NF-κB p65 protein after 24 h. It peaked at 48 h, followed by a decline in a level lower than normal mice with infection ($p<0.05$) (Figure 3B and C).

Measurement of TNF-α, IL-1β and IL-10

The expression of inflammatory cytokines was also measured by Western--Blot.

Analysis of the expression of TNF-α protein

TNF-α in infected normal mice displayed a strong expression, which reached the highest level at the time point of 72 h and restored to normal range. As a comparison, IPA mice had a lower TNF-α expression with mild alternation (Figure 4A and B).

Analysis of the expression of IL-1β protein

IL-1β in IPA mice was lowly expressed and began to gradually increase until 48 h later, which displayed a

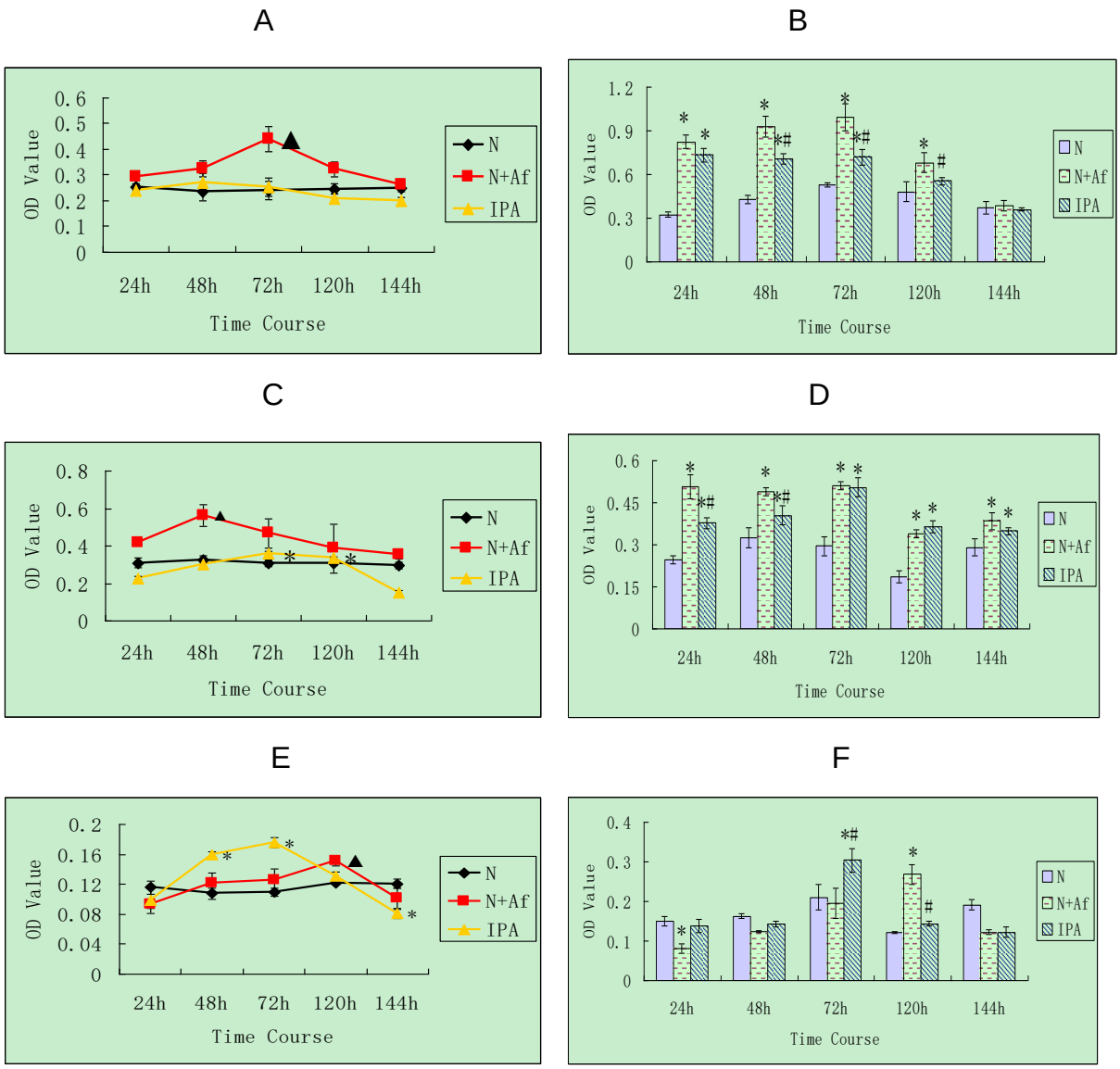

Figure 4. The expression of TNF-α,IL-1β and IL-10. **A.** The expression of TNF-α at different time.▲p<0.05, vs at 24 h in N+ *A. fumigatus* Group; * p<0.05, vs at 24 h in IPA Group. **B.** The expression of TNF-α at the same time point of each group. * p<0.05,vs N; # p<0.05, vs N+ *A. fumigatus*. **C.** The expression of IL-1β at different time. Note:▲p<0.05, vs at 24 h in N+ *A. fumigatus* Group; * p<0.05,vs at 24 h in IPA Group. **D.** The expression of IL-1β at the same time point of each group. Note：* p<0.05, vs N; # p<0.05, vs N+ *A. fumigatus*. **E.** The expression of IL-10 at different time. ▲p<0.05,vs at 24 h in N+ *A. fumigatus* Group; * p<0.05, vs at 24 h in IPA Group. **F.** The expression of IL-10 at the same time point of each group. Note：* p<0.05, vs N; # p<0.05, vs N+ *A. fumigatus*.

decrease expression after 120 h (p< 0.05). On the contrary, the normal mice with *A. fumigatus* infection had a higher expression of IL-1β, which fleetly was elevated and peaked at 48 h (p < 0.05) and then gradually declined (Figure 4C and D).

Analysis of the expression of IL-10 protein

IL-10 in infected normal mice displayed a low expression level and gradually increased and then peaked at the time point of 120 h (p < 0.05) whereas IPA had a strong IL-10 expression in the early stage of infection (before the time point of 72 h) and then reduced to a normal level (Figure 4E and F).

DISCUSSION

Effective innate immunity is the first line of defense to *A.*

fumigatus infection. Phagocytosis of the alveolus macrophages kills inhalational conidia and prevents the formation of hyphae, which can colonize in the host and are associated with lethal infection. Once the conidia escape from phagocytosis and develop into hyphae, neutrophils will take over the defense line. At the same time, macrophages and lung dendritic cells phagocytize conidia and hyphae, present antigens and initiate T cell immune response. Innate immunity not only confers the first line of defense in resistance to *A. fumigatus* infection, but also provides specific signals for initiation of adaptive immunity (Bassetti et al., 2018; Wu et al., 2015). However, the mechanism of innate immunity against the infection of *A. fumigatus* is still largely unknown.

To activate host defense and eliminate invasive pathogens, innate immune response is initiated by pattern recognition, a conserved and pathogen-specific molecular recognition pattern mediated by a series of PRRs that is widely express in macrophages and various cell types. TLRs, the most studied PRRs, belong to type I transmembrane receptors, which broadly is distributed in monocyte-macrophage system, endothelial cell and dendritic cell, etc. By recognizing the associated molecular pattern of invasive pathogens, TLRs activate innate immune response and induce adaptive immune response. TLRs trigger the activation of NF-κB 65 signaling and a series of other downstream networks, leading to the secretion of multiple cytokines and the induction of related biological effects (Portou et al., 2015; De Nardo, 2015; Kang et al., 2018).

Here, in order to systematically mimic patient IPA, the dynamic alternations of TLRs, NF-κB and its downstream cytokines were evaluated in both normal and immunodeficient mice during infection of *A. fumigatus*. Also, cyclophosphamide was used to induce immunosuppression of mice. And, the pathological alternation of pulmonary tissues and culture of *A. fumigatus* was compared in normal mice with those in immunosuppressive mice after nose inhalation of *A. fumigatus* conidia.

Results showed that immunosuppressed mice with nose inhalation of *A. fumigatus* presented pathological alternations similar to clinical IPA cases, indicating the successful establishment of mouse IPA model. Analysis from pulmonary histology combined with CFU assay reminded us that immunosuppressive mice were not able to effectively initiate immune responses, which caused late inflammatory reactions in the early stage for elimination of conidia and suppression of hyphae growth. On the contrary, overreacted inflammatory responses in the late stage of infection led to severe damage of lung tissues.

TLRs/NF-κB signaling is the important network for the regulation of inflammatory and immune response, and also the major pathway for resistance to infection. In this study, we discovered the different dynamic expression pattern of TLR2 and TLR4 mRNA between IPA Group and normal mice with infection. TLR2 mRNA in IPA Group was lowly expressed in the early stage of *A. fumigatus* infection (24 h) but strongly expressed in the late stage (120 and 144 h), while the expression levels of TLR4 mRNA stayed in a low level during infection. Normal mice with infection had a high expression of TLR2 mRNA before 72 h and decreased after that. Until 120 h later, the expression of TLR2 mRNA recovered to a normal level while TLR4 mRNA had a constitutive strong expression during the infection. These results indicate that TLR2 mRNA was abnormally activated in the late stage of IPA, while TLR4 mRNA was always suppressed. We also found that the expression of NF-κB p65 proteins were opposite in these two groups. Twenty-four hours after *A. fumigatus* inoculation, normal mice displayed a gradually increased NF-κB p65 protein, and it declined to a normal level after 72 h; whereas IPA mice had a sharpened increase of NF-κB p65 protein in 24 h, which peaked at 48 h and quickly decreased to a level lower than the average. Previous study showed that the transduction of TLR signaling was under fine regulation of some factors in the normal host, and the produce of NF-κB negatively regulated the activation of TLRs and ensured appropriate intensity of TLR signaling by this complicate feedback mechanism (Carmody et al., 2007). Our results imply a disorder of the regulatory feedback network of TLRs/NF-κB in the IPA host.

Cytokines, an important kind of secretive immune molecules, play roles in diverse biological functions including regulation of cell physiology, mediation of inflammatory responses, involvement of immune reactions, and repair of tissues, etc. The different functions of various cytokines are closely related with the situation and progression of infectious diseases (Peck and Mellins, 2010). During the infection of *A. fumigatus*, there exists a subtle balance between different cytokine types. For an instance, TNF-α, IL-12 and IFN-γ confer resistance to *A. fumigatus* infection in mouse experiment, whereas IL-4 and IL-10 function in opposite direction. In addition, IPA patients went worse when using antibodies of TNF-α and IFN-γ (Rivera et al., 2006). Serum with a high ratio of IFN-γ/IL-10 from clinical IPA patient showed better anti-fungal effects (Hebart et al., 2002). Serum ELIPOT analysis showed *A. fumigatus* -specific T cell in the IPA patient with leukemia and neutropenia produced high levels of IL-10 and low levels of IFN-γ (Potenza et al., 2008). Chronic granulomatous disease (CGD) patient had high levels of pro-inflammatory cytokines after *A. fumigatus* infection compared with normal host, indicating that the disorders of inflammatory and anti-inflammatory responses resulted in increased IPA susceptibility (Shalit et al., 2006).

The disordered activation of TLRs/NF-κB will inevitably lead to abnormal secretion of its downstream cytokines. In this study, we investigated the dynamic alternations of pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokine (IL-10).

Results show that the expression levels of cytokines (TNF-α, IL-1β and IL-10) in mouse lung were closely correlated with pulmonary pathological impairment. Normal mice with *A. fumigatus* inoculation displayed high levels of pro-inflammatory cytokines (TNF-α and IL-1β) in the early stage of infection. Their expression levels all peaked at 48 or 72 h, and thereafter declined to normal level. At the same time, anti-inflammatory cytokine IL-10 elevated in the late stage of infection. Moreover, lung pathology results showed obvious hyperemia and hemorrhage appeared before 72 h, and thereafter inflammatory responses were gradually alleviated, which indicated that secretion of pro-inflammatory cytokines (killing inhalational conidia and preventing the formation of hyphae) was the major inflammatory responses in the early stage of *A. fumigatus* infection, whereas secretion of anti-inflammatory cytokine in the late stage protected tissues from severe impairment caused by overreacted inflammatory responses. In contrast, a lot of secretion of anti-inflammatory cytokine IL-10 in the early infectious stage of IPA mice and a significant decrease of it in the late stage, combined with slow and low secretion of pro-inflammatory cytokines (TNF-α and IL-1β), caused obstacle in timely elimination of conidia and prevention of hyphae formation. Furthermore, excessive secretion of pro-inflammatory cytokines induced overreaction of inflammatory responses and consequent severe lung injury.

Inflammation is one of the necessary parts of effective immune responses in resistance to IPA. Appropriate inflammatory responses can availably eliminate local *A. fumigatus*, whereas improper or overreacted inflammatory responses will cause IPA and associated lung injury (Romani and Puccetti, 2007). Effective inflammatory responses depend on the mutual cooperation or restriction between diverse immunocytes, which ultimately help the host eliminate exotic antigens as well as protect its own tissues by the regulation of the secretions and functions of diverse cytokines. Recognition of pathogens by PRRs is the key of innate and adaptive immunities. Multiple regulations ensure complicate but appropriate activation of signaling pathways. Abnormal activation of upstream and midstream molecules in signaling pathways will affect their downstream networks, and finally cause inflammatory diseases (Kang et al., 2018).

The results indicates that the disorder of TLRs/NF-κB signaling pathway in the immunosuppressed mice with *A. fumigatus* inoculation causes the loss of balance between pro-inflammatory and anti-inflammatory cytokines and eventually led to the incidence and development of invasive aspergillosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Bio-prospecting of macro-algae for potential industrial dyes

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Synthetic dyes have been used for different purposes in various fields, but their continued usage has presented both environmental and health challenges. Therefore, alternative safer dye sources are inevitable. Algae have different pigments with potential for exploitation and usage in different socio-economic sectors. The aim of this study was to bio prospect dyes from macro-algal species of the Indian Ocean marine ecosystem. Six algal samples were collected from Coastal beaches along the Kenyan Indian Ocean during the wet and dry seasons. The samples were processed and used for pigment extraction and screening. Pigments were extracted using ethanol, acetone, diethyl ether and hexane in sequential with distilled water. Subsequently, the crude extracts were analyzed for pigment component using spectrophotometry and qualitatively for presence of active components. From the six species, *Ulva reticulata* was the best dye producer in ethanol and distilled water. All extracts were coloured green except those from *Galaxaura subverticillata* which gave a dark red extract in 80% hexane and a brown extract in distilled water. The crude extracts also contained different active components, with phenols being the most common component in ethanol and acetone extracts. This study demonstrates that macro-algae species from the Indian Ocean ecosystem contain useful pigments for biotechnological exploitation. Future studies should focus on increasing the pigment content through genetic manipulation of macroalgae and analysis of the pigments using modern methods such as the gas chromatography-mass spectrometry (GC-MS).

Key words: Marine biodiversity, marine bio-resources, macro-algae, pigments.

INTRODUCTION

Algae are a wide group of pigmented eukaryotic organisms naturally inhabiting both fresh aquatic and marine ecosystems. They are divided into six wide groups (phyla) based on the dominating pigment which gives the algae its colour (Abayomi et al., 2009). Members of the *Chlorophyta* are green in colour due to the presence of abundant chlorophyll pigment. They contain chlorophyll *a*, *b,* carotenes and xanthophylls.

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Members of *Phaeophyta* comprise brown algae, which contain xanthophylls and chlorophyll *a*. The *Rhodophyta* are made up of red algae and contain chlorophyll *a*, phycoerythrin, phycocyanin, carotenes and xanthophylls. Members of class *Chrysophyta* are made up of diatoms and golden-brown algae, which contain chlorophyll *a* and carotenes. The *Pyrrophyta* consists of dinoflagellates and contain chlorophyll *a,* xanthophylls and carotenes. Members of the *Euglenophyta* are made up of euglena, which contain chlorophyll *a, b*, carotenes and xanthophylls (Bibeau, 2009).

Synthetic dyes are widely used in textile industry, leather tanning, paper production, food technology (Slampova et al., 2001), agricultural research, in light-harvesting arrays, photo electrochemical cells (Wrobel et al., 2001), and hair coloring among other sectors as enumerated by Forgacs et al. (2004). These dyes have an accumulated effect on long-term usage. Almost 89% of hydrogen peroxide hair dyes have been shown to be mutagenic, with half of the components showing various degrees of mutagenicity (Tai et al., 2016). Waste effluent from textile industry mainly consists of excess dyes washed off during the dyeing process and has been reported to cause the highest toxicity to marine life as compared to effluent from pulp and paper industry (Flohr et al., 2012). Allura red, a dye mostly used in baking and beverage industries, has been reported to cause adverse health effects in humans including allergies, cardiac disease and in some cases, multiple sclerosis upon long term use (Rovina et al., 2016). In a study carried out by Sener et al. (2011), Indigotin and methylene blue dyes were reported to cause acute generalized exanthematous pustulosis in some individuals. Thus, it is important to bio prospect for alternative safer dyes.

Due to health and environmental problems caused by the wide usage of synthetic dyes, most industries are considering the adoption of natural dyes and colorants. Recently, green microalgae have gained importance in industries to produce commercially important products such as food ingredients, fertilizers, bio plastics, pharmaceuticals, chemical feed stock and biofuels (Skjånes et al., 2013). Algal pigments have also been quantified using high throughput techniques such as high-performance liquid chromatography and spectrophotometry as an integral part of water quality monitoring and general experimental research on phytoplanktons (Thrane et al., 2015). Of great importance are the chlorophylls, xanthophylls and carotenoids which have been commercially exploited in the pharmaceutical, cosmetic and food industries as demonstrated by Wang et al. *(*2015). Additionally, a recent study by Moldovan et al. (2017) demonstrated the potential usage of phycoerythrin, a red pigment obtained from the macro-algal *Glacilaria* sp. in the textile industry to dye cotton fabrics. Algal pigments are easy to extract as they are either water or organic solvent soluble (Gupta et al., 2013). Chlorophyll and carotenoidss are fat soluble and can be extracted from thylakoid membranes with organic solvents such as acetone, methanol or dimethylsulfoxide. The phycobillins and peridinin are water soluble and can be extracted from algal tissues after the organic extraction of chlorophyll. Large scale extraction of algal pigments involves disintegration of the biomass, followed by treatment with an organic solvent mixture. The pigment can then be extracted from the supernatant of the solvent mixture by various methods including chromatographic methods.

Therefore, since there is dire need for alternative and safer natural dyes, the research team surveyed and documented macro-algal species from the Indian Ocean marine ecosystem while focusing on: (i) determination of solvent suitability for dye extraction among the selected macro-algal species, and (ii) evaluation of dye properties for potential industrial and medicinal applications.

MATERIALS AND METHODS

Study sites and sampling

The algal samples were collected from both public and private beaches along the Kenyan Indian Ocean during the dry season in January 2015 and during the wet season in August 2015. The sample sites included Pyrates, Nyali, Mama Ngina, Kibarani, Malindi, Watamu and Vasco da Gama beaches of the North coast as well as Diani beach in the south coast. Six macro-algal species were targeted as identified using morphological characteristics with comparison to the algae database (http://www.algaebase.org/) and a dichotomous key based on morphological characteristics matching those already documented in the algae encyclopedia. The species were identified as: Ulva *reticulata* (Nyunja et al., 2009), *Rhizoclonium grande* (Gupta, 2012), *Galaxaura subverticillata*, *Ulva pertusa*, *Chaetomorpha viellardi* and *Enteromorpha muscoides* (Silva et al., 1996). Wet algal samples weighing about 150 g were hand-picked, cleaned in sea water and preserved in sterile plastic bags inside cool boxes for transportation to the laboratory at Technical University of Mombasa, Kenya.

Sample preparation and dye extraction

Algal samples were rinsed using distilled water to remove mud and debris. Samples were then dried in a hot air oven maintained at 110°C for a period of 2 h to ensure complete drying. The dry algae were ground separately using a blender then refined to a powder using pestle and mortar. A sequential extraction with organic solvents and distilled water was carried out. First, 10 g of powder from each algae species were weighed and mixed with 100 ml of 80% organic solvent. Two polar solvents (ethanol and acetone) and two non-polar solvents (diethyl ether and hexane) were used. The setup was replicated three times and left in the dark for 24 h to maximize the extraction. Next, the liquid extract was strained using a muslin cloth then the extraction repeated for another 24 h using 100 ml of distilled water to extract the water-soluble pigments. The extracts from overnight incubation were vacuum filtered to obtain debris free dye extracts, then concentrated in a rotor vacuum evaporator (ROVA-2L/3L) to remove excess organic solvents. This was followed by centrifugation at 1800 rpm for 30 min to efficiently separate the organic solvent phase and the dye phase. The liquid dye extract was carefully pipetted from the organic solvent phase and used in subsequent analysis.

Determination of properties of dye extracts

The crude extracts were qualitatively tested for presence/absence of active compounds (glycosides, flavonoids, phenols, alkaloids, saponins and tannins), with prior removal of chlorophyll by saponification method in the presence of 1 M sodium hydroxide as described by Li et al. (2016). To 50 µl extract, 200 µl of 1% sodium hydroxide was added and the mixture was left in the dark at room temperature. The mixture was then centrifuged at 15000 rpm for 5 minutes and the clear layer of the extract pipetted into an Eppendorf tube for analysis. To test for the presence/absence of flavonoids, Shinodas test was used. For phenols, gelatin test was used. For alkaloids, Mayer's test was used. For tannins, phelic chloride test was used. For saponnins, bicarbonate test was used and for glycosides, Keller-kiliani test was used (Senguttuvan et al., 2014; Sheela, 2013).

The unsaponified extracts were tested for pigments composition using calorimetric spectrophotometry. This was done by making a 10x dilution of the concentrated extracts using the solvent used in extraction. The diluted sample was subjected to wavelength scanning using an absorbance spectrophotometer (T70 UV/VIS). Peaks of the absorbance wavelengths were recorded during the progress of the scanning.

RESULTS

Distribution of the macro-algal species

The adoption of conventional taxonomic principles revealed a varying distribution of the six different macro-algal species (*U. reticulata, U. pertusa, R. grande, E. muscoides, G. subverticillata* and *C. viellardi*) in the studied sites. Mama Ngina had four of the species used in the study while Vasco da gamma had three. The rest of the sampling sites were dominated by single species except, Diani beach which did not have any of the algae species. *U. pertusa* was present in four sites sampled: Mama Ngina, Pirates, Nyali and Vasco da Gamma beaches. *U. reticulata* and *C. viellardi* were present in Mama Ngina and Vasco da gamma sites. *R. grande* was exclusively found in Kibarani site as the dominating algal species. *G. subverticillata* was found in Mama Ngina and Malindi sites, while *E. muscoides* only occurred at Watamu site.

Solvent suitability for dye extraction

A comparison of all the extracts from the different solvents showed that, ethanol and distilled water are the best polar solvents for extraction of algal pigments while hexane is the best non-polar solvent for extraction. *U. reticulata* is the best dye producer, followed by *R. grande*, *U. pertusa* and finally *C. viellardi* (Figure 1).

A principle component analysis (Figure 2) showed that the concentration of extracts from *U. pertusa* and *R. grande* using acetone were not significantly different from each other but were significantly different from water extract of *G. subverticillata*. Likewise, *U. reticulata* and *E. muscoides* extracts of hexane and diethyl ether were not significantly different from each other but were significantly different from ethanol extract of *C. viellardi*.

Dye absorbance characteristics

A spectrophotometric analysis determined the minimum and maximum absorbance wavelength for each dye extract, suggesting the possible pigment components. A peak at 444 and 630 nm was present in all species extracts, suggesting a chlorophyll component which is characteristic of most algae (Table 1). Two peaks at 450 and 453 nm were observed in distilled water extracts of *G. subverticillata* and *R. grande.* Acetone extracts of *G. subverticillata* and *E. muscoides* had two similar peaks at 450 and 540 nm. *U. pertusa* acetone extracts had a clear absorbance peak at 480 nm.

Active components of algal dyes

Extracts from the different macro-algal species had different active compounds depending on the solvent used. Tannins were detected in ethanol and acetone extracts of *U. pertusa* and *U. reticulata.* Flavanoids were detected in hexane and diethyl ether extracts of *U. reticulata* and in diethyl ether extract of *R. grande.* Phenols were detected in all extracts of ethanol and acetone. Saponins were present in ethanol and acetone extracts of *U. reticulata, U. pertusa* and *R. grande.* Additionally, saponins were detected in distilled water and acetone extracts of *G. subverticillata.* Notably, alkaloids were only detected in acetone extracts of *U. reticulata* and *U. pertusa,* while Glycosides were present in distilled water extracts of *U. pertusa, R. grande* and *E. muscoides.*

DISCUSSION

The results of this study have shown that the Coastal Indian Ocean of Kenya harbor a diverse macro-algal species that are distributed in different locations. The availability of four of the algae species isolated at Mama Ngina could be attributed to the fact that the site is a shipment area. The ships could possibly harbor different algae species on their surfaces, which find their way into the site. Likewise, the relatively high algae diversity at Vasco da Gama could be attributed to the nature of the site. It is an estuary of the Sabaki River, thus could receive a variety of algae species harbored by the draining waters. The rest of the sites do not experience a mix up of water from different environments, hence the trend in dominance of one species in each site. On the other hand, a lot of human activities at the Diani beach could be responsible for the lack of algae species at the site. The availability of macro-algae species at different sampling sites in all the sampling seasons suggests that

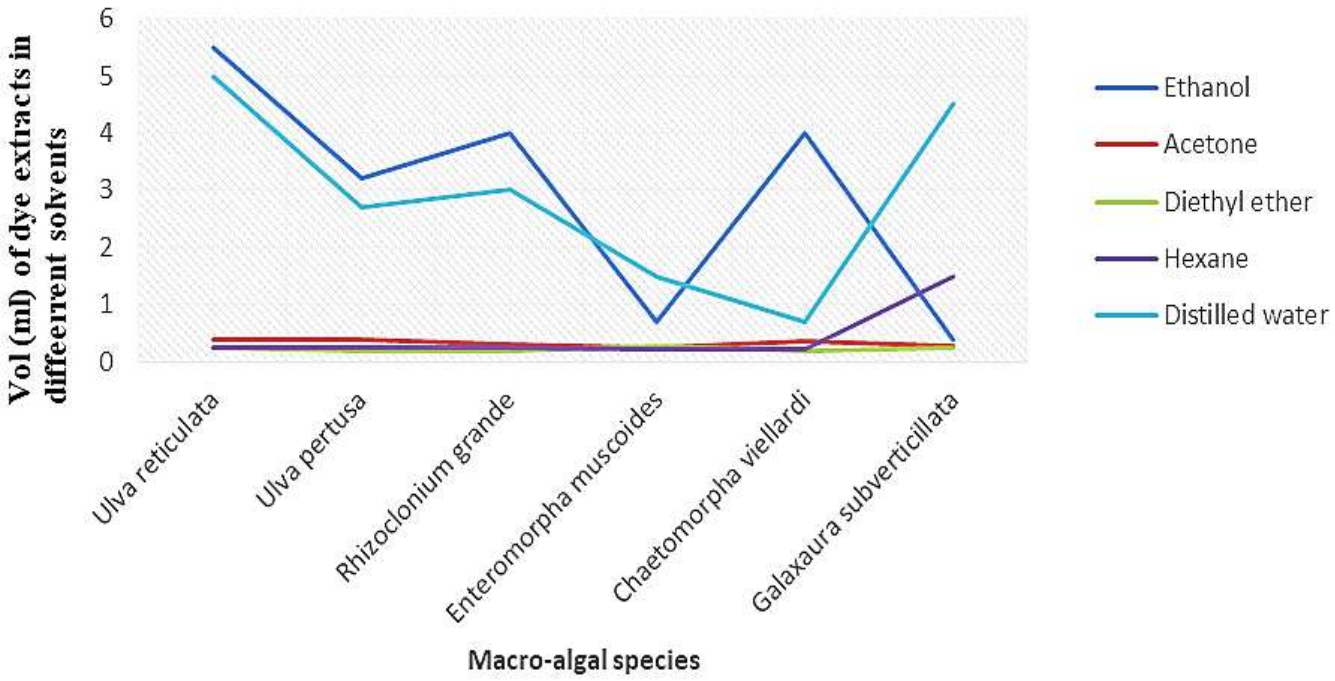

Figure 1. A line graph showing comparison of mean-volume of the dye extracts.

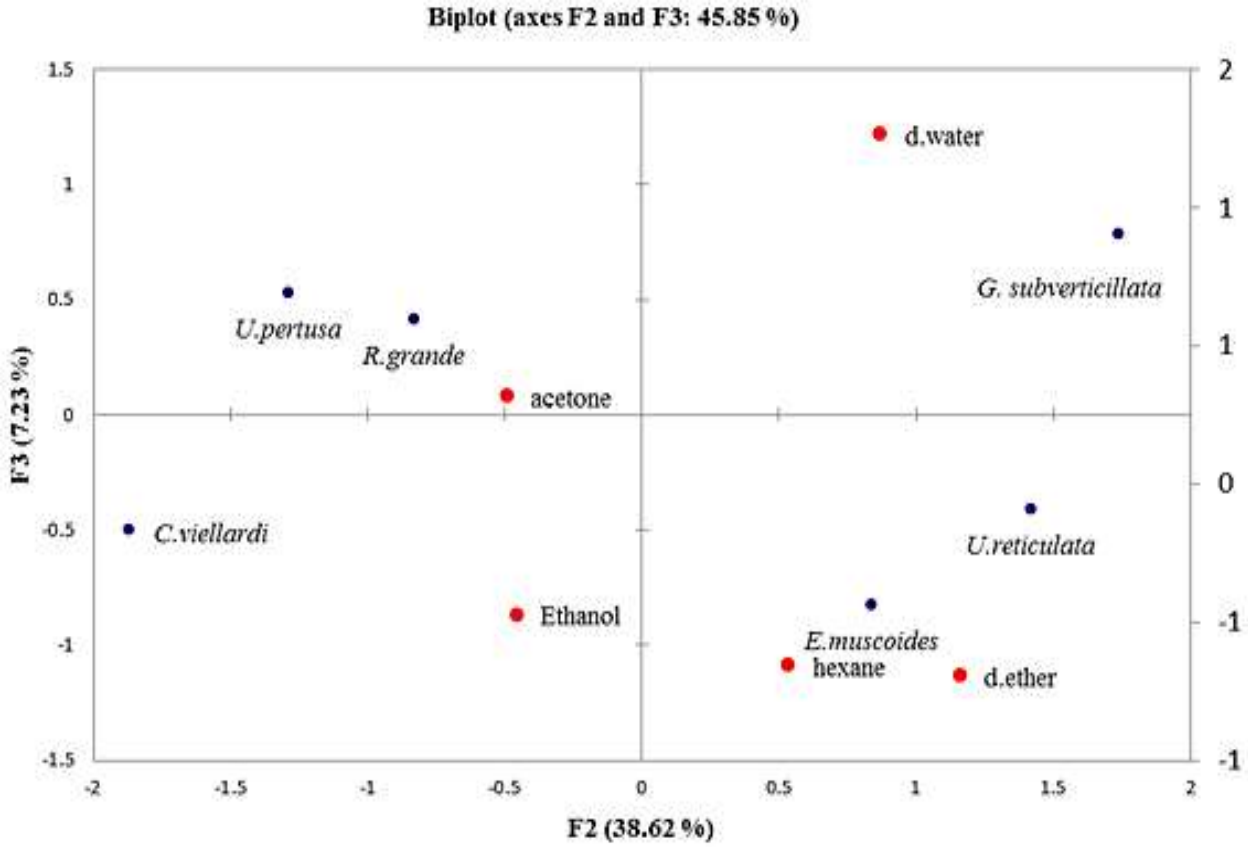

Figure 2. Principle component analysis of species versus extraction solvents. 'd.water' denotes distilled water; 'd.ether' denotes diethyl ether.

Table 1. Wavelengths and pigment identities of the extracts.

Species extract in solvent mixture	Wavelengths (nm) minimal and maximal	Pigment Identity
Ulva reticulata	444, 630	Chlorophyll *c*
	500, 550	Phycocyanin
Rhizoclonium grande	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	450, 453	Carotenoid
Chaetomorpha viellardi	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	500, 550	Phycocyanin
Galaxaura subverticillata	450, 540	Fucoxanthin
	450, 453	Carotenoid
Enteromorpha muscoides	444, 630	Chlorophyll *c*
	450, 540	Fucoxanthin
Ulva pertusa	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	480	Beta xanthin

although the algae could have found their way into the sites using different mechanisms, they have adapted to the conditions and as such become part of the ecosystem. It is also possible that the difference in macro-algae diversity at the sampled sites could be influenced by other environmental conditions including soil and water properties that are beyond the scope of this study.

Most industrial dyes are extracted using chemicals then dissolved in aqueous organic solvents or dimethyl sulfoxide (Valianou et al., 2009). In this study, the dyes were extracted using organic solvents in sequential with distilled water, therefore, by-passing the drying step during concentration. This has an advantage in that it is fast and efficient but has a drawback in that the amount of dyes can only be quantified based on the extent of pigment solubility in the extraction solvent, which could explain the diversity in volumes of pigment extracts observed in this study as shown in Figure 1. The pigment molecules dissolve completely in the solvent and cannot be evaporated to dryness without affecting the pigment structure. Anchoring the pigment extracts on ammonium sulphate is a potential way to capture them in a semi-solid state. The pigments form a dye cake that maintains their stability at room temperature

The principle component analysis in Figure 2, further emphasizes the variation in the solvents capability for pigment extraction. The non-polar solvents cluster together while the polar solvents vary greatly in their ability to extract pigments from different macro-algal species. All the three solvents have varying degree of polarity. It is no wonder each polar solvent cluster separately in the principle component analysis plot. The unmatched extraction efficiency observed with water and ethanol is attributed to the high polarity of these two solvents. Water, being highly polar, is efficient in extraction of lipophilic pigments (Warkoyo and Elfi, 2011). Similarly, ethanol has a high polarity as compared to other organic solvents due to presence of hydroxyl group in its structure, hence the efficiency in extraction as compared to the other solvents (Herrero et al., 2005). However, it should be noted that the high concentrates observed with ethanol and distilled water could be due to the good solvation ability of these two solvents and not necessarily high extraction capabilities. As such, more concentration of crude extracts with more sophisticated techniques like lyophilization is recommended to rule out this possibility.

Different solvents can capture varying pigments based on their hydrophobicity with respect to the solvent. Non-polar solvents are efficient in the extraction of hydrophobic pigments present in high amounts in red algae which could be the reason why hexane only performed best in *G. subverticillata*, a red algae. Polar solvents on the other hand are efficient in extraction of hydrophilic pigments for instance phycoerythrin, phycocyanin and carotenoids as described by Warkoyo and Elfi (2011). This is clearly seen in Table 1, where chlorophylls are observed in all organic solvent extracts, while carotenoids are only observed in distilled water

extracts. Chlorophyll is insoluble in water but soluble in organic solvents. Four types of chlorophylls (*a, b, c1, c2* and *d*) have been documented. Chlorophyll *a* is a blue green pigment that has an absorbance minima and maxima at 430 and 664 nm, while chlorophyll c absorbs at 444 and 630 nm (Torres et al., 2015). They were the only types of chlorophyll identified in most of the extracts, suggesting that they are very essential to most algae species. This is consistent with the findings of Chakraborty and Santra (2008), who observed different levels of chlorophyll *a* and *c* in eight marine macro-algae species.

Chlorophyll *d* was not detected in any of the extracts, although Murakami et al. (2004) suggests that this pigment may sometimes be found in green algae. Besides chlorophyll, the extracts also contain accessory pigments which include fucoxanthin, phycocyanin, phycoerythrin, xanthophylls and carotenoids. Fucoxanthin was only found in distilled water extracts of the red algae, *G. subverticillata* and the green algae, *E. muscoides*. This is because it is a water-soluble pigment. Other studies have shown that red algae contain this accessory pigment in their chloroplasts (Holdt and Stefan, 2011). Phycocyanin is a blue pigment with absorbance wavelengths of 500 and 550 nm (Eriksen, 2008). In this study, the pigment was only present in ethanol extracts of two green algae; *U. reticulata* and *C. viellardi*, although Rahman et al. (2017) reported the presence of phycocyanin in *Cyanidioschyzon merolae*, a red algae. The presence of carotenoids in acetone extract of *R. grande* is consistent with the findings reported by Yoshii et al. (2004), who also extracted carotenoids from the genus, *Rhizoclonium.* Similar findings were also published by Warkoyo and Elfi (2011). With the analysis of crude extracts, background peaks are inevitable.

Mycosporine-like-amino acids have been reported to be the leading contaminants in most pigment extracts (Karsten et al., 2005; Sonntag and Sommaruga, 2007). As such purification with high throughput techniques prior to analysis is highly recommended in future studies to rule out any possibilities of altered wavelength due to mycosporines. Qualitative tests showed presence of a wide variety of phytochemicals. This is supported by Ibañez et al. (2012) and Sahayaraj et al. (2014) they also reported availability of a wide variety of phytochemicals in algae. Although, the amounts of phytochemicals were not quantified in this study, positive qualitative tests suggest that significant amounts could be present, which allows their detection with the qualitative tests. The presence of tannins and flavanoids in this study is consistent with the findings by Thomas et al. (2011), who carried out test for phytochemical compounds in petroleum ether extracts of *U. reticulata.* Phenols were present in all extracts of ethanol and acetone probably due to their polarity caused by the presence of a hydroxyl group in their structure. Similar findings have been reported by Cox et al. (2010) who determined phenolic content of six species of sea Weeds and Vijayavel and Martinez (2010) who studied antimicrobial activity of phenol extracts of *Ulva* species.

Conclusion

The Indian Ocean of the Kenyan Coast harbors macro-algal species which contain useful pigments that can be harnessed for biotechnological applications. From this study, three dyes; green, dark red and brown were obtained from green and red macro-algae species respectively. The dyes have a unique combination of pigments and active components. Future comprehensive studies should be conducted using modern methods such as the gas chromatography-mass spectrometry (GC-MS) to analyze the different pigments detected. This will help to elucidate the molecular structures of the bioactive compounds for further usage. Additionally, we recommend genetic manipulation studies be conducted on such macro-algae species in order to increase their pigment content for industrial applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diversity and genetic identity of pineapple [*Ananas comosus* (L.) Merr.] in Tanzania based on microsatellite markers

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Pineapple [*Ananas comosus* (L.) Merr.] is an important fruit crop cultivated in Tanzania. However, the knowledge on genetic diversity of the pineapple cultivars grown in Tanzania is limited. This study was aimed at determining the genetic diversity and identity of pineapple cultivars from different growing regions in Tanzania using microsatellite markers also known as simple sequence repeat marker (SSR). Ten of the 18 microsatellite markers were polymorphic and generated a total of 22 distinct reproducible bands with an average of 2.2 bands per primer pair. The number of polymorphic bands detected with each primer pair ranged from 1 to 3 with an average of 1.5 per primer pair. The polymorphic information content (PIC) values of each primer pair ranged from 0.17 to 0.79 with an average of 0.41. Two microsatellite loci TsuAC010 and TsuAC039 revealed PIC values higher than 0.50 thus suggesting that such primers have high discriminatory ability. The consensus tree derived from the unweighted pair-group method with arithmetic means (UPGMA) revealed four different groups. Kinole-SCT sub-population formed a distinct group from Madeke-SCT and MD2 hybrid cultivar. Kinole-SC, Mukuranga-SC, and Kiwangwa-SCcultivars were closely related on the cluster analysis. This study demonstrated the existence of low genetic diversity in pineapples cultivated in Tanzania implying that a well-thought-out breeding strategies should be employed for genetic improvements of pineapple. Introduction of exotic clones and employment of modern breeding strategies such as marker assisted selection (MAS) and genetic engineering technologies is recommended. This will widen the current genetic pool of pineapple in Tanzania.

Key words: Pineapple [*Ananascomosus* (L.) Merr.], microsatellite markers, genetic diversity, unweighted pair-group method with arithmetic means (UPGMA), polymorphic information content (PIC).

INTRODUCTION

Pineapple [*Ananas comosus* (L.)Merr.] is a perennial herbaceous fruit crop belonging to the family Bromeliaceae. It is cultivated in tropical and subtropical regions and ranks third in production among non-citrus

tropical fruits, after banana and mango (Coveca, 2002). The annual worldwide production of pineapples reached 24.8 million metric tons in 2013 and the top seven producers (Costa Rica, Brazil, Philippines, Thailand, Indonesia, India, Nigeria, China, Mexico and Colombia) jointly accounted for 90% of the global production (FAO, 2018).

The three most economically important traditional pineapple cultivars in the world are Cayenne, Singapore Spanish, and Queen (d'Eeckenbrugge et al., 1997). Smooth Cayenne is the most important and popular cultivar mainly due to its high yield potential and favorable characteristics as a fresh fruit and for processing and hence preferred for pineapple breeding (Leal and d'Eeckenbrugge, 1996).

Pineapple production in Tanzania is expanding rapidly with increasing tendency toward commercial cultivations through expansion of existing farms and opening new farms. This trend has been caused by increased demand of pineapple in local and internal market particularly in the Middle East. In Tanzania, pineapple production range from 0.4047 to 8.094 ha with yield estimates of 23.37MT/ha in year 2014 (FAOSTAT, 2016). The production is mainly contributed by small holder farmers and few medium scale farmers. Although the larger percent of pineapple produced is consumed locally, few farmers export fresh fruits to markets in Europe and Middle East. However, both local and international markets were faced with several challenges that include short shelf life of the produce, changing consumer preferences, poor branding, packaging and other marketing constraints. Furthermore, farmers, consumers and marketing agents in Tanzania do not know the characteristics of pineapples that are grown in the country. Consequently, the fruits produced has low market value and do not meet the requirements of both local and international markets. In addition, homonyms and synonyms are common among the names of pineapple cultivars and that confuses farmers and restricts the sharing of accurate information and materials among pineapple researchers. Lack of such information on the pineapple identities hamper the use of genetic resources in breeding of pineapple in Tanzania and other major growing areas of the world (Hidayatet al., 2012; Sripaoraya et al., 2001; Zhang et al., 2014).

The current government policy of industrialization is centered around increased production and processing of agricultural goods such as pineapples. To realize this goal, the agricultural sector should focus on improving the production and dissemination of high quality and market/industry preferred pineapple cultivars. Given this challenge, it is important to genetically characterize the locally adapted smooth cayenne cultivars to ascertain their diversity and identities.

Several DNA profiling techniques have been used for cultivar identification and evaluation of genetic diversity in pineapples (Ahmed and Abdel, 2012; Burhooa and Ranghoo-Sanmukhiya, 2012). Among the available molecular markers, microsatellite markers have been widely used for genetic analysis and cultivar identification due to its high polymorphism, abundance, co-dominance inheritance and reproducibility. The technique is simple to assay by Polymerase Chain Reaction (PCR) and gel profiling (Xie et al., 2011). The application of microsatellite markers for plant cultivar characterization have been reported in several crops including grape (Dallakyan et al., 2013), potato (Salimi et al., 2016), rice (Miah et al., 2013), almond (Sorkheh et al., 2017; Zhang et al., 2017), apple (Van Treuren et al., 2010) and wheat (Han et al., 2015; Kroupin et al., 2013). However, DNA profiling in pineapple by microsatellite markers has been rarely studied (Shoda et al., 2012; Alfonso et al., 2013).

The objective of this work was to determine the genetic diversity and identity of pineapple cultivars in Tanzania. This study would contribute to enhancing our knowledge and understanding on the genetic relationship between different cultivars and provide basic information for improving parental selection and other pineapple breeding activities.

MATERIALS AND METHODS

Plant materials

Pineapple germplasm were collected from Kinole in Morogoro, Kiwangwa and Mkuranga in Coast and Madeke in Njombe. These sites represent the major pineapple growing areas in Tanzania. The collected materials (Table 1) were grown and maintained at Chambezi sub-station of Mikocheni Agricultural Research Institute (MARI).

Genomic DNA extraction

Pineapple leaves (10 mm in diameter) were picked and packed in envelopes, labeled and shipped to MARI molecular laboratory for DNA extraction. Pineapple genomic DNA extraction was performed using cetyltrimethyl ammonium bromide (CTAB) method (Charlotte et al., 2016). About 2.5 g of fresh pineapple leaves from each sub-population were pre-chilled in liquid nitrogen, and then crushed using a mortar and pestle to a fine powder before transferring into 1.5 ml of Eppendorf tubes. Then 700 µl of the pre-warmed

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Table 1. List of collected pineapples used in the study.

S/N	Genotypic identity	name	Origin	Fresh colour
1	Kinole-SCT	Kinole (local thorn)	Morogoro	Green-red
2	Madeke-SCT	Njombe (local thorn)	Njombe	Green-red
3	Kiwangwa-SC	Kiwangwa	Pwani	Green
4	Kinole-SC	Kinole	Morogoro	Green
5	Mkuranga-SC	Mkuranga	Pwani	Green
6	Madeke-SC	Njombe	Njombe	Green
7	MD2	MD2 hybrid	Bagamoyo fruit farm	Green

extraction buffer (2% CTAB) mixed with 10% *β*-mercapto-ethanol were added and mixed by vortex. The Eppendorf tubes containing the samples were then incubated at 60°C for 30 min on floater in water bath. Equal volume (700 µl) of phenol: chloroform: isoamyl (25:24:1) were added, mixed by inverting the tubes for 10 min and centrifuged for 10 min at 12000 rpm. Approximately, 500 µl supernatant was transferred into the clean 1.5 ml Eppendorf tubes and 300 µl of cold isopropanol were added. The tubes were incubated at 20°C for 2 h to precipitate the DNA and later centrifuged for 10 min at 12000 rpm to precipitate the DNA. The supernatant was discarded and the pellet washed with 400 µl of 70% ethanol and later air dried at room temperature to complete dryness. The pellets were resuspended in 50 µl of sterile double distilled water and stored in refrigerator at 4°C until needed for PCR.

Determination of pineapple genomic DNA quality

The quality of the genomic DNA was determined by resolving the samples on a 0.8 % (w/v) agarose gel in Tris-acetate EDTA (X1 TAE) (40 mMTris-HCl and one mM EDTA at pH 8.2 adjusted with acetic acid) buffer stained with gel red then ran at 100 V for 40 min. The gel was visualized under UV light and documented using BioDoc-It ^{Tm} Imaging System (Upland, CA, USA).

Determination of pineapple genomic DNA quantity

The DNA concentration of each of the sample was determined using a Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK). A concentration of each sample was standardized to 25 ng/µl (working concentration) by diluting with RNase-free water. The purity check of the DNA was estimated at A260/A280 ratio with the reading between 1.6 and 2.0 considered to be of acceptable quality for further processing.

PCR amplification for detection of SSR markers

Eighteen SSR primers were used for the genetic characterization of the pineapple cultivars. These markers were selected because they revealed great polymorphism in Japanese pineapple genetic diversity study by Shoda et al. (2012). The SSR primer characteristics are depicted in Table 2. Primers were synthesized by Inqaba Biotech East Africa Ltd (Pretoria, South Africa). SSR polymerase chain reaction (PCR) amplification was performed in a 20 µl reaction mixture containing *Accu*Power® PCR Pre Mix including 1 U Top DNA polymerase, 250 µM dNTP, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, Stabilizer and tracking dye (Bioneer Corparation, Daejeon, Republic of Korea), 16 µl RNase-free water, 1.0 µl each of forward and reverse primers and 2.0 µl of 25 ng genomic DNA. DNA was amplified using the following PCR program: 94°C for 2 min initial denaturation and a final denaturation 94°C for 30 s, annealing temperature of 58 to 60°C (Table 2) for 30 s each for 35 cycles and extension at 72°C for 2 min and a final extension at 72°C for 7 min. All PCR reactions were carried out on a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA. USA). PCR products were separated on 2% agarose gels (stained with gel red) at 75 V for 45 min and viewed under UV light and gel documented using BioDoc-It ^{Tm} Imaging System (Upland, CA, USA).

Data management and analysis

The amplified bands were scored for each SSR marker based on the presence or absence of bands, generating a binary data matrix of 1 and 0 for each marker system. Jaccard's similarity coefficient values were calculated and phylogeny based on similarity coefficient values supported with bootstrap were generated using unweighted pair-group method with arithmetic means (UPGMA) using PAST (Past 3.14 version) software. The bootstrap values above 50% were considered of significant. The polymorphism information content (PIC) value for each SSR markers was calculated using the formula by Peakall and Smouse (2012).

$$PIC=1-\sum_{i=1}^{k} Pi^2$$

Where, k is the total number of alleles (bands) detected for one SSR locus and P is the proportion of the cultivars or genotypes containing the allele (band) in all the samples analyzed and *P*i is the frequency of the *i*th allele in the examined test genotypes.

RESULTS

Polymorphic information analysis of SSR primer pairs

Among 18 primers screened, 10 were found to be polymorphic and thus chosen for diversity study and genotype identification. Banding patterns were observed to range from 2 to 4 with an average of 3.1 generated by primer pairs (primer pair TsuAC026 and TsuAC024, and

Table 2. Microsatellite loci with their characteristics.

SSR locus accession	Primer sequence (5′-3′)	Tm (˚C)	Repeat motif	Target size (bp)
TsuAC004 AB716708	F: ATGTTGGTCAAAGGGCTGTT R: gtttcttTCATGATCACACTGGAGATTTG	58	(AG)16	144
TsuAC010 AB716711	F: TGAGTTGTGTCATTGTGTGTCA R: gtttcttGGGGGTCTCCATACATTTTT	58	(GT)14A(AG)12	207
TsuAC013 AB716712	F: TTATGCAGGAAAATAGGGGG R: gtttcttCATGCATCATAAATTCGTGTCC	58	(AGAGAT)3(AG)12	139
TsuAC018 AB716713	F: GCATCGATCTCCATGCAAAC R: gtttcttAAAGGAAACAAGGAGGATGTGA	60	(CA)10A(AC)9	120
TsuAC021 AB716715	F: AATCAAAGTGATTCCCCTTCC R: gtttcttTCTGACATAGGGCTTGCACA	58	(CA)21	141
TsuAC023 AB716716	F: TCGAAAAGAGGATGCTGGAT R: gtttcttTCCGCAGTGTAGGCATGTAA	58	(CA)10(TA)11	143
TsuAC024 AB716717	F: GTCGCCAATCAAATTCCAGT R: gtttcttCTCACGAAACATGAATCACCA	58	(AC)9	126
TsuAC026 AB716718	F: GGGATTAACTTTTCCAGGGG R: gtttcttTTGGATTCCTCGTTTGCATT	60	(AC)8	200
TsuAC039 AB716723	F: CCCTGTATGGGTAGCATTGAA R: gtttcttAAAAGGTATCACGAAAGCGA	60	(AC)8	91
TsuAC041 AB716725	F: CTCTCTTATGGCACAACCCTG R: gtttcttCCTGGTGAGTAATCTATATGCTG	60	(AC)11	279

Table 3. Resolving power values of polymorphic primers.

Primer	Number of amplified bands	Number of polymorphic bands	Number of band patterns	Polymorphic information content (PIC)
TsuAC018	2	1	3	0.40
TsuAC024	2	1	3	0.17
TsuAC010	3	3	4	0.79
TsuAC013	2	1	3	0.36
TsuAC039	3	3	4	0.78
TsuAC026	2	1	3	0.17
TsuAC021	2	1	2	0.26
TsuAC004	2	2	3	0.44
TsuAC018	2	2	3	0.37
TsuAC041	2	1	3	0.36
Total	22	16	3.1	4.1
Average	2.2	1.6	3.1	0.41

primer pair TsuAC039). The ten chosen primers generated a total of 22 unambiguous bands with an average of 2.2 bands per primer pairs (Table 3). The number of polymorphic bands detected with each primer pair ranged from 1 to 3 with an average of 1.6 (Table 3). The polymorphic information content of each SSR marker ranged from 0.17 to 0.79 with an average of 0.41 (Table 3). Markers TsuAC024 and TsuAC026 had the lowest values while marker TsuAC010 and TsuAC039 had the highest value (Table 3).

Unweighted pair group with arithmetic means cluster of the seven pineapple cultivars

The UPGMA clustered the 7 pineapple sub-populations into four major groups with genetic similarity coefficient value ranging from 0.4 to 1 (Figure 1). The phylogenic

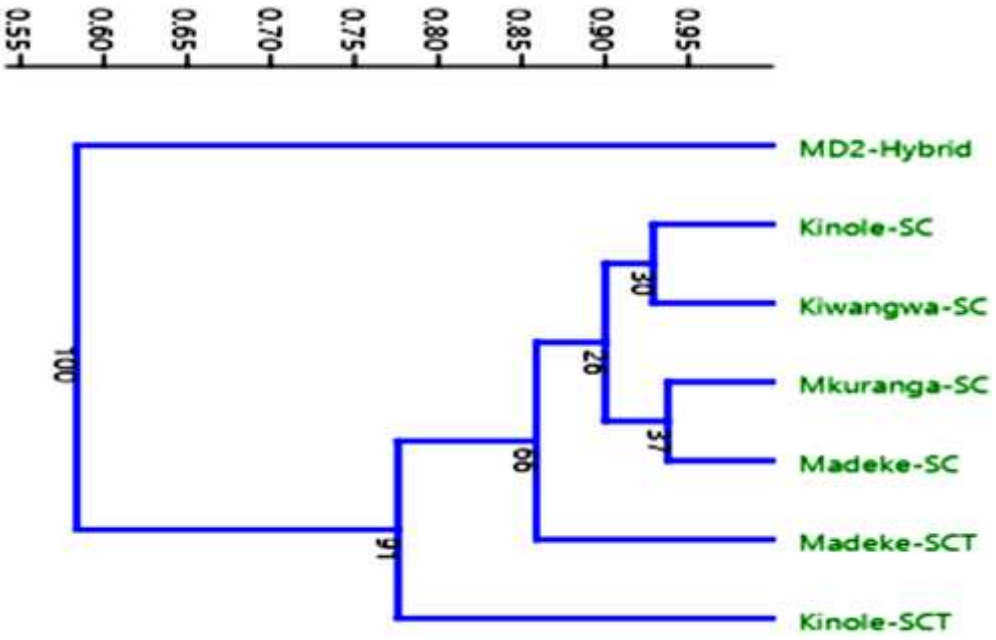

Figure 1. Phylogenetic tree of 7 pineapple sub-populations derived from UPGMA cluster analysis based on dice analysis. All bootstraps with values above 50% were considered to be significantly different.

tree revealed pineapples sub-populations Kinole-SCT, Madeke-SCT and MD2 hybrid were significantly different based on bootstrap and similarity coefficient scale (Figure 1). The first cluster contained MD2 hybrid, the second cluster was Madeke-SCT, the third cluster was made of Kinole-SCT whereas Mkuranga-SC, Madeke-SC, Kiwangwa-SC and Kinole-SC clustered together forming the fourth cluster (Figure 1).

DISCUSSION

Understanding genetic diversity of Tanzanian pineapples is important as it is the first step in harnessing characteristics for cultivar identification at farmer, processor, market and consumer levels as well as their genetic variability for their improvement. This work represents the first study of basic genetic diversity and identity of seven pineapple sub-populations (collections) commonly grown in Tanzania and one imported hybrid pineapple using microsatellite markers. The genetic diversity were measured in terms of polymorphic information content (PIC), total number of amplified bands, number of alleles per locus and number of band patterns. PIC is a measure of the level of polymorphism or diversity in a given population. PIC value is always less than one, and the population with the PIC value of 0.5 to 0.9 are considered to be more diverse. On the other hand, the PIC value less than 0.5 is considered to be of narrow genetic diversity. Of the ten microsatellite markers used in this study, TcuAC010 and TsuAC039 revealed PIC values higher than 0.5, implying that such markers are more powerful to detect the diversity in pineapple populations. Similarly while working on pineapple accessions in Japan using the same markers, Shoda et al. (2012) reported a distinct diversity among the pineapples. The observed polymorphism in the studied pineapple populations suggest that such microsatellite markers could be readily used for genotype identification and genetic diversity studies of pineapple. In the studied pineapple sub populations, the microsatellite markers readily clustered the subpopulation into four distinct groups as revealed by the phylogenetic tree. These findings suggest that there might be more than three groups of smooth cayenne grown in Tanzania.

The MD2 was clearly identified by primer pairs TsuACO10, TsuAC024 and TsuAC26 at the band allele size of 200 bp. The sub-populations Kinole-SC, Kiwangwa-SC, Mkuranga-SC and Madeke-SC clustered together, indicating that these sub-populations were essentially the same but with different names. On the other hand, Madeke-SCT and Kinole_SCT formed its own distinct groups suggesting that the two sub-populations evolved differently. The variations in the pineapple populations detected by the microsatellite markers in this study are complemented by the findings reported by Alfonso et al. (2013) while studying the diversity of pineapple cultivars using SSR markers.

Similar results were also reported using microsatellite markers (Feng et al., 2013). Such variations may result from the combination of self-incompatibility, high levels of somatic mutation, and intraspecific hybridization in pineapple as noted by Kato et al. (2004). The distribution of pineapple cultivars observed in the phylogenetic tree is coherent and clearly shows that the microsatellite markers and the analytical methods used in this study are powerful tools for studying the genetic identity of pineapple.

Conclusion

Microsatellite markers were successfully used to effectively characterize the selected pineapple sub-populations. The study also revealed that the markers can effectively discriminate the different sub-populations. It is therefore important to incorporate microsatellite marker analysis in the selection of genetically distinct sub-populations to identify lineage in pineapple genetic resources conserved. Narrow genetic base of pineapples revealed under this study suggests that a well thought-out breeding program for pineapple improvement is urgently needed. The identified microsatellite markers will reduce the stress of applying many microsatellite markers for the identification of pineapple cultivars in Tanzania (and elsewhere) and hence, saves time and also cuts the cost of research studies for genetic diversity. These findings suggest that further studies should be carried out in identified groups of pineapple for their respective phenotypic characteristics, sensory evaluation and acceptability test for industrial growth and market promotion.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Alternative markers linked to the *Phg-2* angular leaf spot resistance locus in common bean using the *Phaseolus* genes marker database

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Common bean (*Phaseolus vulgaris*) is a rich source of protein, vitamins, and micronutrients and is an important crop for food security throughout Latin America, Asia, and Africa. Among tropical and subtropical regions of the world, where the majority of beans are grown, yield losses due to the pathogenic fungus *Pseudocercospora griseola*, causing angular leaf spot (ALS), can be as high as 80%. The strategic use of marker-assisted selection (MAS) to pyramid multiple resistance genes into a single genetic background with preferred morphological and cooking characteristics is being implemented by six research groups throughout East Africa that make up the African Bean Consortium (ABC). Identifying unique markers that are polymorphic among multiple parents is a major source of marker attrition. In this study, an illustration of how 22 DNA sequences physically linked to the *Phg-2* ALS resistance locus were identified using the PhaseolusGenes marker database and checked for amplification and polymorphism among 16 ABC breeding parents are given. Only three polymorphic markers could be identified following this procedure; one (g796), showed a polymorphism present only in the ALS resistance donor, Mexico 54. The PCR protocol developed to identify the g796 polymorphism was validated among five laboratories. Furthermore, co-segregation analysis of the marker and ALS resistance phenotype in a population of 100 F_2 individuals from the cross between French bean (that is, green bean) genotype Amy and ALS resistance donor Mexico 54 showed the marker is genetically linked (3 cM) to the *Phg-2* locus, in addition to being physically linked. This study suggests that in the near future, genetic resequencing data of diverse common bean accessions, compiled within an easily accessible database, will facilitate identification of markers for MAS, marker/trait association, and candidate gene identification.

Key words: Angular leaf spot resistance, *Phaseolus vulgaris,* marker-assisted selection.

INTRODUCTION

Marker-assisted selection (MAS) is a breeding approach, based on the existence of DNA markers in or closely linked to a gene coding for an important phenotypic trait. Instead of directly selecting for the phenotype, selection

takes place for the linked marker; in a later step, the presence of the phenotype is then verified. This procedure enables the selection process to proceed in the absence of conditions promoting the phenotype for example, low heritability or absence of the pathogen or pest; (Collard and Mackill, 2008) or when phenotypic screening is cumbersome for example, salt tolerance (Ashraf and Foolad, 2013).

While MAS does not replace phenotypic selection, it is an adjunct tool that had been used to facilitate and accelerate breeding, for example in common bean (*Phaseolus vulgaris*). In this crop, MAS has been used mainly to introduce and combine genes of resistance to various fungal, viral, and bacterial diseases (Kelly et al., 2003; Miklas et al., 2006; Duncan et al., 2012; Tryphone et al., 2013; Souza et al., 2014; Meziadi et al., 2016). These diseases are one of the major causes of yield reductions in the bean production regions throughout the world (Schwartz and Pastor-Corrales, 1989; Wortmann et al., 1998). MAS has been used recently in the development of improved common bean germplasm in the U.S. (Singh and Miklas, 2015), Canada (Durham et al., 2013), Brazil (Costa et al., 2010; Souza et al., 2014) and Tanzania (Tryphone et al., 2013).

Common bean is the economically most important domesticated species of the genus *Phaseolus* (Gepts et al., 2008). It originated from two geographically separate domestications, from an already diverged wild ancestor (Gepts et al., 1986). Numerous molecular investigations have confirmed the existence of two geographically diverged gene pools: Andean and Mesoamerican, most recently based on SSR data (Kwak and Gepts, 2009; Okii et al., 2014), SNP data (Cortés et al., 2011; Ariani et al., 2016), and DNA sequences (Schmutz et al., 2014; Vlasova et al., 2016; Rendón-Anaya et al., 2017). A consequence of this diversity organization is that genetic distances within each of the two gene pools are considerably smaller than between gene pools. Hence, it is more difficult to detect polymorphisms within the gene pools. Yet, the sharply increased availability of DNA sequences since the introduction of next-generation sequencing (NGS) and the existence of the PhaseolusGenes genetic marker database make the identification of new markers possible as is illustrated in this article.

The existence of two major geographic gene pools in the common bean host plant is mirrored in the genetic diversity and virulence pattern of some pathogens, including angular leaf spot (*Pseudocercospora griseola)* (Guzmán et al., 1995), anthracnose (*Colletotrichum lindemuthianum)* (Geffroy et al., 1999, 2000), and rust (*Uromyces appendiculatus*) (Araya et al., 2004). These pathogens also have Andean and Mesoamerican gene pools, each of which tends to be more virulent on the bean host from the same geographic area. Thus, breeders seeking to obtain resistance for the Andean gene pool tend to use this resistance in the Mesoamerican gene pool and vice-versa.

Several common bean accessions had been identified from Andean and Mesoamerican gene pools and dominant, monogenic, loci conferring qualitative ALS resistance to specific pathogen races have been mapped in the bean genome. The *Phg-1* locus, identified in AND277, is located on chromosome 1 at a distance of 1.3 cM from marker TGA1.1 (Queiroz et al., 2004; Gonçalves-Vidigal et al., 2011). Mesoamerican resistance locus, *Phg-2*, mapped qualitatively in Mexico 54 is located on chromosome 8 at a distance of 5.9 and 11.8 cM from markers SN02 and OPE04, respectively (Sartorato et al., 2000). The Mesoamerican ALS resistance locus, *Phg-3*, mapped in accession Ouro Negro, is linked to marker g2303 at a distance of 0 cM (Gonçalves-Vidigal et al., 2013). Two dominant, monogenic, resistance loci were also identified in G10909: $Phg_{G10909A}$ and $Phg_{G10909B}$ (Mahuku et al., 2011). These loci are inherited independently from all other known resistance genes, conferring resistance to ALS race 63-63, which overcomes all other known resistance sources. Quantitative trait locus (QTL) mapping had also been used to identify multiple resistance loci in Andean germplasm. Seven QTLs on five chromosomes were identified in a IAC-UNA × CAL 143 recombinant inbred population and named ALS2.1, ALS3.1, ALS4.1, ALS4.2, ALS5.1, ALS5.2 and ALS10.1 (Oblessuc et al., 2012, 2013, 2015). Additionally, three major R genes identified in Andean G5686 (Phg_{G5686A}, Phg_{G5686B} and Phg_{G5686C}) (Mahuku et al., 2009) were later treated as quantitative loci by Keller et al. (2015) and mapped to the same loci as ALS4.1, ALS4.2 and ALS9.1 (Oblessuc et al., 2012; Keller et al., 2015).

The transfer of resistance genes can be facilitated by MAS. Until recently, one of the constraints to marker-assisted selection was the availability of tightly linked DNA sequences from which markers could be developed. The identification of linked RAPD markers and their subsequent conversion into SCAR markers (Adam-Blondon et al., 1994; Melotto et al., 1996; Johnson et al., 1997), while effective in the long run, was slow and generally provided only dominant markers.

This situation has changed markedly with increasing availability of genome sequences, especially whole-genome sequences arranged in pseudomolecules for

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each chromosome and anchored on a dense genetic map (Morrell et al., 2012). In common bean, two whole genome sequences are available, corresponding to the Andean (accession G19833; Schmutz et al. 2014) and Mesoamerican (accession BAT93; Vlasova et al., 2016) gene pools. The Andean whole-genome sequence has been used as the current backbone for a "bean breeder' toolbox", that is, a marker database – Phaseolus Genes (http://phaseolusgenes.bioinformatics.ucdavis.edu), which combines information from both legacy molecular markers and more recent markers resulting from genomic analysis of the common bean genome. Of importance is the anchoring of markers in their respective chromosomal locations via a genome browser (http://abcgb.genomecenter.ucdavis.edu), thus allowing the identification of tightly linked markers in specific regions of the bean genome.

Angular leaf spot is a major driver of common bean yield reduction in Central and South America as well as East Africa, necessitating the need to develop markers linked to resistance genes, which can be used during breeding (Schwartz and Pastor-Corrales, 1989).

In this study, the main goal was to identify new marker alternatives to the SN02 marker tagging the *Phg-2* locus on chromosome Pv08, which confers resistance to Angular Leaf Spot (Nietsche et al., 2000; Sartorato et al., 2000). The SN02 marker is monomorphic in certain crosses, requiring the search for alternative markers as detailed subsequently. The first objective was, therefore, to conduct an *in silico* study in the PhaseolusGenes database to identify tightly linked potential markers to replace SN02. The second objective was to test these potential markers for their suitability across a representative sample of bean lines, including target varieties and disease resistance donors, both at UC Davis and in collaborating laboratories in East Africa. Thirdly, the study sought to demonstrate that the new marker identified was indeed linked to the disease resistance locus, *Phg-2*. In spite of the challenges, mainly the need to identify polymorphisms among evolutionarily close lines, the study is able to identify a new marker exhibiting a robust, easy to score polymorphism and tightly linked to the *Phg-2* resistance gene.

MATERIALS AND METHODS

Plant materials

The 16 parental materials analyzed in this study are part of the breeding projects of the African Bean Consortium (ABC). This consortium groups several breeding projects with a common goal, namely to develop a multiple-disease-resistant version of a local variety that is popular with farmers and consumers (target or preferred variety). Table 1 shows different ABC target varieties and sources of resistance. Seeds of the 16 parental materials used inhybridizations to develop multiple-disease-resistant improved target varieties were obtained from the respective breeding programs and were imported into the U.S. under USDA APHIS Small Seed Quantities permit no. P37-16-00357.

Marker segregation from *Phg-2* locus

After putative alternative markers were identified by PCR amplification of the 16 ABC breeding parents, a F_2 segregating population between a *P. griseola*-susceptible French bean Amy and the resistant parent Mexico 54 was used to confirm the linkage between the g796 marker and the *Phg-2* locus. Amy is a commercially preferred French bean (that is, green bean) genotype with white seeds and round, straight, smooth pods; it is susceptible to ALS. The Mexico 54 parent possesses known resistance to most *P. griseola* races in East Africa; however, it is unadapted to dual growing cycles in Kenya as it only gives flowers under short days. Mexico 54 has medium-sized, pink seeds and is a landrace from Southern Mexico, near the border with Guatemala (CIAT germplasm accession G2595, collected at 16.1167º N. Lat., -92.05º W. Long.). A total of 100 F_2 progeny were genotyped with the g796 STS marker and phenotyped with ALS race 63-39, which was identified by inoculating 12 ALS differential cultivars in triplicate (Pastor-Corrales et al., 1998). Race 63-39 was collected from diseased bean leaves at University of Embu, Kenya and inoculum was produced by isolating spores from single fungal synnemata, grown on V8 medium for 12 days (CIAT, 1987) before inoculating the Amy x Mexico 54 F_2 population. Inoculation of the F_2 population took place in a screenhouse at the University of Embu. Following inoculation, plants were placed underneath screenhouse benches, which were covered with vapor-resistant plastic to maintain relative humidity at 90 to 100% for three days. The temperature during infection and disease development was approximately 20 to 22°C. The phenotypic data for ALS infection with race 63-39 were taken as scores on a 1 to 9 CIAT scale, whereby plants with a disease score of 1 to 3 were considered resistant and those with scores of 4 to 9 were susceptible (CIAT, 1987).

Amy X Mexico 54 F_2 marker genotyping

Leaf tissue from fully expanded first trifoliate leaves were harvested from 100 F_2 plants and their parents, Mexico 54 and Amy, and used for DNA extraction. The harvested leaves were crushed to form a paste and placed on a Whatman FTA® plant card (Sigma Z719730). The cards were air dried at room temperature (20-25°C) for one and half hour then washed with FTA purification reagent as per the manufacturer's procedure. Ten 2 mm FTA® discs were obtained from each sample using a Harris® core punch and placed in 1.5 ml micro-centrifuge tubes. Five hundred microliters of FTA® purification reagent was added to the discs, and incubated at room temperature for five minutes with moderate mixing. The procedure was repeated twice. The discs were washed twice with 500 µl TE before drying for 1.5 h at room temperature. DNA obtained from the discs was adjusted to a concentration of 10 to 50 µg/ul prior to its usage for PCR. The g796 STS marker was amplified from DNA of the 100 F_2 lines and parents (Amy and Mexico 54) to test the linkage relationship between the marker and *Phg-2* resistance locus. PCR amplification of the g796 marker was done using puReTaq ready to go PCR beads®. The PCR reaction was prepared by adding the following to lyophilized PCR beads to make a final volume of 25 µl: 1 µl of each forward and reverse primers (g796 F – 5' GAGAAACTACGGGCTGTTTTACCC 3', g796 R2 – 5' AGTTAAGACCGTTCTGAAGCTTC 3'), 22 µl of sterile water and 1 µl of DNA at concentration of 10 to 50 µg/ul. Marker PCR amplification was performed in an Eppendorf Thermal Cycler® with initial DNA denaturation at 94°C for 3 min followed by 35 cycles of

Table 1. African bean consortium target varieties and sources of disease resistance included in this study.

Target parents to be improved			
Name	**Gene pool**	**Country**	**Attributes**
Kablanketi	Andean	Tanzania	Dry bean; most valuable market class in Tanzania (Mishili et al. 2011); climbing type grown often as bush bean; short cooking time; purple-speckled over cream colored seeds
NABE12 C	Andean	Uganda	Dry bean; climbing type for S.W. Uganda; sugar type (red streaks over cream colored seed
NABE13	Andean	Uganda	Dry bean; bush bean
NABE14	Andean	Uganda	Dry bean; bush bean
Red Wolayta	Mesoamerican	Ethiopia	Dry bean; bush bean
Ibaddo	Andean	Ethiopia	Dry bean; bush bean
Hawassa-Dume	Mesoamerican	Ethiopia	Dry bean; bush bean
Gasilida	Andean	Rwanda	Dry bean; climbing bean
RWV3006	Mesoamerican	Rwanda	Dry bean; climbing bean
Amy	Andean	Kenya	Green bean (that is, French bean) with superior pod quality traits; bush bean
Donor parents			
Name	**Gene pool**		**Disease resistance[1,2] (Genes; Source reference)**
Mexico 54	Mesoamerican		ALS (*Phg-2*; Sartorato et al., 2000)
G2333	Mesoamerican		ANT (*Co-4*^{2}, *Co-5*, *Co-7*; Young et al., 1998)
RWR719	Mesoamerican		Root Rot (*Pythium* spp.) (Nzungize et al., 2011)
VAX3 VAX4 VAX6	Mesoamerican, with introgression from *Phaseolus acutifolius*		CBB (SAP6, SU91; Singh and Muñoz, 1999; Singh et al., 2001; Singh and Miklas, 2015)
Mshindi	Andean		BCMV (*bc-1*^{2}; Nchimbi-Msolla et al., 2008)

1ANT, Anthracnose (C*olletotrichum lindemuthianum);* ALS, Angular leaf spot (P*seudocercospora griseola); B*CMV, Bean Common Mosaic Virus; CBB, Common bacterial blight (X*anthomonas axonopodis);* 2In parentheses, resistance genes targeted by marker-assisted selection.

melting at 94°C for 15 s, primer annealing at 52°C for 20 s, DNA polymerization at 68°C for 20 s, and a final extension at 68ºC for 5 min. After 35 cycles, the amplification products were separated by gel electrophoresis on a 2% (w/v) agarose (Thermo Scientific TopVision Agarose: R0491) gel, stained with ethidium bromide, and visualized under a standard UV transilluminator and photographed using a mounted digital camera. Following PCR, electrophoresis and visualization of marker amplification products, marker genotypes were scored for the parents and 100 Amy x Mexico 54 F_2 individuals, with PCR bands of size 209 base pairs corresponding to the allele from Amy (ALS susceptible parent) and those of size 233 base pairs corresponding to the marker allele from Mexico 54 (ALS resistant parent).

F_2 phenotyping with *P. griseola* race 63-39

Prior to evaluating the F_2 population for symptoms to ALS inoculation, the ALS isolate was identified as race 63-39 by inoculating spores of the fungus onto the ALS differential set of cultivars (Pastor Corrales et al., 1998). Seventeen days after planting, spores of race 63-39 at a concentration of 2×10^4 were sprayed onto the first fully expanded trifoliolate leaves of each of the F_2 and parental plants. Inoculated plants were kept under screenhouse benches covered in vapor resistant plastic to maintain 100% humidity and a range of 20 to 22°C for three days. Plants were then moved to the benchtop and subsequently evaluated for disease symptoms 18 days post inoculation. Phenotypic evaluation of inoculated plants and uninoculated controls were scored on a 1 to 9 scale (CIAT, 1987) with 1 to 3 considered a resistant reaction and 4 to 9 considered a susceptible reaction. A chi-squared analysis was used to test the independence between the marker and gene controlling the host reaction to race 63-39 with an expected segregation ratio of 3:6:3:1:2:1, consistent with independent assortment between a co-dominant marker locus and a dominant resistance gene.

RESULTS

Identification of candidate alternative markers around the SN02 marker

Markers used for MAS in a breeding program must be polymorphic among the parental genotypes and be

cheaply and reproducibly assayed. The SN02 marker previously used to map the *Phg-2* ALS resistance locus (Sartorato et al., 2000) is monomorphic among some of the 16 breeding parents used in the ABC, necessitating the identification of an alternative polymorphic marker. The PhaseolusGenes marker/QTL database is a compilation of all previously identified marker datasets described in *Phaseolus* species (http://phaseolusgenes.bioinformatics.ucdavis.edu/) and is a resource that the bean breeding community can use to predict the physical genetic locations of markers linked to trait-associated loci.

In a first step, the SN02 marker was searched for on the PhaseolusGenes homepage. The marker sequence provided by the database (obtained by sequencing the SN02 amplicon in the common bean breeding line BAT93; J. Kami and P. Gepts, unpublished results) was then aligned to the G19833 reference sequence (*Phaseolus vulgaris* V1.0, Schmutz et al., 2014) using the blast algorithm (Altschul et al., 1990). The physical location of SN02 in the common bean genome was viewed in the UCSC Genome Browser, as implemented in PhaseolusGenes. The UCSC Genome Browser displays all previously published marker datasets aligned to the reference genome, as well as newly developed markers, such as Indel markers (S. M. Moghaddam and P. McClean, pers. comm.) and SSR-motif-containing sequences. The SN02 marker is physically located on chromosome 08 of the G19833 V1.0 reference genome from position 58,535,516 to 58,536,215 bp.

Using PhaseolusGenes, twenty-two alternative SSR, STS, SCAR, Indel and SNP markers were located with an overall bracket of approximately 2.5 Mbp around the SN02 marker (Table 2). These potential alternative markers were chosen due to their close physical linkage to the SN02 SCAR marker, which was previously found to be linked to the *Phg-2* ALS resistance locus at a genetic distance of 5.9 cM in a biparental population derived from the cross between Rudá and Mexico 54 (Sartorato et al., 2000). The markers included seven STSs, six SSRs, five Indels, four SCARs and one SNP spaced at an average distance of ~107,000 base pairs (Figure 1; Mapchart, Voorrips, 2002)

Amplification and polymorphism of potential markers

The 22 markers and SN02 were first tested for PCR amplification and polymorphisms between the ALS resistant parent, Mexico 54, and the three other parents (NABE12C, RWR719, and G2333) utilized in the ABC Ugandan breeding program. NABE12C is a target variety from the Uganda project: It is a climbing variety with large 'sugar'-type seeds (elongated; cream background with red streaks). RWR719 and G2333 are donors of resistance for *Pythium* root rot and anthracnose, respectively. Subsequently, the 23 markers were also analyzed in all 16 lines included in this study (Table 1).

PCR amplification of the 22 alternative markers as well as the SN02 SCAR marker in the Ugandan breeding parents, NABE12C, RWR719, G2333 and Mexico 54 resulted in identification of 13 markers, robustly amplified in all four parents (Table 2). All PCRs were performed using the same cycling parameters which were: 1. 94°C for 3 min; 30 cycles of 2. 94°C for 15 s, 3.50°C for 30 s, 4.68°C for 30 s and 5.68°C for 7 min. Two Indels, NDSU_IND_8_58.8634 and NDSU_IND_8_58.2730, and 1 sequence-tagged site marker, g796, had visible molecular weight differences between Mexico 54 and the other parents. The g796 marker showed a 24-base-pair insertion only in Mexico 54, which was easily assayable by visualization on a gel. The g796 marker was tested in all 16 ABC breeding parents, confirming that Mexico 54 has an insertion in the marker, whereas the other 15 parents do not (Figure 2). The 24-base-pair insertion in the marker of Mexico 54 confers the addition of eight additional amino acids in the predicted protein. The marker (primers and protocol) had been distributed to projects in Uganda, Tanzania, Kenya, and Ethiopia, where the polymorphism has been reproducibly observed (data not shown).

Analysis of linkage relationship between the g796 Marker and the *Phg-2* resistance locus

A test for independent assortment between the g796 marker and the *Phg-2* ALS resistance locus was performed using an F_2 population consisting of 100 individuals from the cross Amy x Mexico 54. First, of the 100 F_2 individuals phenotyped with race 63-39, 73 had phenotypic scores of 1 to 3 (resistant) and 27 had scores of 4 to 9 (susceptible) (Table 3). The observed number of resistant and susceptible individuals in the F_2 was not significantly different than the 3:1 ratio predicted with a single dominant resistance gene conferring resistance ($\chi^2 = 0.213$, df = 1, p = 0.64). Second, the segregation for the g796 marker was tested for an expected 1:2:1 ratio corresponding to a co-dominant locus. No significant departure from the tested ratio was observed ($\chi^2=3.58$, df = 2, p = 0.17). A third chi-square test was then conducted to check for independent assortment between the co-dominant g796 marker and dominant ALS resistance gene using an expected ratio of 3:6:3:1:2:1 (Table 4). The Chi square test statistic was equal to 70.63 with $p < 0.00001$ (df = 5), indicating the marker and resistance gene are linked on chromosome 08 (Table 3). Three individuals display recombination between the marker and resistance gene, which corresponds to an approximate map distance of 3 cM between the marker

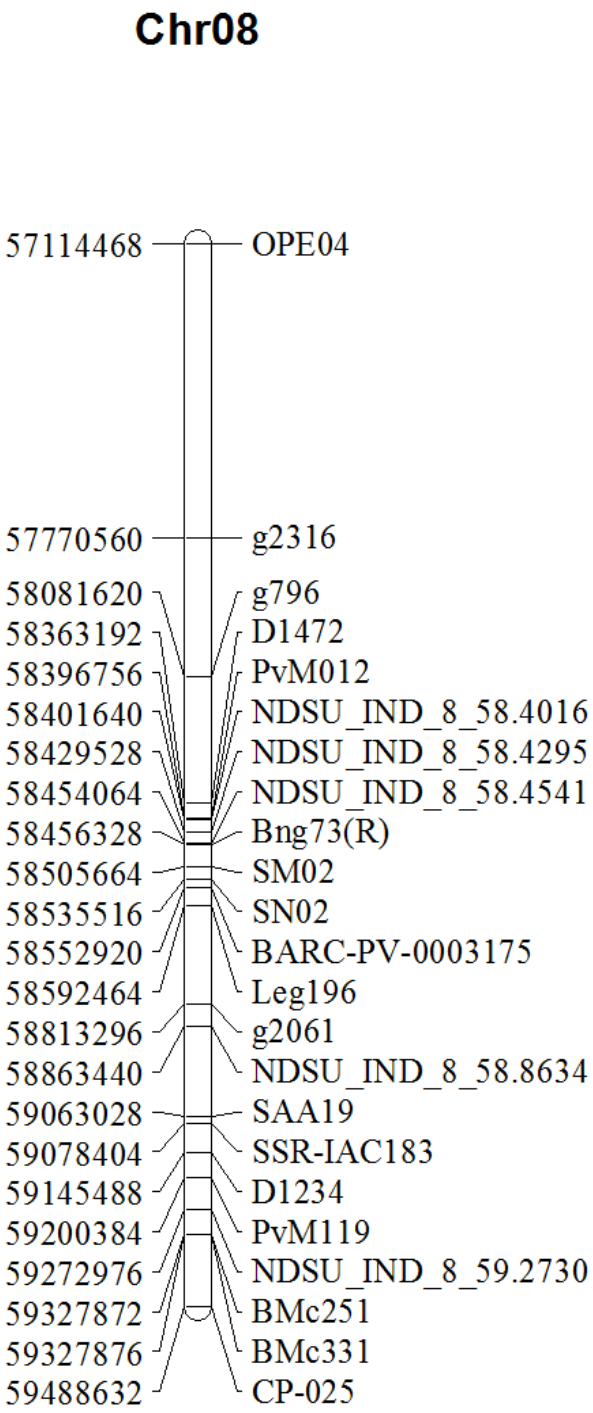

Figure 1. Partial map of chromosome Pv08 of common bean showing twenty-three markers used to screen the Ugandan breeding parents (NABE12C, Mexico 54, RWR719 and G2333) for an alternative to the SN02 marker. On the left of the chromosome are distances expressed in bp according to the G19833 reference map (Schmutz et al., 2014). To the right of the chromosome are the marker sequence names as shown in the PhaseolusGenes gBrowse portal. Markers span a 2.4 Mb region around SN02. More information is given in Table 2.

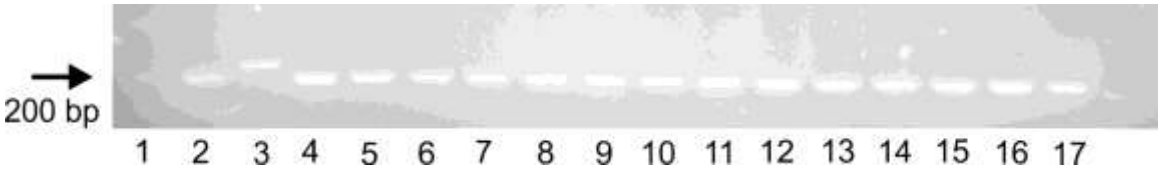

Figure 2. g796 PCR products for 16 ABC parents. Left to right: 1. 1 Kb DNA ladder, 2. NABE12C, 3. Mexico 54, 4. G2333, 5. NABE13, 6. NABE14, 7. Kablanketi, 8. Mshindi, 9. VAX3, 10. VAX4, 11. Ibaddo, 12. HawassaDume, 13. Redwolayta, 14. VAX6, 15. RWR719, 16. Gasilida, 17. TARS-VR-7s (Cultivar Amy is not included in the figure, but shows the same fragment size as the other parents, except Mexico 54).

and gene (3 recombinants / 100 F_2 individuals * 100 = 3 cM).

DISCUSSION

Although the legacy SN02 marker was polymorphic between parents used to identify the *Phg-2* resistance locus (Sartorato et al., 2000), the marker was shown to be monomorphic among the ABC breeding parents from Uganda (Namayanja et al., 2006), and later among all 16 parents, necessitating an alternative marker, which could be used for MAS. A crucial element in the identification of such marker is information on the genetic relationships between parents, which help determine the frequency of polymorphic markers that can be utilized for MAS. In common bean, the well-established divergence between the Andean and Mesoamerican gene pools predicates a preference for Andean x Mesoamerican crosses to maximize polymorphisms at the molecular level (Kwak and Gepts, 2009; Vlasova et al., 2016). However, most crosses in bean breeding often involve parents from the same gene pool or even closely related parents to increase progress from selection and avoid hybrid weakness (Beaver and Osorno, 2009), thereby limiting the potential for polymorphism.

In the ABC project, the multi-parent crosses in each project involve one target (preferred, recurrent) parent and two to three resistance donor parents. Most preferred varieties in each of the projects are of Andean origin, whereas most resistance donors are of Mesoamerican origin. While this situation would seemingly facilitate the identification of markers, polymorphisms should also be identified among the Mesoamerican parents in each project, so that the contribution of each Mesoamerican resistance donor can be monitored in the progenies. The need for polymorphism even within gene pools can be partially be satisfied by the availability of a large number of potential markers as included in the PhaseolusGenes database. It is only such a large number of markers that can withstand the strong attrition imposed by the needs for polymorphism and strong and reproducible amplification, as illustrated in this study. Of the 22 markers selected initially in a region of some ~2.4 Mbp, one - or at most three markers - were found to be suitable as an alternative marker for the SN02 marker because they provided a robust amplification and were polymorphic between the donor parent of the resistance and the three other parents (whether the target recipient or the two other resistant donors).

The PhaseolusGenes marker database has become an essential tool for cataloging bean marker diversity so that the breeding community can utilize marker information from multiple sources to facilitate MAS (Gonçalves-Vidigal et al., 2011, 2013; Reinprecht et al., 2013; Oblessuc et al., 2013, 2015; Aranda et al., 2014; Keller et al., 2015; Sousa et al., 2015; Coimbra-Gonçalves et al., 2016; Perseguini et al., 2016). Because genomic resequencing of breeding parents is today a more routine practice with the lowered cost of sequencing, the PhaseolusGenes database should evolve from a marker database into a sequence database, which includes not only the current markers but also the increasingly large number (> 2,000) of genotypes that have been sequenced, including the 16 parental genotypes of the ABC projects (T. Miller and P. Gepts, unpubl. data).

The development using NGS of markers that are specific to a breeding population has become common practice in crop breeding because the development of reference genome sequences facilitates efficient identification of a large number of physically mapped new markers, as well as different types of markers. *P. vulgaris* reference genomes, based on the Andean landrace G19833 (Chaucha Chuga, Peru), the Mesoamerican breeding line BAT93, and the Mesoamerican variety OAC Rex, introgressed with *P. acutifolius*, have been released recently (Schmutz et al., 2014; Vlasova et al. 2016; http://www.beangenomics.ca/). These resources offer a means (in conjunction with the PhaseolusGenes database and linkage mapping of phenotypic traits by genetic and QTL analyses) to develop new markers for use in MAS and map-based gene isolation. Short genomic sequences for each breeding parent can be mapped to the reference genome and new polymorphisms can be identified, such as SNP, INDELs, or SSR. Moreover, since different breeding programs

Table 2. Twenty-three markers (22 potential alternatives as well as SN02), linked to the *Phg-2* ALS resistance locus on chromosome 8 identified in the *Phaseolus* Genes database (database accessed December 16, 2016).

Marker	Type[a]	Start position (bp)[b]	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplified In	Polymorphic
OPE04	SCAR	57,114,468	TGTGACATGCCAACAACCAC	TGTGACATGCCAACAACC	NMRG	No
g2316	STS	57,770,560	TGAAGGCAATGTTCACAAATATGG	GAGAAACAAGAGTTGGCAACCAAG	NMRG	No
g796	STS	58,081,620	GAGAAACTACGGGCTGTTTTACCC	AGTTAAGACCGTTCTGAAGCTTC	NMRG	Yes
D1472	STS	58,363,192	AGCTTTGCCTCCTGTTGCTA	CACCAATTGATGTGTCGAAAA	NMRG	No
PvM012	SSR	58,396,756	AGCCATTCCCTAAGCCTGTT	CCTTGAACCAAAGGAAACCA	MR	No
NDSU_IND_8_58.4016	InDel	58,401,640	TTATTTGATGGGGGATCCAA	CATCTCTCCAGCATTGCACT	MR	No
NDSU_IND_8_58.4295	InDel	58,429,528	GGGTGTTTTGCAGGTCTT	TCCCCATGATTGTTACCC	NMRG	No
NDSU_IND_8_58.4541	InDel	58,454,064	TCAGGTTGGCAACGGATATT	GGTGCAGTGAATGTGGAAGA	NMRG	No
Bng73 (R)	STS	58,456,328	TTCCCAGTCACGACGTTG	GAATTCGAGCTCGGTACC	none	No
SM02	SCAR	58,505,664	TCTCTTTGCCATCGTTGTTG	AACGCCTCTAAACGGGAGAA	NRG	No
SN02	SCAR	58,535,516	TTTGGAAGTGTGAGCAGTGG	TAGAGGGGCTGAGGACTGAA	NMRG	No
BARC-PV-0003175	SNP	58,552,920	CCGTTGGCATTGGCAATCTGC	AATCTTGGGATGCCTGTCAC	NMRG	No
Leg196	SSR	58,592,464	GTTTTTGCTTGAGCCTCTGC	CTGGTGCGAAGACAATCTGA	NRG	No
g2061	STS	58,813,296	CAAAGCTCTCAATGATCACCATGT	TCAGAAGCAAATGCTTAGACTGTATCA	none	No
NDSU_IND_8_58.8634	InDel	58,863,440	AAATTCTACGGAAATTGAAAGTATAAA	TGCTAGTGAAAGCAAATGACACAGT	NMRG	Yes
SAA19	SCAR	59,063,028	GTCGGGCCAAACTAACAAAG	CAGGCTGGTGGACCTAAAAA	NMRG	No
SSR-IAC183	SSR	59,078,404	TACGCGTGGACTATCCCTCT	GTGACCATCATCTTCGAGCA	MR	No
D1234	STS	59,145,488	TGTTGCAGTGCTCTTCTGCT	TGCTAGTGTTTAAGTGTGTGCCTA	NMG	No
PvM119	SSR	59,200,384	GGGGAGGATAAACCAGAAGG	GTGTAATCCCCTCCAGCAAA	none	No
NDSU_IND_8_59.2730	InDel	59,272,976	CCAGAATGGGAAACAGGATG	AGAGTGAGTTTGGCAGCCAT	NMRG	Yes
BMc251	SSR	59,327,872	TTCAAGGAGGACGTTTGGTC	CATTAACCCCAGCTTTCTCC	none	No
BMc331	SSR	59,327,876	AGGAGGACGTTTGGTCCAG	CCCAGCCTTCTCCCAAAC	NMRG	No
CP-025	STS	59,488,632	GACTTTGGCCTCTCCGTTTTCTTC	ATCTATATGTCCCTTCAATACTGC	NMRG	No
Total genomic range (bp)		2,374,164				

[a] Indel, Insertion-deletion; SCAR: Sequence-characterized amplified region; SNP,Single-nucleotide polymorphism; STS, Sequence-tagged site; bThe starting map position of each marker on chromosome 08 is based on the G19833 Andean whole-genome reference sequence V1.0 (Schmutz et al., 2014) as implemented in the PhaseolusGenes database (phaseolusgenes.bioinformatics.ucdavis.edu; accessed 16 December 2016). Markers were screened for polymorphism between breeding parents NABE12C (N), Mexico 54 (M), RWR719 (R), and G2333 (G). Polymorphic sequences are denoted as those that showed a visible molecular weight difference between Mexico 54 (M) and the other three parents in the Ugandan breeding program (N, NABE12 C; R, RWR719; G, G2333). After confirming the g796 marker was polymorphic between the Ugandan breeding parents, the total 16 parents used in the ABC breeding programs were screened to confirm the polymorphism.

Table 3. F_2 of Amy x Mexico 54 phenotypic and genotypic counts.

Phenotype	n	*g796* genotype		
		$R^{Mex54}r^{Amy}$	$R^{Mex54}R^{Mex54}$	$r^{Amy}r^{Amy}$
Total	χ^2 = 3.58 and 2 d.f., ns	43	33	24
R	73	40	33	0
S	27	3	0	24
χ^2 = 0.21 and 1 d.f., n.s.				

Table 4. Test of independence between marker g796 (A, segregating in co-dominant fashion according to a 1:2:1 ratio) and resistance to *Pseudocercospora griseola* race 63-39 due to *Phg-2* (B, segregating in dominant fashion, 3:1 ratio) in the Amy x Mexico 54 F_2 population.

Class	Genotype	Observed	Expected	χ^2
3	$A^{Mex54}A^{Mex54}B^{Mex54}B^{Mex54}$ $A^{Mex54}A^{Mex54}B^{Mex54}b^{Amy}$	33	18.75	10.83
6	$A^{Mex54}a^{Amy}B^{Mex54}B^{Mex54}$ $A^{Mex54}a^{Amy}B^{Mex54}b^{Amy}$	40	37.50	0.17
3	$a^{Amy}a^{Amy}B^{Mex54}B^{Mex54}$ $a^{Amy}a^{Amy}B^{Mex54}b^{Amy}$	0	18.75	1.0
1	$A^{Mex54}A^{Mex54}b^{Amy}b^{Amy}$	0	6.25	1.0
2	$A^{Mex54}a^{Amy}b^{Amy}b^{Amy}$	3	12.50	7.22
1	$a^{Amy}a^{Amy}b^{Amy}b^{Amy}$	24	6.25	50.41
	Total	**100**	**100**	**70.63**

$P_{df=5}$<0.00001.

have different technologies available to them to detect markers, the development of gel-based, size-polymorphism markers, which are the type routinely used by the ABC breeding programs, can be achieved. Alternative polymorphic markers are useful not only for the selection of loci influencing a specific trait (foreground selection), but also for identifying the genetic composition of recombined individuals after selection (background selection).

Newly developed varieties result from recombination of the genomes of the selected breeding parents Using information about polymorphism present among the parents can allow breeders in the ABC programs to characterize the genetic makeup of the varieties they release into a seed system to estimate their impact. Besides the utility of using polymorphic markers to pyramid multiple genes into a single genetic background (foreground selection), background genomic selection in backcross breeding can be used to select genetic recombinants with the largest amount of their genetic makeup derived from the recurrent parent. Furthermore, improved cultivars with known introgression alleles can be genotyped to verify the identity of an improved cultivar based on its specific genetic signature, or for further improvement during breeding.

The procedure followed here demonstrates the steps to follow to identify new polymorphic markers useful for MAS and the utility of the PhaseolusGenes marker database in a breeding program that seeks to introgress several known resistance alleles into a single genetic background. Furthermore, the combined genomic and transcriptomic resources that are now available to the bean breeding community allow for the identification of genes functionally related to particular traits, such as resistance to ALS. By using the physical location of known markers within annotated genes in reference genome sequences, genes with different putative functions can be identified based on their homology to proteins with known function in other species, such as soybean or Arabidopsis. Polymorphisms identified in annotated genes can then be tested for their association to a particular trait of interest using a population segregating for the trait or with methods such as Virus Induced Gene Silencing to transcriptionally silence

the candidate gene. Overall, the bean community should seek to systematically compile and catalog the corresponding genetic and phenotypic diversity available in *Phaseolus* beans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ABC, African Bean Consortium; **ALS,** angular leaf spot; **BLAST,** basic local alignment search tool; **InDel,** insertion-deletion; **MAS,** marker-assisted selection; **PAMP,** pathogen associated molecular pattern; **PCR,** polymerase chain reaction; **PTI,** PAMP triggered immunity; **RAPD,** random amplification of polymorphic DNA; **SCAR,** sequence-characterized amplified fragment; **SNP,** single-nucleotide polymorphism; **SSR,** simple sequence repeat; **STS,** sequence-tagged site.

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