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African Journal of Biotechnology

Table of Contents: Volume 12 Number 45, 6 November, 2013

ARTICLES

Research Articles

Identification of receptor like kinase genes in coconut and development of a marker for validation of breeding materials resistant to a phytoplasma disease in Ghana
Swarbrick, P. J., Yankey, E. N., Nipah, J. O., Quaicoe, R. and Dickinson, M. J.

Molecular diversity among Turkish oaks (QUERCUS) using random amplified polymorphic DNA (RAPD) analysis
Yılmaz Aykut, Uslu Emel and Babaç M. Tekin

Heterosis expression, interrelationship, direct and indirect effects of component characters on yield in intervarietal crosses of eggplant
S. Ramesh Kumar, T. Arumugam, C. R. Anandakumar, S. Balakrishnan and D. S. Rajavel

Detection of *Acidovorax avenae subsp. avenae* in commercial corn seeds and its correlation with seedling transmission
Krittidetch Anan, Wilawan Chuaboon and Dusit Athinuwat

In vitro comparism of the extracellular secretion of inulosucrase enzyme in potential probiotic *Escherichia coli* 16 and BL-21
Prasant Kumar, Sriram Garg Gopalakrishnan and Naresh Kumar, G

Cloning and characterization of a thermostable and alkaline fibrinolytic enzyme from a soil metagenome
Sun-Yi Lee†, Sun-Nyoung Yu†, Hak-Jong Choi, Kwang-Youn Kim, Sang-Hun Kim, Yong-Lark Choi, Cheol-Min Kim, and Soon-Cheol Ahn

Effects of red monascal rice supplementation on growth, digestive function and oocyte maturation in Siamese fighting fish (Betta splendens Regan, 1910)
Karun Thongprajukaew, Uthaiwan Kovitvadhi, Pisamai Somsueb and Satit Kovitvadhi

Table of Contents: Volume 12 Number 45, 6 November, 2013

Antifungal activity of epithelial secretions from selected frog species of South Africa
D. R. Katerere, A. Dawood, A. J. Esterhuyse, H. F. Vismer and T. Govender

Oil extract from *Gongronema latifolium* leaves exhibit anti-diabetic and anti-ulcer activities
Ezekwe, C. I., Nwodo, O. F. C. and Ezea, S. C.

Population structure and genetic diversity of Sudanese native chickens
Mohy eldein Adam Berima, Ibrahim Abdelsalam Yousif, Herwin Eding, Steffen Weigend and Hassan Hussein Musa

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African Journal of Biotechnology

Full Length Research Paper

Identification of receptor like kinase genes in coconut and development of a marker for validation of breeding materials resistant to a phytoplasma disease in Ghana

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Cape St. Paul Wilt Disease (CSPWD) is a major factor that impacts on coconut productivity in Ghana. Novel markers that might be specific for a promising variety of coconut or that could validate the efficacy of crosses would be valuable for confidence in the identity of palms. This study couples the discovery of such a marker with a high throughput genotyping system based on high resolution melt curve analysis. Using oligonucleotides designed against kinase subdomains of receptor like kinases (RLKs) of other plant species, eight putative RLK genes were isolated from coconut, and the intron sequence of one of these analysed in more detail. Three single nucleotide polymorphisms (SNPs) were identified within this intron that could be used as a tractable marker to differentiate two distinct genotypes, and which could be differentiated using high resolution melt curve analysis. Analysis of different varieties of coconut used in the breeding programme included promising hybrids such as Sri Lanka Green Dwarf x Vanuatu Tall. F1 crosses between these palms had been self pollinated to generate F2 populations. Genotyping of palms at the RLK marker suggested that some F2 offspring of parent F1 palms may have been sired via cross pollination from neighbouring palms, a possibility that would bear significance for such breeding programmes.

Key words: Coconut, genetic markers, receptor-like kinases, high resolution melt curve analysis, single nucleotide polymorphisms (SNPs).

INTRODUCTION

Coconut (*Cocos nucifera* L.; Arecaeae) is an important crop in coastal tropical areas where it supports the livelihoods of many poor people and helps sustain the environment. It can be grown (with minimal capital outlay) in poor soils where no other crops would survive, and is a source of material for food, drink and shelter, providing essential nutrients and also potential income. Twelve million hectares of coconut are grown worldwide and 96% of the farmers are smallholders, tending less than four hectares (Eden-Green, 1999; Dery et al., 2005). A major factor that impacts on coconut productivity in Ghana and throughout Africa, as well as in the Caribbean and

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Abbreviations: LYD, Lethal yellowing disease; **CSPWD,** Cape St Paul wilt disease; **LY,** lethal yellowing; **MYD,** Malayan dwarf; **PNT,** Panama tall; **VTT,** Vanuatu tall; **SGD,** Sri Lanka Green Dwarf; **SSRs,** small sequence repeats; **WAT,** West Africa tall; **CTAB,** cetyl trimethyl ammonium bromide; **BLAST,** Basic local alignment search tool; **PCR,** polymerase chain reaction; **NCBI,** National Center for Biotechnology Information; **ESTs**, expressed sequence tags

Central America, is disease and in particular the Lethal Yellowing (LY) Like diseases caused by phytoplasmas. The symptoms of the disease are characterised by premature fruit drop and blackening of new inflorescences followed by yellowing of the leaves until the crown dies to result in bare trucks. Similar diseases in Africa are referred to as Lethal yellowing like because the phytoplasmas involved are different strains. In Ghana, the disease was first noted in 1932 and is referred to locally as Cape St Paul wilt disease (CSPWD). The disease has since spread westward and the disease front is now close to the Côte d'Ivoire border. Millions of trees have died and LY / LYDs are regarded as the most significant factors impacting on coconut production worldwide (Oropeza et al., 2005). Lethal Yellowing Disease (LYD) is currently going through a second epiphytotic in the Caribbean, in particular in Jamaica, where two thirds of palms (over three million) have been killed because the resistance in the Malayan yellow dwarf (MYD) and MayPan hybrids that were introduced to combat the disease following the first epiphytotic in the early 1970s has broken down. Past breakdown of resistance in coconut breeding programmes against similar diseases have been attributed to pathogen evolution (Broschat et al., 2002), but could also occur through impurities or errors in breeding strategies. In Jamaica, heavy losses of MayPan hybrid palms occurred due to Lethal Yellowing despite them being planted extensively to control spread of the disease. Their failure has been partly explained by the genetic contamination of the Panama tall (PNT), the pollen parent, with pollen from the susceptible Jamaican Tall ecotype and a large percentage of off-types observed in the MYD mother palms (Broschat et al., 2002; Baudouin et al., 2008; Lebrun et al., 2008).

Replanting with resistant palms has proved to be the most effective means to deal with LY/LYD, and certainly in Ghana farmers have been prepared to take the risk of replanting with varieties that are not guaranteed, such is the demand for coconut (Eden-Green, 1999). In the Caribbean, the use of MYD and MYD hybrids (MayPans), had been largely effective until the recent epiphytotic. In Africa, where different strains of phytoplasma occur, different sources of resistance are required. In Ghana's coconut breeding programme, screening and selection for resistance to CSPWD has led to the identification of two promising hybrids. The MYDx Vanuatu tall (VTT) hybrid was thought to be promising for resistance to CSPWD disease, but resistance seems to have been either broken down or compromised. The Sri Lanka Green Dwarf (SGD) xVTT hybrid is now believed to be more promising (Dery et al., 2008), and a population of SGDxVTT F2s has been developed and is being screened for resistance to the disease.

In Ghana, the materials used in the breeding programs have not been characterised and it is expedient to develop a reliable set of molecular markers and techniques that can be used to discriminate between the different varieties. In a disease control context, it is essential that hybrids or supposed resistant/tolerant materials distributed to farmers are exactly what they were claimed to be and this requires characterisation of the breeding materials. Thus, the need to ensure genetic purity in breeding programs is important and this requires having the appropriate tools such as molecular markers for cultivar identification. Use of conserved regions to clone novel genes is a valuable strategy for enabling the discovery of novel markers, especially in species with limited molecular resources available. Microsatellites, or small sequence repeats (SSRs) have been used for evaluating population diversity in several continents (Gunn et al., 2011; Liu et al., 2011; Ribeiro et al., 2010).

However, in this work, we attempted to develop an alternative strategy using single nucleotide polymorphisms in specific genes. The conservation of amino acid sequences has enabled the isolation of resistance gene analogues from different species with degenerate primers (Collins et al., 1998; Shen et al., 1998), and these RLK genes were selected for this study because of their known involvement in defence responses to other types of plant pathogens, and potential involvement in defence against phytoplasmas. Similar approaches, such as the use of SNPs based on WRKY gene sequences have previously been developed in cocoa and coconut (Borrone et al., 2004; Mauro-Herrera et al., 2006). Although SNPs are the most abundant markers, the lack of mass coconut genome sequences has hampered their discovery.

The objectives of this study were to couple the discovery of such a marker with a high throughput genotyping system based on high resolution melt curve analysis. This is a powerful tool for detection of polymerphisms, including those that are subtle from small differences in size or single nucleotide polymorphisms. The aim is to find novel markers that might be specific for a variety of coconut, particularly those used in the coconut breeding programme in Ghana.

MATERIALS AND METHODS

Coconut varieties and sampling of plant material

Palms being used in the coconut breeding programme in Ghana were used in this study, at sites in the Western Region of Ghana. The palms included samples of West Africa tall (WAT; 100 palms from site Fasin), VTT from field site Agona Junction, MYD from site Aiyinase, SGD from Bamiankor and Bonsaso, F1 palms of the cross MYDxVTT (from sites Daboase and Agona Junction), F1 palms of SGDxVTT (from an experimental plot at Agona Junction), and F2 palms of selected SGDxVTT F1 palms (Agona Junction) that were self pollinated in 2008 (SGDxVTT F2 palms at an experimental plot at site Asebu, an area known to be a focus for CSPWD). Coconut trunk tissues were collected following the method of Nipah et al. (2007) with slight modifications as follows: A motorised drill fitted with a sterilised drill bit was used to bore a hole of about 10 cm into the trunk of the coconut at a height of about 1 m above ground level; in this process the phloem tissues are chipped out in the form of sawdust. To prevent cross contamination from palm to palm, the

Table 1. Oligonucleotides used in this study for the discovery of RLK sequences or introns downstream of these sequences, amplifying one intron specifically, identifying which RLK sequence this intron was downstream of, and amplification of a small portion of this intron for the purpose of diagnosing the genotype of three SNPs therein.

Oligonucleotide	Sequence (5'-3')	Source	Purpose
RLKF	ATCGGKAARGGCGGMGCKGGRATYGTSTAC	a	Obtaining RLK coding regions
RLKR	GGSGCGATGTAKCCRTARGAGCCAGC	a	
RLKR4c	GCTGGCTCYTAYGGMTACATCGCSCC	Novel	Obtaining introns downstream of coding regions
KIXR1	AARCTRTASACRTCRCTYTTCTCRTC	Novel	
RLKF	ATCGGKAARGGCGGMGCKGGRATYGTSTAC	a	Identifying the RLK coding region the intron lies downstream of coding regions
KIXR1	AARCTRTASACRTCRCTYTTCTCRTC	Novel	
CnRLKintF1	GGTTGTTATTTGGGATTCAAC	Novel	Amplifying most of intron 1 for comparison of varieties
CnRLKintR1	AACGGAAGAGAATAAATTATGACA	Novel	
CnRLKintF2	CCAACTTTAGCTTATTTGTCAAAC	Novel	Melt curve analysis of SNPs in intron
CnRLKintR2	TCTTCTCGTCCACCTTCAG	Novel	

Oligonucleotides denoted 'a' are sourced from Yamamoto and Knap (2001).

drill bits were washed in water, rinsed in 0.5% sodium hypochlorite and flamed to red hot before cooling in ethanol.

DNA extraction

To obtain genomic DNA from the coconut tissues, a 2 mL tube (Starlab, USA) containing about 6-8 glass beads (Sigma, USA) was half filled with coconut tissue and ground in a fastprep™ machine (Thermo electron corporation, Massachusetts, USA) at 6500 rpm for 45 s. Cetyl trimethyl ammonium bromide (CTAB) buffer (700 µL; Doyle and Doyle, 1990) was added to the tissues and homogenised again at 6500 rpm for 45 s. DNA was then extracted using chloroform/iso-amyl alcohol and precipitated with isopropyl alcohol using a protocol of Daire et al. (1997).

Amplification of putative RLKs by polymerase chain reaction (PCR)

For discovery of putative RLK genes from coconut, the oligonucleotides RLKF and RLKR were used (Table 1; Yamamoto and Knap, 2001). DNA from MYD (as an initial reference sample) was amplified as follows. Reactions (25 µl volume) comprised GoTaq reaction buffer, $MgCl_2$ (at a final concentration of 1.5 mM), dNTPs (0.2 mM each), 0.625 units GoTaq DNA Polymerase, oligonucleotides (0.2 µM each), 0.5 µl template DNA. Reactions were carried out using a Flexigene Thermal Cycler (Techne) with an initial denaturation temperature of 94°C for 3 min, followed by 35 cycles of 94°C for 60 s, 50°C for 60 s and 72°C for 3 min, and a final extension of 72°C for 10 min. Products were analysed by agarose gel electrophoresis (1.5% w/v, as compared to Hyperladder II; Starlab, UK). DNA from SGD, VTT and WAT was also used to obtain further sequences. To obtain sequences downstream of oligonucleotide RLKR, the reverse complement of this sequence was used as a new forward oligonucleotide (RLKRc; Table 1) in combination with a novel reverse oligonucleotide (KIXR1; Table 1), which was designed from alignments of the coding sequences of top matches from the Basic Local Alignment Search Tool (BLAST) searching of putative CnRLK sequences (including soybean, vine, rubber and White Campion), with RLK sequences from Arabidopsis thaliana, rice, bamboo and soybean. DNA from MYD was initially amplified using the standard amplification conditions and analysed by agarose gel electrophoresis as described above. Candidate amplicons were PCR purified (using a PCR purification kit, following the manufacturers instructions; Sigma, Poole, UK) or were gel purified (using a gel purification kit; Sigma). Amplicons were cloned and sequenced as described below.

In order to compare the sequence of a putative intron between coconut varieties, oligonucleotides were designed covering most of the sequence (excluding the conserved regions around RLKRc and KIXR1; using oligonucleotides CnRLKintF1 and CnRLKintR1; Table 1). PCR was carried out following the procedure described above, followed by PCR purification and sequencing, or cloning then sequencing. To find out which putative RLK candidate an intron sequence was downstream of, DNA was also amplified with oligonucleotides RLKF and KIXR1, and cloned sequences of the resulting product (which were greater than 1kb) were compared with sequences of putative RLKs and putative introns. Cloning and sequencing of amplicons of this size is less efficient and reliable than smaller fragments (respectively), which is why amplification was done using RLKRc and KIXR1 in the first instance.

Cloning and sequencing of PCR products

Purified fragments were cloned using the pGEM-T easy vector system (Promega), according to the manufacturer's instructions, transformed into *Escherichia coli* competent cells JM109 (Promega Corp.), cultured, and subjected to blue white selection and reculturing. Cloned fragments were then directly reamplified from *E. coli* colonies by PCR using oligonucleotides M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGAC-3'), and assessed by agarose gel electrophoresis to confirm that ligation had been successful. Samples were purified using a PCR purification kit (Sigma), and sequenced by MWG Eurofins using primers M13F and/or M13R. Sequence alignments were made using Clustalw alignment in Bioedit 7.1.3.0 (Hall, 1999). Sequencing output was also assessed manually by examining SCF fluorescence traces, in order to confirm that sequencing data were of good quality and that bases were unambiguous. This was especially important where a candidate SNP was thought to occur, since in the case where a sample may be heterozygous for a SNP, fluorescence traces could overlap at the base position and base calling alone could not be relied upon.

Sequence analysis

Comparison of the sequences of putative RLKs was carried out by

searching for homology with known sequences of RLKs from other species by performing blastx (translated nucleotide) searches of the National Center for Biotechnology Information (NCBI) protein databases. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011). Phylogeny reconstruction was done with neighbour joining using the 'maximum composite likelihood' stochastic model, and a phylogenetic tree constructed. Sequences were also compared to the rice genome using the Rice Genome Annotation Project database (http://rapdblegacy.dna.affrc.go.jp/tools/blast/) in order to determine whether it may be likely that genomic sequences may contain non-coding regions.

High resolution melt curve analysis

For accurate melt curve analysis of products, a small amplicon is required, so oligonucleotides were designed within the putative intron of CnRLK1 flanking the location of putative SNPs (CnRLKintF2; Table 1, CnRLKintR2; Table 1). CnRLKintF2 was designed within the intron to provide specificity, with CnRLKintR2 straddling coding and non-coding sequence (the priority was that the predicted reaction would be efficient, and amplicon small, and these criteria resulted in a reverse oligonucleotide in a conserved region). A PCR product of 227 base pairs in size was predicted and verified using standard PCR conditions and agarose gel electrophoresis described above. Subsequent reactions were carried out on 96 well opaque reaction plates (BioFire Diagnostics, Inc., formerly Idaho Technology, Utah, USA) with a Flexigene Thermal Cycler (Techne), then melt analysis carried out using a LightScanner (BioFire Diagnostics). Reactions (10 µl volume) comprised 5 µl SensiMix HRM buffer (Bioline), 0.5 µl EvaGreen dye, 0.5 µl template DNA, oligonucleotides to a final concentration of 0.5 µM each. Each reaction was overlaid with 15 µl mineral oil, an adhesive optical lid secured and the plate centrifuged briefly. The following PCR conditions were used: 94°C for 10 min, followed by 40 cycles of 94°C for 15 s, 58°C for 10 s and 72°C for 10 s, and a final step of 25°C for 30 s. Following amplification, a melt curve was immediately performed using a LightScanner (BioFire Diagnostics) to produce the melting profiles of amplicons by detecting fluorescence therein, measured in real-time during a programme ramping the temperature from 60 to 90°C at the default Lightscanner melting rate (0.1°C s^{-1}). Fluorescence data were analysed using LightScanner Software according to the manufacturers' instructions, to obtain melt curves that were normalised for comparison to those generated using reference samples of known genotype. Differences between alleles could easily be discriminated by eye (Figure 2c) and were grouped manually for export.

Mapping of palm locations

To accurately represent the location of palms at field site Agona, GPS mapping of the site was conducted. Garmin eTrex hand held GPS units were used to record the perimeters of plots at field site Agona, and record the locations of certain palms. Mapping data were analysed for presentation using GPSMapEdit.

RESULTS

Discovery and characterization of RLK sequences in coconut

With the aim of identifying SNPs or similar markers in genes of coconut varieties, putative receptor-like kinases were obtained. Oligonucleotides RLKF/RLKR (specific for kinase subdomains of an RLK in soybean; Figure 1a) were used to obtain putative RLK sequences from DNA extracted from four varieties of coconut (MYD, SGD, VTT and WAT). An array of clones was obtained, mostly of the expected size of roughly 500-600 bases. From the cloned sequences analysed, sequences for eight putative unique RLKs were obtained that, upon translated BLAST searching of protein sequences in NCBI, were found to have highly significant homology with RLK protein sequences from species including tomato, vine, *Arabidopsis thaliana*, maize and castor oil plant, *Ricinus communis* (Table 2, Figure 1b). Comparisons between nucleotide sequences of each putative RLK obtained independently from each variety failed to reveal differences between them; there were no differences in the sequences of each putative RLK obtained from cloning of sequences from each variety.

Comparison of the sequence of each putative RLK with the rice genome revealed that five had the highest homology to rice kinases that contained large introns immediately downstream of the region of homology. To determine whether an intron might be present downstream of putative RLK genes in coconut, a novel oligonucleotide was designed within the region of kinase subdomain KIX (downstream of the obtained putative RLKs sequences; 'CnKIXR1'), and this was used together with an oligonucelotide in kinase subdomain VII ('CnRLKR4c', the reverse complement of RLKR; Figure 2a) so that if an intron was present in the nucleotide sequences between these two kinase domains, it would be apparent by a large amplicon.

Using these oligonucleotides, DNA was amplified from a reference sample of MYD, generating three amplicons of approximately 400 to 650 bases, which were cloned and sequenced. Apart from the regions containing the oligonucleotides (kinase subdomains VIII and KIX), there was found to be no homology between sequences, and BLAST searching revealed no significant similarities, which suggested they might be non-coding DNA. One sequence was selected for further analysis and oligonucleotides RLKF and KIXR1 were used to verify that the sequence discovered was from within a coconut RLK gene, and determine which putative RLK lied upstream of it. This was found to be immediately downstream of the sequence obtained for CnRLK1, and therefore appeared to be an intron in gene CnRLK1, of 530 bases in size.

Discovery and analysis of a SNP marker in reference palm samples

Comparison of sequences of the apparent intron in CnRLK1 (by sequencing PCR products of primers CnRLKintF1 and CnRLKintR1) from up to eight samples of each variety gave a completely conserved sequence in samples of SGD, MYD and VTT. However, alignments

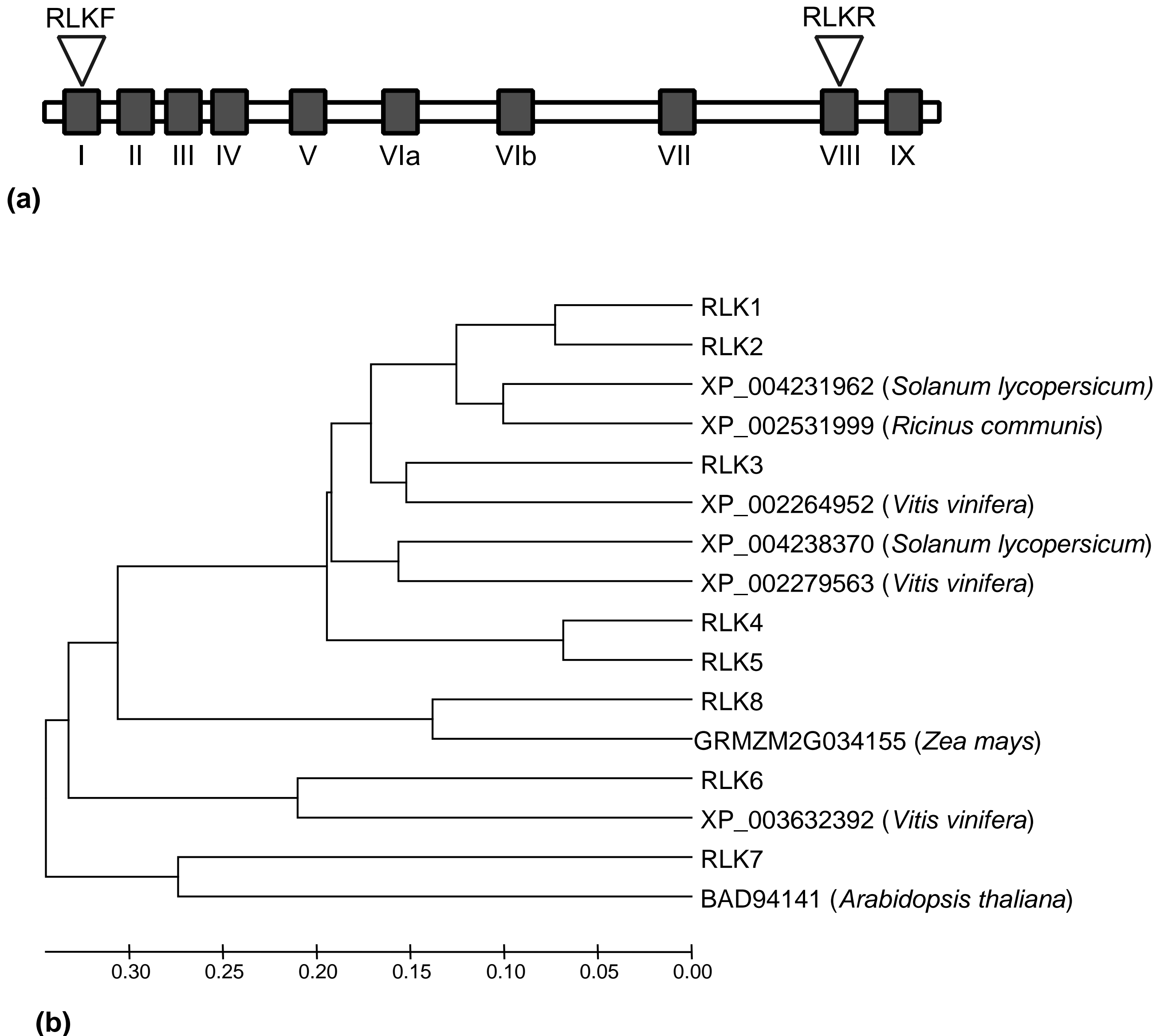

Figure 1. (a) Representation of the protein structure of RLKs from Arabidopsis and soybean, showing the positions of primers RLKF and RLKR, used to isolate coding regions of RLK genes from coconut (after Yamamoto and Knap, 2001, figure not to scale, codons for approximately 200 amino acids lie between the positions of RLKF and RLKR). **(b)** Phylogenetic analysis of the nucleotide sequences of eight unique putative RLK genes isolated from coconut, along with nucleotide sequences with greatest homology to each, from blastx analysis (see Table 2 for details).

showed that sequences from several WAT palms differed from these at three positions: with T, A and A present in positions 460, 491 and 516 in WAT, as opposed to C, G and G in other varieties (Figure 2b). Analysis of sequencing trace data showed that one of the eight WAT samples was from a palm that possessed both the TAA and CGG genotypes, suggestive of a palm that was heterozygous for all three SNPs. This was evident in sequencing trace data, which showed overlapping fluorescence peaks at each SNP (and these peaks were of half the height of surrounding peaks). Cloning of the putative intron in this sample (and two others subsequently identified via melt curve analysis, as described below) showed that sequences cloned from an apparently heterozygous sample were always present as CGG or TAA, not a mixture of polymorphisms at the SNPs, showing that only three genotypes had been found: TAA/TAA, CGG/CGG and TAA/CGG.

A high resolution melt curve analysis method was used to genotype more samples from each site at this marker, with greater throughput than by sequencing. Oligonucleotides were designed that would be specific for the in-

Table 2. BLAST analysis of top protein matches of each of eight unique sequences obtained using oligonucleotides RLKF and RLKR.

Putative RLK*	Top BLAST hit: Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CnRLK1	XP_004231962	Leucine rich repeat receptor-like serine/threonine-protein kinase BAM1-like [*Solanum lycopersicum*]	347	347	99%	3e-110	95%
CnRLK2	XP_002531999	Receptor protein kinase CLAVATA1 precursor, putative [*Ricinus communis*]	338	338	99%	9e-107	95%
CnRLK3	XP_002264952	PREDICTED: Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM3 [*Vitis vinifera*]	313	313	99%	1e-97	87%
CnRLK4	XP_004238370	PREDICTED: receptor protein kinase CLAVATA1-like [*Solanum lycopersicum*]	298	298	100%	3e-92	84%
CnRLK5	XP_002279563	PREDICTED: Receptor protein kinase CLAVATA1-like [*Vitis vinifera*]	308	308	99%	8e-96	87%
CnRLK6	XP_003632392	PREDICTED: leucine-rich repeat receptor-like protein kinase PXL2-like [*Vitis vinifera*]	271	271	99%	7e-82	75%
CnRLK7	BAD94141	Leucine-rich repeat receptor-like kinase At1g09970 [*Arabidopsis thaliana*]	244	244	99%	8e-77	64%
CnRLK8	AFW57819**	Putative leucine-rich repeat receptor protein kinase family protein [*Zea mays*]	306	306	99%	5e-94	82%

Top hit is shown for each putative CnRLK1-8, from translated BLAST (blastx) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=TranslationsandPROGRAM=blastxandBLAST_PROGRAMS=blastxandPAGE_TYPE=BlastSearchandSHOW_DEFAULTS=onandBLAST_SPEC=). *NCBI nucleotide accession numbers are sequentially numbered as follows: CnRLK1-8: KC020611-KC020618. ** Maize Genome Database transcript name GRMZM2G034155_T01: http://maizegdb.org/cgi-bin/displaygenemodelrecord.cgi?id=GRMZM2G034155_P01.

tron, but would amplify a short sequence flanking the three SNPs. With the incorporation of a fluorescent intercalating probe into amplicons, their melting profile was measured in real time following amplification, with higher melting temperature (tm) predicted for samples of CGG, and lower tm expected in samples of TAA. High resolution melting distinguished between genotypes and was found to consistently show three melt curve patterns that were easily distinguishable (Figure 2c). Reactions performed on DNA from palms of known genotype CGG/CGG were found to have the melting profile showing the highest melt temperature. Palms of TAA/TAA genotype clearly showed the lower melting temperature, and reactions from palms shown by sequencing to be heterozygous for TAA and CGG had melt curve profiles intermediate between the other two genotypes. Comparisons of melt curve results and sequencing data, and addition of more samples of each variety, concurred with the initial findings that all tested MYD, SGD and VTT palms were of genotype CGG/CGG (Table 3). Of the 100 WAT palms tested, 97 were TAA and three were heterozygous.

Analysis of hybrid palms at experimental plots

Since the triple-SNP marker discovered in the putative intron of CnRLK1 could discriminate between WAT and other palms, it was decided to test its use for characterising supposed hybrids that were being used on a smallholder farm (MYDxVTT at Daboase) and at experimental trial sites (SGDxVTT at Agona, SGDxVTT F2 palms at Asebu). Using melt curve analyses, genotyping of MYDxVTT palms at Daboase showed that of 82 palms sampled, 50 were CGG/CGG and 32 were TAA/CGG. Melt curve analysis of samples from Agona showed that 47 SGDxVTTs were all of genotype CGG/CGG (Table 3). Another large plot of palms at Agona contains MYDxVTT palms (Figure 3), sixteen of which were genotyped (with 10 being CGG and 6 heterozygous; TAA/CGG; Table 3). The sixteen MYDxVTT palms were taken from the southern edge of the plot, bordering the SGDxVTT plot (Figure 3). Among other palms at the site were three WAT palms of about 30 years old, approximately 20 metres south west of the SGDxVTT plot (Figure 3). These palms were uphill of the plot of SGDxVTTs and separated from them by some mixed vegetation of up to 2.5 metres height. The three WAT palms were found to carry the TAA/TAA genotype.

In 2008, 14 of the SGDxVTT F1 palms at Agona were chosen to be self pollinated for the generation of a population of F2 SGDxVTT palms as part of the coconut breeding programme. The location of these parent palms is shown in Figure 3. The resulting F2 progeny were planted in Asebu later in 2008. In total, approximately 230 palms were planted (on average, 14 F2 palms were obtained

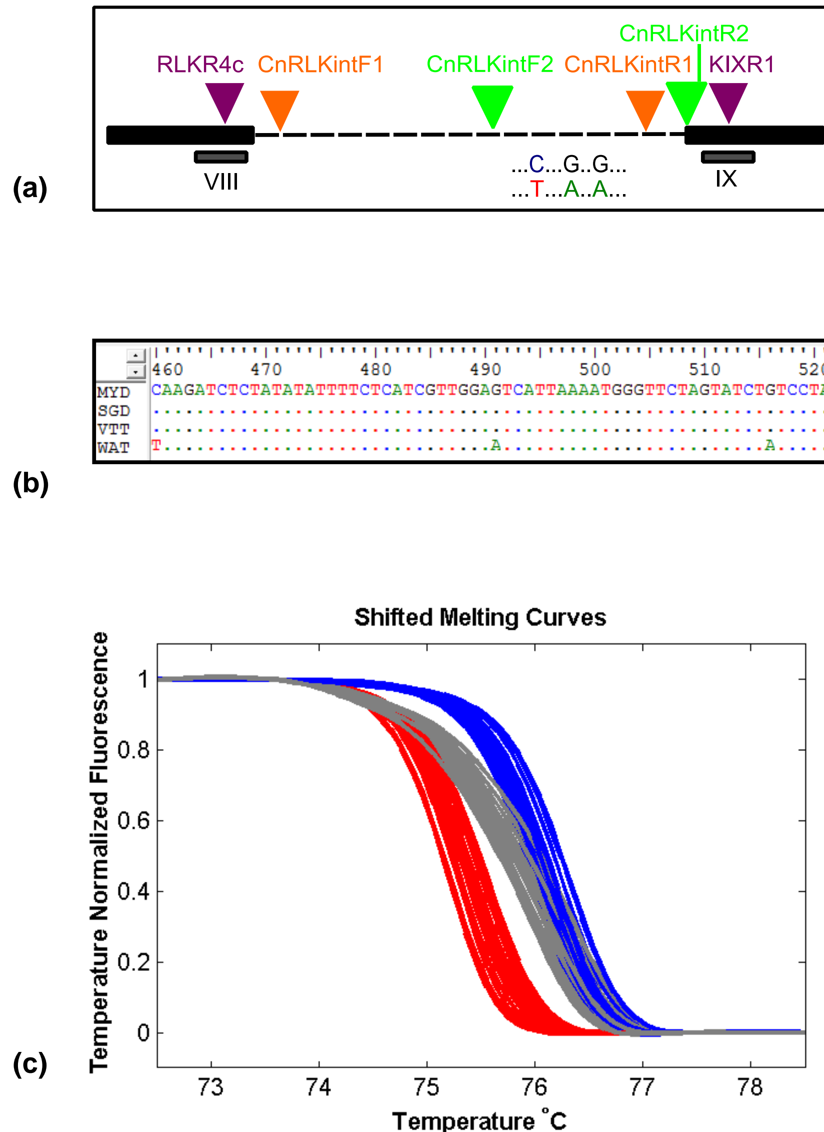

Figure 2. (a). Position of olicognucleotides used to amplify and sequence the intron from CnRLK1 (RLKR4c and KIXR1; coloured purple), and position of oligonucleotides used for specifically amplifying this intron, for comparison between varieties (CnRLKintF1, CnRLKintR1, coloured orange) oligonucleotides used for HRM analyses (CnRLKintF2, CnRLKintR2, coloured green). Black solid bars represent coding regions, dashed line shows intron position and grey boxes indicate the position of kinase subdomains VIII and IX. The approximate position of three SNPs is also indicated. Figure not to scale. (b). Section of sequences of intron CnRLK1 in representative samples of palms of MYD, SGD, VTT and WAT. The position of three SNPs is visible at bases 460, 491 and 516. Sequences containing the intron and partial CDS are presented in GenBank: NCBI nucleotide accession numbers KC020619 and KC020620. (c). Shifted melt curve plots of representative samples from each genotype sampled. Duplicate reactions of 27 extracts are shown, including MYD, SGD, VTT, WAT. TAA/TAA is displayed as red plots, showing the lowest melting temperatures, CGG/CGG is shown as the blue plots (highest melting temperatures) and grey plots show the intermediate melting temperatures of TAA/CGG heterozygous samples.

from each parent palm). Of the 209 palms surviving to produce at least two leaves, sampling and genotyping at the marker in CnRLK1 via melt curve analysis showed that 202 carried the CGG/CGG allele, whereas 7 were found to be heterozygous. The SGDxVTT F2 palms that were found to be heterozygous were sired by SGDxVTT

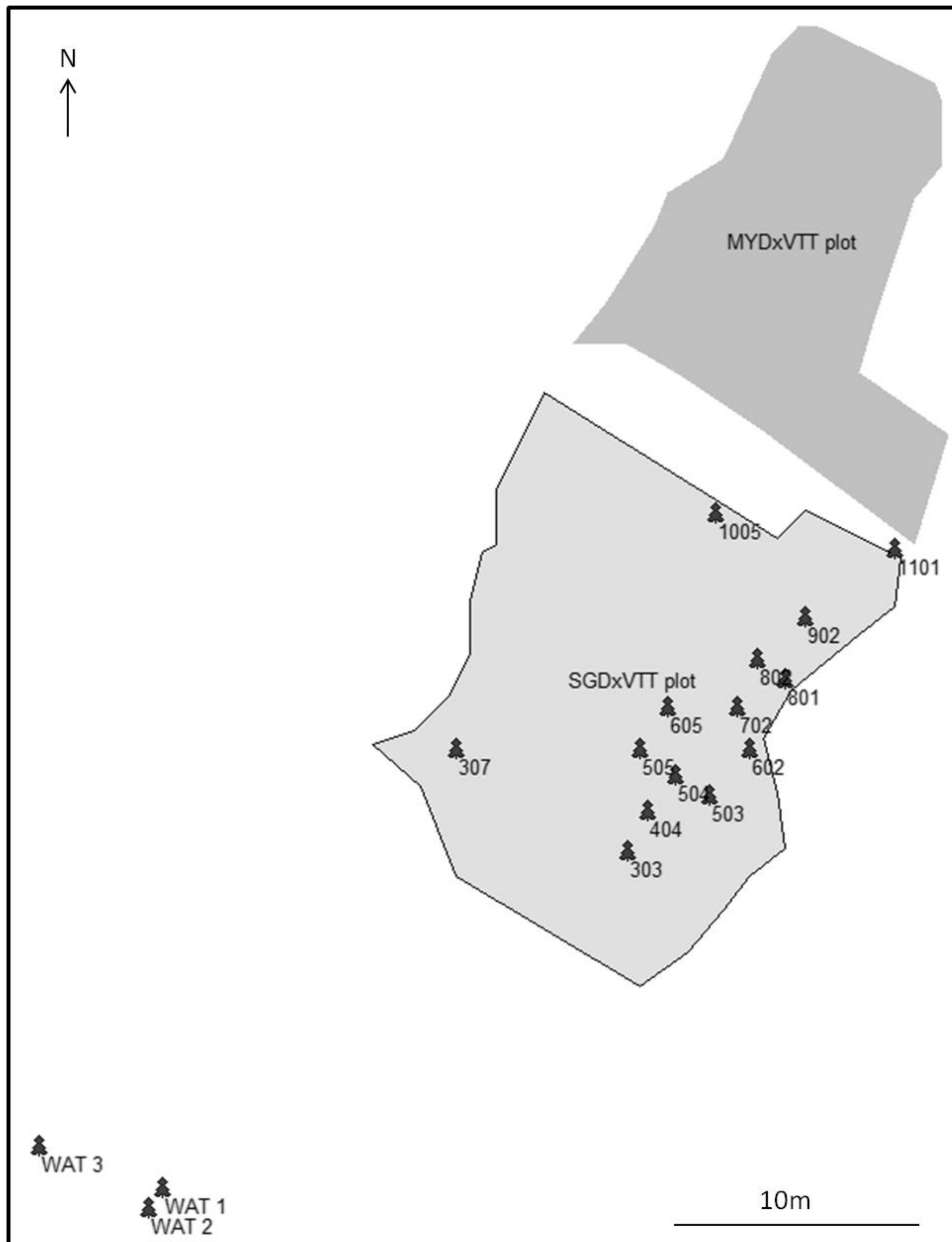

Figure 3. Position of palm plots and experimental palms at field site 'Agona', mapped by GPS. Plots of SGDxVTT and MYDxVTT F1 palms are shown, with the position of those SGDxVTT palms used to generate populations of F2 palms indicated (palms 303-1101). The positions of three WAT palms at this site are also shown. Figure is to scale.

(F1, Agona) palms 303, 307, 702, and 802.

DISCUSSION

This study aimed to find novel markers that might be specific for a variety of coconut, ideal for the development of markers that can allow for the validation of the identity of palms of promising characteristics in their resistance to Cape St. Paul Wilt Disease. Several genes were investigated in order to discover novel markers, following on from analyses of WRKY, ribosomal or plastid genes examined in similar work in coconut and other species (Demesure et al., 1995; Mauro-Herrera et al., 2006;

Table 3. Genotypes of all sampled coconut palms at the marker in the intron of CnRLK1. Genotyping was carried out using high resolution melt curve analysis (oligonucleotides CnRLKintF2/CnRLKintR2).

Variety	n	CGG/CGG	TAA/CGG	TAA/TAA
Reference samples				
MYD	8	8		
SGD	20	20		
VTT*	8	8		
WAT	100		3	97
Daboase				
MYDxVTT	82	50	32	
Agona				
SGDxVTT	47	47		
MYDxVTT	16	10	6	
WAT	3			3
Asebu				
SGDxVTT (F2s)	209	202	7	

* VTT palms also from Agona.

Calvino and Downie, 2007; Meerow et al., 2009). Using oligonucleotides designed against conserved domains of RLKs in other species (Yamamoto and Knap, 2001), this study identified eight putative RLKs in coconut. Whilst the coding sequences identified did not differ between variety, several of these genes were predicted to contain at least one large intron, one of which was analysed in detail and found to contain three SNPs that were present as two different alleles, or in a heterozygous combination. That only two combinations of bases were found at the three SNPs (there were clearly two variants in the population tested since the three SNPs appear as triplets, not a combination thereof) indicates that two versions of the CnRLK1 gene exist in the palms sampled. One allele was found to be characteristic of (though not exclusive to) the Ghanaian West African Tall samples, which are susceptible to Cape St. Paul Wilt disease (CSPWD). Although it is possible that the coconut varieties tested are too heterogeneous to permit discovery of a fully variety-specific marker (as appears likely from the variability visible in studies working with SSR markers in coconut), a simple, tractable maker such as the one described here can be used to great effect in verifying the pedigree of a palm, as compared to its parents.

Introns are excellent candidates for discovery of polymorphisms because they would not cause changes at protein level and so are subject to faster mutation rates. The potential for introns has been studied in intron flanking expressed sequence tags (ESTs) in rice (Tamura et al., 2009), as has the potential for intron length polymorphisms (for example, ILP in resistance gene analogues in wheat; Shang et al., 2010). Use of melt curve analysis in genotyping these markers is becoming increasingly acknowledged for its potential.

The discovery of individuals among the F2 population of SGDxVTT palms that were heterozygous for the marker in the intron of CnRLK1, despite all fourteen parent palms being homozygous for the CGG allele, suggests that cross pollination of some of the parent palms may have occurred. Tall varieties are primarily outbreeding via wind pollination (or potentially pollinated via insects) whereas dwarf varieties of coconut are mostly self-pollinating (Baudouin and Santos, 2005; Ramanatha Rao, 2005). There is a further possibility that those F2 palms found to be CGG/CGG could also have cross pollinated with neighbouring SGDxVTT or MYDxVTT palms at Agona. The discovery of three WAT palms at the Agona site was a further cause for concern, since they were likely to be around thirty years old and may have been releasing pollen when the SGDxVTT parent palms were selected for self pollination. The prevailing winds in the coastal region of Ghana are from the south west making the prospect for pollen spread across the plot of SGDxVTTs feasible in this wind pollinated species. MYDxVTTs were often found to be heterozygous, so could also be a source for the possible contamination detected at Agona. Development and analysis of further markers will establish the extent to which this may have occurred.

The possibility of cross pollination could have implications for the coconut breeding programme since it suggests that at least some palms of the promising SGDxVTT hybrid used for generation of material for screening for resistance to CSPWD may have been com-

promised, and possibly from susceptible palms. However, it should be noted that careful precautions are taken at sites such as these to prevent cross pollination; normally, to prevent extraneous pollen from contaminating the crosses, all palms within 300 metres are castrated since under natural conditions coconut pollen can travel over 300 metres (Mantriratne, 1965; de Nucé de Lamothe and Rognon, 1975; Ramanatha Rao, 2005). Furthermore, while assisted pollination (without precautionary bagging) can result in pollen contamination (unless very large plot sizes are used) (de Nucé de Lamothe and Rognon, 1975; Ramanatha Rao, 2005) the crosses carried out are followed by bagging of tassels. Therefore the occurrence of cross pollination is not confirmed and requires more attention: similar markers will be identified to further investigate this. It is also possible that the purity of the MYDxVTT hybrid palms at Daboase, which were deployed in a farm in an area of disease pressure, may also have been compromised. For example, planting material may have been supplemented with self-seed from local WATs, and it is these WATs rather than the MYDxVTT that have succumbed to the CSPWD. This is also under examination.

The mechanism with which to identify markers that might be used to validate the pedigree of a palm is, at present, valid and valuable in a situation where plant or DNA material from both parent and offspring is available. The collection of eight RLK sequences obtained during this study will be useful for further marker discovery and we are assessing these and introns therein with the aim of identifying further useful markers for the coconut breeding programme, using such high throughput genotyping methods as those described here. Simple genotyping strategies will be valuable in breeding still, especially transferable technologies: melt reactions similar to those carried out in this work can be done on increasing number of machines, such as portable amplification machines including isothermal amplification (for example, using the Optigene Genie II machine). Such work could be carried out in-country on portable, affordable machines, and we are assessing the feasibility of such an application.

Ultimately it is hoped that, since these palms are planted in the diseased area of Asebu, markers associated with resistance or tolerance can be identified based on a marriage of the genotyping and diagnostics being developed (Yankey et al., 2011). The advance of sequencing technologies will be used to facilitate the discovery of useful novel sequences, but for subsistence crops (as compared with cash crops) amenable strategies such as the one described here offer a valuable resource. The work here has shown a strategy for the discovery of SNPs in introns, coupled with a high throughput analysis method that could be adapted for use in breeding programmes. In future work, it should be possible to identify SNP-based markers specifically linked to phytoplasma resistance, and to develop a strategy in which breeding materials and/or planting materials distributed to growers are tested to confirm that they are resistant. Such markers would be able to identify contaminated breeding material before it is provided to growers so that the situation with resistance breakdown in the MayPan palm can be avoided.

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Full Length Research Paper

Molecular diversity among Turkish oaks (*QUERCUS*) using random amplified polymorphic DNA (RAPD) analysis

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The genus *Quercus* (Fagaceae) includes the most important woody plants with decidious and evergreen species in Northern hemisiphere. They have a problematic taxonomy because of widespread hybridization between the infrageneric taxa. Turkey is one of the most important region of the world according to oak species number and variation. In this study, species belonging to evergreen oaks in Turkey were investigated to solve taxonomic problems and to design the limit of taxa by using random amplified polymorphic DNA (RAPD) data. Here, three species of evergreen oaks known as *Quercus coccifera*, *Quercus ilex* and *Quercus aucheri* were studied in all area located and made the comparison within and among species studied using ten RAPD markers. As a result; it can be stated that the presence of the three species in *Ilex* section is clear. Furthermore, existence of two infraspecific taxa or two seperate taxa in species level within *Q. coccifera* may be quite possibly considered.

Key words: *Quercus ilex*, *Quercus coccifera*, *Quercus aucheri*, random amplified polymorphic DNA (RAPD).

INTRODUCTION

The genus *Quercus* is one of the most diversified groups of the trees of temperate zone in north Hemisiphere with more than 500 species (Govaerts and Frodin, 1998; Tovar-Sanchez and Oyama, 2004; Olfat and Pourtahmasi, 2010; Maryam Ardi et al., 2012). Govaerts and Frodin (1998) state that the genus *Quercus* is represented by 531 species in the world and 250 of these species in America, 125 of these in Asia and remaining species in Europe, North Africa and Macaronesia. The area including South East Asia and Pacific islands is the center of morphological variation of Fagaceae, altough this area does not contain the most species of *Quercus* (Kaul, 1985).

Oaks are the woody, widespread, long-lived, outcrossing and wind-pollination species. For this reason, oaks can spread too wide geographic regions and as a result of this, they show high variations comparison to other woody plant species (Kremer and Petit, 1993; Hokanson et al., 1993; Bacilieri et al., 1996; Neophytou et al., 2010). It is well known that extensive hybridization behaviors may occur among species (Bacilieri et al., 1996; Manos et al., 1999; Samuel, 1999; Jensen et al., 2009; Neophytou et al., 2010) in the same group or section in the genus *Quercus*, because of weak reproductive barriers between oak species. Consequently, hybrid species spring up. Therefore the genus *Quercus* is taxonomically one of the most problematic groups (Bacilieri et al., 1996). The most of species in Turkey and all distributed countries have taxonomic problems. Taxonomic problems can be solved by molecular studies in addition to morphological and cytological studies and so genetic diversity and the limits of taxa can be deter-

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mined more clearly (Borazan and Babaç, 2003; Yılmaz et al., 2008; Simeone et al., 2009; Alam et al., 2009; Papini et al., 2011; Yılmaz et al., 2011).

Turkey is one of the most important region for oaks according to the species number and geographical distribution. Oaks in Turkey have a natural distribution of about 6.5 million ha area represented by 18 species in three different section (Davis, 1982; Yaltırık, 1984; Kasapligil, 1992) as white oaks (*Quercus* L.), red oaks (*Cerris* Loudon.) and evergreen oaks (*Ilex* Loudon.). Here, the species analysed were *Quercus coccifera*, *Quercus aucheri* and *Quercus ilex* known as evergreen oaks. These are very problematic species in Turkey in the comparison to other members of the genus. The distribution area for *Q. aucheri* is only south weast region of Turkey and in the Greek island like Rhodos in the world.

However; *Q. aucheri* is confused with the another member of *Ilex* section, (*Q. coccifera*). As a result, it can be stated that it is not very well known species for bio-systematic features and species limit. Moreover, it is controversial subject that *Q. coccifera* and *Quercus calliprinos* Webb. are seperate species or *Q. coccifera* has two subspecies known as *Q. coccifera* subsp. *coccifera* and *Q. coccifera* subsp. *calliprinos* (Toumi and Lumaret, 2001; Salvatore and Paola, 1976). Distribution area for *Q. calliprinos* is east mediterranean region and seperated from *Q. coccifera* with different living area. These taxonomical problems indicate that the real phenetics and fylogenetics relations within *Ilex* section have not still been fully explained. Hybridization and vegetative variations cause problems and make difficult to determine the borders of taxa.

Random amplıfıed polymorphıc DNA (RAPD) is a polymerase chain reaction (PCR) based technique used to show polymorphism among species. Especially this method is very helpful for systematics purposes and phylogenetic relation. For this aim, RAPD was used in this study as molecular technique (Kumar and Gurusubramanian, 2011). In order to solve this problem, variations within and between populations of taxa were pointed out by using some statistical analyses such as Statistica version 8.0 for principal component analysis (PCA) and cluster analysis (CA) using an unweighted pair group method (UPGMA) analysis and Popgen 32. According to the results of statistical analyses, it was attempted to draw the most possible borders of taxa based on the DNA bands obtained from RAPD analyses (Sesli and Yegenoglu, 2009; Açık et al., 2009; Kavalcıoglu et al., 2010) and a better phenetic classification by using molecular characters showing high correlations with each other.

MATERIALS AND METHODS

Study materials are composed of three species (*Q. coccifera*, *Q. ilex* and *Q. aucheri*) belonging to *Ilex* section of Turkey oaks. Totally 26 populations were represented to show variations within and among species (Table 1 and Figure 1). Leaves were used as material to show the differences in the molecular study. *Q. coccifera* was represented by 16 populations and other two species (*Q. ilex* and *Q. aucheri*) were represented by 5 populations. Especially, fresh and young leaves were preferred as material. Collected leaves were put into plastic bags filled silica gel and dried for the DNA isolation.

DNA extraction

Firstly, leaves in plastic bags filled silica gel were ground in liquid nitrogen using a mortar. DNA was extracted using a DNAeasy Plant Mini Kit (Qiagen). Extracted DNAs were kept at 4°C. Quality of each DNA sample were controlled by running on agarose gel before being used in PCR.

RAPD-PCR and gel electrophoresis

Molecular analysis was performed using RAPD method (Williams et al., 1990; Welsh and McClelland, 1991). Totally 30 primers, studied in oaks previously, were selected to find primers that exhibit polymorphism and give reproducible results. After the initial screening, 10 primers giving the best results among 30 primers were selected for further analysis (Table 2).

Amplification reactions were carried out in a 25 µl mix. The reaction mixture was prepared using PCR Buffer, $MgCl_2$, dNTP mixture containing dATP, dCTP, dGTP and dTTP, 10- base RAPD primer and taq DNA polymerase. After the primer selection, PCR conditions was determined. The program consisted of 40 cycles as fallows: Denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extention at 72°C for 2 min. A final extention at 72°C for 10 min was included.

The amplification products were electrophoresed in 1.4% agarose gels with TBE buffer at 100 V for 1 h and 30 min and stained with ethidium bromide. Gels with amplification fragments were visualized and photographed under ultraviolet light. RAPD bands were estimated by reference to a 100-bp ladder (Fermentas).

Data analysis

In order to score the RAPD products, amplified fragments were recorded as present (1) or absent (0) in all individuals for each fragment. Then the tables were constructed containing number and size of the DNA fragments for each populations. Polymorphic bands were determined for all populations. Molecular diversity among populations and species was evaluated by calculating the percentage of polymorphic fragments. The comparison of genetic distance and genetic similarity were calculated according to Nei (1972). RAPD data were evaluated by using two different statistical programs. Statistica version 8.0 were used for PCA and CA using an unweighted pair group method (UPGMA) analysis. Popgen 32 was used for genetic similarity and genetic distances.

RESULTS

In the RAPD analysis, 156 individuals representing 26 populations were used. A total 217 polymorphic bands were scored using the 10 RAPD primers. The size of the amplication products was between 150 to1600 base-pair. Table 2 shows the total number of polymorphic bands provided from each primers. The minimum and maximum size of amplification products provided from different

Table 1. Populations sampled (C = *Q. coccifera*, A = *Q. aucheri*, I = *Q. ilex*).

Pop. No	Location	Coordinates		Altitude (m)
		N	E	
C1	İzmir-Balıkesir border area, Altınova barrage road	39° 12.903	026° 49.302	70
C2	İzmir-between Dikili-Çandarlı, 20 km. to Çandarlı	39° 01.253	026° 55.505	40
C3	Manisa-between Kırkağaç-Akhisar, 1-2 km. after Çandarlı	39° 05.800	027° 40.257	190
C4	Çanakkale-Ezine-Bozcaada pier	39° 47.950	026° 12.115	50
C5	Gökçeada-between Gökçeada-Dereköy	40° 09.689	025° 49.586	60
C6	Mersin-5-10 km. after Seratvul	36° 50.997	033° 18.402	1400
C7	Karaman-between Mut-Ermenek, 45 km. before Ermenek	36° 37.276	032° 55.182	1300
C8	Antalya-between Korkuteli-Bucak, 25 km. before Bucak	37° 15.582	030° 19.362	920
C9	Aydın-Eski Çine, Ovacık village	37° 32.889	028° 05.310	300
C10	Aydın-Söke, between Bağarası-Akçakaya village	37° 40.350	027° 31.347	40
C11	Muğla-between Muğla-Kale, 59 km. before Kale	37° 08.142	028° 32.157	800
C12	Denizli- between Kale-Tavas, 1-2 km. before Tavas	37° 33.069	029° 03.150	940
C13	Uşak-between Sivaslı-Uşak, 12 km. after Sivaslı	38° 34.259	029° 36.303	825
C14	Gaziantep- between Yavuzeli-Araban	37° 22.975	037° 33.292	740
C15	Kahramanmaraş- between k.maraş- göksun	37° 43.514	036° 40.038	1075
C16	Hatay-between Kırıkhan-Hassa	36° 36.554	036° 23.591	350
A1	Antalya-between Kemer-Kumluca	36° 25.429	030° 25.447	530
A2	Aydın-Çine,Across from the cemetery Kuruköy	37° 33.558	028° 04.047	180
A3	Aydın-Priene-Söke	37° 44.967	029° 16.369	90
A4	İzmir-Selçuk-Zeytinköy	37° 59.569	027° 17.226	65
A5	Muğla-between Milas-Bodrum, Dörttepe village	37° 11.242	027° 37.142	8
I1	Zonguldak-Alaplı, Sabırlı village	41° 08.901	031° 23.147	180
İ2	Zonguldak-between Alaplı-Düzce	41° 08.443	031° 20.596	4
I3	Düzce- between Yığılca-Alaplı	41° 09.136	031° 23.627	60
I4	İstanbul-between Anatolian Fortrees-Kavacık	41° 04.220	029° 05.085	65
I5	Gökçeada-between Gökçeada-Dereköy	40° 09.689	025° 49.586	60

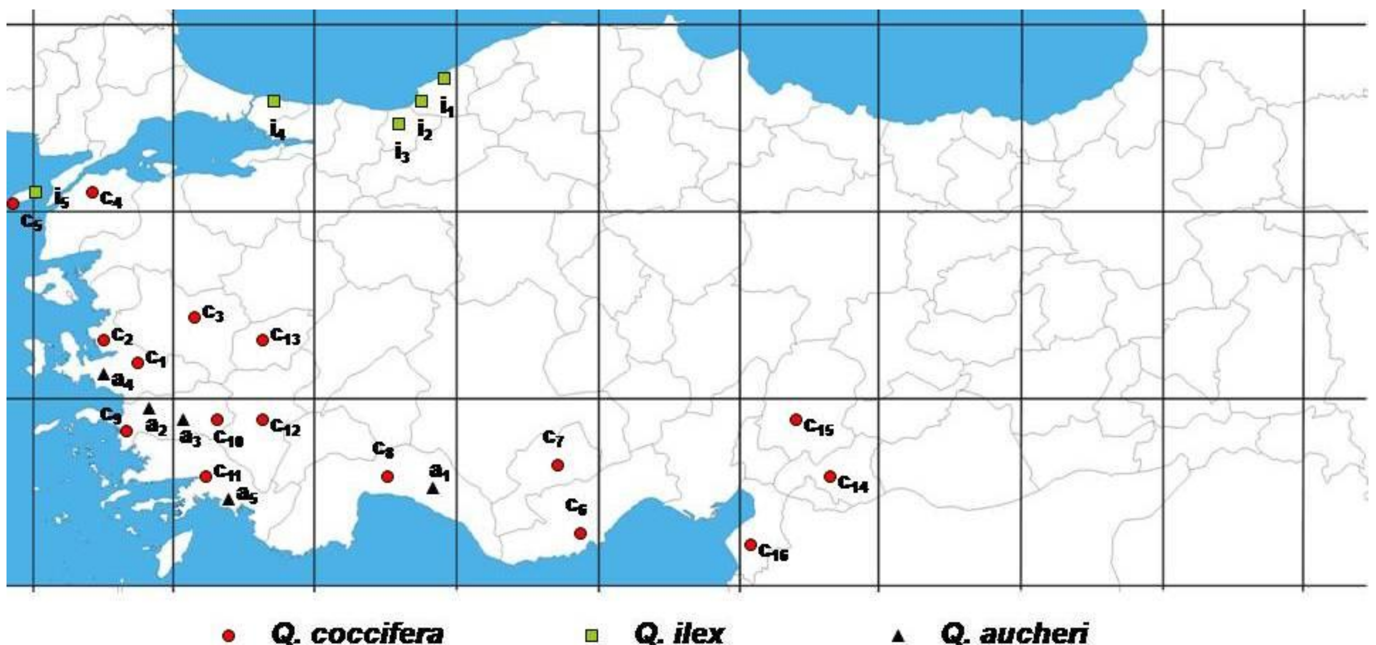

Figure 1. Distribution of studied populations of *Q. coccifera*, *Q. ilex* and *Q. aucheri* in Turkey.

Table 2. The list of primers used in RAPD and analysis of PCR amplification products by selected primers.

Prımer	Sequence (5'-3')	Number of bands	Amplification products (bp)
OPA-01	CAGGCCCTTC	20	300 to 1400
OPA-08	GTGACGTAGG	18	200 to 1400
OPA-09	GGGTAACGCC	21	250 to 1400
OPB-04	GGACTGGAGT	22	200 to 1400
OPX-04	CCGCTACCGA	23	150 to 1400
OPC-03	GGGGGTCTTT	22	150 to 1600
OPC-09	CTCACCGTCC	19	300 to 1300
OPS-09	TCCTGGTCCC	25	200 to 1500
OPS-18	CTGGCGAACT	23	200 to 1400
OPU-01	ACGGACGTCA	24	200 to 1400
Total	10	217	150 to 1600

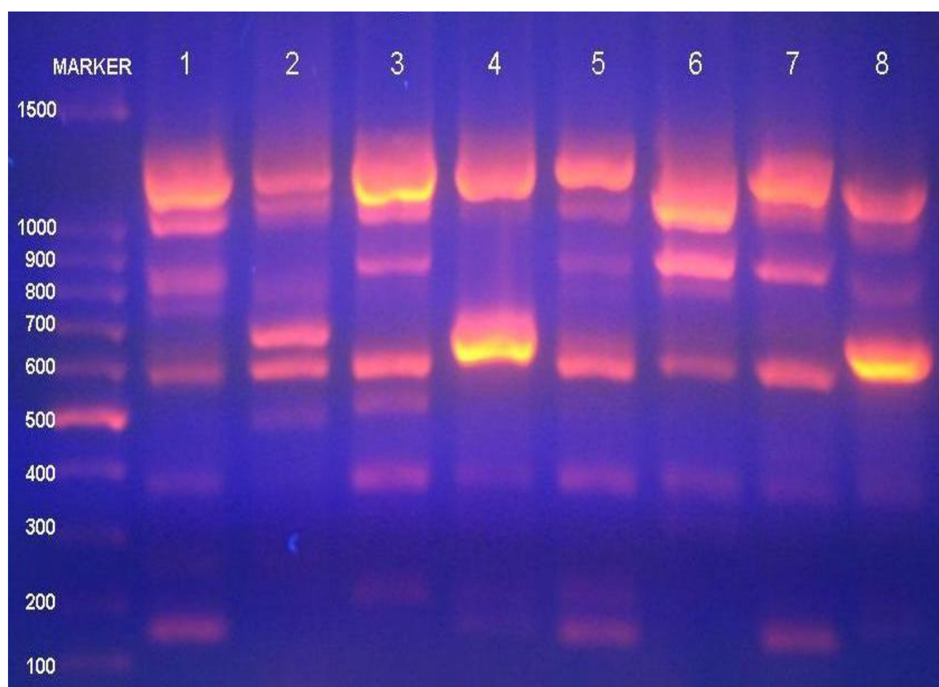

Figure 2. RAPD products in C13 population with OPX-04 primer.

primers were also listed in Table 2.

In order to score the RAPD products, individuals of each population were run separately for each primer (Figure 2). Additionally, RAPD products of six individuals from every population were bulked and run together, to see all populations products in the same gel for each primer (Figure 3). CA and PCA were carried out for the analysis of variations within and among studied species. According to these results, *Q. ilex* and *Q. aucheri* were observed as close two separate groups. Populations of *Q. coccifera* showed more differences than populations of *Q. ilex* and *Q. aucheri*. But fundamentally, three studied species showed differences from each other. When the each species were evaluated separately, generally geographically close populations showed more similarity than geographically distant ones (Figures 4 and 5). Populations belonging to *Q. ilex* were separated into two sub-groups in CA pehenogram. The first of these was I1, I2 and I3 populations.

The second sub-group of *Q. ilex* was composed of I4 and I5 populations. Other species, *Q. aucheri* was separated into two sub-groups like *Q. ilex* but here C7 population of *Q. coccifera* showed the high similarity with the populations of *Q. aucheri*. Finally, when the populations of *Q. coccifera* were examined, it drew attention that *Q. coccifera* was separated into three sub-groups. When these three sub-groups are observed attentively, they were separated as geographically from each other

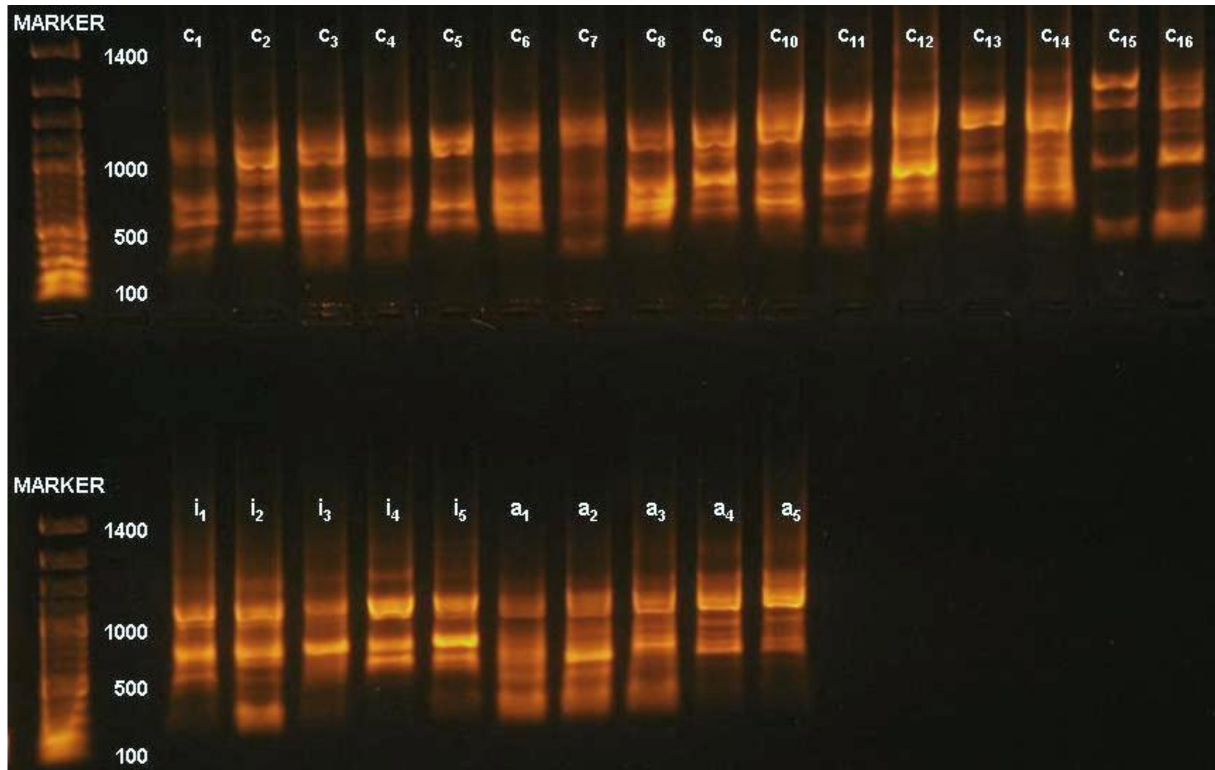

Figure 3. Visualization of all population's RAPD products with OPB-04 primer.

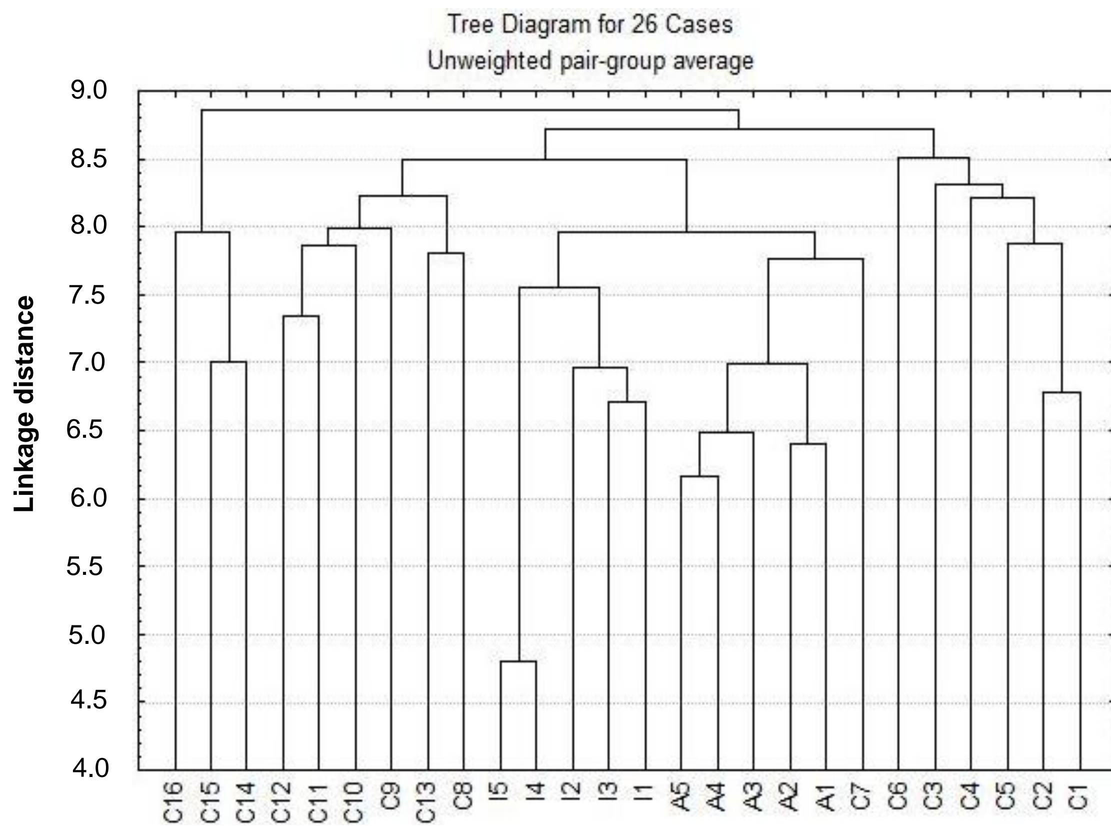

Figure 4. Phenogram resulting from cluster analysis with UPGMA.

Table 3. The comparison of genetic distance (below diagonal) and genetic similarity (upper diagonal) (Nei, 1972).

	c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12	c13	c14	c15	c16	i1	i2	i3	i4	i5	a1	a2	a3	a4	a5
c1	***	0.998	0.985	0.991	0.991	0.992	0.993	0.991	0.996	0.991	0.992	0.988	0.990	0.985	0.988	0.986	0.985	0.990	0.987	0.991	0.995	0.995	0.994	0.990	0.990	0.996
c2	0.001	****	0.981	0.990	0.989	0.992	0.990	0.987	0.993	0.987	0.991	0.985	0.987	0.982	0.985	0.983	0.981	0.986	0.982	0.986	0.992	0.991	0.992	0.986	0.987	0.991
c3	0.014	0.018	****	0.971	0.973	0.987	0.986	0.987	0.980	0.987	0.990	0.984	0.982	0.973	0.990	0.989	0.976	0.986	0.987	0.985	0.988	0.979	0.986	0.989	0.973	0.981
c4	0.008	0.010	0.029	****	0.985	0.990	0.989	0.983	0.994	0.985	0.980	0.978	0.982	0.974	0.973	0.977	0.981	0.977	0.984	0.985	0.987	0.992	0.985	0.981	0.993	0.991
c5	0.009	0.010	0.026	0.014	****	0.987	0.990	0.992	0.989	0.987	0.989	0.995	0.993	0.976	0.981	0.981	0.981	0.980	0.985	0.989	0.991	0.991	0.989	0.986	0.989	0.992
c_6	0.007	0.007	0.012	0.009	0.013	****	0.990	0.987	0.992	0.987	0.988	0.985	0.987	0.977	0.986	0.986	0.977	0.979	0.985	0.986	0.988	0.986	0.988	0.982	0.985	0.988
c_7	0.006	0.009	0.013	0.011	0.009	0.009	****	0.993	0.991	0.989	0.990	0.990	0.990	0.981	0.990	0.990	0.986	0.992	0.989	0.992	0.992	0.994	0.990	0.991	0.989	0.993
c_8	0.008	0.012	0.012	0.016	0.007	0.012	0.006	****	0.986	0.991	0.990	0.997	0.994	0.977	0.987	0.989	0.982	0.984	0.992	0.989	0.994	0.994	0.993	0.994	0.987	0.991
c_9	0.003	0.006	0.019	0.005	0.011	0.007	0.008	0.013	****	0.992	0.990	0.983	0.989	0.985	0.987	0.983	0.985	0.988	0.987	0.992	0.992	0.994	0.992	0.983	0.992	0.996
c_{10}	0.008	0.012	0.013	0.015	0.012	0.012	0.011	0.008	0.007	****	0.992	0.990	0.996	0.989	0.992	0.991	0.988	0.983	0.985	0.992	0.991	0.989	0.989	0.987	0.983	0.989
c_{11}	0.007	0.008	0.009	0.020	0.010	0.011	0.009	0.009	0.010	0.007	****	0.990	0.990	0.984	0.991	0.988	0.990	0.990	0.988	0.989	0.993	0.987	0.991	0.990	0.982	0.989
c_{12}	0.011	0.014	0.015	0.021	0.004	0.014	0.009	0.002	0.016	0.009	0.009	****	0.995	0.972	0.983	0.986	0.979	0.981	0.988	0.991	0.993	0.989	0.990	0.993	0.985	0.989
c_{13}	0.009	0.013	0.017	0.017	0.006	0.012	0.010	0.005	0.011	0.003	0.009	0.004	****	0.987	0.989	0.993	0.987	0.977	0.983	0.991	0.989	0.988	0.986	0.986	0.981	0.988
c_{14}	0.014	0.017	0.026	0.026	0.024	0.023	0.019	0.022	0.014	0.010	0.015	0.028	0.012	****	0.992	0.989	0.987	0.974	0.965	0.975	0.973	0.977	0.975	0.968	**0.964**	0.976
c_{15}	0.011	0.014	0.009	0.026	0.019	0.013	0.009	0.012	0.012	0.008	0.008	0.016	0.010	0.007	****	0.993	0.982	0.988	0.979	0.984	0.983	0.983	0.985	0.980	0.973	0.984
c_{16}	0.013	0.017	0.010	0.022	0.018	0.013	0.009	0.010	0.016	0.008	0.011	0.014	0.006	0.010	0.006	****	0.989	0.977	0.979	0.985	0.982	0.980	0.978	0.982	0.969	0.980
i_1	0.015	0.018	0.023	0.018	0.018	0.022	0.013	0.017	0.014	0.011	0.009	0.020	0.012	0.012	0.018	0.011	****	0.977	0.981	0.983	0.982	0.980	0.976	0.981	0.974	0.981
i_2	0.010	0.013	0.013	0.023	0.020	0.020	0.007	0.015	0.012	0.017	0.009	0.018	0.022	0.025	0.012	0.023	0.022	****	0.986	0.988	0.991	0.989	0.991	0.989	0.985	0.991
i_3	0.012	0.018	0.012	0.015	0.014	0.014	0.010	0.007	0.012	0.014	0.011	0.011	0.016	0.034	0.021	0.020	0.018	0.013	****	0.989	0.994	0.991	0.992	0.992	0.991	0.993
i_4	0.008	0.013	0.014	0.015	0.010	0.013	0.008	0.010	0.007	0.007	0.010	0.008	0.009	0.024	0.015	0.014	0.017	0.011	0.011	****	0.994	0.990	0.988	0.991	0.988	0.994
i_5	0.004	0.007	0.011	0.012	0.008	0.011	0.007	0.005	0.007	0.008	0.006	0.006	0.010	0.026	0.016	0.017	0.017	0.008	0.005	0.005	****	0.995	0.997	0.997	0.993	0.996
a_1	0.004	0.008	0.020	0.007	0.008	0.013	0.005	0.006	0.006	0.010	0.013	0.010	0.011	0.023	0.017	0.020	0.020	0.010	0.008	0.009	0.004	****	0.996	0.991	0.996	**0.998**
a_2	0.005	0.007	0.013	0.014	0.010	0.011	0.009	0.006	0.007	0.010	0.008	0.009	0.013	0.025	0.014	0.021	0.023	0.008	0.007	0.011	0.002	0.003	****	0.992	0.994	0.996
a_3	0.009	0.013	0.010	0.018	0.014	0.017	0.008	0.005	0.016	0.012	0.009	0.006	0.013	0.032	0.019	0.017	0.018	0.010	0.007	0.008	0.002	0.008	0.007	****	0.988	0.991
a_4	0.009	0.013	0.027	0.006	0.011	0.015	0.010	0.012	0.007	0.016	0.017	0.014	0.018	0.036	0.027	0.031	0.025	0.014	0.008	0.011	0.006	0.003	0.005	0.011	****	0.996
a_5	0.003	0.008	0.018	0.009	0.008	0.012	0.006	0.008	0.003	0.010	0.010	0.010	0.011	0.023	0.016	0.020	0.019	0.008	0.006	0.005	0.003	0.001	0.003	0.008	0.003	****

(Figure 4).

Populations belonging to *Q. coccifera* evaluated in the West and South West region of Turkey are C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 and C13 (Figure 1) and results from cluster analysis with UPGMA showed that the popula-tions from these regions were similar in the comparison to the remaining (Figures 4 and 5). Eventually, populations having the highest differences of *Q. coccifera* were C14, C15 and C16. These popu-lations originated from East Mediterranean region (Figure 1). The molecular analysis with CA and PCA revealed a high degree of separation between the species.

When the tables of genetic distance and similarity was investigated, the lowest genetic distance was observed between C1-C2 and A1-A5 populations (Table 3). In other words, the highest genetic similarity was observed between C1-C2 and A1-A5 populations. The highest gene-tic distance was between C14-A4 and C14-I3 populations, respec-tively. Therefore, the lowest genetic similarity was between C14-A4 and C14-I3 populations.

DISCUSSION

This is the first report of RAPD data analysis for assessing relationships between these three taxa. But there are some studies that used RAPD data in different sections of oaks (Bruschi et al., 2003; Gonzalez-Rodriguez et al., 2004; Franjic et al., 2006; Ardi et al., 2012). Here, taxonomies of the studied species are not well known. Especially *Q. aucheri* is known as "forgotten oak tree'' (Yaltırık, 1984), because it is distributed only in South West Turkey and in a few East Aegean Islands (Davis,

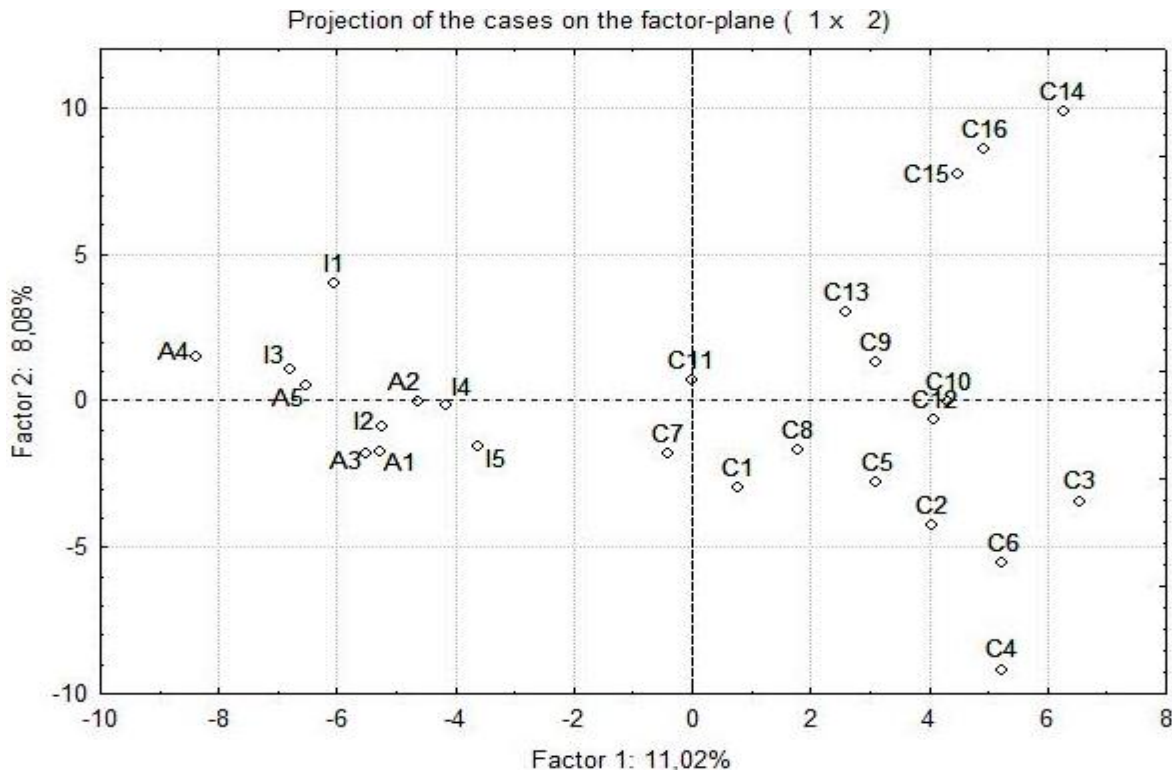

Figure 5. The resulting projection of principal component analysis.

1982).By this study; the lack of molecular properties of *Ilex* section is completed in detail. The results gave the satisfactory findings for phenetic groupings of taxas. The significant differences are found on the all studied species. Firstly, *Q. ilex* is separated from the other species. Due to similarities among the some *Q. coccifera* and *Q. aucheri* populations, they are well separated with each other. This is the first study that shows *Q. coccifera* is separated into two geographical groups in Turkey. The first group has the populations sampled from West and South West regions of Turkey. The populations sampled from C14, C15 and C16 belonging to East Mediterranean region included into the second group. This geographically separation within populations of *Q. coccifera* suggests that there are sub-groupings or different species in this taxon. The most common group is *Q. coccifera* found in many regions, while the less and restricted group is found only in the East Mediterranean region. The second group is geographically closer to Syria, Israel and Palestine. In addition, these two groups are represented as a single *Q. calliprinos* species and its two subspecies as *Q. calliprinos* subsp. *coccifera* and *Q. calliprinos* subsp. *calliprinos* in Flora of Palestine (Zohary, 1966). When the studied populations are compared with each other according to the genetic similarity, it can be said that genetically distant populations are also located geographically in different and far regions (Figures 4 and 5; Table 3). The most high genetic similarity are found between C1-C2 and A1-A5 populations which are also geographically close populations. On the contrary, genetically the most distant populations, C14-A4 and C14-I3 are the two different species which are geographically located very distant.

As a result of this study, it might be suggested that: (1), The results showed the presence of the second group within *Q. coccifera* but this needs to be supported in a study including *Q. calliprinos* samples from Syria, Israel and Palestina; (2), the groupings based on molecular studies support the presence of the three species in *Ilex* section; (3), the two groups showing geographical differentiations within *Q. coccifera* may strengthen the existence of two infraspecific taxa such as *Q. coccifera* subsp. *coccifera* and subsp. *calliprinos* or two different taxa at species level.

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Full Length Research Paper

Heterosis expression, interrelationship, direct and indirect effects of component characters on yield in intervarietal crosses of eggplant

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Heterosis has been amply exploited in developing hybrids in brinjal. In India, only 17.8% area of brinjal cultivation is under hybrid seed due to lack of appropriate hybrids for specific area and purpose. In the present investigation, 14 parents were selected on the basis of divergence and mated in line (L) × tester (T) design hybrids and parents were raised to measure heterosis for different yield attributes. 40 hybrids resulting from a L × T mating design comprised of 10 lines (female) and 4 testers (males) were studied to determine the magnitude of heterosis and genotypic correlation between yield and yield characters. Expression of superiority over the commercial check occurred in 7 crosses, which ranged from -56.36 ($L_3 \times T_4$) to 34.07% ($L_7 \times T_2$). The hybrid ($L_7 \times T_2$) had good heterosis values for growth and yield and is recommended as the most promising combination for developing high yielding hybrid eggplant varieties. Most crosses involving T_2 as tester parent had significant positive heterosis over the mid-parent and standard variety. There were strong correlations between fruit yield and numbers of branches per plant, average fruit weight and number of fruit per plant. Path analysis indicated that plant height, number of branches per plant, fruit length, fruit pedicel length, number of fruit per plant, average fruit weight and little leaf incidence had direct and positive effects on yield per plant, but negative and direct effects occurred for days to first flowering, fruit circumference, calyx length, shoot borer infestation, fruit borer infestation, ascorbic acid content and total phenol content. Simple selection would be effective for improvement of fruit yield.

Key words: *Solanum melongena,* association analysis, hybrid vigour.

INTRODUCTION

Brinjal (*Solanum melongena* L.), also known as eggplant and an important vegetable crop of India. It is widely cultivated in both temperate and tropical regions of the globe mainly for its immature fruits. In Tamilnadu (India), the productivity of brinjal is less as compared to national average, owing to use of low yielding cultivars grown for

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local preferences and their susceptibility to pests and diseases (Nalini, 2007). The present production is not proportionate with the demand. So, brinjal deserves a deep deliberation for improvement. It should be highly pragmatic by the fact that, India being the centre of origin and diversity of brinjal, it should pave the way for bringing about a kind of plant type, which could enhance its quality and productivity without sacrificing the consumer needs (Shafeeq et al., 2007). Quality and productivity of eggplant can be improved through heterosis breeding (Kakikazi, 1931). The estimation of heterosis for yield and its component characters would be useful to judge the best hybrid combination for exploitation of superior hybrids. Genotypic correlations exist between yield and yield attributing characters are important in breeding. Yield is the end product of many correlated characters. Selection for yield would be more effective when it is based on component characters which are positively correlated. When more number of variables is considered in correlation, the association becomes more complex. Use of path coefficient analysis makes clear direct and indirect associations and identifies the most reliable yield contributing characters (Daliya and Wilson, 2002). The study was under taken to study the extent of heterosis in different crosses and their utilization in future crop improvement.

MATERIALS AND METHODS

The experiment was conducted during June to October (*kharif)* 2010 to 2011 at College Orchard, Agricultural College and Research Institute, Madurai, India, situated at 9°5 N latitude and 78°5 E longitudes at 147 m above MSL. Ten lines were crossed with 4 testers through Line × Tester mating design to derive 40 F_1 hybrids (Table 1). The hybrids and 14 parents were established in a sandy loam soil and arranged in a Randomized Complete Block Design with three replications. Three ploughings were done with cultivator to make the soil fine tilth. Thirty (30)-day-old seedlings raised in the nursery beds were transplanted on ridges with a spacing of 60 × 60 cm. 30 plants were maintained for each hybrid and parent in each replication. Cultural practices were followed uniformly for all hybrids and (TNAU Crop Production Guide, 2005). Observations were recorded from 5 randomly selected plants in each replication. The data recorded for the traits plant height, days to first flowering, number of branches per plant, fruit length, fruit circumference, number of fruit per plant, average fruit weight and fruit yield per plant for estimating heterosis. Selections were made in F_1 hybrids based on fruit shape, color, size and fruit yield per plant. Superior hybrids were selected and selfed. Seed were collected from selfed fruit and stored for further breeding.

The magnitude of heterosis in hybrids was expressed as percent of increase or decrease of a character over mid-parent (d_i), better parent (d_{ii}) and standard hybrid (d_{iii}) and estimated following the formula of Fonseca and Patterson (1968). The significance of magnitude of the relative heterosis, heterobeltiosis and standard heterosis was tested with the formula suggested by Turner (1953). Correlation coefficients were computed using formulae of Johnson et al. (1955). Path coefficients were obtained following the method of Dewey and Lu (1959).

RESULTS AND DISCUSSION

The analysis of variance revealed that parents showed significant differences for all characters (Table 2). Variance due to lines was significant for all traits indicating existence of genetic variability for growth and yield attributes among lines (females) and testers (males). The interaction between lines × testers was also significant for all the growth and yield traits.

Average performance and magnitude of heterosis for different quantitative characters varied (Table 3). In parents, T_2 (66.90 cm) had the shortest plants and L_2 (98.83 cm) was tallest. The hybrid $L_1 \times T_4$ recorded tallest plants height, followed by $L_2 \times T_1$ with positive heterosis over mid, better and standard parents indicating over-dominance. Among the 40 hybrids, 15 had significant positive over the mid-parent for plant height. Three hybrids had significant positive heterosis and one hybrid had significant negative heterosis ($L_6 \times T_3$) over the better parent. Twenty-seven (27) of 40 hybrids had significant positive heterosis over standard variety and the extent of heterosis over the standard variety. Similar findings were reported by Preneetha (2002) and Thangamani (2003).

Earliness is considered an important character in any crop improvement programme, which is manifested in F_1 hybrids and preferred for commercial cultivation when high yield is coupled with earliness. The hybrid $L_8 \times T_1$ had the shortest period of days to first flower and $L_8 \times T_4$ had the longest period. For days to first flowering negative heterosis is desirable. Of 40 hybrids, 8 ($L_1 \times T_1$, $L_4 \times T_1$, $L_5 \times T_1$, $L_6 \times T_1$, $L_6 \times T_3$, $L_7 \times T_3$, $L_8 \times T_1$ and $L_{10} \times T_1$) had significant heterosis in the desirable direction over the mid-parent. Seventeen (17) hybrids had significant heterobeltiosis in the negative direction (desirable). As many as 29 hybrids had heterosis in the desirable direction over the standard variety; only 2 ($L_3 \times T_2$ and $L_8 \times T_4$) had significant positive heterosis over the standard variety.

Number of branches per plant influences yield. Of 40 crosses, the highest number of branches per plant was for $L_6 \times T_2$. Of 40 hybrids 21 had significant positive heterosis while three hybrids (L_2 x T_1 and $L_8 \times T_4$) exhibited significant negative heterosis. Seventeen hybrids had significant heterosis over the better parent, of which 10 had heterosis in the positive direction and 7 (L_2 x T_1, L_2 x T_3, L_4 x T_3, $L_8 \times T_4$, $L_{10} \times T_1$, L_{10} x T_3 and L_{10} x T_4) had heterosis in the negative direction. In 18 hybrids, there was significant heterosis in the negative direction, where-as three hybrids (L_5 x T_2, L_6 x T_2 and L_7 x T_2) had significant positive heterosis over standard variety. These results agree with findings of Preneetha (2002).

Fruit length is an important character to be considered while selecting eggplant for high yield. The longest fruit was in T_1 followed by L_8 and shortest fruit were in T_2. The hybrid L_6 x T_1 exhibited good performance and heterobeltiosis for fruit length. 21 hybrids had significant negative

Table 1. Features of parents used in study.

Name of local type	Flower bearing	Fruit bearing	Fruit shape	Fruit color	Calyx type	Calyx spininess	Source	Symbol
Lines								
Alavayal Local	Cluster	Cluster	Round	Light purple	Persistent	Non- spiny	Alavayal, Madurai D.t ,Tamil Nadu	L_1
Sedapatty Local (Green)	Cluster	Cluster	Oval	Purplish green	Persistent	Non- spiny	Sedapatty, Madurai D.t, Tamil Nadu	L_2
Kariapatty Local	Cluster	Cluster	Round	Green striped	Persistent	Non- spiny	Kariapatty, Virdhunagar D.t, Tamil Nadu	L_3
Alagarkovil Local	Cluster	Cluster	Round	Green striped	Persistent	Non- spiny	Alagarkovil, Madurai D.t, Tamil Nadu	L_4
Palamedu Local	Cluster	Cluster	Round	Light blue	Persistent	Non- spiny	Palamedu, Madurai D.t, Tamil Nadu	L_5
Melur Local	Cluster	Cluster	Round	Purple	Persistent	Non- spiny	Melur, Madurai D.t, Tamil Nadu	L_6
Keerikai Local	Cluster	Cluster	Oval	Purplish green	Persistent	Non- spiny	Sempatty, Dindigul D.t, Tamil Nadu	L_7
Nilakottai Local	Cluster	Cluster	Oblong	Green striped	Persistent	Non- spiny	Nilakottai, Dindigul D.t, Tamil Nadu	L_8
Singampunari Local	Cluster	Cluster	Round	Purplish green	Persistent	Non- spiny	Singampunari, Sivagangai D.t, Tamil Nadu	L_9
Sedapatty Local (Blue)	Cluster	Cluster	Round	Purple striped	Persistent	Non- spiny	Sedapatty, Madurai D.t, Tamil Nadu	L_{10}
Tester								
Annamalai	Cluster	Cluster	Long	Purple	Non persistent	Non- spiny	Vegetable Research Station, Palur, Tamil Nadu	T_1
KKM 1	Cluster	Cluster	Egg shaped	White	Persistent	Non- spiny	Agricultural College and Research Institute, Tuticorin, Tamil Nadu	T_2
Punjab Sadabahar	Cluster	Cluster	Long	Purple	Non persistent	Non- spiny	Tamil Nadu agricultural university, Coimbatore	T_3
EP 65	Cluster	Cluster	Oval	Dark purple	Non persistent	Non- spiny	Vegetable Research Station, Palur, Tamil Nadu	T_4

heterosis over the mid-parent.

A total of 27 hybrids had significant heterosis over the better parent in desirable direction (negative). Useful heterosis was exhibited by all 40 hybrids over the standard variety. The cross combination exceed-ing the superior parent is a valuable character in heterosis breeding (Patil et al., 2001). Similar findings were reported by Kaur et al. (2001).

Fruit circumference contributes to improved yield and parents L_1, L_5, T_3, T_4, and L_9 had the largest fruit. The highest fruit circumference was recorded in L_6 x T_4 followed by L_1 x T_2 and the lowest was in L_3 x T_3. The observations were positive and significant for 14 hybrids. Positive and negative non-significant heterosis were observed in seven and four hybrids, respectively. Seven hybrids had significant and heterobeltiosis,

Table 2. Analysis of variance for parents and hybrids for vegetative and reproductive characters.

Source	df	PH [a]	DFF	NB/P	FL	FC	NF/P	AFW	FY/P
Hybrids	39	346.5306*	54.8730*	17.4039*	5.4570*	9.9952*	161.9557*	149.5525*	0.6288*
Lines	9	973.0851*	76.5412*	40.2728*	4.9768*	20.0498*	197.8137*	328.0857*	0.9020*
Testers	3	66.6626*	17.7451*	12.1612*	9.7032*	16.6776*	234.2205*	72.4168*	0.4214*
Line x Testers	27	168.7756*	51.7756*	10.3635*	5.1453*	5.9011*	141.9736*	98.6121*	0.5608*
Error	78	85.0412	3.3519	4.3914*	0.0933	0.4478	1.8176	4.0138	0.0100

*Significant at 5% level; [a]PH, plant height (cm); DFF, days to first flowering; NB/P, number of branches per plant; FL, fruit length (cm); FC, fruit circumference (cm); NF/P, number of fruit per plant; AFW, average fruit weight (g) and FY/P, fruit yield per plant (kg).

Table 3. Average performance and magnitude of heterosis for plant height and days to first flowering in eggplant.

Entry	Average value	Plant height (cm)			Average value	Days to first flowering		
		MP	BP	SV		MP	BP	SV
Alavayal Local	93.97				78.51			
Sedapatty Local (Green)	98.83				75.43			
Kariapatty Local	90.60				77.67			
Alagarkovil Local	91.93				78.02			
Palamedu Local	83.53				73.33			
Melur Local	95.20				76.89			
Keerikai Local	96.33				71.28			
Nilakottai Local	88.93				78.63			
Singampunari Local	96.60				73.11			
Sedapatty Local (Blue)	84.93				79.62			
Annamalai	73.17				81.27			
KKM 1	66.90				68.24			
Punjab Sadabahar	79.93				73.32			
EP 65	96.43				71.45			
L_1 X T_1	95.47	14.24	1.60	30.48**	73.67	-7.79**	-9.35**	-9.35**
L_1 X T_2	102.47	27.39**	9.05	40.05**	77.18	5.19**	-1.69	-5.03**
L_1 X T_3	104.77	20.49**	11.49	43.19**	79.28	4.43*	0.98	-2.45
L_1 X T_4	125.13	31.44**	29.76**	71.03**	79.60	6.16**	1.39	-2.05
L_2 X T_1	124.20	44.42**	25.67**	69.75**	81.13	3.55*	-0.17	-0.17
L_2 X T_2	102.13	23.24**	3.33	39.58**	76.13	5.98**	0.92	-6.32**
L_2 X T_3	110.20	23.29**	11.50	50.62**	83.64	12.46**	10.88**	2.92
L_2 X T_4	101.80	4.27	3.00	39.13**	76.20	3.76*	1.02	-6.24**
L_3 X T_1	112.27	37.11**	23.91**	53.44**	82.85	4.26*	1.94	1.94
L_3 X T_2	100.50	27.62**	10.93	37.36**	84.83	16.28**	9.22**	4.38*
L_3 X T_3	99.22	16.36*	9.51	35.61**	75.16	-0.45	-3.23	-7.52**
L_3 X T_4	96.70	3.40	0.28	32.16**	74.48	-0.11	-4.10*	-8.35**
L_4 X T_1	100.17	21.34**	8.96	36.90**	70.73	-11.19**	-12.97**	-12.97**
L_4 X T_2	91.63	15.38	-0.33	25.24*	71.50	-2.22	-8.35**	-12.02**
L_4 X T_3	101.20	17.77*	10.08	38.31**	75.00	-0.89	-3.87*	-7.72**
L_4 X T_4	94.20	0.02	-2.32	28.75**	74.67	-0.09	-4.29*	-8.12**
L_5 X T_1	86.90	10.91	4.03	18.77	70.89	-8.29**	-12.77**	-12.77**
L_5 X T_2	85.53	13.72	2.39	16.90	74.26	4.91**	1.26	-8.63**
L_5 X T_3	95.60	16.97*	14.45	30.66**	78.12	6.54**	6.53**	-3.87*
L_5 X T_4	83.90	-6.76	-13.00	14.67	72.65	0.36	-0.93	-10.61**
L_6 X T_1	91.77	9.01	-3.61	25.42*	74.28	-6.07**	-8.60**	-8.60**
L_6 X T_2	85.90	5.98	-9.77	17.40	70.94	-2.24	-7.74**	-12.71**
L_6 X T_3	72.33	-17.40*	-24.02**	-1.14	71.87	-4.31*	-6.53**	-11.57**
L_6 X T_4	93.90	-2.00	-2.63	28.34**	73.55	-0.83	-4.34*	-9.50**
L_7 X T_1	96.73	14.14	0.42	32.21**	78.66	3.13	-3.21	-3.21
L_7 X T_2	103.20	26.44**	7.13	41.05**	76.05	9.02**	6.69**	-6.42**
L_7 X T_3	106.93	21.33**	11.00	46.15**	69.25	-4.22*	-5.55**	-14.79**
L_7 X T_4	97.23	0.88	0.83	32.89**	76.45	7.13**	7.00**	-5.93**
L_8 X T_1	93.77	15.69*	5.43	28.15**	68.69	-14.08**	-15.48**	-15.48**
L_8 X T_2	86.60	11.14	-2.62	18.36	78.50	6.90**	-0.17	-3.41
L_8 X T_3	96.67	14.49	8.70	32.12**	75.46	-0.68	-4.03*	-7.15**
L_8 X T_4	96.83	4.48	0.41	32.35**	87.98	17.25**	11.90**	8.26**
L_9 X T_1	86.53	1.94	-10.42	18.27	75.51	-2.18	-7.09**	-7.09**

*, **Significant at 5 and 1% level.

Table 3. Contd.

Entry	Average value	Number of branches per plant			Average value	Fruit length (cm)		
		MP	BP	SV		MP	BP	SV
L_9 X T_2	93.20	14.01	-3.52	27.38**	73.94	4.62*	1.14	-9.02**
L_9 X T_3	87.29	-1.11	-9.64	19.30	77.70	6.13**	5.97**	-4.39*
L_9 X T_4	84.33	-12.62	-12.70	15.26	72.22	-0.08	-1.22	-11.14**
L_{10} X T_1	84.25	6.57	-0.81	15.14	75.31	-6.38**	-7.33**	-7.33**
L_{10} X T_2	85.33	12.40	0.47	16.63	76.38	3.32	-4.07*	-6.01**
L_{10} X T_3	87.27	5.86	2.75	19.27	78.54	2.71	-1.36	-3.36
L_{10} X T_4	82.50	-9.02	-14.45	12.76	82.21	8.84**	3.25	1.16
SEd	7.25	6.23	7.20	1.52		1.31	1.51	
Alavayal Local	21.13				7.44			
Sedapatty Local (Green)	22.32				7.54			
Kariapatty Local	16.36				6.93			
Alagarkovil Local	20.49				6.53			
Palamedu Local	20.04				7.87			
Melur Local	21.89				7.14			
Keerikai Local	22.11				7.79			
Nilakottai Local	21.74				10.23			
Singampunari Local	17.88				5.41			
Sedapatty Local (Blue)	21.62				6.92			
Annamalai	24.18				11.61			
KKM 1	15.31				5.25			
Punjab Sadabahar	18.50				6.85			
EP 65	18.01				8.07			
L_1 X T_1	26.36	16.36**	9.03	9.03	6.58	-30.74**	-43.28**	-43.28**
L_1 X T_2	23.70	27.71**	12.15	-1.99	6.92	9.30**	-6.57*	-40.41**
L_1 X T_3	20.02	1.03	-5.25	-17.19**	6.83	-4.16	-7.74*	-41.15**
L_1 X T_4	19.63	0.32	-7.08	-18.79**	8.17	5.58*	1.20	-29.61**
L_2 X T_1	19.12	-17.74**	-20.90**	-20.90**	7.78	-18.73**	-32.97**	-32.97**
L_2 X T_2	19.74	3.11	-11.53	-18.34**	5.86	-8.39**	-22.28**	-49.51**
L_2 X T_3	18.81	-7.82	-15.70*	-22.18**	6.63	-7.85**	-12.07**	-42.88**
L_2 X T_4	19.60	-2.82	-12.19	-18.94**	8.00	2.48	-0.91	-31.07**
L_3 X T_1	22.87	12.83*	-5.40	-5.40	6.16	-33.57**	-46.96**	-46.96**
L_3 X T_2	20.71	28.08**	26.58**	-14.33*	6.52	7.03*	-5.92	-43.83**
L_3 X T_3	16.80	-3.63	-9.21	-30.51**	6.97	1.16	0.58	-39.95**
L_3 X T_4	19.46	13.22	8.03	-19.51**	6.54	-12.77**	-18.95**	-43.62**
L_4 X T_1	26.52	18.75**	9.69	9.69	6.34	-30.11**	-45.40**	-45.40**
L_4 X T_2	25.50	39.84**	24.45**	5.47	7.34	24.62**	12.46**	-36.76**
L_4 X T_3	17.25	-11.54	-15.83*	-28.66**	5.88	-12.14**	-14.21**	-49.37**
L_4 X T_4	18.09	-6.05	-11.73	-25.19**	9.84	34.79**	21.88**	-15.22**
L_5 X T_1	23.09	4.45	-4.49	-4.49	6.00	-38.41**	-48.33**	-48.33**
L_5 X T_2	27.09	50.41**	35.19**	12.04*	6.41	-2.29	-18.52**	-44.77**
L_5 X T_3	26.78	38.97**	33.65**	10.77	6.74	-8.40**	-14.32**	-41.93**
L_5 X T_4	25.92	36.24**	29.36**	7.21	7.86	-1.38	-2.64	-32.28**
L_6 X T_1	23.07	0.16	-4.58	-4.58	4.36	-53.46**	-62.41**	-62.41**
L_6 X T_2	28.53	50.67**	30.33**	18.01**	10.15	63.75**	42.09**	-12.55**
L_6 X T_3	25.08	24.18**	14.57*	3.74	6.32	-9.72**	-11.57**	-45.58**
L_6 X T_4	24.20	21.29**	10.55	0.10	5.44	-28.54**	-32.66**	-53.16**
L_7 X T_1	23.69	2.35	-2.03	-2.03	5.58	-42.47**	-51.92**	-51.92**
L_7 X T_2	27.53	44.55**	24.51**	13.87*	7.88	20.80**	1.11	-32.11**
L_7 X T_3	22.75	12.02*	2.88	-5.91	5.74	-21.60**	-26.35**	-50.55**
L_7 X T_4	23.85	18.87**	7.85	-1.36	7.90	-0.46	-2.19	-31.96**
L_8 X T_1	26.83	16.88**	10.99	10.99	6.87	-37.10**	-40.84**	-40.84**
L_8 X T_2	23.02	22.07**	5.90	-4.77	5.89	-23.90**	-42.41**	-49.25**
L_8 X T_3	22.22	10.43	2.21	-8.09	10.70	25.32**	4.63*	-7.81**
L_8 X T_4	16.21	-18.45**	-25.44**	-32.95**	8.83	-3.46	-13.62**	-23.89**
L_9 X T_1	28.24	34.29**	16.81**	16.81**	5.50	-35.34**	-52.61**	-52.61**
L_9 X T_2	19.49	15.14*	9.02	-19.37**	5.69	6.82	5.30	-50.95**
L_9 X T_3	22.91	25.94**	23.82**	-5.24	6.78	10.63**	-1.02	-41.59**
L_9 X T_4	20.75	15.60*	15.17	-14.19*	6.74	0.00	-16.52**	-41.93**
L_{10} X T_1	15.29	-33.24**	-36.77**	-36.77**	5.91	-36.20**	-49.08**	-49.08**
L_{10} X T_2	19.97	6.25	-7.60	-17.39**	6.30	3.45	-9.01**	-45.75**
L_{10} X T_3	18.83	-6.15	-12.91*	-22.13**	5.12	-25.64**	-26.01**	-55.89**
L_{10} X T_4	18.15	-8.40	-16.04*	-24.93**	5.62	-25.03**	-30.39**	-51.58**
SEd	1.65	1.22	1.41		0.23	0.20	0.23	
CD at 1%	4.25				0.60			

*, **Significant at 5 and 1% level.

Table 3. Contd.

Entry	Average value	Average fruit weight (g)			Average value	Fruit yield per plant (kg)		
		MP	BP	SV		MP	BP	SV
CD at 5%	3.23		0.46	CD at 5%	3.23			
Alavayal Local	66.32				1.72			
Sedapatty Local (Green)	56.09				1.86			
Kariapatty Local	41.05				1.27			
Alagarkovil Local	46.01				1.26			
Palamedu Local	81.66				1.91			
Melur Local	55.72				1.75			
Keerikai Local	65.76				1.79			
Nilakottai Local	50.01				1.27			
Singampunari Local	34.94				1.16			
Sedapatty Local (Blue)	54.17				1.37			
Annamalai	55.9				2.12			
KKM 1	42.42				1.46			
Punjab Sadabahar	50.34				1.56			
EP 65	53.38				1.36			
L_1 X T_1	69.40	13.57**	4.65	24.15**	2.47	28.65**	16.33**	16.33**
L_1 X T_2	54.62	0.47	-17.63**	-2.28	1.35	-15.13**	-21.55**	-36.58**
L_1 X T_3	65.78	12.77**	-0.81	17.67**	1.35	-17.60**	-21.36**	-36.42**
L_1 X T_4	51.83	-11.14**	-21.84**	-7.27*	1.45	-5.42	-15.34**	-31.55**
L_2 X T_1	48.00	-14.28**	-14.42**	-14.13**	2.07	3.77	-2.67	-2.67
L_2 X T_2	54.32	10.29**	-3.15	-2.82	1.42	-14.17**	-23.48**	-32.97**
L_2 X T_3	57.82	8.64**	3.08	3.43	2.14	24.95**	14.87**	0.63
L_2 X T_4	52.24	-1.83	-6.86*	-6.54*	1.56	-2.80	-15.95**	-26.37**
L_3 X T_1	43.50	-10.26**	-22.18**	-22.18**	1.45	-14.45**	-31.71**	-31.71**
L_3 X T_2	44.44	6.49	4.76	-20.50**	0.99	-27.05**	-31.81**	-53.22**
L_3 X T_3	61.99	35.65**	23.13**	10.89**	1.74	23.11**	11.54*	-18.05**
L_3 X T_4	47.41	3.76	-5.82	-15.19**	0.93	-29.35**	-31.70**	-56.36**
L_4 X T_1	60.91	19.54**	8.97**	8.97**	2.40	41.73**	13.03**	13.03**
L_4 X T_2	52.64	19.04**	14.39**	-5.84*	2.32	70.34**	59.04**	9.11*
L_4 X T_3	53.38	10.79**	6.03	-4.51	1.49	5.79	-4.27	-29.67**
L_4 X T_4	58.18	20.76**	15.57**	4.08	1.92	46.31**	41.28**	-9.73*
L_5 X T_1	66.13	-3.85	-19.02**	18.30**	1.71	-15.44**	-19.62**	-19.62**
L_5 X T_2	59.64	-3.87	-26.97**	6.68*	2.34	38.87**	22.30**	10.20**
L_5 X T_3	56.04	-15.10**	-31.38**	0.24	2.36	36.08**	23.52**	11.30**
L_5 X T_4	51.17	-22.47**	-37.34**	-8.47**	2.21	35.17**	15.51**	4.08
L_6 X T_1	51.64	-7.47**	-7.62**	-7.62**	1.48	-23.65**	-30.30**	-30.30**
L_6 X T_2	55.96	14.05**	0.44	0.11	2.24	39.56**	27.76**	5.49
L_6 X T_3	53.48	0.84	-4.02	-4.34	1.35	-18.31**	-22.81**	-36.26**
L_6 X T_4	51.00	-3.83	-8.47**	-8.77**	1.68	7.82	-4.37	-21.04**
L_7 X T_1	56.55	-7.04**	-14.01**	1.16	1.13	-42.25**	-46.78**	-46.78**
L_7 X T_2	69.49	28.46**	5.67*	24.31**	2.85	75.36**	59.03**	34.07**
L_7 X T_3	54.50	-6.11*	-17.12**	-2.50	1.43	-14.43**	-19.93**	-32.50**
L_7 X T_4	57.67	-0.65	-12.30**	3.17	1.74	10.38*	-2.98	-18.21**
L_8 X T_1	42.04	-20.61**	-24.79**	-24.79**	2.26	33.14**	6.28	6.28
L_8 X T_2	42.74	-7.51*	-14.53**	-23.54**	1.66	21.91**	13.96*	-21.82**
L_8 X T_3	40.84	-18.60**	-18.87**	-26.94**	1.04	-26.18**	-33.12**	-50.86**
L_8 X T_4	49.54	-1.27	-1.60	-11.38**	2.06	57.31**	52.09**	-2.83
L_9 X T_1	46.66	2.73	-16.53**	-16.53**	2.29	39.15**	7.69*	7.69*
L_9 X T_2	46.87	21.17**	10.49**	-16.15**	1.73	31.81**	18.54**	-18.68**
L_9 X T_3	56.43	32.34**	12.09**	0.95	1.22	-10.40*	-21.79**	-42.54**
L_9 X T_4	50.54	18.54**	0.40	-9.58**	1.42	12.43*	4.42	-33.28**
L_{10} X T_1	57.01	3.59	1.98	1.98	1.12	-35.82**	-47.25**	-47.25**
L_{10} X T_2	52.21	8.11**	-3.61	-6.60*	1.85	30.81**	26.77**	-13.03**
L_{10} X T_3	61.70	18.07**	13.91**	10.38**	1.43	-2.51	-8.55	-32.81**
L_{10} X T_4	56.59	8.29**	4.47	1.23	1.45	6.73	6.34	-31.55**
SEd	1.60	1.39	1.60		0.08	0.07	0.08	
CD at 1%	4.15				0.21			
CD at 5%	3.15				0.16			

*, **Significant at 5% and 1% level.

24 had significant and negative heterosis. Thirty-one (31) hybrids had significant and positive heterosis over the standard variety. Four hybrids had non-significant and negative values. The findings corroborate the results of

Table 4. Genotypic correlation between fruit yield and growth characters in eggplant.

Parameter	Days to first flowering	Number of branches per plant	Fruit yield per plant (kg)
Plant height (cm)	0.454*	-0.125	0.007
Days to first flowering		-0.385*	-0.211
Number of branches per plant			0.598*

*Significant at 5% level.

Nalini (2007).

The number of fruit per plant influences yield. The lowest number of fruit per plant was in L_5 and the highest in T_1 followed by L_9 among parents. Nineteen crosses, L_1 x T_1, L_2 x T_1, L_2 x T_3, L_4 x T_1, L_4 x T_2, L_4 x T_4, L_5 x T_2, L_5 x T_3, L_5 x T_4, L_6 x T_2, L_6 x T_4, L_7 x T_2, L_7 x T_4, L_8 x T_1, L_8 x T_2, L_8 x T_4, L_9 x T_1, L_9 x T_2 and L_{10} x T_2 exhibited positive heterosis in the desirable direction over the mid-parent. Expression of heterosis over the better parent in the positive direction occurred in 14 crosses. Significant heterosis over comer-cial check was recorded in 33 hybrids with 9 had positive and 23 had negative heterosis. Considerable positive heterosis in different cross combinations were also reported by Chowdhury et al. (2010).

The highest single fruit weight was for L_7 x T_2 followed by L_1 x T_1 and the lowest was in L_8 x T_3. Of 40 hybrids, 17 had significant desirable heterosis over the mid-parent in the positive direction. Only 7 hybrids had significant heterobeltiosis. In 8 hybrids, heterosis was significant and in the positive direction over the standard variety. The heterosis over the better and standard parents was negative for fruit weight. Similar findings reported by Nalinidharwad et al. (2011) and Patil et al. (2001) lend support to the present results. This trend is not a constraint, because smaller sized eggplant fruit are preferred in South India and hybrids with smaller and more fruit could be selected.

The ultimate interest of the breeder is to get high yield. An appreciable amount of heterosis in F_1s over the mid-parent value occurred for fruit yield per plant. The T_1 parent had the highest yield followed by L_5 and L_2, while the lowest yield was for L_9. Fruit yield per plant showed a wide range among hybrids. Among the 40 hybrids, 19 had significant positive heterosis over the mid-parent. The hybrid from the cross L_7 x T_2 had the highest magnitude of heterosis in the positive direction followed by L_4 x T_2, L_8 x T_4 and L_4 x T_4. Most crosses involving T_2 as tester parent had significant, positive, heterosis over the mid-parent. This agrees with Ramesh Kumar et al. (2013).

Association analysis

Genotypic correlation coefficients varied depending on character (Tables 4 to 8). Of 15 characters studied, only numbers of fruit per plant, number of branches per plant and average fruit weight had significant association with fruit yield at phenotypic and genotypic levels (Prabakaran, 2010).

Fruit yield had significant negative association with fruit borer infestation at both levels. This trait could be considered as an important criterion for selection for fruit yield. Total phenol content had considerable non-significant positive correlation with fruit yield per plant followed by fruit length, fruit circumference and shoot borer infestation. During selection these traits can be considered to improve fruit yield. There were significant, and positive, correlations for plant height with days to first flowering, while days to first flowering significantly, and negatively, correlated with numbers of branches per plant. As days to first flowering decreases, there will be an increase in number of branches per plant. Fruit pedicel length had a significant, and positive, correlation with clayx length. Fruit circumference was significantly, and positively, associated with average fruit and average fruit weight may result in simultaneous improvement of fruit yield per plant (Thangamani and Jansirani, 2012). These cha-racters are highly reliable components of fruit yield and could be utilized as yield indicators during selection. Days to first flowering, fruit pedicel length, calyx length, fruit borer infestation and ascorbic acid, an important quality trait, were negatively associated with fruit yield per plant. Selection for this trait will result in reduction weight. Similarly, the association between shoot borer infestation and little leaf incidence was positive and significant. Selection for this trait will result in reduction of fruit yield, number of branches per plant, number of fruit per plant of fruit yield.

Path coefficient analysis

The path coefficient analysis permits the separation of direct effect from indirect effects through other related traits by partitioning genotypic correlation coefficients. Plant height, number of branches per plant, fruit length, fruit pedicel length, number of fruit per plant, average fruit weight and little leaf incidence had positive direct effect on yield (Table 9).

Direct selection for these characters are likely will bring about an overall improvement in fruit yield per plant. The residual effect determines how causal factors account

Table 5. Genotypic correlation between fruit yield and fruit characters in eggplant.

Parameter	Fruit pedicel length (cm)	Fruit circumference (cm)	Calyx length (cm)	Number of fruits per plant	Average fruit weight (g)	Fruit yield per plant (kg)
Fruit length (cm)	0.101	-0.185	0.181	0.139	0.094	0.200
Fruit pedicel length (cm)	-	0.034	0.248*	-0.185	0.088	-0.124
Fruit circumference (cm)	-	-	-0.011	0.061	0.273*	0.154
Calyx length (cm)	-	-	-	-0.094	0.161	-0.023
Number of fruit per plant	-	-	-	-	-0.148	0.836*
Average fruit weight (g)	-	-	-	-	-	0.380*

*Significant at 5% level.

Table 6. Phenotypic correlation between fruit yield and fruit characters in eggplant.

Parameter	Fruit pedicel length (cm)	Fruit circumference (cm)	Calyx length (cm)	Number of fruits per plant	Average fruit weight (g)	Fruit yield per plant (kg)
Fruit length (cm)	0.084	-0.169	0.147	0.150	0.085	0.205
Fruit pedicel length (cm)	-	0.027	0.247*	-0.186	0.084	-0.125
Fruit circumference (cm)	-	-	-0.019	0.064	0.247*	0.147
Calyx length (cm)	-	-	-	-0.108	0.161	-0.034
Number of fruit per plant	-	-	-	-	-0.140	0.831*
Average fruit weight (g)	-	-	-	-	-	0.381*

*Significant at 5% level.

Table 7. Genotypic correlation between fruit yield, pest and disease incidence and quality characters in eggplant.

Parameter	Fruit borer infestation (%)	Little leaf incidence (%)	Ascorbic acid content (mg/100g)	Total phenols content (mg/100g)	Fruit yield per plant (kg)
Shoot borer infestation (%)	-0.007	0.400*	-0.053	0.141	0.102
Fruit borer infestation (%)	-	-0.048	0.084	0.187	-0.280*
Little leaf incidence (%)	-	-	0.048	0.208	0.043
Ascorbic acid content (mg/100 g)	-	-	-	-0.151	-0.045
Total phenols content (mg/100 g)	-	-	-	-	0.227

*Significant at 5% level.

Table 8. Phenotypic correlation between fruit yield, pest and disease incidence and quality characters in eggplant.

Parameter	Fruit borer infestation (%)	Little leaf incidence (%)	Ascorbic acid content (mg/100 g)	Total phenols content (mg/100 g)	Fruit yield per plant (kg)
Shoot borer infestation (%)	-0.009	0.393*	-0.047	0.137	0.094
Fruit borer infestation (%)		-0.054	0.065	0.168	-0.275*
Little leaf incidence (%)			0.057	0.203	0.047
Ascorbic acid content (mg/100 g)				-0.151	-0.036
Total phenols content (mg/100 g)					0.215

* Significant at 5% level.

Table 9. Direct and indirect effects of different characters on fruit yield in eggplant.

Character	X1[a]	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	Fruit yield per plant
X1	0.0072	-0.0123	-0.0022	0.0000	-0.0006	-0.0012	0.0019	0.0429	-0.0058	0.0025	0.0119	0.0061	-0.0040	-0.0055	0.039
X2	0.0027	-0.0328	-0.0130	0.0001	-0.0002	0.0037	0.0012	-0.0581	-0.0455	0.0015	-0.0035	0.0042	0.0005	0.0027	-0.138
X3	-0.0002	0.0073	0.0583	-0.0000	-0.0002	-0.0046	0.0012	0.0370	0.0864	0.0000	-0.0096	0.0041	-0.0039	-0.0045	0.166
X4	0.0008	-0.0021	0.0152	0.0003	-0.0005	0.0025	0.0005	-0.0304	0.1915	0.0025	0.0111	-0.0018	0.0013	-0.0025	0.169
X5	0.0001	-0.0047	-0.0030	0.0011	0.0004	0.0070	-0.0011	0.0384	0.0539	0.0034	0.0084	-0.0042	0.0029	0.0027	0.100
X6	-0.0010	0.0016	-0.0035	0.0001	0.0047	-0.0012	-0.0015	-0.1185	0.0503	0.0034	0.0035	-0.0062	-0.0013	0.0055	-0.062
X7	0.0002	0.0032	0.0071	-0.0002	0.0001	-0.0379	0.0000	0.0898	0.1571	0.0020	0.0064	-0.0002	-0.0000	-0.0001	0.229
X8	-0.0022	0.0065	-0.0115	0.0002	0.0011	0.0004	-0.0064	-0.0532	0.0928	0.0055	-0.0005	-0.0118	0.0013	0.0058	0.030
X9	0.0003	0.0022	0.0025	0.0000	-0.0006	-0.0040	0.0004	0.8516	-0.0525	-0.0004	0.0002	0.0011	-0.0004	-0.0007	0.801*
X10	-0.0000	0.0026	0.0087	0.0001	0.0004	-0.0103	-0.0010	-0.0777	0.5758	0.0024	0.0206	-0.0003	-0.0015	-0.0040	0.509*
X11	-0.0015	0.0041	-0.0004	-0.0003	-0.0013	0.0061	0.0028	0.0275	-0.1143	-0.0124	0.0010	0.0088	0.0010	-0.0024	-0.077
X12	-0.0015	-0.0020	0.0098	-0.0001	-0.0002	0.0042	-0.0000	-0.0042	-0.2072	0.0002	-0.0572	-0.0001	-0.0010	0.0025	-0.253*
X13	0.0019	-0.0060	0.0106	-0.0002	-0.0012	0.0004	0.0033	0.0408	-0.0074	-0.0048	0.0002	0.0229	-0.0003	-0.0044	0.057
X14	0.0020	0.0012	0.0154	-0.0002	0.0004	-0.0001	0.0005	0.0237	0.0615	0.0009	-0.0038	0.0005	-0.0148	0.0035	0.093
X15	0.0021	0.0046	0.0137	-0.0001	-0.0013	-0.0003	0.0019	0.0324	0.1216	-0.0016	0.0075	0.0053	0.0027	-0.0191	0.167

*Significant at 5% level; Residual effect, 0.102; Diagonal values, direct effects; Half diagonal values, indirect effects. [a] X1, Plant height (cm); X2, Days to first flowering; X3, Number of branches per plant; X4, Fruit length (cm); X5, Fruit pedicel length (cm); X6, Fruit circumference (cm); X7, Calyx length (cm); X8, Number of fruit per plant; X9, Average fruit weight (g); X10, Shoot borer infestation (%); X11, Fruit borer infestation (%); X12, Little leaf incidence (%); X13, Ascorbic acid content (mg/100g); and X14, Total phenols content (mg/100g).

for variability of the dependent factor, that is, fruit yield per plant in this study. The residual effect was only 0.102, accounting for 97.50% of variability in fruit yield per plant, was explained by the 15 variables included in the study.

The characters studied by path analysis for yield appear to be appropriate. Plant height, number of branches per plant, fruit length, fruit pedicel length, number of fruit per plant, average fruit weight and little leaf incidence are important characters to bring about overall improvement in fruit yield per plant. Similar trend of findings were also obtained by Thangamani and Jansirani (2012) for fruit yield per plant, number of branches per plant and average fruit weight in brinjal.

Conclusion

Promising lines are to be used to obtain higher yield, earliness and increased fruit number. Due to high heterosis the importance of non-additive genetic effects in expression can be inferred. The establishment of a population with a wide genetic base, using recurrent selection methods for increasing combining ability, will lead to future new lines which result in hybrids superior to those studied. The hybrids L7xT2, L1xT1, L4 xT1, L5xT3, L5xT2, L8 x T4 and L4 x T2 had the highest values over standard variety heterosis. These hybrids can be utilized for selecting superior desirable segregants in later generations. Correlation and path analysis indicated that more fruit per plant with heavier weight are contributors to improved yield. At the outset comprehensive results obtained from the correlation and path analysis indicated that more number of fruits per plant with more fruit weight are outstanding contributors made by the hybrids for yield per plant. Therefore, while selecting the hybrids due weightage may given to the above said traits for the overall improvement of fruit yield.

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Detection of *Acidovorax avenae* subsp. *avenae* in commercial corn seeds and its correlation with seedling transmission

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***Acidovorax avenae* subsp. *avenae* (*Aaa*) caused bacterial leaf streak of corn. To minimize the risk of introducing this pathogen, sorbitol neutral red (SNR) agar was integrated with bio-polymerase chain reaction (PCR) to detect *Aaa* in commercial corn seeds. A set of 32-random samples was initially detected by sorbitol neutral red (SNR). Colony forming units (CFU) of *Aaa* from SNR plates and seed extracts was confirmed diagnostic tools by bio-PCR. The bio-PCR produced the positive band from 6, 8 and 10 samples of waxy corn, sweet corn, and field corn with 4, 4, 4, 6, 30, and 40%; 12.5, 14, 16, 23, 30, 30, 30, and 68.2% and 2, 4, 6, 10, 14.3, 20, 20, 30, 30, and 30% respectively, that correlated with the incidence of *Aaa* detected on SNR agar. We also demonstrated *Aaa* transmission from sweet corn seeds to seedlings by plant bioassay. As expected, seeds did not show evident symptoms of bacterial leaf streak, but typical *Aaa* fluid colonies were reisolated indicating seedling transmission of *Aaa*. The two methods studied provide an effective quantification of *Aaa* in corns seeds. This suggests that certification of corn seeds against *Aaa* should be developed.**

Key words: Bio-PCR, sorbitol neutral red (SNR) agar, contaminated seeds, waxy corn, sweet corn, field corn.

INTRODUCTION

Acidovorax avenae subsp. *avenae* (*Aaa*) is an important pathogen of several hosts including oat, corn, wheat, sugarcane, millet, foxtail, and rice (Kadota, 1996; Prathuangwong et al., 2004). Bacterial leaf streak of corn caused by *Aaa* was first reported in the major corn growing areas of Thailand by Prathuangwong et al. (2004). Economic losses of 30% caused by *Aaa* on sweet corn susceptible cultivar, Insee2, have been reported (Techati, 2008). The symptoms of the disease are long streak lesions, water soaking and haloes parallel with leaf vein. These symptoms are similar to other corn diseases such as Stewart wilt caused by *Pantoea stewartii* subsp. *stewartii*.

Since the pathogen does not always produce distinct symptoms under field conditions, the disease is very difficult to diagnose (Prathuangwong et al., 2004). The causal agent was reported as a serious seedborne pathogen of various plants such as rice (Shakya, 1987), watermelons, and cantaloupes (Schaad et al., 1978). It can be recovered from seeds using general agar medium (Song et al., 2000; 2001) but seedborne contamination of corn was not detected. Moreover, the infected seeds are an important source of primary inoculum. However, *Aaa* is predominated by other saprophytic bacteria, thus, it is difficult to isolate and identify. Some methods such as blotter test (Shakya and Chung, 1983), serological test

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Abbreviations: ***Aaa*,** *Acidovorax avenae* subsp. *avena*e; **PCR,** polymerase chain reaction; **SNR,** sorbitol neutral red; **BLAST,** Basic Alignment Search Tool; **ITS,** internal transcribed spacer.

(Kadota et al., 1991; Shakya, 1987), and selective media (Kadota, 1996; Zeighler and Alvarez, 1989) have been developed for the detection of *Aaa,* but none have become widely used. A selective medium would be useful for quantitative recovery and monitoring the pathogen in seed lots. Some selective media (Kadota, 1996; Summer and Schaad, 1977; Zeigler and Alvare, 1989) have limited success in detecting the pathogen directly from seeds or differentiating the bacteria from other seedborne bacteria. Pathogenicity tests are very time-consuming and need further testing to confirm the identity of pathogen.

Plant pathogenic bacteria have been identified quickly by polymerase chain reaction (PCR)-based detection methods (Henson and French, 1993; Song et al., 2004). Although PCR could be a very quick, and clean method for plant pathogenic bacteria detection, it has limitations with non-purified samples such infected seeds or plant leaf samples. Non-purified samples consisted of numerous compounds and saprophytic cells which disturbed the PCR reaction. All these problems were reduced by purification of the target bacteria and extraction of DNA. Moreover, the interfering of classical PCR reaction cause by dead cells and/or free DNA resulted in false positives.

One way to avoid the causal agent of false positives of PCR is to increase the target bacterium on semiselective agar or in liquid media prior to PCR, a technique termed bio-PCR (Schaad et al., 1995; Song et al., 2004). Therefore, the purpose of this study was to combine the use of a modified semi-selective medium, sorbitol neutral red (SNR) with bio-PCR for the specific detection of *Aaa* in commercial corn seeds.

MATERIALS AND METHODS

Bacterial strains and pathogenicity test

Aaa strains were isolated from thirty-four leaf samples of corn seedling at 10 days after planting by routine practice. Virulence of all *Aaa* strains was assessed on 10 days old corn of susceptible cultivar Insee2, using previously described quantitative methods (Techati, 2008). Briefly, aqueous cell suspensions (OD_{600} = 0.2, ca. 10^8 cfu/ ml of each) were sprayed on leaves of plants, covered with plastic bag for 48 h, and maintained in greenhouse. Control plants were inoculated with sterile water in the same manner. The experiment was done in randomized compete block design (RCBD). At four to seven days after inoculation, disease severity was assessed using a scoring method adapted from Pataky et al. (1997) based on the percentage of tissue area affected by *Aaa* at 3 levels. Disease severity score comprised: A, Short streak lesions and irregular margin, percent of tissue infected area less than 10%; B, long streak lesions, water soaking and haloes parallel with leaf vein, percent of tissue infected area 11-25%; and C, long streak lesions and leaf blight observed, percent of tissue infected area more than 25%. Nine corn leaves, collected from three different plants, were evaluated for each strain.

Recovery of *Aaa* from infected seeds

The experiment compared the efficiency of the modified SNR [3.0 g of K_2HPO_4, 1.0 g of NaH_2PO_4, 1.0 g of KNO_3, 0.2 g of $MgSO_4{\cdot}7H_2O$, 10 ml of neutral red (0.2% aqueous solution 69% active), and 15 g of agar, after autoclaving, 2.0 ml of sterile solution of cyclohexamide (100 mg/ml 75% ethanol), 50 ml of sterile solution of D-sorbitol (10% aqueous)were added, and supplemented with ampicillin 100 µl/mg] in the recovery of *Aaa* from corn seed extracts with Luria Bertani (LB) agar. 20 g of corn seeds from infected and healthy plants, respectively, were surface disinfected before treatment with 95% ethanol for 3 min and washed with sterile distilled water 5 times to remove the 95% ethanol. They were then added to 50 ml of the SNR broth and shaken at 28°C for 15 h. The 10^{-1} - 10^{-3} dilution of infected and non-infected seed extracts and the virulent *Aaa* strain (Techati, 2008) in LB broth were prepared. 0.1 ml of each dilution was inoculated onto ten plates of SNR and LB media by using agar plating method. The plates were incubated at 28°C for 2-4 days and then recovery colony numbers were determined. The experiment was done in RCBD and repeated three times.

Sensitivity of bio-PCR with *Aaa*

To verify the minimum number of *Aaa* colonies that could make the PCR positive, representative *Aaa* strain was inoculated in LB agar. After 15 h at 28°C, an *Aaa* colony was removed from the plates using a sterile toothpick and transferred to a PCR tube and 100 µl of sterile water was added. 1, 2, 3, and 10 µl of bacterial suspension were spread on LB agar for counting the bacterial concentration. The experiment was done with three replications and was repeated three times. Also, 1, 2, 3, and 10 µl of bacterial suspension in sterile water without DNA extraction were added to the PCR cycles. PCR-amplification was performed with primers AcAVF 5'-GGC TGG ATC ACC TCC TTT C-3' and AcAVR 5'-ACT TGC GAG GTC TTT CAC C- 3' designed from 16S-23S internal transcribed spacer (ITS) of *Aaa* accession number EU368726.1. DNA fragments were amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10X ThermoPol rection buffer (100 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% NP-40, and 50% glycerol), 1 µl of 10 pmol of each primer, 2 µl of dNTP mixed, 0.5 µl of *Taq* DNA Polymerase (BioLabs, USA). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 15 s of denaturation at 94°C, 30 s of annealing at 58°C, 1 min of extension at 72°C and the last extension step at 72°C was extended to 5 min. Amplified DNA was determined by agarose gel electrophoresis on a horizontal, 0.8% agarose gel in Tris/Borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) at 70 volts constant for 45 min using 1X TBE as a running buffer. The gel was stained with 0.5 µg of ethidium bromide per ml for 10 min and briefly then washed in water before visualizing under UV light and being photographed over a transilluminator (GDS 800, Complete Gel Documentation Analysis System). Moreover, sensitivity of bio-PCR was tested with corn seeds from infected mature plants compared with seeds from healthy plants. 20 g seeds were surface disinfected as described above, and added to 50 ml of SNR broth and shaken at 28°C for 15 h. One µl of seed extracts in SNR broth was added to the PCR cycles. PCR condition and reaction was as described above.

Detection of *Aaa* from naturally contaminated commercial corn seeds

Thirty-two seed samples were randomly collected from commercial corn seeds in Thailand including 6, 9, and 17 seed samples of waxy corn, sweet corn, and field corn, respectively. Fifty seeds from each sample were planted and the disease severity on the leaves at 10 days after planting was observed using the modification method as described above (Pataky et al., 1997). 20 g of each seed sample

Figure 1. Representative of disease severity score (%) of bacterial leaf streak caused by *Acidovorax avenae* subsp. *avenae* including, Group A = short streak lesions and irregular margin (A), Group B = long streak lesions, water soaking and haloes parallel with leaf vein (B), Group C = long streak lesions and leaf blight observed (C).

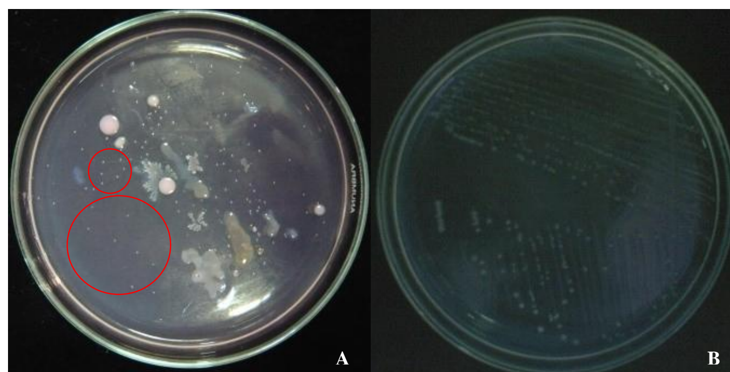

Figure 2. *Acidovorax avenae* subsp. *avenae* (*Aaa*) colony on SNR were white, shiny, round, smooth, convex, and 1.0 - 1.5 mm in diameter after 4 days in the circle that showed different morphology with saprophytic bacteria (A). Colonies of *Aaa* on LB were showed typically 1.0 - 2.0 mm after 2 days (B).

and 0.5 g of leaves from corn seedlings (10 days after planting) in the same samples were added to 50 and 5 ml of SNR broth, respectively, and shaken at 28°C for 15 h. One microliter (1 µl) of seed or leaf extracts in SNR broth was added to the PCR cycles. PCR condition and reaction was as described above. The presence of the PCR product was correlated with disease incidence and severity determinations.

DNA sequencing and evaluation

The PCR product was chosen for direct sequencing of the region of 16S-23S ITS. DNA sequencing was done with an Automated DNA Sequencer at the Bioservice Unit, National Science and Technology Development Agency (NSTDA), Thailand. Analysis of sequences was conducted using Basic Alignment Search Tool (BLAST) and the DNASTAR Lasergene software package (DNASTAR, WI, USA). The MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW. MegAlignment was then calculated for 16S-23S ITS sequences as available from GenBank for selected *Aaa*.

RESULTS

Bacterial strains and pathogenicity test

Forty-nine *Aaa* strains were isolated from leaves of corn seedling (Figure 1). Creamy white, circular smooth with entire margins, glistening *Aaa* colonies 1-2 mm in diameter on LB medium were isolated consistently from those diseased plants (Figure 2B). Among isolates, *Aaa* strains were classified by disease severity based on their aggressiveness on Insee2 cultivar into 3 groups. Group A, including 10 weak strains, caused short streak lesions and irregular margins. Group B, including 29 moderately virulent strains, caused long streak lesions, water soaking and haloes parallel with leaf vein. Group C, comprising highly virulent strains, caused long streak lesions and leaf blight. The population of *Aaa* in this group was 10 strains (Figure 1).

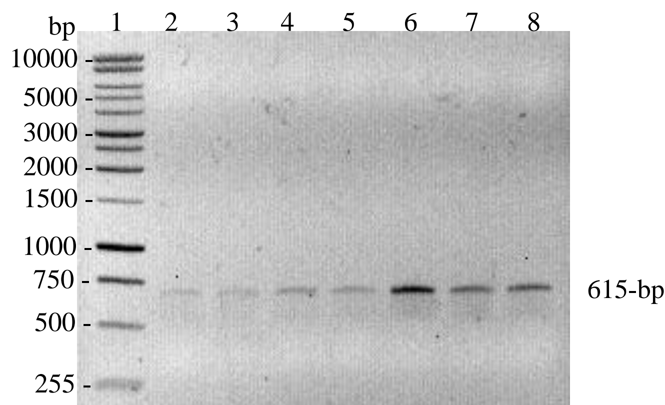

Figure 3. Bio-PCR amplification of the 615-bp fragment of the internal transcribed spacer region of the 16S-23S rDNA of *Acidovorax avenae* subsp. *avenae* in infected seed samples with primer AcAVF and AcAVR. PCR products were separated by electrophoresis on a 0.8% agarose gel. Lane 1, 1 kb DNA ladder; lanes 2-7, PCR products amplified; lane 8, 615-bp fragment from genomic DNA of causal agent of bacterial leaf streak.

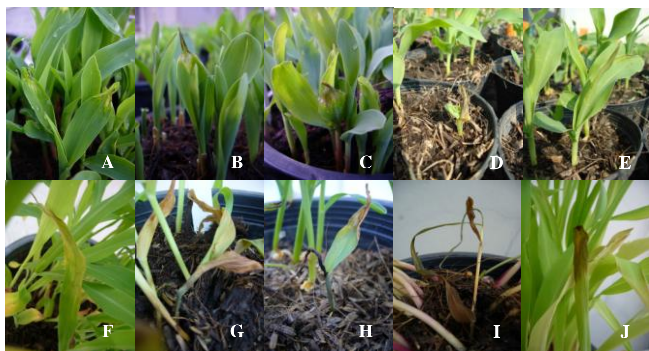

Figure 4. Seedling disease of waxy corn (A and F), field corn (B and G), and sweet corn (C, D, E, H, I, and J) caused by *Acidovorax avenae* subsp. *avenae* at 10 (A-E) and 21 (F-J) days after planting, respectively.

Recovery of *Aaa* from infected seeds

Modified SNR was the differential medium. *Aaa* colonies were white, shiny, round, smooth, convex, and 1.0 - 1.5 mm in diameter after 4 days and showed different morphology from saprophytic bacteria. Colonies of *Aaa* on LB were typically 1.0 - 2.0 mm diameter after 2 days (Figure 2). The recovery of *Aaa* on the SNR medium was compared with those on LB. The SNR medium supported good growth of *Aaa*. The recovery numbers of *Aaa* ranged from $4x10^3$ and $6x10^4$ cfu/ml at dilution of 10^{-2} and 10^{-3}, respectively, on SNR. While no *Aaa* was detected on LB, saprophytic bacteria of corn seed extracts predominate growth on LB. Also, *Aaa* was not detected from non-infected seeds by SNR. This indicated that SNR is specific or sensitive enough for reliable detection of *Aaa* from corn seeds.

Sensitivity of bio-PCR with *Aaa*

One aim of the research was to verify the efficacy of bio-PCR for *Aaa* detection with specific primers AcAVF and AcAVR at the various volume of bacterial suspension template. The results showed 1, 2, and 3 µl of *Aaa* suspension (consisting of 20, 30, and 100 cfu/ml, respectively) made the PCR positive (Figure 3), whereas in the 10 µl sample of *Aaa* suspension (consisting of 4 x 10^2 cfu/ml), PCR was negative. Moreover, bio-PCR with 1 µl of *Aaa* suspension template consistently detected *Aaa* in seeds from infected plants across all samples (100%) and did not detect *Aaa* in seeds from non-infected plants (100%). This indicated that bio-PCR was able to detect *Aaa* in corn seeds at concentrations of at least 20 cfu/ml. The sensitivity of bio-PCR with low number of bacterial cells such as 5 to 10 cfu/ml would be developed.

Detection of *Aaa* from naturally contaminated commercial corn seeds

Aaa was detected in 24 of 32 commercial corn samples (75%) using bio-PCR with the semiselective SNR liquid medium. The bio-PCR assay produced the positive band 615-bp (Figure 3) from 6, 8, and 10 samples of waxy corn, sweet corn, and field corn, respectively, and correlated with the incidence of *Aaa* detected on SNR agar and disease symptoms of corn (observed at 10 days after planting). The infected seeds sample exhibited disease severity on waxy corn, sweet corn, and field corn with 4, 4, 4, 6, 30, and 40%; 12.5, 14, 16, 23, 30, 30, 30, and 68.2%; and 2, 4, 6, 10, 14.3, 20, 20, 30, 30, and 30%, respectively (Figure 4).

DNA sequencing and evaluation

Alignment analysis of 16S-23S ITS sequence of *Aaa* isolated from infected corn seeds by MegAlign program (DNAstar inc) revealed that the nucleotide shares identity with 16S-23S ITS sequence (100% at nucleotide levels) of *Aaa* strain FC-320 (EU368726.1). This confirms that the pathogen isolated from infected corn seeds is *Aaa*.

DISCUSSION

Among *Aaa* strains isolated in this study was a heterogeneous species causing varying disease severity on susceptible corn cultivar Insee2. The research supports results reported by Techati (2008) that classified disease severity on corn plants into 3 groups based on the modified disease assessment from Pataky et al. (1997) and correlated with Insee2 inoculated with *Aaa*. In

addition, Insee2 cultivar was the suitable cultivar for classifying *Aaa* into different groups which correlated with genetic classification by Techati (2008).

However, seeds contaminated by *Aaa* were indistinguishable from healthy seeds based on morphology. Therefore, the best practice for detection of *Aaa* in corn seeds should be developed. *Aaa* in infected seeds was detected by SNR with added 100 mg/ml of ampicillin which inhibited growth of other seedborne bacteria where *Aaa* is resistance to ampicillin. SNR worked well with *Aaa* corn strains but not with rice and other strains (Song et al., 2000). The bacterial populations recovered from infected seed samples enriched in SNR liquid media and detected by semiselective SNR agar ranged from 4 $x10^3$ to 6 x 10^4 cfu/ml but were not detected in LB agar. Because of the rapid growth of saprophytic bacteria and other seedborne in LB liquid medium after enrichment for 15 h, colonies of *Aaa* were not visible when the resulting growth was plated onto LB agar. In contrast, growth of saprophytic bacteria and other seedborne was reduced enough in semiselective SNR agar to allow visual detection of *Aaa* colonies in most samples when plated onto SNR agars. We confirmed the value of using d-sorbitol as the sole carbon source in SNR medium for isolating *Aaa* of corn in Thailand (Summer and Schaad, 1977).

This SNR media result was correlated with the disease incidence and bio-PCR analyzes. Therefore, both techniques were effective techniques to detect *Aaa* in corn seeds and could be used for detection of *Aaa* in commercial seeds.

Corn seeds contaminated with *Aaa* are important sources of primary inoculum and a means of dissemination of the pathogen to new areas. Although methods including serology (Kadota, 1996; Shakya and Chung, 1983), blotter test and inoculation technique (Techati, 2008) are available for *Aaa* detection, none are specific or sensitive enough for reliable detection and are time-consuming. Besides being relatively insensitive, serological techniques may detect dead cells or closely related species. In contrast, bio-PCR detected live cells only. The disadvantages of pathogenicity methods or blotter test include time and increased possibilities for cross-contamination.

Based on availability of specific primers, PCR has become a popular technique for identification of bacteria (Schaad et al., 2001). The ITS regions are non-functional elements located between the 16S and 23S genes in prokaryotic rDNA loci and exhibited sequence variation useful for designing specific primers for identifying bacteria at the species level (Neefs et al., 1990; Kostman et al., 1992; Song et al., 1997). Also, the information from this study indicated that AcAVF and AcAVR were specific for *Aaa* strain from corn. Sensitivity of this bio-PCR technique is high for detecting *Aaa* with 20 cfu/ml in naturally infected corn seeds. For bio-PCR to be successful, a medium is needed that allows sufficient growth of the target bacterium to allow detection by PCR (generally $1x10^3$ cfu/ml or greater) before being overgrown by other bacteria (Schaad et al., 1995). The low number of *Aaa* that bio-PCR can detect is important for future research.

Therefore, SNR medium and bio-PCR techniques greatly improved the detection of *Aaa* in commercial corn seeds. Bio-PCR, using SNR medium to enrich the target bacteria in liquid media, allows for rapid and sensitivity for detecting low numbers of the *Aaa* pathogen in contaminated corn seeds. If high numbers of saprophyte bacteria are encountered after enrichment in solid or liquid media, a DNA extraction step could be included to reduce possible inhibition of PCR. In these cases, the presence of large numbers of other microorganisms prevents easy isolation of the pathogen on semiselective agar. The enrichment time necessary to detect 20 cfu/ml by bio-PCR is only 18 h (15 h enrichment and 3 h PCR reaction). These results show that bio-PCR can be applied to seeds with moderately high levels of background microflora and still detect low levels of *Aaa*. Furthermore, saprophytic bacteria, overgrow the target bacterium in corn seed samples, and can inhibit the PCR reaction from plant extracts. Bio-PCR using a semi-selective medium overcomes these problems and provides a highly sensitive assay for detecting *Aaa* in comercial corn seeds. The seed assay can be completed within 18 h, which is shorter than the 14-21 days required for traditional culturing, pathogenicity test, and subsequent bacterial identification.

The potential risk of the dissemination of *Aaa* in corn seeds in the international exchange of corn germplasm is of serious concern. Our results indicates *Aaa* is seed-borne pathogen and transmitted by seed into seedling. SNR medium and bio-PCR protocol as tools greatly improved the detection of *Aaa* in commercial corn seeds. These results show that SNR and bio-PCR can be applied to seeds with moderately high levels of background microflora and still detect low levels of *Aaa*. Bio-PCR may be a valuable tool for detecting *Aaa* in seed lots, monitoring natural bacterial spread, tracking the pathogen in field studies and detecting waterborne or airborne cells caught on filters.

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Full Length Research Paper

In vitro comparism of the extracellular secretion of inulosucrase enzyme in potential probiotic *Escherichia coli* 16 and BL-21

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***Escherichia coli* 16 has potential probiotic properties including antimicrobial activity due to extracellular secretion of colicins E1/1a1b. Inulosucrase (InuJ) enzyme catalyses the polymerization of a fructose moiety of sucrose leading to the formation of fructooligosaccharides. The present investigation compared the activity of InuJ enzymes cloned into pMAL-p2ΔlacI^Q a deletion vector and transformed into *E. coli* 16 and standard strain that is, *E. coli* BL21. Specific activities of InuJ enzyme were estimated in supernatant, periplasm and lysate. Specific activities of InuJ activity in cell lysate were similar in *E. coli* 16 and *E. coli* BL21 without induction of tac promoter with isopropyl thio-β-D-galactoside (IPTG). InuJ activity is mainly present in the periplasm of *E. coli* BL21 whereas in *E. coli* 16, most of the activity is in the supernatant. Superantant of *E. coli* 16 strain also showed good antibacterial activity due to colicin E1/Ia1b. Colicin E1/1a1b transport system could allow extracellular secretion of InuJ proteins in probiotic *E. coli* 16.**

Key words: Colicin, extracellular, *E. coli*, fructooligosaccharide, inulosucrase, prebiotic, probiotic.

INTRODUCTION

Prebiotics are a category of nutraceutical product that has the ability to promote the growth of specific beneficial gut bacteria (Kelly, 2008). In 2007, Roberfroid defined prebiotics as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gut microflora that confers health benefit”. Fructooligosaccharides (FOS) has been used as prebiotic and is considered as a functional food ingredient (Cherbut, 2002; Fanaro et al., 2005; Bouhnik et al., 2006; Roberfroid, 2007; Paineau et al., 2008). FOS is the common name for fructose oligomers that are mainly composed of 1-kestose (GFS2), 2-nystose (GF3) among others in which fructose units are bound at the β-2, 1 position of sucrose through the transfructosylating enzymes such as glucosyltransferases, fructosyltransferases and inulosucrase (Yun et al., 1996). Inulosucrase has been previously shown to be involved in the synthesis of FOS (Van Hijum et al., 2006).

Escherichia coli, a Gram negative bacterium is widely used as a host strain for recombinant protein production

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Abbreviations: FOS, Fructooligosaccharides; **BRP,** bacteriocin release proteins; **IPTG,** isopropylthio-β-galactoside; **MBP,** maltose binding protein.

Table 1. Bacterial strains and plasmids used.

Strain	Relevant characteristics	Reference/source
Plasmid		
pET15b- inuJ	Expression vector, derived from pET15b by insertion of a inulosucrase inuj gene; Ap^r	Anwar et al.,2008
pMAL-p2ΔlacIQ	deletion of lacIQ from periplasmic expression vector pMal-p2; Ap^r	This study
pMAL-p2ΔlacIQ - inuJ	derived from periplasmic expression vector pMAL-p2Δ$lacI^Q$ by insertion of inuJ ; Ap^r	This study
Bacterial		
E. coli BL21	F′ ompT hsdSB (rB- mB-) gal dcm	Sambrook and Russell, 2001
E. coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ-	Sambrook and Russell, 2001
E. coli s16	Wild type	Kumar et al., 2009

in industry. Some natural *E. coli* strains secrete protein extracellulally but their mechanisms of secretion are not clearly understood, nor are they widely exploited for recombinant protein production and metabolic engineering (Ni and Chen, 2009). In some case, recombinant proteins directed to the periplasm were found in the medium but the process is not known (Choi and Lee, 2004; Mergulhao et al., 2005). Sommer et al. (2010) constructed a plasmid which contains bacteriocin release proteins (BRP) that allow secretion of recombinant protein from the periplasm into the culture medium (Sommer et al., 2010). It was known that BRP or lysis proteins are responsible for the release of colicins such as A, E1, E2, K, N, U, and Y (Cascales et al., 2007; Singh et al., 2012). Previously, we had reported *E. coli* 16 isolated from rat fecal matter to possess characteristic such as acid tolerance, antibiotic sensitivity, no pathogenicity and antimicrobial activity against the members of *Enterobacteriacae* family. Antimicrobial activity was due colicin E1/1a 1b which is secreted into the culture media in *E. coli* 16 (Kumar et al., 2009). All these above characteristic attributed *E. coli* 16 to be a potential probiotic. In this study, we compared the heterologous expression of Inulosucrase from a periplasmic expression vector in the *E. coli* 16 and *E. coli* BL-21.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

All the *E. coli* strains and plasmids used in this study are listed in Table 1. The bacterial strains used in this study were *E. coli* DH5α, *E. coli* 16 and *E. coli* BL-21. Strains of *E. coli* were grown at 37°C in the Luria Bretani medium (Hi-Media Laboratories,Mumbai, India). The minimal medium used had the following composition: 12.8 g/l $Na_2HPO_4{\cdot}7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 mg/l $CaCl_2$, 1 mM $MgSO_4$, thiamine and trace elements. Antibiotics were used at the following final concentrations: ampicillin 100 μg ml^{-1}. Plasmid pET15b-*inuJ* was a generous gift from Dr. Dijkhuizen. *E. coli* DH5α was used for constructing recombinant plasmids. *E. coli* BL21and *E. coli* 16 were used for expressing the proteins under *in-vitro* condition.

Construction of plasmids: pMAL-p2Δ$lacI^Q$ and pMAL-p2Δ$lacI^Q$-*InuJ*

pMAL-p2 was digested with Mlu I/EcoRV, end filled and further self ligated, leading to disruption of *$lacI^Q$* gene to obtain constitutive pMAL-p2Δ$lacI^Q$ vector. Confirmation of disrupted clones of pMAL-p2Δ$lacI^Q$ was done on X-gal plate without isopropylthio-β-galactoside (IPTG). Plasmid pET15b-*InuJ*, containing inulosucrase gene, was digested with NcoI/BamHI to insert the 1.7 kb DNA fragment containing the *inuJ* gene into pMAL-p2Δ$lacI^Q$ for periplasmic expression. The recombinant plasmid was confirmed by restriction digestion. All the above DNA manipulations were carried out according to the procedures described by Sambrook and Russell (2001). Further confirmation of *E. coli* harbouring inulosucrase constructs cells were inoculated in M9 media (Hi-Media Laboratories, Mumbai, India) containing 20 mM sucrose as a sole source of carbon.

Transformation of pMAL-p2Δ$lacI^Q$ and pMAL-p2Δ$lacI^Q$-*InuJ* plasmid in both *E. coli* 16 and *E. coli* BL-21

The plasmids pMAL-p2Δ$lacI^Q$ and pMAL-p2Δ$lacI^Q$-*InuJ* were independently transformed into *E. coli* 16 using the $CaCl_2$ method. Similarly, plasmids pMAL-p2Δ$lacI^Q$ and pMAL-p2Δ$lacI^Q$ -*InuJ* were also independently transformed into *E. coli* BL-21using the $CaCl_2$ method.

Preparation of *E. coli* cell extracts and *InuJ* activity assay

E. coli BL-21and *E. coli* 16 harboring pMAL-p2Δ$lacI^Q$ and pMAL-p2Δ$lacI^Q$ *InuJ* constructs were grown overnight and harvested by “centrifugation (Heraeus Fresco refrigerated centrifuge, Thermo Scientific, Germany) at 9,200 g for 2 min at 4°C. The cell pellet was washed twice with 50 mM phosphate buffer (pH=7. 0) followed by re-suspension in the same buffer. The cells were then subjected to sonication (Branson Sonifier Model 450) for a total period of 1 min at a pulse rate of 15 s in an ice bath, followed by centrifugation at 9,200 g at 4°C for 30 min to remove cell debris. The cell free extract was used for the inulosucrase assays. Periplasmic fraction (Ames et al., 1984) and extracellular fraction were also checked for the presence of the enzyme activity. The initial rate of the inulosucrase reaction was measured at 37°C in 50 mM potassium phosphate buffer (pH 7.0) in the presence of 500 mM sucrose. The enzyme

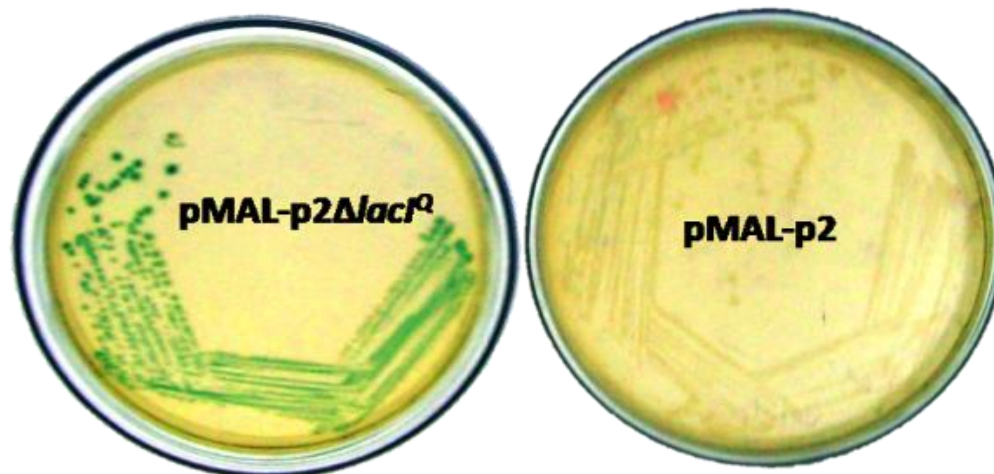

Figure 1. Phenotypic confirmation of *E. coli*16 harbouring pMAL-p2Δ*lacI*^{Q} plasmid on X-gal without addition of IPTG. X-gal breakdown leads to blue colour indicate deletion of lacI^{Q} where as control plasmid without deletion showed colourless.

inulosucrase catalyzes the formation of fructose polymers from sucrose in turn liberating glucose and thus this can be used as an indicator of the enzyme activity. Glucose was estimated using the 3, 5- dinitrosalicylic acid (DNSA) method (Miller et al., 1959).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native gel activity staining of recombinant inulosucrase

SDS PAGE gel was performed as mentioned by Sambrook and Russell (2001) to detect inulosucrase polypeptide. Samples were mixed with an equal amount of 2X sample buffer (0.125 M Tris-HCl [pH 6.8], 1% SDS, 20% glycerol, and incubated at 90°C for 5 min, centrifuged at 10,000 rpm for 20 s, and loaded onto a 12% slab gel. After electrophoresis, the gel was stained with Coomassie blue. Native gels were incubated at ambient temperature in McIlvaine's buffer (pH 7.0) with 10% sucrose. Formation of FOS within the gel led to white, turbid bands indicating the position of active enzyme. Prolonged incubation caused bursting of the gel due to excessive FOS formation at these sites (Hettwer et al., 1995).

Statistical analysis

Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and the results were expressed as mean ± SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, California, USA.

RESULTS

Molecular and phenotypic confirmation of *E. coli* transformants of pMAL-p2Δ*lacI*^{Q} and pMAL-p2Δ*lacI*^{Q}-*InuJ* plasmids

Deletion of repressor binding protein from pMAL-p2 plasmid gave rise to "constitutive phenotype" (Figure 1). The deleted plasmid clones and the original plasmid clones were plated on X-gal plates without IPTG. It was found that the deletion clone cleave X-gal showed blue colonies while the plasmid control does not cleave X-gal showed white colonies. Deletion of repressor binding protein in pMAL-p2 to form pMAL-p2ΔlacI^{Q} vector and insertion of inulosucrase gene in pMAL-p2Δ*lacI*^{Q} was confirmed by the release of 2.3 kb fragments upon digestion with SalI and BglII enzymes. Phenotypic confirmation of the clones containing the functional enzyme indeed grew on sucrose without IPTG while the vector controls and wild types did not grow in the presence of sucrose (Figure 2).

SDS-PAGE and activity of inulosucrase enzymes

The SDS-PAGE of cell free extracts of *E. coli* BL21 (DE3) containing pET-*InuJ* showed the presence of 63 kD protein band where as pMAL-p2Δ*lacI*^{Q}- *InuJ* band was seen at 106 kD (Figure 3). This is because InuJ protein is obtained as maltose binding protein (MBP)s-InuJ as a translational fusion protein with a molecular weight of 106 kD. In native gel loaded with the supernatant of *E. coli* 16 harboring pMAL-p2Δ*lacI*^{Q}- *InuJ* incubated in McIlvaine's buffer (pH 7.0) with 10% sucrose showed clearly a white turbid band indicating the activity of inulosucrase enzymes (Figure 4). Specific activity of inulosucrase enzyme in the supernatant, periplasm and lysate of *E. coli* 16 and BL-21 strain harbouring pMAL-p2ΔlacI^{Q}-*InuJ* were monitored (Figure 5a, b and c). In lysate the inulosucrase activities were similar in both the strains harbouring the plasmids. *E. coli* BL21strain harbouring pMAL-p2ΔlacI^{Q}-*inuJ* and pET-*InuJ* activity were found in the periplasm. While In *E. coli* 16 strain the activity was mostly found in the supernatant.

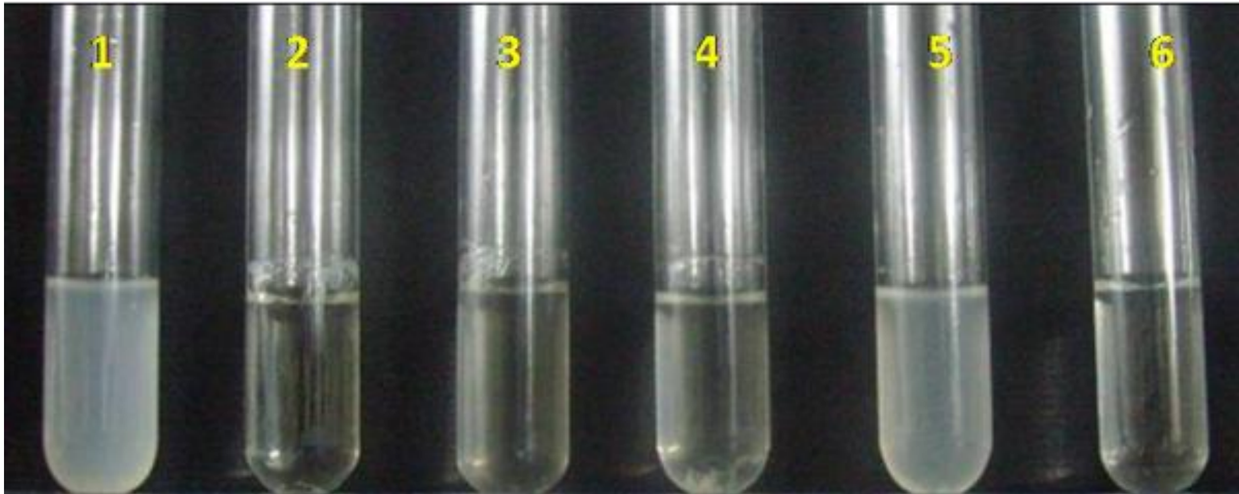

Figure 2. Growth of inulosucrase transformants in presence of M9 medium containing sucrose as a sole carbon source. Lane 1, *E. coli* BL21 (DE3) containing pET-15b-*inuJ* with IPTG; Lane 2, *E. coli* BL21 (DE3) containing pET-15b-*inuJ* without IPTG; Lane 3, *E. coli* BL21; Lane 4, *E. coli* 16 containing pMAL-p2Δ*lacI*^{Q}; Lane 5, *E. coli* 16 containing pMAL-p2Δ*lacI*^{Q} *inuJ*; Lane 6, *E. coli* 16.

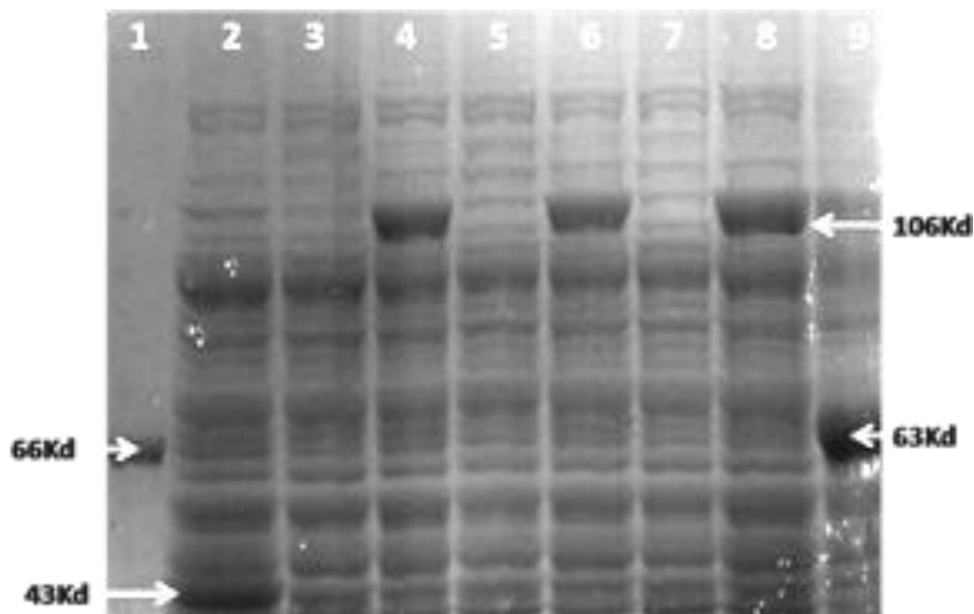

Figure 3. Protein profiles of *E. coli* BL21 expressing inulosucrase enzymes, resolved on 10% resolving gel by SDS-PAGE. Lane 1, BSA 66 kd; Lane 2, pMAL-p2 control; Lane 3, pMAL-p2ΔlacI^{Q}; Lane 4, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 5, pMAL-p2ΔlacI^{Q}; Lane 6, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 7, pMAL-p2ΔlacI^{Q}; Lane 8, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 9, pET-*inuJ* 63kd.

DISCUSSION

Modernization of diet resulted in a significant increase in the amount of added sugar such as sucrose and fructose, in food products leading to its daily consumption amounting to ≥ 100 g of sugar per day (Basciano et al., 2005). Intake of sweetener above 25% of total energy consumed will cause hypertriglyceridemia and gastrointestinal symptoms (Tappy and Kim-Anne, 2010). High amount of sucrose and fructose in diet ultimately leads to various metabolic disorder such as obesity, diabetes and hypertension

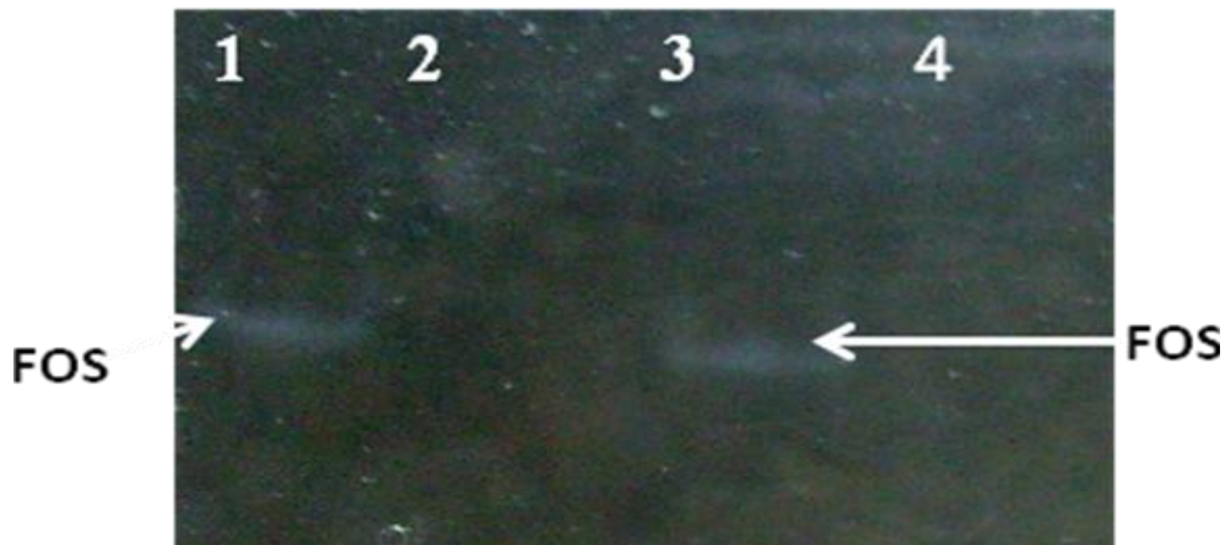

Figure 4. Native PAGE to show the activity of inulosucrase enzyme in supernatant of *E. coli* 16. White turbid band indicate FOS generation due to the activity of inulosucrase enzymes. Lane 1, pMALp2ΔlacI^{Q}-InuJ; Lane 2, pMALp2ΔlacI^{Q}; Lane 3, pMALp2ΔlacI^{Q}-InuJ; Lane 4, pMALp2ΔlacI^{Q}.

among others. Complex sugars like FOS on the other hand are beneficial and classified as functional food ingredient (Roberfroid, 2007; Kelly, 2008, 2009). FOS are also known for its antilipogenic effects and are useful in reducing blood glucose level in humans (Delzenne and Kok, 2001).

Interestingly, FOS can be produced from sucrose by the action of enzymes like inulosucrase which break down sucrose and polymerize it into FOS. In this case, we decided to genetically engineer the previously characterized (Kumar et al., 2009) potential probiotic *E. coli* 16 and *E. coli* BL-21 to express and secrete InuJ without induction with IPTG. As under *in-vivo* condition it is not possible to induce the gene by external factors such as IPTG. Similarly we cannot also use such promoter which constitutively expressed and form inclusion bodies. Hence we deleted the repressor protein of pMal-p2, that lead to moderate expression of Inulosucrase without induction with IPTG. Under *in-vivo* condition, this periplasmic InuJ could now access sucrose in the diet and polymerize it into FOS.

To demonstrate this characteristic we construct the plasmid and transformed into the host such as potential probiotic *E. coli* 16 and *E. coli* BL-21 and measured the activity of InuJ by using the DNSA methods to detect free glucose as an indicator of the sucrose hydrolysis (Figure 5). We also checked for direct indication of polymer synthesis using native gel electrophoresis (Figure 4). Surprisingly the heterologous protein even though targeted to the periplasm by the expression vector found its way into the extracellular medium in the case of *E. coli* 16 but not in *E. coli* BL-21. Comparisons of specific activity of inulosucrase cloned into periplasmic expression vector, transformed and expressed in *E. coli* 16 and *E. coli* BL21 demonstrated that extracellular expression is achieved only in colicin producing microorganism. Various reports suggested that BRP protein is responsible for the release of colicin such as colicin A, E1, E2, K, N, U, and Y into the extracellular medium (Cascales et al., 2007; Sommer et al., 2010; Singh et al., 2012). There are reports that extracellular secretion could be achieved in *E. coli* strain by co-expression of a lysis-promoting protein (Sommer et al., 2010). *E. coli* cells having the outer membrane generally do not help to secrete periplasmic proteins into the culture medium.

Here, we have shown a potential probiotic *E. coli* 16 that expresses E1/1a1b Colicin and its transport system mediates inulosucrase into the culture medium, that leads to the conversion of sucrose into FOS. Extracellular secretion of InuJ enzymes is better than the periplasmic secretion under *in-vivo* condition. Engineered *E. coli*16 strain produce colicin and FOS thus its give health benefits; colicin fight against enteropathogens and FOS a prebiotic product activate the beneficial microbes present in gut.

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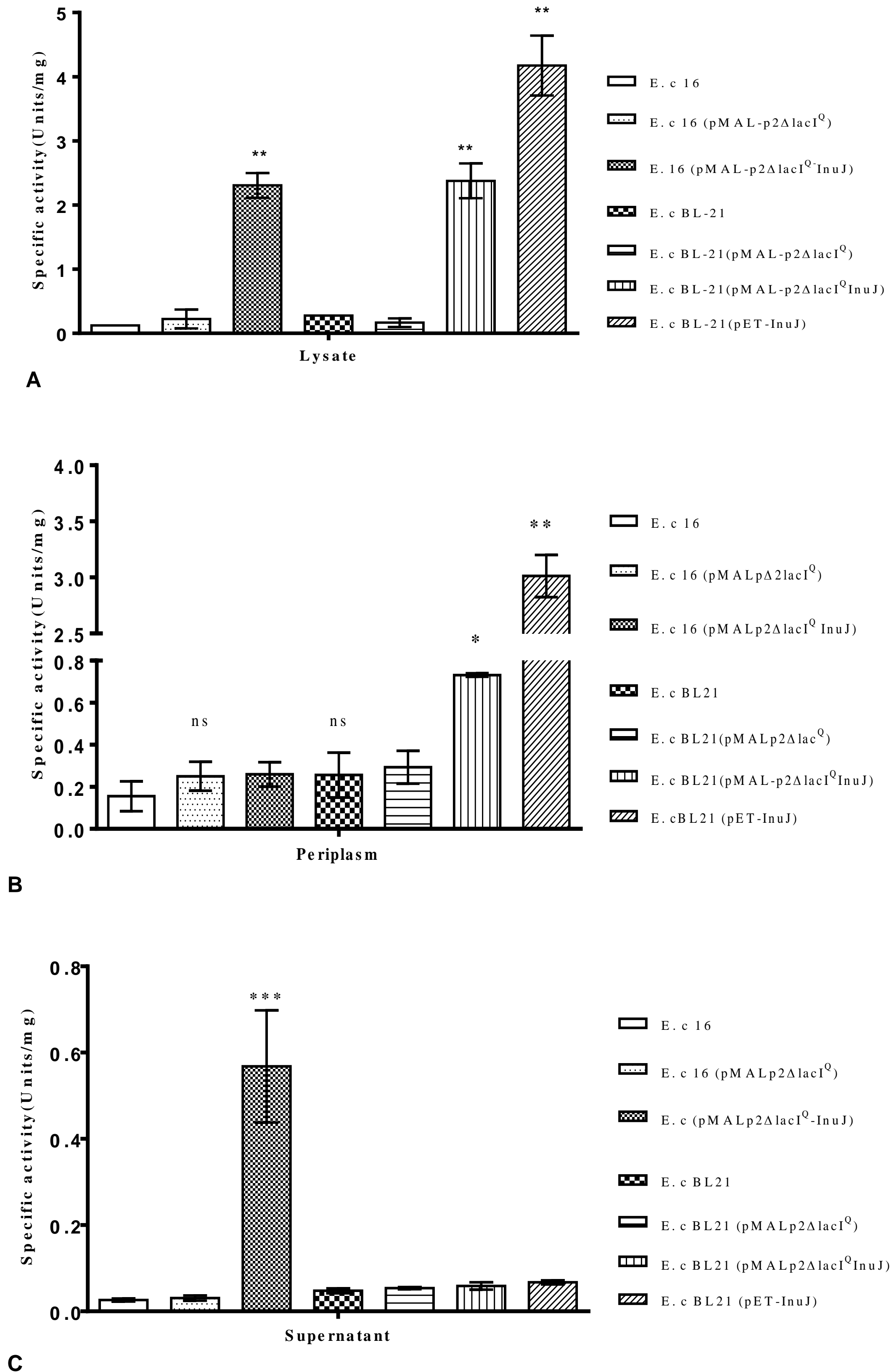

Figure 5. Specific activity of inulosucrase enzyme in a, Lysate; b, periplasm and c, supernatant of *E. coli* 16 and BL-21 strain containing (pMAL-p2ΔlacIQ-InuJ). All values are expressed as mean ± SD (n=4-6) each group and analysis was done using ANOVA. ***p<0.001, **p<0.01 and *p<0.05 compare to *E. coli* 16.

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Full Length Research Paper

Cloning and characterization of a thermostable and alkaline fibrinolytic enzyme from a soil metagenome

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A sequence-based polymerase chain reaction (PCR) was employed to screen fibrinolytic enzymes from soil metagenomes. A basic alignment search tool (BLAST) sequence homology analysis of 15 positive amplicons indicated a high degree of nucleotide sequence identity (>98%) to a fibrinolytic enzyme, nattokinase in *Bacillus* sp. Among the positive clones, KSL79_FE was selected for further characterization. Sequence analysis showed that its open reading frame (ORF) consisted of 1,146 nucleotides encoding 375 amino acids, of which two differed from the nattokinase (T268S and V298A). To overexpress the fibrinolytic enzyme, we transformed the plasmid pET28a/KSL79_FE into *E. coli* BL21 Codon (+) cells, leading to yield optimal expression by using a 9-h induction with 30 uM isopropyl thio-β-D-galactoside (IPTG) at an OD_{600} of 0.5. The resulting KSL79_FE enzyme exhibited caseinolytic and fibrinolytic activities similar to those of nattokinase. In contrast to the nattokinase which showed the optimal conditions for proteolytic activity at 37°C and pH 8.0, KSL79_FE enzyme displayed maximal proteolytic activity at 50°C and pH 9.0. In addition, the enzyme activity of KSL79_FE was inhibited by Zn^{+2} ions, but not by Cu^{+2} ions, not similar to nattokinase. The two residues varied from amino acid sequence of nattokinase which might change the biochemical properties and optimal enzyme activity of KSL79_FE.

Key words: Nattokinase, proteolytic activity, metagenome, fibrinolytic activity, cloning and expression.

INTRODUCTION

Blood clots form when thrombin proteolytically converts fibrinogen into fibrin, which subsequently forms insoluble clots. A balance between fibrin clot formation and fibrinolysis is critical for maintaining healthy circulation, but improper fibrin accumulation in the blood vessels (thrombosis) can seriously impair blood circulation, leading to acute myocardial infarction, ischemic heart disease, and high blood pressure. Thrombosis is

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Abbreviations: tPA, Plasminogen activator; **PCR,** polymerase chain reaction; ***FE*,** fibrinolytic enzyme gene; **IPTG,** isopropyl thio-β-D-galactoside; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **PVDF,** polyvinylidene fluoride; **IgG,** immunoglobulin G; **TCA,** trichloroacetic acid; **EDTA,** ethylenediaminetetraacetic acid; **EGTA,** ethylene glycol bis (2-aminoethyl ether) tetraacetic acid; **PMSF,** phenylmethylsulfonyl fluoride; **DTT,** DL-dithiothreitol; **BLAST,** Basic Alignment Search Tool; **ORF,** open reading frame; **NA,** *p*-nitroaniline; **DGGE,** denaturing gradient gel electrophoresis.

accumulation in the blood vessels (thrombosis) can seriously impair blood circulation, leading to acute myocardial infarction, ischemic heart disease, and high blood pressure. Thrombosis is prevalent in modern life and is the main contributor to death throughout the world.

As therapeutic agent for thrombosis, fibrinolytic enzymes, such as tissue plasminogen activator (tPA), urokinase, and the bacterial plasminogen activator streptokinase have been extensively studied. Among them, urokinase has been used to prevent and treat cardiac and cerebrovascular diseases for many years. However, this enzyme has a short half-life, low specificity to fibrin, and causes excessive bleeding and recurrence (Wong and Mine, 2004).

In the search for potential alternatives to traditional anti-thrombosis drugs, previous studies have identified and characterized several effective thrombolytic agents from microorganisms (Kim et al., 1996), earthworms (Wang et al., 2005), snake venom (De-Simone et al., 2005; Jia et al., 2003), centipede venom (You et al., 2004), insects (Ahn et al., 2003), and leeches (Chudzinski-Tavassi et al., 1998). These intriguing agents, which are usually plasminogen activators or plasmin-like proteases, have proven useful in understanding the fibrinolytic mechanism and have shown promise of potential therapeutic drugs. In recent, fibrinolytic enzymes were discovered from traditional fermented product, such as Natto in Japan, Chungkookjang in Korea, and Douchi in China (Peng et al., 2005).

According to a previous report (Sumi et al., 2003), oral intake of the fibrinolytic enzymes in fermented foods and tPA levels in plasma is also increasing the solubilization of fibrin in the blood, suggesting that oral intake of anti-thrombotic agents may be effective or more effective than intravenous injection. As a result, recent intensive research has sought to develop efficient methods to screen for fibrinolytic enzymes which are suitable for oral administration.

In this regard, traditionally fermented foods are recognized as a source of fibrin-specific fibrinolytic enzymes. However, harvest of fibrinolytic enzymes directly from fermented foods is hampered by the difficult isolation and cultivation steps, such as expression of the proteins and characterization of their biological functions. Therefore, it is worthwhile to investigate with new approaches and useful environmental resources that may yield interesting fibrinolytic enzyme (Hugenholtz and Pace, 1996).

For many decades, microbiologists have been fascinated by the observation that the vast majority of natural environmental microorganisms (99% of the organisms in some environments) cannot yet be cultivated *in vitro* (Lu et al., 2013; Liles et al., 2003). Only one percentage of microorganisms has been analyzed from biosphere and yet most microbes in nature have not been studied. Conventional culturing methods have the limits to those that grow under laboratory conditions (Sharma et al., 2010). The recent research provides compelling evi-evidence for the existence of many novel types of microorganisms in the environment in numbers and varieties that dwarf those of the comparatively few microorganisms amenable to laboratory cultivation. Countable numbers of enzymes, such as protease, lipase and amylase, have been isolated and characterized from collective genomes of the total microbiota found in nature, termed as the metagenome. The pool of combined genomes obtained from communities of microbial organisms in their natural environments provides access to the world of uncultured microorganisms and vastly more genetic information than one of the cultivable subset of microbiota (Lee et al., 2006). Here we isolated and characterized a fibrinolytic enzyme from environmental soil metagenomic libraries beyond conventional fermented foods.

MATERIALS AND METHODS

Materials

Enzymes and chemicals were purchased from BioRon (Ludwigshafen, Germany), and vectors and reagents for the cloning process were purchased from Takara Korea Biomedical (Seoul, Korea). Polymerase chain reaction (PCR) products and plasmid purification kits were from Bioprogen (Daejeon, Korea). Other fine chemicals and reagents used in this study were purchased from commercial grade.

Construction of plasmids for the expression of the recombinant fibrinolytic enzme

The PCR primers used in this study are listed in Table 1. The putative fibrinolytic enzyme gene (*FE*) from the KSL79 clone was generated by PCR using the Pro/Bam_F plus Pro/Eco_R primer set, as previously described (Cho et al., 2004). The gene product was purified with a PCR purification kit (Bioprogen) and cloned into the pMD18-T vector (Takara Korea Biomedical). The resulting plasmid was transformed into *Escherichia coli* DH5 DH5α. The coding fragment was excised from pMD18/KSL79_FE by treatment of *Bam*HI and *Eco*RI, and the pET28a vector (Novagen; Madison, WI, USA) was linearized with the same restriction enzymes. After the purification, the two fragments were ligated together using T4 DNA ligase (Takara). The final recombinant pET28a/KSL79_FE was confirmed by sequencing analysis (Genotech Co.; Daejeon, Korea).

Induction and preparation of the recombinant fibrinolytic enzyme

E. coli cells harboring pET28a/KSL79_FE were cultured in Luria-Bertani (LB) medium containing 50 μg/ml kanamycin at 37°C, and cell growth was determined by measuring the optical density of the culture at 600 nm (OD_{600}) with a spectrophotometer (U-2800 UV-Vis-Spectrophotometer; Hitachi High-Technologies Co.; Tokyo, Japan). To express the recombinant fibrinolytic enzymes, 30 μM of isopropyl thio-β-D-galactoside (IPTG) was added to the cultures, and subsequently incubated for 9 h under aerated conditions until the OD_{600} of the cultures reached 0.5. After centrifugation at 10,000 rpm for 15 min, the cell pellet was resuspended in a phosphate buffer (20 mM sodium phosphate in 500 mM NaCl (pH 7.4)) and subjected to three cycles of sonication for 10 min with a Vibra-Cell VCX 500 (SONICS; Newtown, CT, USA) at 40% maximum amplitude followed by a 10-min chill. The lysate was centrifuged at

Table 1. Primers used in this study.

Primer	Sequence (5' → 3')
Pro0771_F	CGC W[1]GC CGG AAA CGA AGG TTC
Pro0998_R	ACG TGA GGA GTC GCC ATK[2] GAC G
Pro0044_F	CGT TAA TCT TTA CGA TGG CGT TC
Pro0419_R	CCG CTG TCG ATA ACM GCT AC
Pro0001_F	GCG TGA GAA GCA AAA ATT GTG G
Pro1146_R	GCT TAT TGT GCA GCT GCT T
Pro/Bam_F	GCG GAT CC[3]G TGA GAA GCA AAA AAT TGT GGA TCA GC
Pro/Eco_R	GCG AAT TCT TAT TGT GCA GCT GCT TGT ACG TTG A

[1] W, A or T; [2] K, G or T. [3] Underlined capitals mean restriction sites of corresponding enzyme.

10,000 rpm for 15 min, and the supernatant was used as the crude enzyme preparation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

Cell pellets were incubated with 1 x lysis buffer (Novagen) at 37°C for 1 h. SDS-PAGE was carried out on a 10% polyacrylamide gel at 80 V for 5 h, and the proteins were transferred at 80 V for 2 h onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences; Buckinghamshire, England). The membrane was blocked with 5% (w/v) fat-free milk in TBST buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.01% (v/v) Tween-20) for 1 h, followed by incubation for 1 h in a 1:5,000 dilution of an anti-nattokinase antibody, which was provided from Professor Gal in Jinju Industry University, Korea (Lee et al., 2005). After three washes with TBST, the membrane was incubated for 1 h in a 1:2,000 dilution of an anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (HRP; Koma Biotech Inc.; Seoul, Korea). Immunoreactive proteins were visualized using an ECL Western blotting detection reagent (Amersham) and photographed with a fluorescence scanner (LAS 3000; Fuji Film Life Science; Stamford, CT, USA).

Assay for caseinolytic and fibrinolytic activity

Caseinolytic activity was determined colorimetrically as follows (Lee et al., 2006): A reaction mixture containing 50 µl of 3.0% (w/v) casein, 400 µl of 67 mM sodium phosphate buffer (pH 7.4), and 50 µl enzyme solution was incubated for 1 h at 37°C. To colorimetrically observe fibrinolytic activity, a reaction mixture containing 250 µl of 1.2% (w/v) fibrin, 200 µl of 67 mM sodium phosphate buffer (pH 7.4), and 50 µl enzyme was incubated at 37°C for 3 h (Lee et al., 2006). Both reactions were stopped by the addition of 750 µl trichloroacetic acid (TCA) solution (0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) for 30 min at room temperature. The reaction mixture was centrifuged at 15,000 rpm for 15 min, and 80 µl of culture free supernatant was incubated with 200 µl of 0.55 M Na_2CO_3 and 20 µl Folin-Ciocalteu (Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 30 min. The absorbance of the mixtures was read at 660 nm. One unit of caseinolytic or fibrinolytic activity was defined as the amount of enzyme that, in one minute, yielded an increase in absorbance at 660 nm equivalent to 1 g of tyrosine.

Effects of metal ions and chemical reagents on caseinolytic activity

The influence of various metal ions was investigated by pre-incubation the enzymes in presence of $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ at final concentration of 5 and 10 mM in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 30 min. To examine the effect of protease inhibitors, the enzymes were preincubated with the different chemical reagents such as ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis (2-aminoethyl ether) tetraacetic acid (EGTA) (metal-chelating agents), phenylmethylsulfonyl fluoride (PMSF) (a serine protease inhibitor), and DL-dithiothreitol (DTT) (a reducing agent) at final concentration of 5 and 10 mM in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 30 min. The percentage of relative activity was calculated by considering the activity of the enzymes without metal ions or chemical reagents as 100%. Under the standard conditions with 3% (w/v) casein.

RESULTS AND DISCUSSION

Preparation of clones encoding fibrinolytic enzyme

In a previous study, we have constructed metagenomic libraries from soil specimens. Using these metagenomic libraries, we employed a sequence-based polymerase chain reaction (PCR) method to screen for genes encoding fibrinolytic enzymes from soil metagenomic libraries. The specific PCR primers for fibrinolytic enzyme gene were Pro0001_F, Pro0044_F, Pro0771_F, Pro0419_R, Pro0998_R, and Pro1146_R originated from genes encoding fibrinolytic enzymes that belong to the subtilisin group of serine/threonine proteases (Table 1)(Nakamura et al., 1992). The fibrinolytic enzyme gene from *B. subtilis* var. *natto* (nattokinase) was used as a positive control.

Analysis of clone pools of soil metagenomic libraries with each primer set was carried out and their positive PCR products revealed the bands ranged from 228 to 1,146 bps in size, depending on used primers. Pro0001_F and Pro1146_R primer set yielded the full sequences of 15 putative fibrinolytic enzymes.

Analysis of the putative fibrinolytic genes

We subsequently analyzed the DNA sequences of the 15 PCR products generated with Pro0001_F and Pro1146_R by direct complete sequencing. Using a Basic Alignment Search Tool (BLAST) program analysis, these PCR

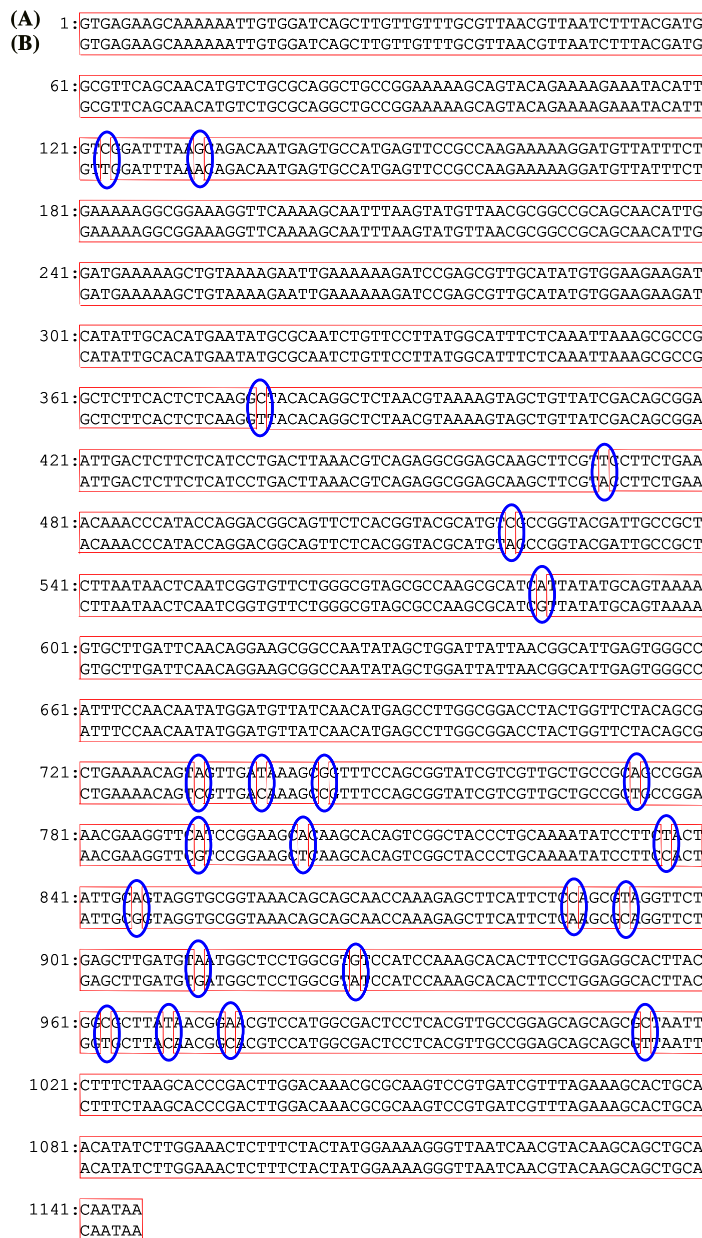

Figure 1. Comparison of nucleotide sequences of KSL79_FE and nattokinase from *B. subtilis* var. *natto*. Nucleotide sequences of KSL79_FE (Genbank accession number FJ950748) (A) and nattokinase (Genbank accession number AF368283) (B) were aligned and numbered from the GTG start codon at the 5' end of the fibrinolytic enzyme gene. The circle indicated difference sequences.

products showed 98% (964-965 bases matched/979 bases total) and 99% (950 bases matched/955 bases total) sequence similarities to the *nattokinase* gene from *Bacillus subtilis* var. *natto* (GenBank accession number AF368283). One clone, KSL3_FE, was 100% identical to the *B. subtilis* gene *aprE*, which encodes a subtilisin precursor (GenBank accession number AJ539133); the 14 other clones were 99.5% identical to the *B. subtilis* gene *Nk1*, encoding a thermostable mutant of the nattokinase (GenBank accession number AY940162).

The KSL79_FE PCR product, one of *Nk1*-like clones, consisted of 1,146 bps open reading frame (ORF), extending from a GTG initiation codon to a TAA termination sequence, which encodes a polypeptide comprised of 382 amino acid residues. We found 22 and 6 nucleotide differences between the ORF regions of KSL79_FE and the *nattokinase* and *Nk1* genes, respectively (Figure 1), but the deduced amino acid

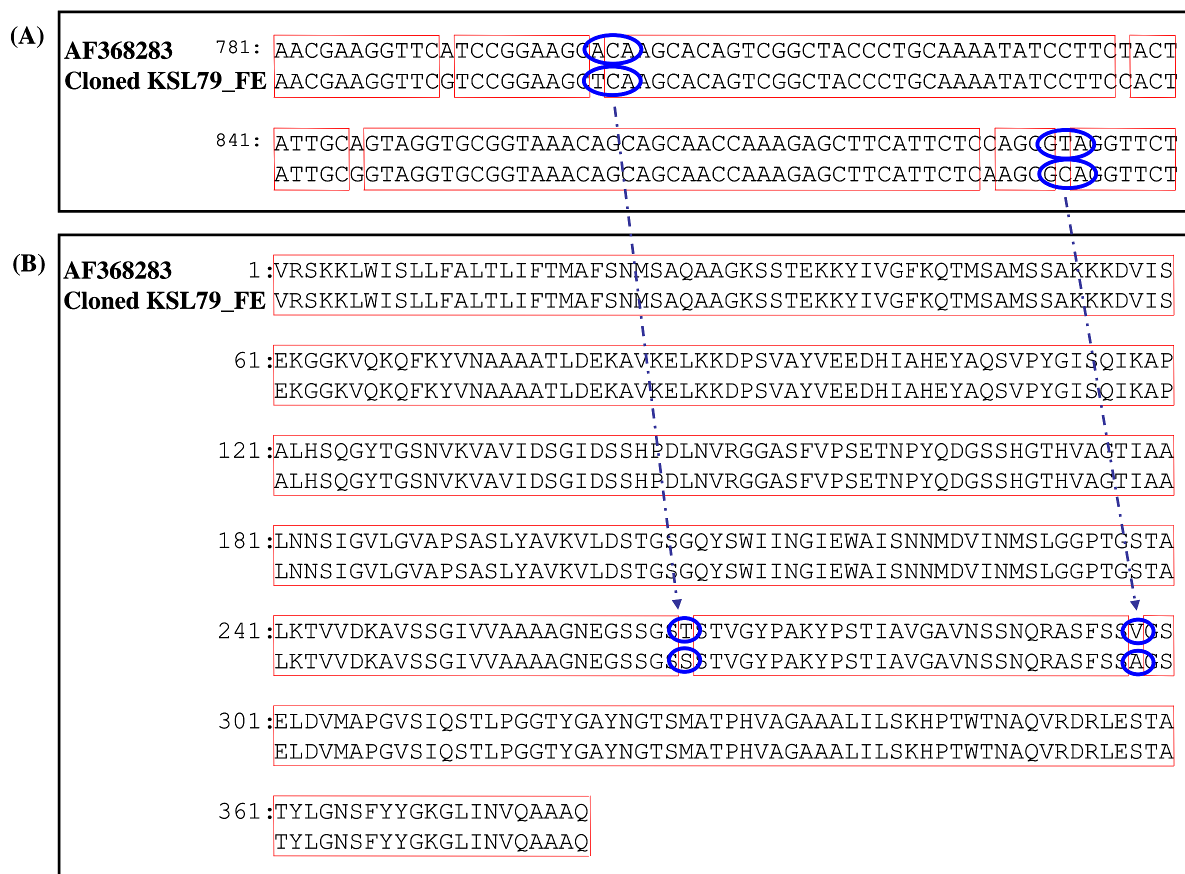

Figure 2. Comparison of the deduced amino acid sequences of KSL79_FE and nattokinase from *B. subtilis* var. *natto*. Differences of nucleotide sequences (A) and deduced amino acid sequences (B) between KSL79_FE (Genbank accession number FJ950748) and nattokinase from *B. subtilis* var. *natto* (Genbank accession number AF368283) were aligned.

sequence of KSL79_FE was 100% identical to the *Nk1* sequence. In contrast, although 20 of the nucleotides different from the *nattokinase* sequence were silent mutations, the remaining two nucleotides created new codons translatable to other amino acids: serine replaced threonine at position 268, and alanine replaced valine at position 298 (Figure 2).

A previous report identified the *arpN* as a novel fibrinolytic enzyme in *B. subtilis*, even though its sequence was almost 98% homologous to one of subtilisins in *B. subtilis* (Wong and Doi, 1986). Also another report isolated, purified, and characterized the thermostable fibrinolytic Nk1 protein (Chang et al., 2000). Deduced amino acid analysis of KSL79_FE showed that it had a typical 29-amino-acid-long signal peptide (pre-sequence) with the signal peptidase cleavage sequence of AlaGlnAlaAla at the N terminus as that of subtilisin. It was also followed by a 77-amino-acid-long hydrophilic sequence (pro-sequence) required for the production of enzymatically active form and the appropriate folding of the enzyme molecule. These results suggest that KSL79_FE protease belongs to subtilisin family protease.

Expression of the fibrinolytic enzyme gene KSL79_FE

To amplify the encoding region of putative fibrinolytic enzyme from the selected KSL79 clone, we performed PCR using the Pro/Bam_F primer, which contained a *Bam*HI restriction site just before the start codon, and the Pro/Eco_R primer, which contained an *Eco*RI site after the stop codon (Table 1). We then digested these restriction sites-anchored amplicon, ligated it into the pMD18-T vector, and transformed the resulting plasmid (pMD18-T/KSL79_FE) into *E. coli* DH5α (Figure 3). After DNA sequence verification, we subcloned the *KSL79_FE* ORF into the pET28a expression vector (Figure 3).

SDS-PAGE and Western blotting analyses were performed to determine the pET28a/KSL79_FE expression level in *E. coli* BL21 Codon Plus, as regulated by the *lac* promoter and induced by IPTG. We observed

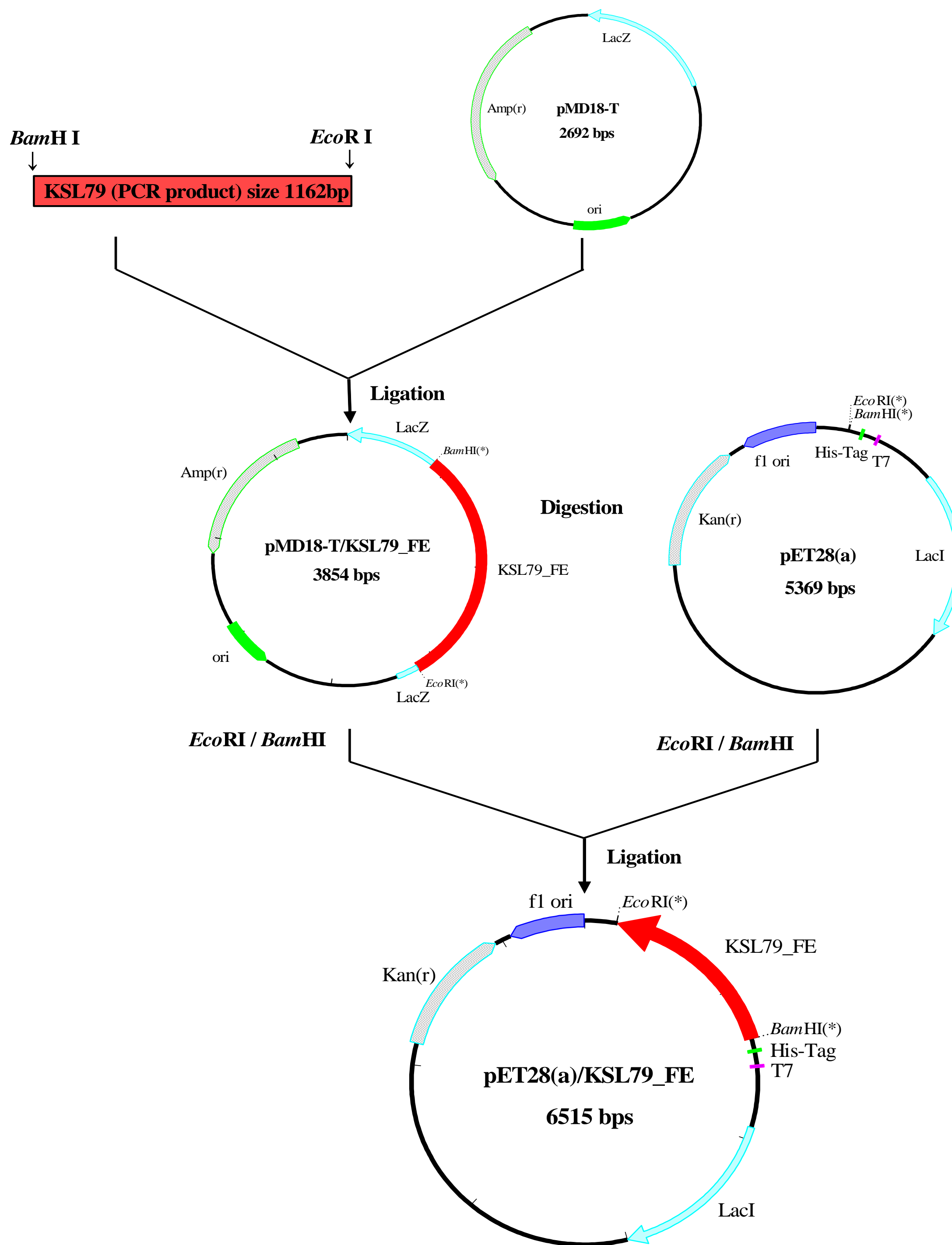

Figure 3. Construction of pET28a/KSL79_FE recombinant plasmid. The KSL79_FE PCR fragment excised from the pMD18/KSL79_FE plasmid was ligated into the multi-cloning site of pET28a expression cloning vector for prokaryote genes.

an optimal recombinant enzyme expression in *E. coli* BL21 Codon Plus cell harboring pET28a/KSL79_FE after optimal induction with 30 μM IPTG for 9 h (Figure 4A and B), exhibiting a molecular weight of approximately 41.8 kDa, which was immunoreactive with a nattokinase antibody (Lee et al., 2005). In studies using *Bacillus* expression systems, it has been reported that the active fibrinolytic enzyme such as subtilisin-type protease is produced in form of 275 amino acid residues, upon deletion of the pre-sequence and autolysis of pro- sequence,

Figure 4. Effect of host cell strain, IPTG concentration, induction time, and cell density on pET28a/KSL79_FE expression. (A) BL21 Codon Plus (1), XL1-blue (2), JM109 (3), or DH5α was transformed with recombinant pET28a/KSL79_FE. Transformants were induced at 0.5 of $O.D_{600}$ with 50 μM IPTG for 5 h; (B) BL21 Codon Plus transformed with recombinant pET28a/KSL79_FE was induced for 5 h with 0.0, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 μM IPTG, respectively. (C) Transformants were induced with 30 μM IPTG at an OD_{600} of 0.5 for 0, 3, 6, 9, and 20 h, respectively. (D) Transformanst were induced with 30 μM IPTG for 5 h after the OD_{600} reached 0.1, 0.3, 0.5, 0.8, and 1.0, respectively. Control(C) means untransformed BL21 Codon Plus.

exhibiting two major characteristic protein bands with molecular masses of approximately 40 and 43 kDa on SDS-PAGE analysis, even though subtilisin would be synthesized as a pre-pro-enzyme (Staley and Konopka, 1985). However, in this experiment with *E. coli* as host cells, only one protein band with a molecular mass of approximately 43 kDa was observed as an active enzyme (Figure 4B).

Effect of temperature and pH on the proteolytic activity of KSL79_FE

To compare the proteolytic activity of KSL79_FE to that of nattokinase, we first evaluated the temperature dependency of proteolytic activity under standard conditions at various temperatures (20, 30, 37, 50, 60, and 70°C) and pH 7.0, using casein or fibrin as substrates. As shown in Figure 5A, the optimum temperatures for caseinolytic activity of the KSL79_FE enzyme and nattokinase were 50 and 37°C, respectively. Likewise, we observed similar optimum temperatures for fibrinolytic activity of KSL79_FE enzyme and nattokinase (Figure 5B). We next determined the optimal pH for proteolytic activity of KSL79_FE and nattokinase enzymes by varying the pH of the reaction mixture from pH 3.0 to 11.0, using 0.5 mM citrate buffer (pH 3.0 to 5.0), 0.1 mM Tris-HCl buffer (pH 5.0 to 9.0), or 0.1 mM carbonic acid buffer (pH 9.0 to 11.0). In contrast to nattokinase that exhibited maximum activity at pH 7.0 - 8.0, the optimum enzyme activity of KSL79_FE for fibrin or casein hydrolysis was observed at pH 9.0 (Figure 6A and B). Taken together, these results indicate that the KSL79_FE enzyme requires higher temperature and pH than nattokinase to maximally hydrolyze casein or fibrin.

Substrate specificity of KSL79_FE

To compare substrate specificity of the two enzymes, we investigated the ratio of casein hydrolysis to fibrin hydrolysis. As shown in Table 2, the KSL 79_FE enzyme hydrolyzed both casein and fibrin with substrate specificities that resembled those of nattokinase, which is known to be more specifically hydrolyzing to fibrin. Enzymatic kinetics was determined by measuring the release of *p*-nitroaniline (NA) from the chromogenic substrate N-succinyl-AAPF-*p*NA. The Michaelis-Menten constrants (K_m and V_{max}) of the KSL 79_FE were obtained from the plot of the fibrinolytic activity with fibrin concentration. All of these kinetic parameters were similar with previous data of Nk1 protein, which was identical to amino acid sequences of the KSL 79_FE (data not shown) (Chang et al., 2000).

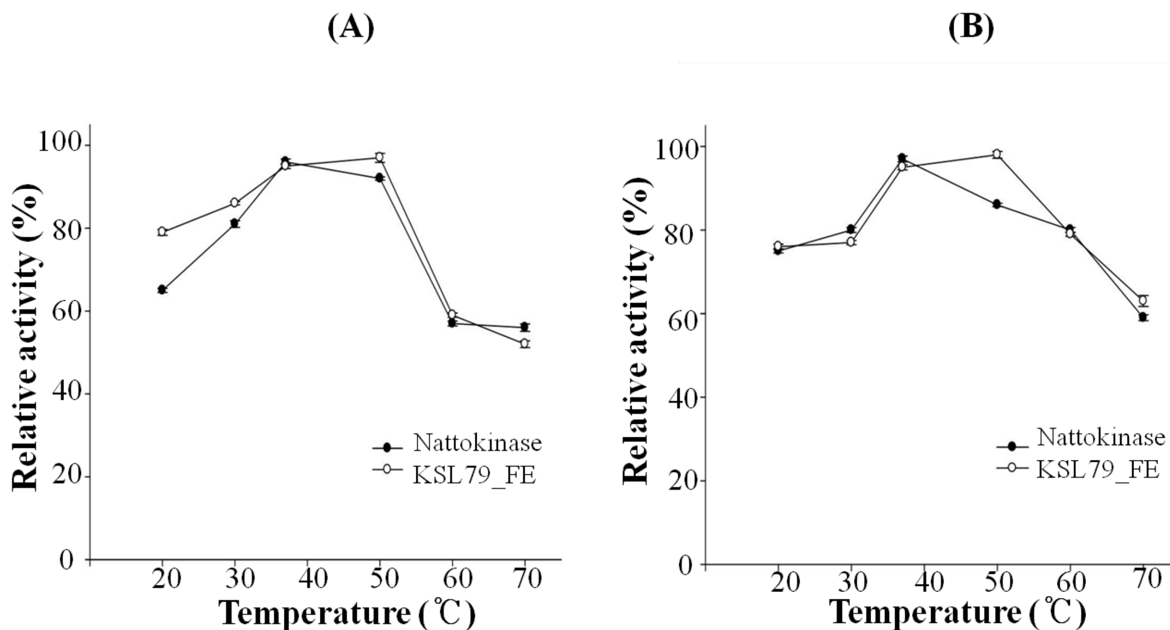

Figure 5. Optimal temperatures of enzymes for maximal proteolytic activity. The optimal temperature for proteolytic activity of the KSL79_FE and nattokinase enzymes was determined under standard conditions at different temperatures (20, 30, 37, 50, 60, and 70°C) and pH 7.0, using casein (A) or fibrin (B) as substrates.

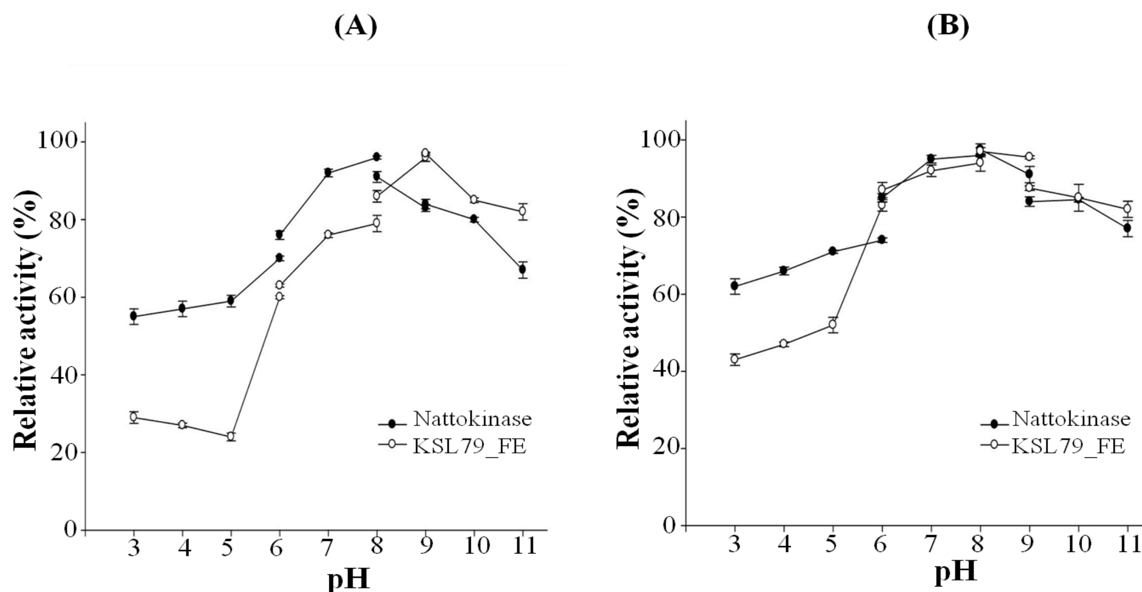

Figure 6. Optimal pH of enzymes for proteolytic activity. The optimal pH for proteolytic activity of KSL79_FE and nattokinase enzymes was determined by varying the pH of the reaction mixture from pH 3.0 to 11.0, using 0.5 M citrate buffer (pH 3.0 to 5.0), 0.1 M Tris-HCl buffer (pH 5.0 to 9.0), or 0.1 M carbonic acid buffer (pH 9.0 to 11.0), and incubating the enzymes at their respective optimal temperatures using casein (A) or fibrin (B) as substrates.

Effects of metal ions and additives on the proteolytic activity of KSL79_FE

The proteolytic activity of KSL79_FE was estimated in the presence of different metal ions. As shown in Figure 7B, all metal ions at 5 mM concentration did not or slightly affect the activity of KSL79_FE protease, whereas it was partially inhibited in the presence of 10 mM Fe^{2+} ions.

Table 2. Substrate specificity of nattokinase and KSL 79_FE enzymes.

Enzyme	Caseinolytic acitivity (U/ml)[2]	Fibrinolytic acitivity (U/ml)[2]	Substrate specificity[3]
Nattokinase[1]	0.6290	0.4847	1.29
KSL79_FE	0.7555	0.5789	1.30

[1]A fibrinolytic enzyme produced from *B. subtilis* var. *natto* was used as a control; [2]proteolytic activity by colorimetric method when samples were treated with equal enzyme concentrations at $O.D_{600}$; [3]caseinolytic activity/fibrinolytic activity.

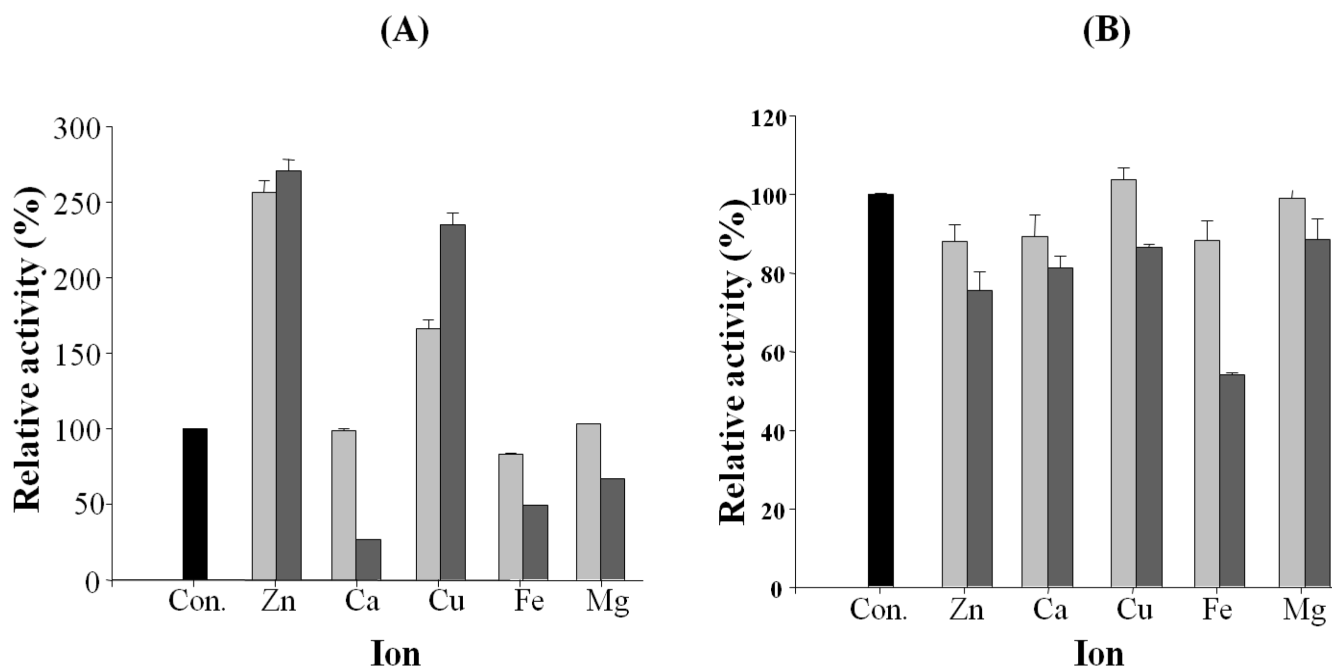

Figure 7. Effects of metal ions on the activity of nattokinase and KSL79_FE. The nattokinase (A) and KSL79_FE (B) were pre-incubated in both the absence and the presence of 5 mM or 10 mM metal ions in 50 mM Tris-HCl (pH 7.0) at 37°C. After 30 min of pre-incubation, residual protease activity was measured with 3.0% (w/v) casein.

Unlike KSL79_FE, the enzyme activities of nattokinase were enhanced by Zn^{2+} and Cu^{2+} ions and inhibited by Ca^{2+}, Fe^{2+}, and Mg^{2+} ions (Figure 7A).

Next, we determined the effects of chemical reagents on the enzyme activities of nattokinase and KSL79_FE. The results displayed that the addition of EDTA (10 mM), EGTA, PMSF, or DTT strongly inhibited the proteolytic activity of KSL79_FE (Figure 8B), whereas the enzyme activity of nattokinase was reduced by only PMSF treatment (Figure 8A), collectively, these results indicate that KSL79_FE protease from metagenomic library requires different metal ions and stability for proteolytic activity as compared to those of nattokinase.

Nucleotide sequence accession number

Nucleotide sequence of the KSL79_FE fibrinolytic enzyme gene from soil metagenome was deposited in GenBank database under the accession number FJ950748.

Fibrinolytic enzymes have been isolated and studied from various resources, especially from traditional fermented Asian foods. These well-known fibrinolytic enzymes chiefly have been administrated orally to convey their effects. Moreover, recent reports suggested that the original producer isolated from these sources, *B. subtilis*, expresses recombinant fibrinolytic enzymes at a low level. These issues necessitate studies to investigate, isolate, and express novel fibrinolytic enzymes, to characterize new approaches, and to explore various other sources besides fermented food. Our approached one largely untapped resource, the environmental metagenome; as such, the study here in is the first to our knowledge to screen for fibrinolytic enzyme from soil metagenome.

Metagenome analysis is a powerful technique for exploring unknown but useful living cells in the environ-

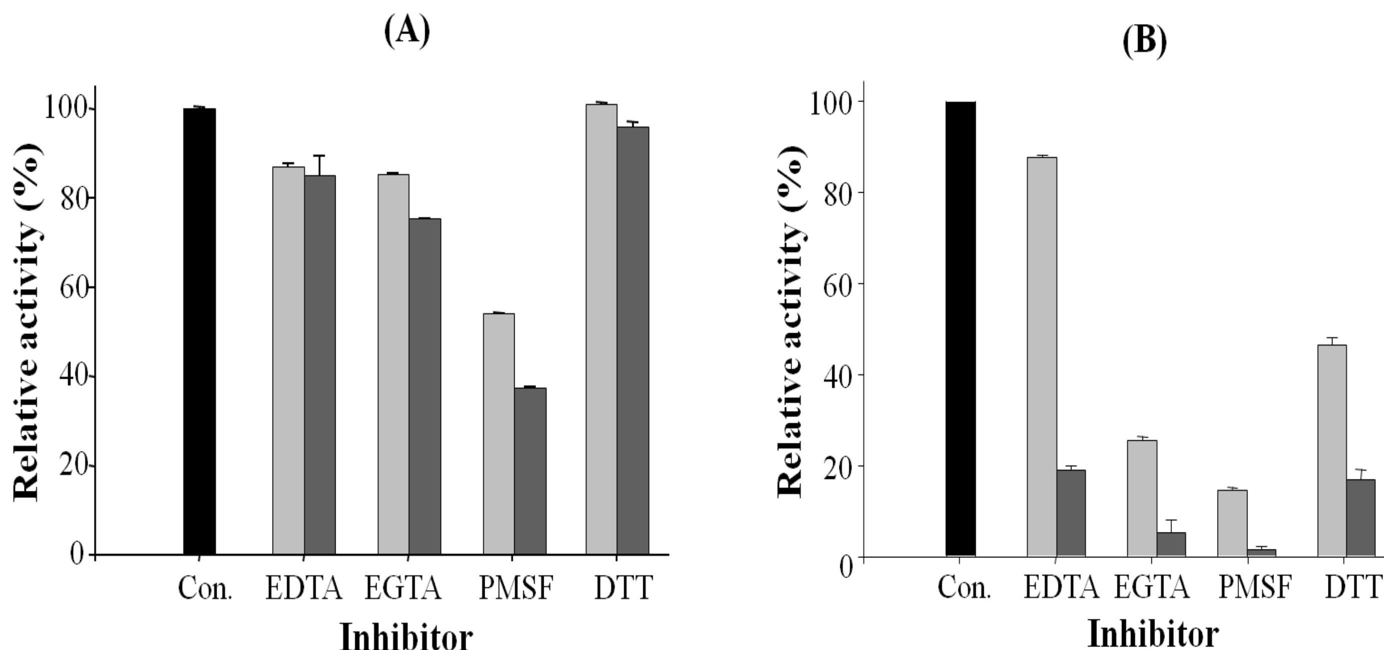

Figure 8. Effects of chemical reagents on the activity of nattokinase and KSL79_FE. The nattokinase (A) and KSL79_FE (B) were pre-incubated in both the absence and the presence of 5 mM or 10 mM chemicals in 50 mM Tris-HCl (pH 7.0) at 37°C. After 30 min of pre-incubation, residual protease activity was measured with 3.0% (w/v) casein.

ment.Existing methods used to conduct molecular metagenomics, such as PCR amplification, PCR denaturing gradient gel electrophoresis (PCR-DGGE) analysis, and microarray using specific probes, represent functional approaches specific to the environmental microorganisms, but they generally yield low active clone recovery rates. Even though current *in vitro* cultivation techniques have improved our ability to grow previously uncultivable microorganisms, the overwhelming majority of microbes in nature have not been characterized (Lee et al., 2006). Moreover, activity-based screening after cultivation is problematic, because there is so little information regarding optimal cultivation and expression conditions.

In this study, we isolated 15 positive clones from this soil DNA library by PCR. One of the clones, KSL79_FE, revealed sequence differences from *B. subtilis* var. *natto* nattokinase but was identical to the thermostable nattokinase mutant Nk1, in which two amino acids at positions 268 and 298 are altered. Nevertheless, the KSL79_FE enzyme exhibited the optimal hydrolytic conditions of 50°C and pH 9.0, compared to 37°C and pH 7.0 to 8.0 for nattokinase, suggesting that the two amino acids that differed from the nattokinase sequence may convey changes in the optimal pH and temperature required for maximal proteolytic activity. Despite these differences, both enzymes displayed similar substrate specificity. Future studies will characterize how the proteolytic activity of the expressed recombinant enzyme depends on temperature and pH, and they will elucidate the relationship between amino acid sequence and proteolytic activity in this enzyme.

ACKNOWLEDGEMENT

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African Journal of Biotechnology

Full Length Research Paper

Effects of red monascal rice supplementation on growth, digestive function and oocyte maturation in Siamese fighting fish (*Betta splendens* Regan, 1910)

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Evaluation of red monascal rice supplementation on growth, digestive function and oocyte maturation were investigated in Siamese fighting fish (*Betta splendens*). Completely randomized design with different dietary levels of red monascal rice (0.00, 0.25, 0.50, 1.00 and 2.00%) was conducted for six weeks. The growth of fish fed a control diet was not statistically different ($P > 0.05$) from a diet containing 0.25% of red monascal rice. However, significantly lower values ($P < 0.001$) were observed in fish fed more than 0.50% red monascal rice, in a dose-dependent manner. Muscle RNA concentrations were higher in fish fed control diet than in fish fed red monascal rice, while protein concentration and RNA/protein ratio were similar. Body composition and fatty acid profiles (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, total omega-3 and total omega-6) were unchanged among dietary treatments. Digestive functions were perturbed by decreasing the specific activities of amylase, total protease, trypsin and chymotrypsin ($P < 0.001$), and increasing the specific activity of lipase ($P < 0.005$). Reproductive consummation of oocytes was significantly different in fish fed red monascal rice, due to increasing RNA concentration and RNA/protein ratio ($P < 0.003$) and decreasing specific activities of trypsin- and chymotrypsin-like enzymes ($P < 0.03$). These findings suggest the toxicological effects of red monascal rice by interfering with growth, digestive function and oocyte maturation in Siamese fighting fish.

Key words: Digestive enzyme, fatty acid, growth, muscle, oocyte, red monascal rice, Siamese fighting fish.

INTRODUCTION

Red monascal rice is a product of ordinary rice fermented with the fungal genus *Monascus*. It has been widely used as a food additives for coloring meat (Bakosova et al., 2001), fish (Takatsuki et al., 1988) and chicken eggs (Wang and Pan, 2003). Monascal rice provides at least six pigments-yellow (ankaflavin and monascin), orange

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Abbreviations: SGR, Specific growth rate; **DSI,** digestosomatic index; **GSI,** gonadosomatic index; **MOFA,** monounsaturated fatty acid; **PUFA,** polyunsaturated fatty acid.

(monascorubrin and rubropunctanin) and red (monascorubramine and rubropunctamine), as well as other seconddary metabolites, antioxidant compounds (Yang et al., 2006), hypertensive agents and antihypercholesterolemic agents (Su et al., 2003; Wang and Pan, 2003).

However, a toxic chemical, citrinin, is often a by-product of fer-mentation, depending on culture conditions; for example, fermentation of rice by foodstuff-relevant *Monascus* sp. produces up to 2.5 g kg^{-1} citrinin, while liquid culture has reached as high as 56 mg kg^{-1} (Eisenbrand, 2006). This compound has been found to induce reproductive abnor-malities in male gametes (Qingqing et al., 2012), and to reduce the rate of oocyte maturation and fertilization (Chan, 2008); it also has a teratogenic effect (Chan and Shiao, 2007; Singh et al., 2007a; Chan, 2008), and has induced maternal toxicity in pregnant rats (Singh et al., 2007b). Moreover, it has been associated with cytoto-xicity (Liu et al., 2005) and the activation of apoptosis by the mitochondrial pathway (Yu et al., 2006).

Recently, *in vitro* screening of feedstuffs for the liberation of various pigments based on crude enzyme digestion, standardized with trypsin activity, indicated that red monascal rice is an appropriate source for promoting coloration in Siamese fighting fish (Thongprajukaew et al., 2012). Moreover, supplementation of aquaculture feed with red monascal rice for rearing juvenile Siamese fighting fish has been reported (Thongprajukaew et al., 2011). Safety evaluation of red monascal rice for use as a food supplement has been studied in chickens (Wang and Pan, 2003), rats (Chan and Shiao, 2007; Kumari et al., 2009) and rabbits (Wei et al., 2003). However, *in vivo* observation of red monascal rice supplementation in aquatic animals has not yet been performed.

The objective of the present study was to investigate the effects of red monascal rice supplementation on growth, digestive function and oocyte maturation in aquatic species. Unique combinations of biochemical parameters were used as indicators: digestive enzyme specific activities (amylase, lipase, total protease, trypsin and chymotrypsin); oocyte qualities (trypsin-like enzyme specific activity, chymotrypsin-like enzyme specific activity, RNA, protein and RNA/protein ratio); and muscle qualities (RNA, protein, lipid, RNA/protein ratio and protein/lipid ratio). Siamese fighting fish (*Betta splendens* Regan, 1910) was chosen as a representative model because they generate the highest income among sales of exported ornamental fish in Thailand. The findings of this study could provide additional information regarding red monascal rice supplementation as a food additive for rearing aquatic species.

MATERIALS AND METHODS

Diet preparation and biochemical compositions

The ingredients of experimental diets and their biochemical composition are shown in Table 1. Mixtures of specified feedstuffs (fish meal, soybean meal, wheat gluten, squid meal and wheat flour) were modified using microwave irradiation to enhance digestive enzyme hydrolysis. The diets were produced by mixing the modified feed mixtures with red monascal rice ("Mona" rice; Fame Biotech Co, Ltd., Thailand) in different concentrations (0.00, 0.25, 0.50, 1.00 and 2.00%), together with other additives and sufficient water (30%) to achieve an appropriate moisture content. The glutinous mixtures were passed through a hand pelletizer, dried at 60°C for 3 h, and then stored at 4°C until used for feeding. For biochemical composition analysis, the diets were dried at 105°C for 24 h before determining protein, lipid, fiber, and ash contents, in accordance with AOAC standard method (2005). Nutritional values of the diet were expressed as percentage on a dry matter basis. Carbohydrate contents were calculated by the difference.

Fish husbandry and sample collection

Solid red females, 1.5 months old, were obtained from a private farm in Nakhon Pathom province, Thailand. The fish were individually acclimatized in a plastic aquarium (8 cm diameter × 11 cm height) for 10 days before starting the experiment. A control diet (without red monascal rice) was fed two times daily during fish acclimatization. The fish (0.40 ± 0.01 g initial weight and 3.44 ± 0.05 cm initial length) were distributed into individual aquariums (7 cm width × 7 cm length × 15 cm height, with a 10 cm water level). The experiment was conducted for six weeks under a 12-h light/12-h dark cycle. Twenty (20) fish in each dietary treatment were fed *ad libitum* at 8:00 a.m. and 6:00 p.m. At the end of the experiment, the fish were starved for 24 h prior to sampling to avoid metabolic interference after diet ingestion. All fish were sacrificed by chilling in ice according to "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes," National Research Council, Thailand.

Fifteen (15) fish (n = 15) were used for measurement of growth, muscle quality, digestive enzyme specific activity and oocyte quality. Body weight and total length were individually measured before carefully collecting the epaxial white muscle, digestive tracts, and oocytes. Pooled body samples from fish dissection were used to analyze biochemical composition (three pooled fish per sample, n = 5). Four fish from each treatment (n = 4) were randomly sampled for fatty acid determination. All tissues were then stored at -80°C until determinations were performed. Parameters for measuring growth and development of female fish were calculated by the following formulae:

Condition factor (CF, g cm^{-3}) = 100 × (live body weight/total $length^3$)

Digestosomatic index (DSI, %) = 100 × [digestive tract weight/body weight]

Gonadosomatic index (GSI, %) = 100 × [oocyte weight/body weight]

Specific growth rate (SGR, % day^{-1}) = 100 [e^g-1]

Where, g = $(\ln W_t - \ln W_0)/(t - t_0)$; W_t = mean weight at month t; W_0 = mean weight at month t_0.

Weight gain (WG, g) = final body weight - initial body weight

Water quality management

Water was changed at a rate of 75% every other day. Water qualities were measured weekly using a water analyzer (556 MPS

Table 1. Feedstuff ingredients and biochemical composition of experimental diets with different supplementation levels of red monascal rice.

Ingredient and composition	Red monascal rice (%)				
	0.00	0.25	0.50	1.00	2.00
Ingredient (%)					
Fish meal	24	24	24	24	24
Soybean meal	20	20	20	20	20
Wheat gluten	11	11	11	11	11
Squid meal	5	5	5	5	5
Wheat flour	31	30.75	30.50	30	29
Lecithin	1	1	1	1	1
Fish oil	2	2	2	2	2
Soybean oil	1	1	1	1	1
Mineral mixture*	0.05	0.05	0.05	0.05	0.05
Vitamin mixture**	0.25	0.25	0.25	0.25	0.25
Vitamin C	0.1	0.1	0.1	0.1	0.1
Fermented red rice	0.00	0.25	0.50	1.00	2.00
Cellulose	5.1	4.6	4.6	4.6	4.6
Composition (%)					
Moisture	8.1	8.6	7.7	6.6	6.5
Crude protein	39.1	41.6	40.5	42.7	40.1
Crude lipid	4.5	3.5	3.2	3.7	4.2
Nitrogen free extract	33.5	32.3	33.9	32.3	34.5
Crude fiber	3.5	3.2	3.8	3.5	3.6
Ash	11.3	10.8	10.9	11.2	11.1
Gross energy (kJ/g)	16.8	16.8	16.7	17.1	17.1

*Mineral mixtures in 1 kg of feed contained 30 mg iron, 20 mg zinc, 25 mg manganese, 5 mg copper, 5 mg iodine and 0.2 mg selenium. **Vitamin mixtures in 1 kg of feed contained 4,000 IU vitamin A, 2,000 IU vitamin D_3, 50 mg vitamin E, 10 mg vitamin K, 20 mg thiamine, 20 mg riboflavin, 20 mg pyridoxine, 200 mg calcium panthothenate, 150 mg niacin, 2 mg biotin, 5 mg folic acid, 0.2 mg vitamin B_{12}, 400 mg inositol and 200 mg ethoxyquin.

Multi Probe System, YSI Inc., Yellow Springs OH, USA). During the whole experimental periods the average temperature was 28.44 ± 0.07°C, pH was 7.45 ± 0.02, and dissolved oxygen was 7.56 ± 0.05 mg L^{-1}. Ammonia content (0.0044 ± 0.0001 ppm) was measured according to APHA, AWWA and WPCF (1998). All analysis was performed in triplicate.

Digestive enzyme activities

Digestive enzyme extraction

Digestive enzymes were extracted from whole digestive organs and oocytes in the presence of 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl, using a micro-homogenizer (THP-220; Omni International, Kennesaw GA, USA). The homogenate was then centrifuged at 13,000 × *g* for 20 min at 4°C. The lipid portion on the upper layer of the supernatant was carefully removed. The supernatant was collected and then kept at -80°C for digestive enzyme assay. Protein concentration in the enzyme extract was determined according to Lowry et al. (1951) using bovine serum albumin as a standard curve.

Digestive enzyme assays

Specified optimal conditions for studying digestive enzyme activities in Siamese fighting fish were used as described in Thongprajukaew et al. (2011). Amylase activity was determined based on Areekijseree et al. (2004) using starch solution as a substrate. Amylase specific activity was expressed as µmol maltose produced h^{-1} mg protein^{-1}. Lipase activity was analyzed based on Winkler and Stuckmann (1979) using *p*-nitrophenyl palmitate as a substrate. The specific activity of lipase was expressed as µmol *p*-nitrophenol produced h^{-1} mg protein^{-1}. Total protease activity was assayed using azocasein as a substrate, based on Areekijseree et al. (2004). The specific activity of total protease was expressed as mU mg protein^{-1}. One unit (U) of total protease activity was defined as the amount of enzyme producing an increase of 1.0 absorbance unit at 440 nm. Trypsin and trypsin-like activities from the digestive tract and oocyte extracts, respectively, were assayed using *N*-benzoyl-*L*-arginine-*p*-nitroanilide (BAPNA) as a substrate. Chymotrypsin and chymotrypsin-like activities from the digestive tract and oocyte extracts, respectively, were assayed using *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (SAPNA) as a substrate. The assays of these serine proteases were performed according to Rungruangsak-Torrissen (2007), with specific activities expressed as µmol *p*-nitroanilide produced h^{-1} mg protein^{-1}.

Muscle, body and oocyte compositions

Moisture of the body was determined using an automatic infrared

moisture analyzer (MA 30; Sartorius, Göttingen, Germany). Concentrations of RNA and protein in the muscle, body and oocytes were determined using TRIzol® reagent (Invitrogen, Carlsbad CA, USA), as described in Rungruangsak-Torrissen (2007). The extinction coefficient for calculating RNA is E_{260} = 40 µg RNA ml^{-1}, and for protein is E_{280} = 2.1 mg protein ml^{-1}. Lipid contents were extracted using ethyl acetate, as described by Supannapong et al. (2008). Ratios of RNA/protein and protein/lipid were calculated from the amounts of RNA, protein and lipid from the same sample. All values were expressed on wet weight basis.

Fatty acid determination

Fish samples (n = 4) were dried using a freeze dryer (Heto FD3; Heto-Holten Allerød, Denmark) under dark conditions for 48 h before analyzing fatty acid profiles. Lipids for fatty acid analysis were extracted according to Kates (1986). Fatty acid methyl esters were prepared using sodium methoxide, as described by Gandhi and Weete (1991). Fatty acid profiles were analyzed using a gas chromatography (GC-14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel packed SS column and flame ionization detector. Injector temperature was increased from 205 to 250°C at a rate of 2°C min^{-1}. Detector temperature was maintained at 275°C. Nitrogen was used as a carrier gas at 2 kgf cm^{-2}. Menhaden oil methyl esters (National Marine Fisheries Service, Seattle WA, USA) were used as internal standards.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Significant differences among averages were ranked using Duncan's Multiple Range Test (DMRT) at a 95% significance level. Relationships among selected parameters were calculated using Pearson's product moment correlation.

RESULTS

Growth, body compositions and muscle quality

Survival rate of fish in all dietary treatments was 100% (Table 2). All growth indicators, including total length, body weight, weight gain and specific growth rate (SGR), were similar between fish fed control diet and those fed the diet with 0.25% red monascal rice (Table 2, $P > 0.05$). Nearly all parameters of growth were significantly affected by red monascal rice supplementation ($P < 0.05$), and were decreased in a dose-dependent manner (except the condition factor).

Body biochemical composition, include-ing moisture, lipid, protein, protein/lipid ratio and total minerals, were not statistically different (Table 2, $P > 0.05$) among dietary treatments. Protein concentration and RNA/ protein ratio in the white muscle of untreated and treated fish were similar, while the RNA concen-tration showed a significant decrease ($P = 0.008$) in fish fed red monascal rice when compared to the control (Table 2).

Digestive indices and digestive enzyme specific activities

Digestive tract weight was significantly lower in fish fed the diet containing 2.00% red monascal rice ($P < 0.05$), while the digestosomatic index (DSI) fluctuated among dietary treatments (Table 3). Supplementation of red monascal rice had significant effects on the specific activities of digestive enzymes for protein ($P < 0.001$), carbohydrate ($P < 0.001$) and lipid ($P = 0.004$). Specific activities of amylase, total protease, trypsin and chymotrypsin were significantly decreased ($P < 0.05$), while specific activity of lipase was significantly increased, in the presence of red monascal rice ($P < 0.05$).

Activity ratios of amylase to trypsin (A/T ratio) and trypsin to chymotrypsin (T/C ratio) were similar among dietary treatments ($P > 0.05$), whereas the slope of trypsin to chymotrypsin (slope T/C ratio calculated by the slope of regression between chymotrypsin (X-axis)) and trypsin (Y-axis) specific activities of the same sample according to Rungruangsak-Torrissen et al. (2009) was significantly decreased in treated groups when compared with control ($P = 0.002$).

Reproductive indices and oocyte qualities

The maturation rate of fish in all dietary treatment was 100% (Table 4), as indicated by the presence of oocyte in all collected fish. Oocyte weight progressively decreased ($P < 0.05$) in a dose-dependent manner, while the gonadosomatic index (GSI) slightly decreased (pooled data, $P < 0.05$) when fish received red monascal rice. Protein synthesis (RNA concentration) and protein turn-over (RNA/protein ratio) in the oocytes were significantly increased with administration of red monascal rice. Supplementation of red monascal rice had potent effects on specific activities of trypsin-like ($P < 0.03$) and chymotrypsin-like ($P < 0.008$) enzymes; there was a slight decrease in enzyme activities with red monascal rice doses of 0.25 and 0.50%, and a dramatic decrease at doses of 1.00 and 2.00%.

Relationship between growth and digestive capacities

Specific growth rate (SGR) of fish showed positive correlations with digestive tract weight and specific activities of amylase, total protease, trypsin and chymo-trypsin (Table 5). However, specific activity of lipase and the T/C ratio were negatively correlated with SGR. The changes of SGR, digestive tract weight, specific activities of all digestive enzymes, and A/T ratio were closely related. For muscle qualities, RNA concentration showed positive correlations with SGR, protein concentration, and specific

Table 2. Effect of different dietary levels of red monascal rice on survival rate, growth, body composition and muscle quality of female Siamese fighting fish. Analysis was conducted using individual fish from each treatment.

Parameter	*P*	Red monascal rice (%)				
		0.00	**0.25**	**0.50**	**1.00**	**2.00**
Survival rate (%)	-	100	100	100	100	100
Growth (*n* = 15)						
Total length (cm)	**< 0.001**	4.78 ± 0.06^{a}	4.84 ± 0.06^{a}	4.65 ± 0.06^{ab}	4.49 ± 0.07^{bc}	4.37 ± 0.12^{c}
Body weight (g)	**< 0.001**	1.21 ± 0.04^{a}	1.22 ± 0.03^{a}	1.09 ± 0.03^{b}	1.04 ± 0.04^{b}	1.01 ± 0.05^{b}
Weight gain (g)	**< 0.001**	0.81 ± 0.04^{a}	0.82 ± 0.03^{a}	0.69 ± 0.03^{b}	0.64 ± 0.04^{b}	0.61 ± 0.05^{b}
Condition factor (CF, g cm^{-3})	**0.037**	1.10 ± 0.02^{a}	1.08 ± 0.03^{a}	1.08 ± 0.03^{a}	1.15 ± 0.03^{ab}	1.22 ± 0.06^{b}
Specific growth rate (SGR, % day^{-1})	**< 0.001**	3.15 ± 0.10^{ab}	3.20 ± 0.07^{a}	2.86 ± 0.07^{bc}	2.72 ± 0.11^{c}	2.61 ± 0.15^{c}
Body composition (*n* = 5, %)						
Moisture	0.104	76.12 ± 0.95	76.34 ± 0.76	77.94 ± 0.68	78.68 ± 0.65	77.37 ± 0.34
Protein	0.926	13.51 ± 0.56	12.97± 0.89	12.92 ± 0.43	12.77 ± 0.65	12.80 ± 0.79
Lipid	0.918	3.62 ± 0.42	3.82 ± 0.41	3.77 ± 0.32	3.56 ± 0.40	3.41 ± 0.23
Protein/lipid ratio	0.938	3.90 ± 0.53	3.46 ± 0.30	3.51 ± 0.43	3.77 ± 0.59	3.80 ± 0.33
Ash	0.389	4.12 ± 0.19	4.33 ± 0.48	4.07± 0.28	4.34 ± 0.20	4.62 ± 0.03
Muscle quality (*n* = 15)						
RNA (µg g^{-1})	**0.008**	2,460 ± 42^{a}	2,270 ± 45^{b}	2,283 ± 48^{b}	2,298 ± 40^{b}	2,216 ± 64^{b}
Protein (mg g^{-1})	0.922	300 ± 13	299 ±13	287 ± 9	295 ± 12	291 ± 12
RNA/protein ratio (µg mg^{-1})	0.496	8.38 ± 0.31	7.74 ± 0.28	8.04 ± 0.24	7.98 ± 0.39	7.70 ± 0.23

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$).

activities of amylase, total protease, trypsin and chymotrypsin; while RNA/protein ratio showed positive and negative relationships with A/T ratio and protein concentration, respectively (Table 5).

Relationship between growth and reproductive parameters

Increased oocyte weight occurred concurrently with increase of body weight and decrease of RNA concentration (Table 6). Oocyte weight showed a negative relationship with protein synthesis and protein turnover. Trypsin-like specific activity was highly correlated with chymotrypsin-like enzyme activity.

Both protease enzymes showed positive correlations with protein synthesis capa-city (RNA and RNA/protein ratio). However, no significant relationship was observed between protein concentration and other reproductive parameters.

Fatty acid profiles of fish

The main fatty acids found in the bodies of female Siamese fighting fish were C16: 0, C18: 1*n*9 and C18: 2*n*6 (Table 7). Fatty acid content was relatively higher in fish fed red monascal rice compared to fish fed control diet ($P > 0.05$). Total amounts of saturated fatty acid (SFA) monounsaturated fatty acid (MOFA), polyunsaturated fatty acid (PUFA), Σ*n*-3 and Σ*n*-6 were similar among

Table 3. Effect of different dietary levels of red monascal rice on digestive growth and digestive enzyme expressions in female Siamese fighting fish. Analysis was conducted using individual fish from each treatment.

Parameter	*P*	Red monascal rice (%)				
		0.00	**0.25**	**0.50**	**1.00**	**2.00**
Digestive growth (*n* = 15)						
Digestive tract weight (g)	**0.001**	0.11 ± 0.00^{a}	0.11 ± 0.00^{a}	0.10 ± 0.01^{a}	0.10 ± 0.00^{a}	0.08 ± 0.00^{b}
Digestosomatic index (DSI, %)	0.103	8.84 ± 0.32	8.74 ± 0.37	9.35 ± 0.48	9.63 ± 0.47	8.06 ± 0.43
Digestive enzyme (*n* = 15)						
Amylase [A][a]	**< 0.001**	359.45 ± 23.86^{a}	274.33 ± 23.90^{b}	265.38 ± 19.83^{b}	191.17 ± 14.07^{c}	199.51 ± 19.78^{c}
Lipase[b]	**0.004**	4.17 ± 0.12^{a}	4.40 ± 0.15^{ab}	4.36 ± 0.16^{ab}	4.87 ± 0.28^{bc}	5.25 ± 0.31^{c}
Total protease[c]	**< 0.001**	70.81 ± 3.27^{a}	53.57 ± 4.81^{b}	56.41 ± 4.06^{b}	45.15 ± 3.93^{c}	42.96 ± 4.67^{c}
Trypsin [T][d]	**< 0.001**	15.71 ± 1.03^{a}	11.64 ± 0.97^{b}	11.67 ± 0.86^{b}	9.70 ± 0.90^{bc}	8.40 ± 0.93^{c}
Chymotrypsin [C][d]	**< 0.001**	128.20 ± 6.23^{a}	98.03 ± 8.83^{b}	100.18 ± 6.96^{b}	80.42 ± 8.19^{bc}	78.19± 7.79^{c}
A/T ratio	0.722	23.71 ± 1.84	24.47 ± 1.66	23.80 ± 1.70	21.47 ± 2.44	25.25 ± 1.91
T/C ratio	0.126	0.12 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.12 ± 0.01
Slope T/C ratio	-	0.1409	0.1037	0.1047	0.1070	0.0950

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$).
[a]expressed as μmol maltose h^{-1} mg protein^{-1}; [b]expressed as μmol *p*-nitrophenol h^{-1} mg protein^{-1}; [c]expressed as mU mg protein^{-1}; [d] expressed as μmol *p*-nitroanilide h^{-1} mg protein^{-1}.

Table 4. Effect of different dietary levels of red monascal rice on reproductive growth and oocyte quality in female Siamese fighting fish. Analysis was conducted using individual fish from each treatment (*n* = 15).

Parameter	*P*	Red monascal rice (%)				
		0.00	**0.25**	**0.50**	**1.00**	**2.00**
Reproductive growth (*n* = 15)						
Maturation (%)	-	100	100	100	100	100
Oocyte weight (g)	**0.002**	0.24 ± 0.01^{a}	0.25 ± 0.01^{a}	0.21 ± 0.01^{ab}	0.19 ± 0.01^{b}	0.18 ± 0.02^{b}
Gonadosomatic index (GSI, %)	0.095	20.23 ± 0.95	20.01 ± 0.79	19.07 ± 1.07	17.68 ± 0.79	16.98 ± 1.26
Oocyte quality (*n* = 15)						
RNA (μg g^{-1})	**0.002**	5,210 ± 209^{c}	5,208 ± 209^{c}	5,359 ± 186^{bc}	6,055 ± 190^{ab}	6,443 ± 420^{a}
Protein (mg g^{-1})	0.078	290 ± 13	316 ± 15	279 ± 11	294 ± 13	269 ± 5
RNA/protein ratio (μg mg^{-1})	**0.002**	18.37 ± 0.94^{ab}	17.19 ± 1.05^{a}	19.81 ± 1.29^{ab}	20.93 ± 0.94^{bc}	24.06 ± 1.61^{c}
Trypsin-like specific activity*	**0.026**	0.40 ± 0.01^{a}	0.38 ± 0.02^{ab}	0.38 ± 0.02^{ab}	0.33 ± 0.02^{b}	0.33 ± 0.02^{b}
Chymotrypsin-like specific activity *	**0.007**	0.44 ± 0.02^{a}	0.41 ± 0.02^{a}	0.40 ± 0.02^{ab}	0.34 ± 0.02^{b}	0.35 ± 0.02^{b}

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$).
*expressed as μmol *p*-nitroanilide h^{-1} mg protein^{-1}.

Table 5. Pearson correlation coefficient (*r*) among SGR, digestive enzyme expressions and muscle qualities (RNA, protein and RNA/protein ratio) in female Siamese fighting fish at the end of the experiment. Data were calculated from fish in all treatments (*n* = 75).

Parameter	SGR	DTW	Amylase	Lipase	Total protease	Trypsin	Chymotrypsin	A/T ratio	T/C ratio	RNA	Protein
SGR	1										
DTW	**0.520****	1									
Amylase	**0.470****	0.231	1								
Lipase	**-0.478****	**-0.324****	**-0.382****	1							
Total protease	**0.428****	**0.470****	**0.690****	**-0.468****	1						
Trypsin	**0.489****	**0.537****	**0.682****	**-0.488****	**0.950****	1					
Chymotrypsin	**0.491****	**0.530****	**0.650****	**-0.536****	**0.962****	**0.945****	1				
A/T ratio	-0.065	**-0.359****	**0.311****	**0.242***	**-0.381****	**-0.434****	**-0.415****	1			
T/C ratio	**-0.279***	-0.159	-0.199	**0.477****	**-0.370****	-0.228	**-0.464****	0.147	1		
RNA	**0.263***	0.066	**0.357****	-0.171	**0.259***	**0.291***	**0.251***	0.037	-0.071	1	
Protein	0.068	0.138	-0.014	-0.186	0.121	0.124	0.128	-0.201	0.046	**0.394****	1
RNA/protein ratio	0.108	-0.109	**0.252***	0.077	0.037	0.064	0.033	**0.237***	-0.090	0.211	**-0.804****

SGR, specific growth rate; DTW, digestive tract weight. Significant correlation coefficients between measurement parameters are indicated by bold values (*$P < 0.05$, ** $P < 0.01$).

Table 6. Pearson correlation coefficient (*r*) between body weight and oocyte parameters in female Siamese fighting fish at the end of the experiment. Data were calculated from fish in all treatments (*n* = 75).

Parameter	Body weight	Oocyte weight	Trypsin-like	Chymotrypsin-like	RNA	Protein
Body weight	1					
Oocyte weight	**0.796****	1				
Trypsin-like	0.182	-0.122	1			
Chymotrypsin-like	0.212	-0.046	**0.920****	1		
RNA	**-0.508****	**-0.738****	**0.322****	**0.259***	1	
Protein	0.133	0.150	-0.231	-0.137	-0.073	1
RNA/protein ratio	-0.045	**-0.570****	**0.385****	**0.266***	**0.773****	**-0.666****

Significant correlation coefficients between measurement parameters are indicated by bold values (*$P < 0.05$, ** $P < 0.01$).

Table 7. Fatty acid compositions in carcasses of female Siamese fighting fish. Analysis was conducted from four fish ($n = 4$) in each treatment.

Fatty acid (%)	Significance	Red monascal rice (%)				
		0.00	0.25	0.50	1.00	2.00
Fatty acid (% total lipid)	0.119	97.13 ± 0.95	98.35 ± 0.09	98.55 ± 0.35	99.03 ± 0.34	97.78 ± 0.29
C14:0	0.256	1.63 ± 0.05	1.68 ± 0.05	1.85 ± 0.09	1.70 ± 0.09	1.75 ± 0.06
C15:0	0.179	0.25 ± 0.05	0.45 ± 0.05	0.40 ± 0.06	0.45 ± 0.05	0.45 ± 0.05
C16:0	0.359	28.70 ± 0.59	29.35 ± 0.90	31.63 ± 0.70	30.03 ± 0.78	29.55 ± 1.69
C16:1	0.149	2.37 ± 0.24	2.78 ± 0.17	3.35 ± 0.27	3.15 ± 0.33	3.25 ± 0.29
C18:0	0.322	6.35 ± 0.75	6.48 ± 0.51	5.53 ± 0.22	5.05 ± 0.25	5.73 ± 0.67
C18:1*n*9	0.647	28.73 ± 1.14	27.35 ± 1.20	28.95 ± 1.59	30.70 ± 2.16	28.23 ± 1.45
C18:2*n*6	0.094	17.23 ± 0.82	17.35 ± 0.46	15.05 ± 0.37	17.85 ± 1.01	17.20 ± 0.63
C18:3*n*3	0.139	1.05 ± 0.06	0.90 ± 0.07	0.85 ± 0.03	0.80 ± 0.13	0.78 ± 0.05
C18:4*n*3	**0.041**	0.98 ± 0.09^{ab}	0.93 ± 0.03^{ab}	0.80 ± 0.04^{b}	1.08 ± 0.07^{a}	0.85 ± 0.05^{b}
C20:1*n*9	**0.018**	0.60 ± 0.00^{a}	0.38 ± 0.00^{bc}	0.48 ± 0.05^{ab}	0.45 ± 0.03^{bc}	0.33 ± 0.06^{c}
C20:2*n*6	0.152	0.53 ± 0.05	0.68 ± 0.05	0.60 ± 0.07	0.53 ± 0.02	0.63 ± 0.02
C20:3*n*6	**0.007**	1.78 ± 0.19^{a}	2.08 ± 0.11^{a}	1.75 ± 0.21^{a}	1.10 ± 0.14^{b}	1.60 ± 0.07^{a}
C20:4*n*6	0.114	1.35 ± 0.10	1.33 ± 0.11	1.20 ± 0.11	0.98 ± 0.15	1.38 ± 0.06
C22:4*n*3	**0.002**	0.43 ± 0.02^{a}	0.45 ± 0.03^{a}	0.37 ± 0.03^{b}	0.30 ± 0.00^{c}	0.30 ± 0.00^{c}
C22:4*n*6	0.866	0.78 ± 0.05	0.80 ± 0.04	0.83 ± 0.06	0.73 ± 0.15	0.83 ± 0.03
C22:5*n*3	0.477	0.37 ± 0.03	0.40 ± 0.00	0.30 ± 0.00	0.28 ± 0.09	0.25 ± 0.05
C22:6*n*3	0.564	5.00 ± 0.44	5.33 ± 0.27	4.85 ± 0.54	4.35 ± 0.53	5.13 ± 0.21
Σ SFA	0.235	36.80 ± 0.62	37.73 ± 0.45	39.30 ± 0.76	37.00 ± 0.93	37.25 ± 1.09
Σ MUFA	0.512	30.95 ± 1.22	30.50 ± 1.30	32.78 ± 1.70	34.30 ± 2.44	31.78 ± 1.22
Σ PUFA	0.428	29.38 ± 1.43	30.13 ± 1.07	26.43 ± 1.31	27.73 ± 2.18	28.73 ± 0.75
Σ *n*-3	0.519	7.73 ± 0.45	7.90 ± 0.44	7.00 ± 0.56	6.73 ± 0.85	7.10 ± 0.24
Σ *n*-6	0.300	21.65 ± 0.99	22.23 ± 0.68	19.43 ± 0.77	21.00 ± 1.38	21.63 ± 0.61

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; nd = not detected. Data with different superscripts in each row indicate significant difference ($P < 0.05$). Significant values in each fatty acid are indicated by bold letters ($P < 0.05$).

all dietary groups ($P > 0.05$).

DISCUSSION

Effect of red monascal rice supplementation on growth

Growth performance studies indicated the acceptance of red monascal rice at 0.25% whereas the higher levels resulted in a significant decrease in growth, as indicated by the reduction of nearly all growth indicators (except condition factor) (Table 2). This observation is in agreement with the report of Wang and Pan (2003), which found the greatest reduction of body weight and egg production, and increases of feed consumption and feed conversion, in laying hens fed with 8% red monascal rice.

Wei et al. (2003) reported significant decreases in weight gain in rabbits when the concentration of red monascal rice was increased. However, Kumari et al. (2009) observed no significant differences in food intake, body weight and relative organ indices in rats fed diets with acute doses of red monascal rice. The progressively increased condition factor with the dose of red

monascal rice indicated an alteration of the morphometric relationship between weight and length. Fish fed with monascal rice decreased weight and length. The skeletal growth (length) of the fish fed monascal rice decreased faster than their weight (9.9 to 16.5% vs 2.7 to 8.6%). Moisture content of the fish body increased when fish received red monascal rice (Table 2). This might lead to a reduction of body strength, which is in agreement with the poorer performances for movement and display in treated fish (behavioral observation) when compared with the control.

The correlation coefficient indicated that SGR of fish was positively related to RNA concentration in white muscle (r = 0.263, P < 0.05). This finding explains the lower growth of fish when RNA synthesis is prohibited. Toxicological effects of citrinin, a secondary metabolite produced by several fungal species including *Monascus* sp. and *Penicillium* sp., have been reported, including increasing apoptosis and decreasing the number and viability of treated cells (Liu et al., 2005; Chan and Shiao, 2007). Citrinin increased DNA fragmentation in HL-60 cells (Yu et al., 2006), which might lead to a reduction of the muscle RNA (Table 3) thereby inducing lower cell proliferation (Chan and Shiao, 2007).

This phenomenon suggests a lower growth rate of treated fish. All observations indicate a retarding effect from chronic administration of red monascal rice on the growth in female.

Effect of red monascal rice supplementation on digestive function

Digestive processes of the fish were statistically affected (P < 0.002) by supplementation levels of red monascal rice, as also indicated by lowering digestive tract weight (Table 3). A positive correlation between body weight and gastrointestinal weight was observed in mature female (r = 0.523, P < 0.01). This phenomenon indicates that fish growth is driven by digestive capacities. This illustrates a tendency for suppressing digestion, absorption and utilization of nutrients in fish fed monascal rice. Toxicological evaluation of red monascal rice for interfering with digestive functions was clearly observed, using digestive enzymes as indicators.

Decreased specific activities of amylase and proteolytic enzymes in fish fed red monascal rice (Table 3) indicated digestive dysfunctions in the utilization of carbohydrate and protein, respectively. This correlated with a reduction of growth and development, as shown by lowered expression levels of serine proteases, trypsin and chymotrypsin (Chan, 2008); this governed a decrease of the slope T/C ratio (Rungruangsak-Torrissen et al., 2009), a key digestive enzyme factor for predicting growth and feed utilization in aquatic animals. Increased lipase specific activity in treated fish might be due to the use of lipids as a main energy source when carbohydrate and protein digestion is inhibited. Perturbation of lipid metabolism by supplementation of red monascal rice could affect the maturation of females, as shown by a negative correlation coefficient between lipase specific activity and oocyte weight (r = -0.414, P < 0.01). These findings are in agreement with previous reports on the reduction of cholesterol and triglycerides in animals after dietary supplementation with monascal rice (Wang and Pan, 2003; Wei et al. 2003). Moreover, upregulation of lipase activity might increase total fatty acids in the bodies of treated fish (Table 7). However, some fatty acid might be obtained from the monascal rice (Juzlova et al., 1996).

Effect of red monascal rice supplementation on fish reproduction

Red monascal rice induced a significant reduction in the reproductive indices by decreasing oocyte weight and GSI (Table 4). Effects of the mycotoxin citrinin on inhibiting the maturation of oocytes, fertilization efficiency and fetal development have been investigated (Chan and Shiao, 2007; Chan, 2008). Moreover, citrinin plays a potent role in inducing maternal toxicity (Singh et al., 2007b) and teratogenic effects in Wistar rats (Singh et al., 2007a). This is similar to its toxic effect on the male reproductive system, increasing the number of abnormal spermatozoa and decreasing the number of live spermatozoa (Qingqing et al., 2012). However, in a study on dietary supplementation of monascal rice in rat (Kumari et al., 2009), histopathological examination found no differences in the relative weights of sex organs (testes and ovaries).

Increased oocyte RNA and RNA/protein ratio in fish fed red monascal rice indicated a slower development of oocytes by increasing genetic materials and protein turnover rate for cell proliferation and differentiation in the earlier developmental stage. Similar results were confirmed by negative correlations between oocyte weight and RNA (r = -0.738, P < 0.01, Table 6), and between oocyte weight and RNA/protein ratio (r = -0.570, P < 0.01).

Significantly, lower specific activities of trypsin-like and chymotrypsin-like enzymes were observed in fish that received monascal rice. Both enzymes are reported to play an integral role in yolk formation and degradation (Hiramatsu et al., 2002), and embryogenesis and hatching (Sveinsdottir et al., 2006). Hydrolytic activities of serine proteases govern the abundance of amino acids for regulating osmotic pressure in oocytes and maintaining the buoyancy capacity of newly hatched juvenile fish (Finn et al., 2002). Sveinsdottir et al. (2006) discussed that increases in trypsin-like and chymotrypsin-like activities at first feeding may be possible to increase

larval survival of Atlantic cod (*Gadus morhua*). However, variation of trypsin-like enzyme in fish oocytes has been found to occur due to differences in diet quality (Rungruangsak-Torrissen, 2007). Therefore, these findings point to the reproductive effect of red monascal rice, by interfering with oocyte development in female Siamese fighting fish.

Conclusions

Supplementation of red monascal rice resulted in a significant reduction in growth, digestive function and oocyte maturation in female Siamese fighting fish, as indicated by the negative alterations of various biochemical parameters. These findings indicate an adverse effect of red monascal rice supplementation on somatic and reproductive differentiation in female Siamese fighting fish. They provide sufficient data for deciding on the merits of using red monascal rice as a food additive for rearing Siamese fighting fish.

Furthermore, the use of these biochemical parameters for measuring the effect of red monascal rice in higher animals should be of interest.

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Antifungal activity of epithelial secretions from selected frog species of South Africa

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Resistance to antibiotics has been acknowledged as a major global public health problem. The use of antimicrobial peptides to provide alternatives to combat multi-drug antibiotic resistance is beginning to attract increasing attention. The high diversity of amphibian skin peptides renders anurans an important potential source for the discovery of novel pharmacophores. This study aimed to investigate the antifungal activity of skin secretions from selected frogs (*Amietia fuscigula, Strongylopus grayi* and *Xenopus laevis)* and one toad (*Amietophrynus pantherinus)* of the south Western Cape Province of South Africa. Initially, different extraction techniques for the collection of skin secretions were tested and optimized, thereafter the extracts were tested against three fungal species of medical and agricultural importance that is, *Candida albicans*, *Fusarium verticillioides* and *Aspergillus flavus.* Chemical stimulation gave the best yield by mass, and secretions from *A. fuscigula* showed the best activity with an MIC of 40 µg / ml against *C. albicans* and 200 µg / ml against *A. flavus*. In general, *C. albicans* and *A. flavus* were the most sensitive while *F. verticillioides* was the most resistant. From this study it appears that bioprospecting of South African frog species has the potential to yield potential therapeutic lead agents.

Key words: Antifungal, African anurans, antimicrobial peptides (AMP), *Candida albicans*, *Aspergillus flavus*, bioprospecting, minimum inhibitory concentrations (MIC).

INTRODUCTION

Fungi are an important cause of human, animal and plant disease. Fungal infections in particular, have increased dramatically due to their opportunistic occurrence as a result of reduced immune status associated with the human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) pandemic, cancer chemotherapy and transplantation surgery (Ascioglu et al., 2002). In particular, oral and genital candidosis due to *Candida albicans* and related species is a significant cause of morbidity and mortality (Jarvis, 1995). Treatment is limited by the narrow range of effective antifungal agents available, their toxicity (Feldmesser,

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Abbreviations: HIV/AIDS, Human immunodeficiency virus/acquired immunodeficiency syndrome; **PPE,** porcine pulmonary edema; **NTDs,** neural tube defects; **HBV,** hepatitis B virus; **AMPs,** antimicrobial peptides; **EDTA,** ethylene diaminetetraacetic acid; **PDA,** potato dextrose agar; **PDB,** potato dextrose broth; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MIC,** minimum inhibitory concentration.

2003), emergence of resistance strains (Klepser, 2001) and relapse and re-infection (De bruyne, 1997).

With all the research focus on human disease, very little attention has been given to fungi and the toxins they produce that pose a threat to agriculture, and thereby cause disease in humans. Subsistence farming is widespread in rural Africa and is a strategy by poor rural households to reduce expenditure on food and ensure food security (Watkinson and Makgetla, 2002). Food production and storage therefore play an important role in stabilising seasonal food production (Bankole et al., 2006).

The fungus, *Fusarium verticillioides,* is one of the most common seed - borne fungi associated with corn / maize used for human and animal consumption throughout the world (Marasas et al., 1984). *F. verticillioides* and *Fusarium moniliforme* produce fumonisins which have been implicated as a risk factor in the development of oesophageal cancer in the Transkei region of South Africa as well as in the Cixian and Linxian counties of the People's Republic of China (Sydenham et al., 1996) and is a cause of leukoencephalomalacia in horses and porcine pulmonary edema (PPE) (Marasas et al., 1988). More importantly fumonisin exposure has been associated with neural tube defects (NTDs) (Marasas et al., 2004). Another important fungus is *Aspergillus flavus* which grows on crops left on the ground or stored in poor conditions and produces the aflatoxin mycotoxins. Aflatoxins are field and storage mycotoxins which are potent carcinogens, mutagens and immuno-suppressing agents (Katerere et al., 2008). They can act in synergy with the Hepatitis B virus (HBV) to increase the risk of hepatocellular carcinoma (Bhat and Vasanthi, 2003). Perinatal exposure to aflatoxins has been shown to stunt growth (low height for age) and may contribute to infant mortality as a result of protein energy malnutrition (Gong et al., 2002).

For centuries plants have been the major source of active compounds for pharmaceutical products (Springfield and Weitz, 2006). In recent years, the search for new pharmaceuticals of natural origin has intensified and been extended to include sources other than plant material (Clarke, 1997). The utilization of animal - based medicines also has a long history from ancient times (Weiss, 1947; Angeletti et al., 1992; Rosner, 1992) and scientists are increasingly exploring the use of metabolites from animals for antimicrobial activity.

Amphibians in particular offer an attractive source of novel antimicrobials because they exist in microorganism - rich environments, causing them to produce potent antimicrobial peptides (AMPs) as an innate form of defence (Govender et al., 2012). Given the respiratory and antimicrobial functions of the amphibian skin, it is likely that some of the molecules found in amphibian secretions may be of use in the treatment of skin and respiratory infections (Clarke, 1997). Extensive studies have been conducted on AMP of frogs belonging to the genus *Rana* (Che et al., 2008; Simmaco et al., 1998a).

However there have been few studies on the antimicrobial activity of African frog species despite the large number of species and high endemicity which is typical of tropical West Africa, southern Africa and the Madagascari islands (Channing, 2001; Glaw and Vences, 2007; Poynton, 1999; Rödel, 2000). South Africa alone is home to over 100 anuran species (Minter et al., 2004) of which nearly half are found in the Western Cape Province and 27 species are endemic to the south Western Cape region (De Villiers, 2008). This large diversity and density may correlate to a great molecular diversity creating high potential for the discovery of novel therapeutic peptides.

This study aimed to extract and test secretions of frog and toad species found in the South Western Cape Province for antifungal activity. The first phase investigated the optimum extraction method. This method was then used to obtain secretions from three frog (*Amietia fuscigula, Strongylopus grayi* and *Xenopus laevis)* and one toad species (*Amietophrynus pantherinus)* which were then tested for activity against three fungal species using the microplate bioassay method.

MATERIALS AND METHODS

Ethical considerations

An application was made to and approved by the Cape Peninsula University of Technology (CPUT) Health and Applied Sciences Research Ethics committee. A permit from the Cape Nature Conservation of South Africa was obtained for the collection of the specimens used. Upon capture the species were identified by Dr Abeda Dawood, a qualified zoologist.

Field collection

A pond at the University of the Western Cape, Cape Town, South Africa, was identified for the collection of specimens of *X. laevis*. A total of 15 medium sized *X. laevis* frogs were collected, of which 12 weighing between 22 and 30 g were randomly selected for the testing of extraction techniques. These specimens of frog were collected using a home-made bucket trap with a narrowing entry hole during May 2008. This entry hole is large enough to let the frog in but not out. Ox liver was used as bait to attract the frogs into the bucket. The bucket with ox liver in hosiery material was submerged overnight and then collected in the morning. All frogs captured, besides the three frogs used in the skin harvesting technique, were released back where they were collected.

Four specimens from three species of frogs (*A. fuscigula, S. grayi* and *X. laevis)* and one toad species (*A. pantherinus)* were collected in the wild from the Oude Molen Village, Pinelands, Cape Town, South Africa during June 2008. The numbers collected were limited by the number of frogs available. Frogs and collecting sites are illustrated in Figures 1 and 2.

Evaluation of extraction techniques of frog and toad secretions

The four techniques tested to extract the frog skin secretions were swabbing, physical stimulation, tissue harvesting and chemical stimulation. Three X. laevis were used for each technique. The

Figure 1. A map of South Africa showing the location of the study site.

extracts obtained were freeze-dried and then weighed and the yield calculated per body mass. The extracts were in each case re-constituted into 100 ul of sterile distilled water.

Swabbing - a novel approach

This was a new method developed for this study. The dorsal surface of three frogs was massaged gently with a cotton swab. The swabs were then placed in Eppendorf tubes, sealed and stored at -20°C. A 100 µl of distilled water was used to wash off the secretions on the swab and this sample was used further in the analysis

Physical stimulation - a novel approach

A frog was placed in a Ziploc® bag with 30 ml of double distilled water as illustrated in Figure 3 and was shaken in the bag, gently for 5 min. The distilled water containing the skin secretions was then poured from the bag into 50 ml centrifuge tubes and an additional 10 ml of double distilled water that was used to rinse the frog in the bag was added to the 50 ml centrifuge tube. The 40 ml samples were freeze-dried and then stored at -20°C for further analysis.

Tissue harvesting

The tissue harvesting technique of Goraya et al. (2000) was modified in this study. Frogs were individually euthanized by placing the frog in a bottle that contained tricaine methane sulfonate (MS 222) dissolved in distilled water. As much of the dorsal skin of the frogs was then removed and stored at -20°C. The tissue was homogenized in 10 ml of distilled water and freeze dried.

Chemical stimulation

This technique was adapted from Che et al. (2008). Cotton wool was soaked in anhydrous ether and placed into a tube. An individual frog was placed in a clean 5 l glass bottle. The plastic test tube with the cotton wool soaked in anhydrous ether was put into the bottle and the lid was tightened. The frog was left in the bottle for 2 min. The plastic test tube was removed from the bottle and the frog was rinsed with 15 ml of buffer (0.1 M phosphate buffer, containing 5 mM ethylene diaminetetraacetic acid (EDTA), pH 6.0) to wash off the skin secretions. The buffer containing the secretions was then freeze-dried.

Testing for anti-fungal activity

Three fungal species of medical and agricultural importance were used viz: *F. verticillioides* (MRC 826), *A. flavus* (MRC 3954), *C. albicans* (MRC 8907). These fungi were obtained from the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit of the South African Medical Research Council (MRC). The fungal isolates were grown on potato dextrose agar (PDA) for five days at 25°C and then stored in an incubator at 4°C. Fungal suspensions were prepared by suspending the spores in a solution of 0.05% tween 20. Prior to use, the suspensions were diluted in potato dextrose broth (PDB) standardized to 0.5 McFarland. The microtitre plate method described in detail by Katerere and Eloff (2005) and Thembo and co-workers (2010) was used. The indicator ρ-INT was included at the beginning of the experiment and visual inspection of the plates was done every 24 h and MIC recorded for up to five days.

Figure 2. Specimens collected from the Oude Molen area. **A)** *Amietia fuscigula,* **B)** *Strongylopus grayi,* **C)** *Amietophrynus pantherinus* and **D)** *Xenopus laevis.*

Figure 3. The physical stimulation technique being applied to the *X. laevis.*

Table 1. Secretion yields of anurans collected from the Oude Molen area, Pinelands, Cape Town.

Catalogue number	Family	Species	Common name	Mass of species (g)	Absolute yield (mg)	Yield (mg/g)
AD 326	Ranidae	*Amietia fuscigula*	Cape River frog	80.08	140	1.75
AD 327	Bufonidae	*Amietophrynus pantherinus*	Western Leopard toad	6.06	130	21.45
AD 328	Ranidae	*Strongylopus grayi*	Clicking Stream frog	10.28	80	7.78
AD 330	Pipidae	*Xenopus laevis*	Platanna	2.01	90	44.78

RESULTS

Extraction technique selection

The amount of secretions obtained from the swabbing, skin harvesting and physical was so small that it was impossible to analyse using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the Bradford assays. In contrast, the chemical stimulation technique yielded a larger quantity of secretions which. A total of 50 mg was obtained which translated to a yield of 0.66 mg/g. Due to its superiority and ease of use, the chemical stimulation method was selected as the method of choice for subsequent use. The four species collected and the yield obtained are shown in Table 1.

A wide variation in the yield of secretions was observed. It would have been expected that the larger the frog the greater the skin surface area and number of secretory glands, therefore more secretions would be obtained. The results indicate that this was not the case. Unfortunately, due to the limitations on our collecting permit we could not collect more than one specimen for each species. It might be worth investigating whether the yield is repeatable with a larger sample size.

In this study, the frogs were stimulated once and released back into their natural environment. Mangoni et al. (2001) stated that frogs that have been pharmacologically depleted of skin antimicrobial peptides will not easily recover unless the animals are gradually exposed to bacteria or fungi in their environment. In their depleted state these frogs will succumb to infection if suddenly exposed to otherwise innocuous microbes. This seems to indicate that AMPs are inducible rather than constitutive compounds.

Antifungal activity for the different frog and toad species was found to be between 0.04 and 12.5 mg/ml (Table 2). Good inhibition was shown by all frog secretions on *C. albicans* with minimum inhibitory concentration (MIC) values of between 0.04 and 0.19 mg/ml after 120 h. Apparent biofilm formation was evident with *C. albicans* after 72 h of exposure to the extract. This makes the potency of extracts from *A. fuscigula* (AD 326) all the more interesting. Candida species are well known for biofilm formation which results in reduced sensitivity (d'Enfert, 2006). Similar results were obtained against *A. flavus,* where relatively low concentrations of 0.19 to 0.39 mg/ml were obtained after five days. *F. verticillioides* was generally resistant to all extracts.

In the case of isolates of *Aspergillus* spp. conidium formation was enhanced on plates after five days. Conidial formation may be an indication of stress and it might ironically result in increased biosynthesis of mycotoxin (Guzman-de-Penã and Ruiz-Herrera, 1997). In stability graphs, the lower and more horizontal (flatter) the curve, the better the activity of the test extract. A more horizontal line depicts fungicidal activity as opposed to fungistatic activity, that is, the ability of a test extract to inhibit fungi for a certain time and lose activity thereafter (Thembo et al., 2010). It is evident that the extracts are particularly stable and maybe fungicidal against *C. albicans*, with MIC not exceeding 0.19 mg / ml after five days. AD 326 is particularly stable and potent.

DISCUSSION

Frog skin secretions were previously obtained by either electrical stimulation (Dourado et al., 2007; Kim et al., 2007; Nascimento et al., 2007), chemical stimulation using norepinephrine (Conlon et al., 2007; Nascimento et al., 2007; Rollins-Smith and Reinhart, 2005) or skin harvesting (Goraya et al., 2000; Roseghini et al., 1989). More recently the irritant chemical stimulation technique (Che et al., 2008) has been used to good effect. In the Che et al. (2008) study, 30 frogs of the same species were used, whereas in the present study a single frog provided sufficient yield for the bioassays. Furthermore, the skin secretions could be obtained in the field and the animals could be released at their site of capture. This reduced the amount of stress imposed on the frogs and ensured that the frogs were released back on their original collection sites.

Following the discovery in 1987 of the magainins in skin secretions of the African clawed frog *X. laevis* (Zasloff, 1987), attention has been increasingly focused upon the skins of anurans as a potential source of

Table 2. Antifungal activity of anuran extracts against three fungal species.

Extract species	MIC (mg ml^{-1})											
	48 h			72 h			96 h			120 h		
	Fv[a] *826	Ca[b] *8907	Af[c] *3954	Fv 826	Ca 8907	Af 3954	Fv 826	Ca 8907	Af 3954	Fv 826	Ca 8907	Af 3954
Amieta fuscigula (AD 326)	0.39	0.04	0.02	1.56	0.04	0.19	1.56	0.04	0.19	12.5	0.04	0.19
Amietophrynus pantherinus[#] (AD 327)	0.39	0.04	0.02	1.56	0.19	0.19	1.56	0.19	0.19	1.56	0.19	0.39
Strongylopus grayi (AD 328)	0.39	0.04	0.02	3.12	0.09	0.09	3.12	0.19	0.09	12.5	0.64	0.39
Xenopus laevis (AD 330)	0.39	0.02	0.02	3.12	0.09	0.09	3.12	0.19	0.09	12.5	0.19	0.39
Amphotericin B*		0.52			1.17			6.38			21.0	

[a]*Fusarium verticillioides* (MRC 826); [b]*Candida albicans* (MRC 8907); [c], *Aspergillus flavus* (MRC 3954); *average MIC in ul/ml; [#]denotes toad species.

novel antibiotics (Nicolas and Mor, 1995; Simmaco et al., 1998b). Che et al. (2008) stated that the skin of amphibians, particularly those belonging to the families of Pipidae, Hylidae, Hyperoliidae, Pseudidae and Ranidae, synthesize and secrete a diverse array of antimicrobial peptides. Similar to the study of Zasloff (1987), the present study found that low concentrations of *X. laevis* secretions were able to inhibit growth of *C. albicans*. Skin secretions from the frog species *A. fuscigula,* (Pyxicephalidae), *A. pantherinus* (Bufonidae) and *S. grayi* (Pyxicephalidae) have not been previously tested for antifungal activity and this study is the first to do so. Peptides have been isolated from the skin secretions of toads belonging to the family *Bufonidae* (Clarke, 1997; Maciel et al., 2003, 2006) however, none of these studies focused on the species *A. pantherinus* or any other South African bufonid representatives.

Previous studies have reported that secretions from *Rana septentrionalis* (Bevier et al., 2004), *Rana areolata* (Ali et al., 2002), *Amolops loloensis* (Wang et al., 2008) showed no activity against *C. albicans.* In the present study, the frog skin secretions were active against not only *C. albicans* but also A. *flavus*. Strains of *Candida* spp have been used in previous similar studies (Ali et al., 2001; Basir et al., 2000; Wang et al., 2007) and the activity found ranged from 0.03 to >0.10 mg/ml. The results are similar to those obtained in this study. There is a need to isolate more secretions from the most active species, re-test them and isolate and elucidate the bioactive peptides for possible further research and development.

Conclusion

This study demonstrates the potential of finding novel antimicrobial peptides from anurans of the south Western Cape and South Africa. The use of peptides to provide alternative approaches to combating multi-drug resistant organisms is gaining momentum. This study presents a strong case for broadening research focus from bioprospecting for antimicrobials in medicinal plants to Africa's amphibian species, which are largely unexplored and may present a treasure trove for finding novel therapeutics.

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Full Length Research Paper

Oil extract from *Gongronema latifolium* leaves exhibit anti-diabetic and anti-ulcer activities

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Air-dried, pulverized leaves of *Gongronema latifolium* were subjected to step wise fractionation using first, ethanol and subsequently fractionation of the dried ethanol extract using solvents of increasing polarity, n-hexane, chloroform, ethylacetate and ethanol. Column fractionation of the n-hexane extract using graded solvent mixture of n-hexane and ethylacetate in specified ratios, yielded four fractionates, one of which F2 was an oil. The F2 (oil) fraction was characterized by gas chromatography/mass spectral analysis. The effects of this oil fraction on alloxan- induced diabetes mellitus and indomethacin - induced ulceration were studied. The results obtained reveals that oil extract evoked significant inhibition of both alloxan- induced diabetes and indomethacin- induced ulcer in rats. The oil on analysis revealed content of essential fatty acyl esters and aromatic dicarboxylic acids, all of which are implicated in inhibiting hyperglycaemia and ulcer formation probably through oxidative reaction or through production of prostaglandins.

Key words: *Gongronema latifolium*, essential fatty acids, diabetes mellitus, ulcer, prostaglandins.

INTRODUCTION

Extractable oil, an important nutritive component of plants, have not been properly projected and utilized. Fats and oils are naturally occurring organic compounds of plants whose function is mostly to supply energy and serve as structural components of membranes and in pharmaceuticals as drug delivery (Ajalli, 2004; Garret and Grimsham, 2005; Ezekwe, 2013; Odo et al., 2013). The amount and type of fat in diet (solid or oil) have important implication because a diet containing large amount of saturated fat is linked to an increase in risk of atherosclerosis and subsequent heart disease and stroke (BMA, 2002). On the other hand, animals maintained on fat free diet develop poor growth, poor wound healing and dermatitis (Ajalli, 2004).

Plants essential oils and their components have been associated with biological activities such as possessing antimicrobial and anti pests (Dongmo et al., 2007; Amvam Zollo et al., 1998), anti-oxidant (Lemos et al., 2006), anti-flammatory, anti-ulcer (Olivierra et al., 2004; Siani et al., 1999) activities and protection against cardiovascular disease (Ezekwe, 2013; Odo et al., 2013). Linoleic acid component of oils, is the precursor of prostaglandins which act as hormone to protect the lining of the stomach against ulceration, lowers blood pressure and stimulate contraction of uterine walls (BMA, 2002). However, other works suggest that excess of polyunsaturated fats is central to the development of degenerative diseases. They are universally toxic to energy producing systems and act as “misleading signal” channelling cellular adaptation down self-defeating pathways and diabetes is one of the terminal diseases that can be caused by the polyunsaturated vegetable oils (Peat, 1996). Even impaired insulin access to tissues may be one of the initial consequences of elevated free fatty acids (Arbeeny et al., 2001; Garrett and Grimsham, 2005).

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It is based on these conflicting views that this work was undertaken in order to ascertain if dietary oils from a vegetable could exhibit protection against some chronic disease such as ulcer and diabetes. And especially, as it has been observed by the World Health Organization (WHO, 2003; Ezekwe, 2013; Odo et al., 2013), that insufficient consumption of fruits and vegetables, a major source of these oils, is among the major cause of diseases of chronic conditions such as diabetes mellitus, cardiovascular diseases, cancers, especially of the gastrointestinal tract.

Hence, the advocacy by the WHO promoting increase in fruit and vegetable intakes. *Gongronema latifolium* is one of the perennial herbal plants of Nigeria that have aromatic properties of important medicinal value (Arbeeny et al., 2001). It is listed among the medicinally important leafy vegetables of South West Nigeria (Ayodele, 2008) and as Africa leafy vegetables (Smith and Eyzaguirre, 2007; Siani et al., 1999). It has been implicated with anti-diabetic and anti-oxidant properties (Ezekwe, 2005; Ugochukwu and Babbady, 2003; Amvam Zollo et al., 1998), anti-inflam-matory properties (Esterbauer and Puhl, 1991) and intestinal muscle relaxation (Gamaniel and Akah, 1996; Cavanagh, 2007). The anti-diabetic and anti-ulcer effects of the oil extract, from the leaves of this plant, were determined on rats.

MATERIALS AND METHODS

Plant materials

Leaf samples of *G. latifolium* were air-dried, pulverized and stored in plastic containers at -10°C.

Animals

Thirteen (13) albino mice weighing between (19 to 30 g) were purchased from the Departmental Animals House, University of Nigeria, Nsukka. They were housed in metal cages under standard conditions of 12 h light/dark cycles, fed pelleted feed and water *ad libitum.*

Rats

Inbred Wistar albino rats weighing (150 to 250 g) were purchased from Departmental Animal House and housed under same conditions as the mice.

Extraction procedure

Crude ethanol extract

Air-dried pulverized leaves (1 kg) were macerated in 5.0 L of 96% ethanol for 48 h. The filtrate from Whatman No. 1 filter paper was dried at 40°C and the Crude Ethanol Extract (CEE) and stored for further use.

Fractionation of crude ethanol extract (CEE)

The dried CEE (75 g) was adsorbed on silica gel G (1:2 w/w) and extracted with n-hexane. The fraction was dried and stored for biochemical determinations.

Fractionation of n-hexane fraction

The dried n-hexane fraction (20 g) was chromatographed on silica gel (70 to 230) mesh packed into a glass column (4 × 120) cm, with a bed of 60 cm height. Graded solvent mixtures of n-hexane and ethylacetate were used for the elution that is, n-hexane, n-hexane: ethylacetate 19:1, 9:1. Aliquots of 50 ml were collected and concentrated. Similar fractions were pooled by thin layer chromatography and on further concentration yielded fractions 1 and 2.

F1 - white waxy substance,
F2 - yellowish brown oil.

Animal studies

Determination of the effect of F2 on alloxan - induced diabetes mellitus

Four groups of five rats were used representing: 1) Normoglycaemic, 2) diabetic F2-treated, 3) diabetic glibenclamide-treated, and 4) diabetic non-treated. Diabetes was induced by the method of Abdel-Hassan et al. (2000) by i. p. administration of alloxan (150 mg/kg b. w.).

Diabetes was confirmed after 72 h of persistent hyperglycaemia above 300 mg/kg b. w. as shown previously by Al-Hadar et al. (1994). Blood glucose levels were monitored at intervals of 0, 1, 3, 6, 12 and 24 h. The % reduction in blood glucose is computed after 24 h.

Determination of the effect of F2 (oil on indomethacin-induced ulceration)

The method of Uridishani et al. (1979) was used. Three groups of four rats were used namely: GP 1 - saline, Gp 2 - F2 and GP 3 - ranitidine. Food was removed from the rats (12 h) and water just before the experiment. The different groups received their drugs (extract) 30 min before oral administration of 40 mg/kg indomethacin. After 72 h, rats were sacrificed in ether chamber and their stomachs excised, dissected, washed, fixed in formal saline and mounted on slab. Ulcer crater or wounds were counted using a magnifying (×10) lens and the mean ulcer indices computed.

Structural elucidation of F2

F2 was analysed by ‘gas chromatograph/mass' spectral analysis using agilent 5973N mass selective detector coupled to Agilent 6890N gas chromatograph with specified parameters. Column size (30 × 0.2 mm, film thickness 0.25 μm). Operating conditions - carrier gas helium with a flow rate of 2 ml/min, column temperature (60 to 275° at 4°C/mm), injection and detector temperature (280°C), injector volume (2 μl), split ratio (1:5).

The MS operating parameters were as follows: ionization potential (70 eV), ionization current (1A), ion source temperature (200°C) and resolution of 1000. Identification of F2 was based on comparison of the retention times and computer matching of MS fragments with NISTO2 library.

Statistical analysis

The results obtained were analysed by SPSS version 18 using one way analysis of variance (ANOVA) and subjected to Fischer LSD

Table 1. The chemical composition of sample F2.

Compound	RT (min)	Molecular weight	Molecular formula	MS Fragment ions
Ethyl hexadecanoate (palmitic acid ester)	18.25	284	$C_{18}H_{36}O_2$	284, 255, 241, 157, 151, 88, 55
Ethyl 9, 12 - octadecadienoate (linoleic acid ester)	21.03	308	$C_{20}H_{36}O_2$	308, 277, 263, 250, 164, 149, 135, 121, 108, 95, 81, 67, 55
(Z,Z,Z) ethyl 9, 12, 15 - octadecatrienoate (linolenic ester)	21.08	306	$C_{20}H_{34}O_2$	306, 277, 261, 250, 237, 203, 191, 173, 149, 135, 121, 108, 95, 79, 67, 55
Ethyl oleate (oleic acid ester)	21.15	310	$C_{20}H_{38}O_2$	310, 284, 264, 246, 235, 222, 180, 166, 152, 137, 123, 110, 97, 83, 69, 55
Ethyl octadecanoate (stearic acid ester)	21.68	312	$C_{20}H_{40}O_2$	312, 269, 213, 157, 101, 88, 83, 73, 61, 55
Diisoctyl - 1, 2 - benzenedicarboxylate	26.82	390	$C_{24}H_{38}O_4$	390, 279, 167, 149, 132, 113, 93, 83, 71, 57

Table 2. Effect of F2 fraction on diabetes mellitus.

Treatment group	Dose	Mean blood glucose concentration (mg/100 ml)/time						Maximum % reduction
		O h	1 h	3 h	6 h	12 h	24 h/time	
Normoglycaemic	5 ml/kg	119.70^a ± 7.0	109.00 ± 8.0	93.00 ± 9.0	78.70 ± 7.0	74.00 ± 4.0	55.00 ± 5.0	53.78%
Diabetic F2- treated	100 mg/kg	429.75 ± 30.3	390.50 ± 49.3	370.00 ± 40.1	212.25 ± 43.1	137.50 ± 22.1	83.75 ± 12.9	80.51
Diabetic glibenclamide treated	100 mg/kg	460.00 ± 23.0	257.0 ± 22.0	200.00 ± 10.0	156.00 ± 24.0	118.0 ± 28.0	68.0 ± 9.0	85.22
Diabetic untreated (negative control)	5 ml/kg	356.00 ± 23	409.50 ± 38.8	363.5 ± 27.0	382.50 ± 18.0	341.29 ± 22.0	270.0 ± 19.0	24.60

P<0.05 against negative control.

post HOC. Results were expressed as mean ± SEM. Differences between means were considered significant at P < 0.05.

RESULTS

Table 1 shows that F2 is composed of six esters of fatty acids, of which two are essential fatty acids (linoleic, linolenic acids), one mono unsaturated (oleic acid), two saturated (stearic and palmitic acids and an aromatic fatty acid). Diisoctyl 1, 2 - benzenedicarboxylate. The standard anti-diabetic drug glibenclamide inhibited hyperglycaemia significantly (P < 0.05). The F2 fraction equally inhibited hyperglycaemia significantly (P < 0.05). The maximum reduction of hyperglycaemia in the three treated groups of animals was 56.1, 80.51 and 85.22%, respectively (Table 2). The glibenclamide significantly (P < 0.05) lowered the blood glucose level over time. The fall in blood glucose was more prominent

Table 3. Effect of F2 on indomethacin- induced ulcer in rats.

Group	Dose	Mean ulcer index	% inhibition of ulcer
Saline	3 ml	6.10 ± 0.94	
F2-treated	100	*0.77 ± 0.27	87.38
Ranitidine	100	*0.75 ± 0.26	87.70

*P < 0.05.

from the 3rd hour of the experiment. Similarly, the F2 isolate (the oil fractions) significantly (P < 0.05) reduced hyperglycaemia. The drop in blood glucose was more prominent between the 5 and 6th hours. The n-hexane extract lowered blood glucose but it was not significant (p > 0.05). The standard drug, ranitidine, significantly (P < 0.05) hindered ulceration of rat stomach mucosa (Table 3). Similarly, F2 fraction hindered ulceration in rat stomach significantly (P < 0.05). The inhibition of ulcer by the n-hexane fraction at 400 mg/kg was below that of the F2 and ranitidine.

DISCUSSION

The phytochemical screening of the crude ethanol extract revealed an abundance of phytochemicals in the crude ethanol extract many of which have been implicated in disease remedies. Shimizu et al. (2001) and Williams et al. (2007) reported that triterpenoids of *Gymnema inodorum* inhibited the absorption of glucose while Abdel-Hassan et al. (2000) demonstrated that alkaloids and saponins in aqueous extract of *Citrullus colocythis* exerted hypoglycaemic effect on diabetic rabbits. Flavonoids of *Equisetum myriochaetum* have also been implicated in reduction hyperglycaemia in streptozotocin diabetic rats (Ezekwe, 2005; Williams et al., 2007; Ezekwe, 2013; Odo et al., 2013). The result from this work, showed that hyperglycaemia induced by alloxan, was significantly (P < 0.05) inhibited by the oil extract, (F2), of *G. latifolium.* The magnitude of inhibition was 56% for n-hexane, 80% for the oil (F2) and 85% for the standard drug. This work also confirms the family relationship between *G. latifolium* and *Gymnema sylvestre* as both of them significantly reduced hyperglcaemia. Studies carried out on *G. sylvestre* showed that oil extracts possessed hypoglycaemic activity (Punitha et al., 2005). GC/MS analysis revealed that two of the components of F_2 are essential oils (linoleic and linolenic acids), in addition to an aromatic oil, saturated and mono unsaturated fatty acyl esters, which tend to suggest that these components, must have contributed to the total reduction in blood glucose level. Such complications as nerve and kidney disorders in diabetes are known to improve when evening rose oil, a source of r-linolenic acid was administered to patients (Day, 1998) but no direct link between dietary oil and protection from diabetes mellitus have been established yet. However, (Peat, 1996) indicated that polyunsaturated fats were central to the cause of degenerative diseases such as diabetes mellitus, whereas coconut oil proved to be protective. This work also showed that the F2 oil evoked a significant (P < 0.05) inhibition of ulcer in drug-induced ulcer.

The ulcer inhibition was comparatively close between the standard drug (87.70%) and the F2 (87.38%). This work vitiates the studies carried out on *G. latifolium* by Ogunwande et al. (2007) who showed that *G. latifolium* contained essential oils which had anti-microbial activity. Essential oils facillitate gastroprotection as a result of tendency to generate prostaglandins from arachidonic acid, a product of action of cyclooxygenase on linoleic acid (Snowdon and Philips, 1985; Amvam Zollo et al., 1998). Several works have implicated prostaglandin in inhibition of ulcer as a result of bicarbonate mechanism of gastric protection by the mucosa (Bottling and Salzamnn, 1974; Lauritzen et al., 2001). An important inference from this study is that the extract was non-toxic even at the highest dose level (5000 mg/kg b. w.). This therefore does not place any limitation to the use of the vegetable.

In conclusion, the oil extract (F2) from *G. latifolium* was effective in protecting against diabetes and ulcer, two pathological conditions of degenerative nature. These two conditions, usually attributed to oxidative reactions, might suggest that the oil extract functioned as antioxidant. This possibility is under investigation. Hence, oil extract of *G. latifolium* did not exhibit deleterious effects in the animal studied but rather inhibited hyperglycaemia in alloxan-induced diabetic rats and protected against ulcer in indomethacin-induced ulcerative rats.

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Population structure and genetic diversity of Sudanese native chickens

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The objectives of this study were to analyze genetic diversity and population structure of Sudanese native chicken breeds involved in a conservation program. Five Sudanese native chicken breeds were compared with populations studied previously, which included six purebred lines, six African populations and one Sudanese chicken population. Twenty-nine (29) microsatellite markers were genotyped individually in these five populations. Expected and observed heterozygosity, mean number of alleles per locus and inbreeding coefficient were calculated. A model based cluster analysis was carried out and a Neighbor net was constructed based on marker estimated kinships. Two hundred and one alleles were detected in all populations, with a mean number of 6.93 ± 3.52 alleles per locus. The mean observed and expected heterozygosity across 29 loci was 0.524 and 0.552, respectively. Total inbreeding coefficient (F_{IT}) was 0.069±0.112, while differentiation of subpopulations (F_{ST} 0.026±0.049) was low indicating the absence of clear sub-structuring of the Sudanese native chicken populations. The inbreeding coefficient (F_{IS}) was 0.036±0.076. STRUCTURE software was used to cluster individuals to 2 ≤ k ≤ 7 assumed clusters. Solutions with the highest similarity coefficient were found at K=5 and K=6, in which Malawian, Zimbabwean, and purebred lines split from Sudanese gene pool. The six Sudanese native chicken populations formed one heterogeneous cluster. We concluded that Sudanese native chickens are highly diverse, and are genetically separated from Malawian, Zimbabwean chickens and six purebred lines. Our study reveals the absence of population sub-structuring of the Sudanese indigenous chicken populations.

Key words: Genetic diversity, microsatellites, population structure, Sudanese native chickens.

INTRODUCTION

In recent years, animal biodiversity management has become an important issue in the international community because of changes in large-scale production systems (FAO, 2007). In the absence of comprehensive breed characterization data and documentation of the origin of breeding populations, molecular marker informa-

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Abbreviations: LBZ, Large beladi from Zalingei; **LBDa,** large beladi Dammzein; **BAL,** large beladi Khartoum; **BNAb,** bare neck Abu Naama; **BNOb,** bare neck Obeid; **BT,** Betwil are six Sudanese populations; ZA, ZB, ZC, ZD, ZE , five Zimbabwe eco-types; **MA,** Malawi; **BRS_A,** broiler sire line A; **BRD_A,** broiler A; **BL_A,** brown egg layer line A; **BL_C,** brown egg layer line C; **WL_A,** white egg layer line A; **PCR,** polymerase chain reaction; **BRD,** broiler dam.

Table 1. The geographical description of the study area and sample size.

Agro-ecological zone	Rainfall (mm)	Temperature (°C)	Geographical region	Chicken population	Sample size
I-Damzein	600-800	28 - 30	High rainfall Savannah	LBDa	17
I-Abu Naama	400-600	28- 30	High rainfall Savannah	BNAb	18
II-Abassia					
II-Rashad					
II-Tajmala	600-800	26 - 28	High rainfall mountain Savannah	BTNm	36
II- Dalinj					
II- El farshi					
II-El Obeid	200-400	26 - 28	Low rainfall Savannah	BNOb	12
III-Zalingei	400-600	24 - 26	High rainfall avannah	LBZ	16

Sources: IPCC and CRU; SIM (Sudan Interagency Mapping); vmaplv0, NIMA; UN Cartographic Section. LBZ, Large beladi from Zalingei; LBDa, large beladi Dammzein; BAL, large beladi Khartoum; BNAb, bare neck Abu Naama; BNOb, bare neck Obeid; BT, Betwil are six Sudanese populations.

tion may provide reliable estimates of genetic diversity within and between a given set of populations (Zanetti et al., 2010; Granevitze et al., 2007). Molecular marker information has been used to monitor genetic diversity of populations (DeMarchi et al., 2006), and to valorize genetic resources using genetic traceability systems (Dalvit et al., 2007).

Among molecular markers, microsatellites have been intensively used over the last two decades as they are well dispersed in the genome and highly polymorphic (Cheng et al., 1995). They have been used in many countries to study the genetic relationships among local chicken breeds (Muchadeyi et al., 2007; Dalvit et al., 2009). Microsatellite markers have also been used to assess population structure and diversity of a number of native chickens in Africa (Mtileni et al., 2011; Mwacharo et al., 2011). Several molecular studies of local chicken populations in Africa have been done separately for different countries (Muchadeyi et al., 2007; Mtileni et al., 2011).

More than 1.3 billion chickens are found in Africa today, producing approximately 1.7 and 2.1 million metric tons of eggs and meat, respectively, of which 80% are from indigenous stocks (FAO, 2006). In Sudan, the traditional sector comprises 70% of the total chicken's annual production of 20.1 million birds and 900 million eggs (Sulieman, 1996). The Sudanese fowls with various types, which collectively are called Beladi (means native), were characterized by Desai (1962). These birds are commonly classified as Large Beladi (LB), Bare-Neck (BN) and Betwil (BT) ecotypes (Desai, 1962). Indigenous fowl is less productive compared to exotic breeds, but play an integral role in the smallholders farming systems. The aim of the present study was to evaluate the genetic variability within and between Sudanese native chickens, and study the level of population differentiation between Sudanese native chicken and other village chicken populations from similar extensive systems of production in Africa and pure bred populations with known breed history.

MATERIALS AND METHODS

Geographical description of the study area

Different areas were selected for samples collection, namely Damazein, Abu Naama, Abassia, Nuba Mountains El Obeid and Zalingei (Table 1). These areas are located between 10° N and 15° N latitude, 23° E and 35° E longitude, and 453 and 1350 m above sea level. The rainfall ranges from 200 to 800 mm and the average temperatures are between 24 and 30°C.

Sampling of household

Five Sudanese native chicken populations including two large Beladi chicken populations from Zalingei (LBZ =16; 6 ♀ + 10 ♂) and Damazein region (LBDa = 17; 10 ♀ + 7 ♂), two populations of Bare Neck chicken from Abu Naama (BNAb =18; 8 ♀ + 10 ♂) and EL-Obeid (BNOb = 12; 7 ♀ + 5 ♂), and one population of Betwil from Nuba Mountains (BTNm = 36; 19 ♀ + 17 ♂) were collected based on the phenotypic characteristics of each local breed (Desai, 1962). DNA samples of the sixth Sudanese population (BAL) used in this study were originally collected by Muchadeyi et al. (2007) from Khartoum state.

Reference populations

Microsatellite data of six populations were selected from AVIANDIV project. These consisted of one broiler dam (BRD) and one broiler sire (BRS) lines, two brown egg layers (BL-A and BL-C) and two white egg layers (LS-S and WL-A) with 30 individuals per population. The broiler dam and sire lines, brown egg layers and white egg layer line A (WL-A) were commercial lines, whereas the other white egg layer (LS-S) was an experimental White Leghorn line-Rs maintained at the Institute of Farm Animal Genetics as a conservation flock (Hartmann, 1987).

The pure lines were managed as closed populations with known pedigree and breed history. These characteristics made them well suited to be used as reference populations for comparison with the Sudanese chickens studied.

In addition, data of seven populations were collected from previous studies conducted by Muchadeyi et al. (2007), including five local chicken populations from Zimbabwe eco-zone ZA, ZB, ZC, ZD and ZE with sample sizes of 50, 51, 50, 50 and 37, respectively, and 60 birds from a scavenging chickens population, sampled in Malawi (MA).

Table 2. Nei's estimation of expected and observed heterozygosity, mean number of alleles per population and locus and F-statistics over all loci.

Population	H_E	H_O	No of alleles per locus	F_{IT} ± SE	F_{ST} ± SE	F_{IS} ± SE
Sudanese breeds	0.552	0.524	5.3	0.069±0.112	0.026±0.049	0.036±0.076
African breeds	0.646	0.595	6.28	0.074±0.121	0.013±0.024	0.059±0.104
Commercial breeds	0.439	0.424	3.18	0.336±0.428	0.317±0.403	0.007±0.066
Over all mean	0.546	0.514	-	0.187±0.237	0.137±0.177	0.050±0.083

H_E, Expected heterozygosity; H_O, observed heterozygosity; F_{IT}, total of inbreeding coefficient; F_{ST}, inbreeding of subpopulation relative to the total population; inbreeding coefficient.

Collection of blood samples and DNA extraction

From Sudanese chickens, a drop of blood was sampled from the wing vein (brachial vein) onto Whatman FTA® classic filter cards (Whatman International Ltd). Blood samples were air dried and stored in original packaging box at room temperature. Genomic DNA was extracted at the International Central Lab, Ministry of Science and Technology (Souba-Sudan) using phenol-chloroform method as described by Sambrook and Russel (2001).

DNA polymorphism

A set of 29 microsatellite markers were used to examine the genetic variability, twenty-eight of which are part of the 30 microsatellites recommended by FAO-ISAG (2004) in measurement of Domestic Animal Diversity (MoDAD) Project for assessing chickens genetic diversity. MCW80 is not included in FAO list but had been previously used together with some of FAO markers in multiplex reaction for the AVIANDIV populations. Polymerase chain reaction (PCR) was used to amplify the specific DNA fragments containing microsatellites as described elsewhere (Muchadeyi et al., 2007). The DNA fragments produced by amplification were visualized on 8% polyacrylamide gel, which was performed with a LI-COR semi-automated DNA analyser (LI-COR Biotechnology, Division, Lincoln, NE68504). Electrophoregram processing and allele-size scoring was done using the RFLPscan software package (Scanalytics, Division of CSP, Billerica, USA).

Statistical analyses

Marker polymorphism and population diversity

Total number of alleles, allele frequencies, average number of alleles per locus, observed heterozygosity, expected heterozygosity and inbreeding coefficients (F_{IS}) for each population across loci were determined using Microsatellite-Toolkit for Excel (Park, 2001). The Weir and Cockerham (1984) estimate of Wright's fixation indices (F_{IT}, F_{ST} and F_{IS}) was calculated in order to quantify the partitioning of variance between and within populations using FSTAT Software (Goudet, 2001). Standard errors for fixation indices were generated using bootstrapping over loci and population. Between populations pairwise F_{ST} estimates and Nei's standard genetic distances (Nei, 1972) were calculated.

Assignment of individuals to populations

The algorithm implemented in STRUCTURE software was used to cluster individuals based on multilocus genotypes (Pritchard et al., 2000). The analysis involved an admixture model with correlated allele frequencies. The model was tested using 20 000 iterations of burn-in phase and 50 000 iterations for each $2 \leq K \leq 7$ assumed cluster with 100 runs for each *K* value. A pairswise comparison between runs for each number of clusters defined a priory was done by SIMCOEFF software (Rosenberg et al., 2002). The solutions with over 95% similarity were considered identical. The most frequent solution for each K-value was considered to be the probable clustering for the given number of assumed groups and visualized by DISTRUCT software (Rosenberg et al., 2004). Clustering was done in two data sets, the full set of all populations under study, and the Sudanese chicken populations only.

Estimation of the optimal number of cluster in structure

To determine the optimum number of clusters for each K-value, a method described by Evanno et al. (2005) was applied to determine the number of clusters that fits best to underlying structure of these populations. In the present study, of the 100 runs for a given value of K, some outliers were detected in the distribution of log likelihoods. Runs of which the log likelihood deviated from the mean with more than three standard deviations, were removed from analyses. All removed runs showed a log likelihood that deviated downwards from the mean, indicating that these runs got stuck in a local optimum.

Marker estimate kinships

Similarity indices between and within populations were calculated from allele frequencies using Malecot's definition of similarity (Eding and Meuwissen, 2001). A network tree was constructed from the MEK using SPLITSTREE-4 software packages (Hudson and Bryant, 2006).

RESULTS

Genetic diversity within and between populations

The total number of alleles among the six Sudanese native chicken populations across all loci was 201. All microsatellite loci were polymorphic. The number of alleles PER locus ranged from three (MCW103, MCW098, MCW248, MCW1650 to 17 (LEI234). The mean number of alleles per locus for the Sudanese chicken populations was higher than that of the reference populations, and lower than that of the other African breeds (Table 2). In addition, the other African chicken breeds showed higher estimates of both expected and observed heterozygosity followed by Sudanese breeds

Table 3. Mean number of alleles per locus, expected (H_{E}), observed (H_O) and F_{IS} for Sudanese chicken populations.

Population	Sample size	No of alleles ± SD	F_{IS}	H_E ± SD	H_O ± SD
LBDa	17	4.76 ± 2.0	0.054	0.560 ± 0.024	0.531 ± 0.023
LBZ	16	4.10 ± 1.4	0.093	0.507 ± 0.031	0.461 ± 0.023
BT	36	5.00 ± 2.0	0.040	0.562 ± 0.028	0.540 ± 0.015
BNAb	18	4.62 ± 2.1	0.032	0.535 ± 0.031	0.518 ± 0.022
BNOb	12	4.00 ± 1.6	0.005	0.581 ± 0.026	0.578 ± 0.027
BAL	48	5.62 ± 2.5	0.081	0.561 ± 0.025	0.517 ± 0.013

Table 4. Marker estimated kinship (above the diagonal) and pair wise F_{ST} (below the diagonal) within and between Sudanese native chicken populations.

Pop	BAL	LBDa	LBZ	BT	BNAb	BNOb
BAL	0.063	0.043	0.000	0.028	0.053	0.043
LBDa	0.008	0.069	0.005	0.039	0.049	0.048
LBZ	0.105	0.098	0.159	0.021	0.042	0.028
BT	0.030	0.011	0.076	0.061	0.036	0.046
BNAb	0.008	0.006	0.114	0.026	0.091	0.045
BNOb	0.013	0.007	0.080	0.007	0.020	0.094

BAL, Large Beladi from Khartoum; LBDa, large Beladi Damazein; LBZ, large Beladi Zalingei; BT, Betwil form Nuba Mountains; BNAb, bare neck Abu Naama; BNOb, bare neck El-Obied.

and commercial lines. The observed and expected heterozygosity estimates and inbreeding coefficient (F_{IS}) of each of the six Sudanese native chicken populations are shown in Table 3. The average observed and expected heterozygosity across 29 loci was 0.524, and 0.552, respectively. The inbreeding coefficient (F_{IS}) within populations ranged from (0.005 to 0.093). The mean inbreeding coefficient F_{IS} for the Sudanese native chicken populations was lower than that of African populations but it was greater than that of purebred lines. The mean fixation coefficient of Sudanese sub-population F_{ST} was slightly higher than that of other of African populations, but both of them were much lower than the value found for purebred lines.

Genetic distances

Kinship coefficient estimated within and between the Sudanese chicken populations (above the diagonal) as well as pairwise F_{ST}-value below the diagonal) are shown in Table 4. The highest kinship was calculated between individuals within LBZ (0.159), while lowest kinship was estimated between BAL and LBZ (o.ooo). Pairwise-F_{ST}-value of Sudanese chicken populations revealed the lowest genetic differentiation (0.006) between LBDa and BNAb populations while it was highest between LBZ and BNAb (0.114) populations.

Cluster analysis

Results STRUCTURE clustering are displayed in Figure 1. At K = 2, the six Sudanese native chicken populations clustered together with the five Zimbabwean populations, the Malawian chicken population and the two broiler lines, whereas the two white egg layer lines and the two brown egg layer lines formed a separate group. At K = 3, for the most likely solution (N = 29) the six Sudanese native chicken populations split from African chickens while the broiler lines clustered with African chickens from Zimbabwe and Malawi. At K = 4, the white egg layers and the brown egg layers split from each other. The most stable solutions with the highest similarity coefficient between runs (97 and 99 identical runs, respectively) were observed at K = 5 and K = 6, respectively.

At K = 5, the purebred lines (the white egg layers, brown egg layers and broilers) and the six Sudanese native chicken populations clustered into four distinct clusters and were separated from the other African populations. At K = 6, the Sudanese chicken, Malawian chicken, and purebred chicken lines made up independent clusters whereas the five Zimbabwean eco-types grouped together.

At K=7 (and above K=8, 9 data not shown) similarity coefficients decreased considerably. The purebred lines and Malawi chickens remained as homogenous and distinct clusters, while the five Zimbabwe eco-types appeared as a heterogeneous group. At these K values, the six Sudanese chicken populations did not show any sub-structuring. In the same manner, analysis was done for the Sudanese populations only and results supported the non-existence of a population sub-structure (data not shown).

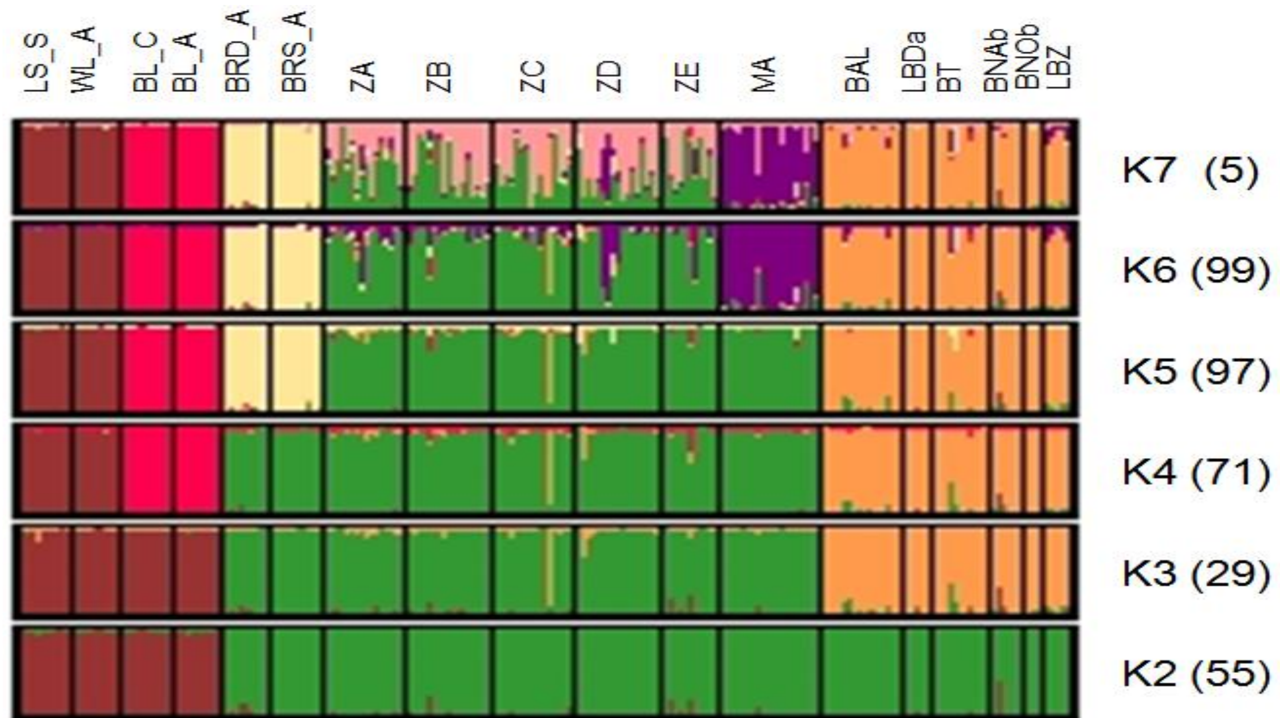

Figure 1. STRUCTURE clustering of Sudanese native chickens in reference to the extensively raised chickens (Zimbabwian and Malawian) and purebred lines (broilers, white and brown egg layers). LBZ, Large beladi from Zalingei; LBDa , large beladi Dammzein; BAL, large beladi Khartoum; BNAb, bare neck Abu Naama; BNOb, bare neck Obeid; BT, Betwil are six Sudanese populations; ZA, ZB, ZC, ZD, ZE , five Zimbabwe eco-types; MA, Malawi; BRS_A , broiler sire line A; BRD_A , broiler A; BL_A , brown egg layer line A; BL_C brown egg layer line C; white egg Layer experimental line and WL_A , white egg layer line A.

Network tree

Network tree based on marker estimated kinships is shown in Figure 2. The clustering shows the separation of broiler lines from the layer lines, with the African populations being clustered in between. Sudanese chicken populations from their own separate cluster, shows LBZ being the most distinct population.

DISCUSSION

Sudanese native chickens were highly polymorphic compared to purebred lines. The average number of alleles per locus for the Sudanese native chicken populations was lower than that for Zimbabwean chicken ecotypes and other African chickens as reported earlier using the same 29 microsatellite markers (Muchadeyi et al., 2007; Granevitze et al., 2007), and for Italian local chicken breeds (5.6 ±2.1) (Zanetti et al., 2010; Bianchi et al., 2011), whereas it was higher than that estimated for the Japanese-native chickens (Nagoya breed) which ranged from 2.35 to 2.85 (Tadano et al., 2012). Variation in number of alleles per locus could be due to sample size such that sampling strategies of each study should be taken into consideration when comparing results from different studies. In addition, African chicken breeds showed higher Nei's estimation of expected and observed heterozygosity followed by Sudanese breeds and commercial breeds. The average observed and expected heterozygosity across 29 loci for Sudanese native chicken populations was higher than the Japanese-native chickens (Nagoya breed), the average observed and expected heterozygosity was 0.438 and 0.433, respectively (Tadano et al., 2012). Granevitze et al. (2007) studied 65 populations using 29 markers and found the expected heterozygosity was 0.52 which was lower than that of Sudanese chicken breeds. On the other hand, the high heterozygosity levels, ranging from 0.51 to 0.67, were reported for chickens across Cameroon, Benin, Ghana, Cote d'Ivoire, and Morocco, corresponding to the values usually found in scavenging populations worldwide (Leroy et al., 2012). Mtileni et al. (2011) reported that village chicken populations were more diverse than conservation flocks. Sudanese indigenous chickens in different rural districts are raised under a typical extensive production system with feed scarcity, disease prevalence and absence of extension services

The F_{IS} values indicate a reduction of the observed heterozygosity compared to what is expected under random mating and serves as an indication of inbreeding

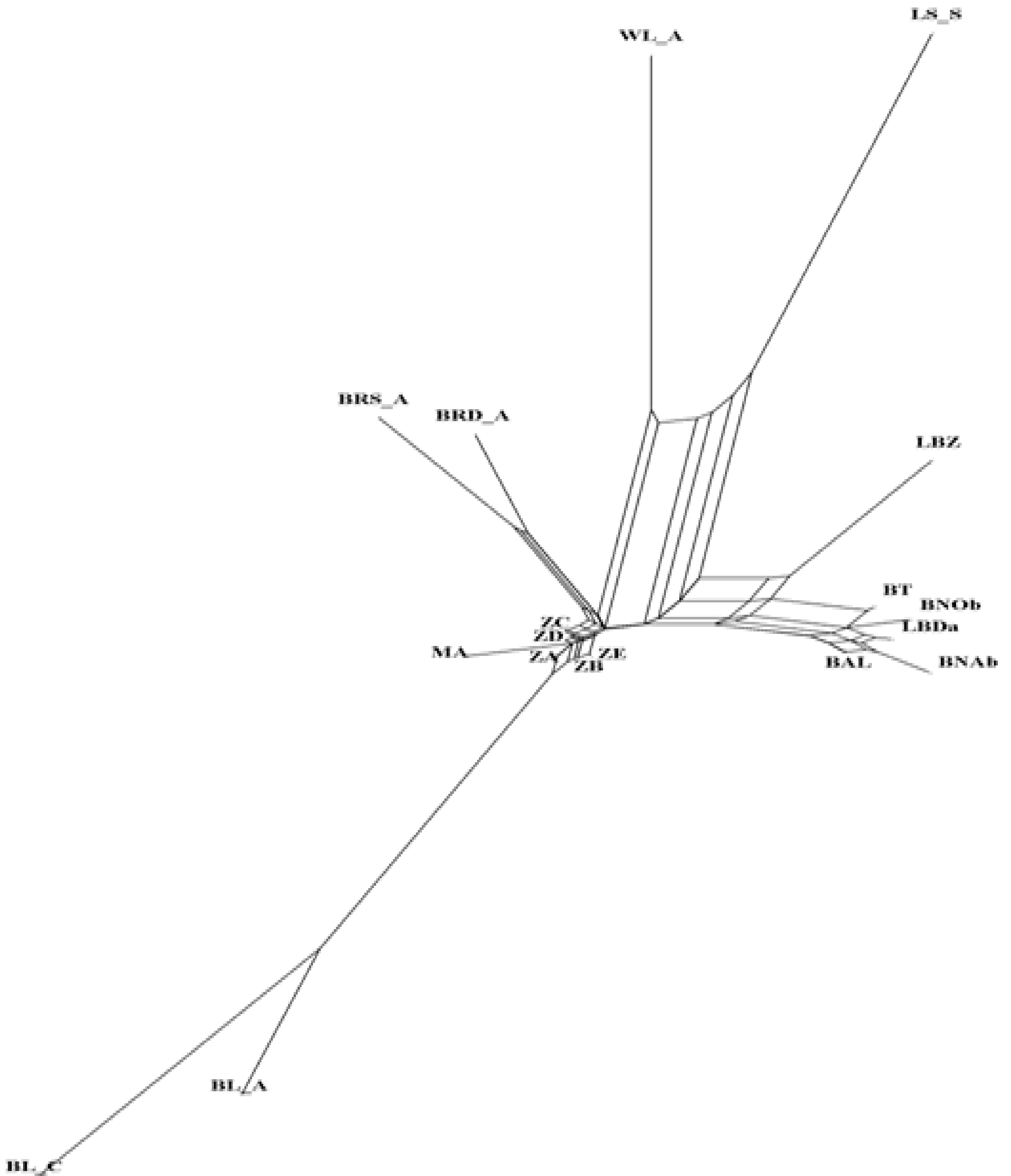

Figure 2. Network tree derived from marker estimated kinships. LBZ, Large beladi from Zalingei; LBDa , large beladi Dammzein; BAL, large beladi Khartoum; BNAb, bare neck Abu Naama; BNOb, bare neck Obeid; BT, Betwil are six Sudanese populations; ZA, ZB, ZC, ZD, ZE , five Zimbabwe eco-types; MA, Malawi; BRS_A , broiler sire line A; BRD_A , broiler A; BL_A , brown egg layer line A; BL_C brown egg layer line C; white egg Layer experimental line and WL_A , white egg layer line A.

within the population (Hartl, 1998). The mean F_{IS} for the Sudanese native chicken populations was lower than that of other African populations but was greater than that of the purebred lines. Pure lines are managed as closed populations but nevertheless Fis estimates are lower than in Sudanese chickens, this might be due to sub-strucuture within the populations avoiding non-random mating (Wahlund effect). The exotic Wyandotte chicken breed was introduced into Sudan earlier in 1926 by a British Veterinarian to improve poultry production. Some other exotic breeds such as White Leghorn, Rhode Island Red, New Hampshire and Sussex were imported shortly after the establishment of Kuku Research Centre in 1962. Recently, several large scale integrated poultry projects have been established using a number of modern commercial chicken strains. In the present study, the marked difference in F_{IS} between the Sudanese chicken populations and the reference pure bred lines implies the fact that these groups were selected from areas that have not been subjected to governmental programs of upgrading indigenous chickens with exotic strains.

On the other hand the subdivision of the lines (F_{ST}), as an indication of genetic differentiation among the lines, revealed a moderate to high differentiation among these groups. Population differentiation as determined from pair-wise F_{ST} values between all combinations of six Sudanese native chicken populations was low but it is slightly greater than that obtained for other African populations. The smallest and largest genetic distances were obtained for LBDa vs BNAb and LBZ vs BNAb, respectively. These patterns of distances may relate to differences among the agro-ecological zones. The LBDa and BNAb populations were collected from almost similar zone with short distance apart where nomadic herders move freely with their herds and carrying with them their animal companions including birds, therefore admixture of the flocks are possible. On the other hand, BLZ population was obtained from a different agro-ecological zone of the Western part of Sudan. This area is surrounded by the Marrah mountain which considered as the highest mountainous region in Sudan (10000 Feet above the sea level), thus representing a natural geographical barrier and minimizing the possibility for any flocks exchange. Eltanany et al. (2011) assessed the genetic diversity of three Egyptian local chicken strains (Fayoumi, Dandarawi and Sinai) and six synthetic breeds derived from Fayoumi and Sinai, and showed that the global inbreeding (F_{IT}) was 0.11, among-population differentiation (F_{ST}) was 0.07, and within-population differentiation (F_{IS}) was 0.04.

Applying the method of Evanno et al. (2005) suggested the most stable clustering solutions at $K = 5$ and K = 6. Mtileni et al. (2011) found the most probable clustering at K = 5 (95% identical runs) in South African indigenous chicken populations. Structure based clustering further supports the low level of differentiation among the Sudanese native chicken. The lack of observed sub-structuring among Sudanese native chicken populations at K value = 6 suggests that the Sudanese native chickens do not separate into different sub-population. The early separation of the Sudanese chickens at $K \leq 3$ from the African gene pool and purebred lines in STRUCTURE based clustering suggest the differentiation of the Sudanese native chickens from populations located in the southern part of the African continent. Leroy et al. (2012) indicated that from K = 2, most African chicken populations appeared clearly differentiated from commercial lines and the Moroccan population, with Cameroon chicken populations showing intermediate results but indicated that, these results could not be generalized for African chicken populations at the individual level, and there was a relatively high heterogeneity of membership coefficients within populations, particularly in comparison with commercial lines (Leroy et al., 2012). Sudanese chickens make up a gene pool that is separated from other African chickens as well as pure bred lines.

Conclusions

We concluded that Sudanese native chickens are less diverse, and are genetically separated from Malawian, Zimbabwean and six purebred lines. Microsatellite marker revealed the absence of population sub-structuring in Sudanese native chickens.

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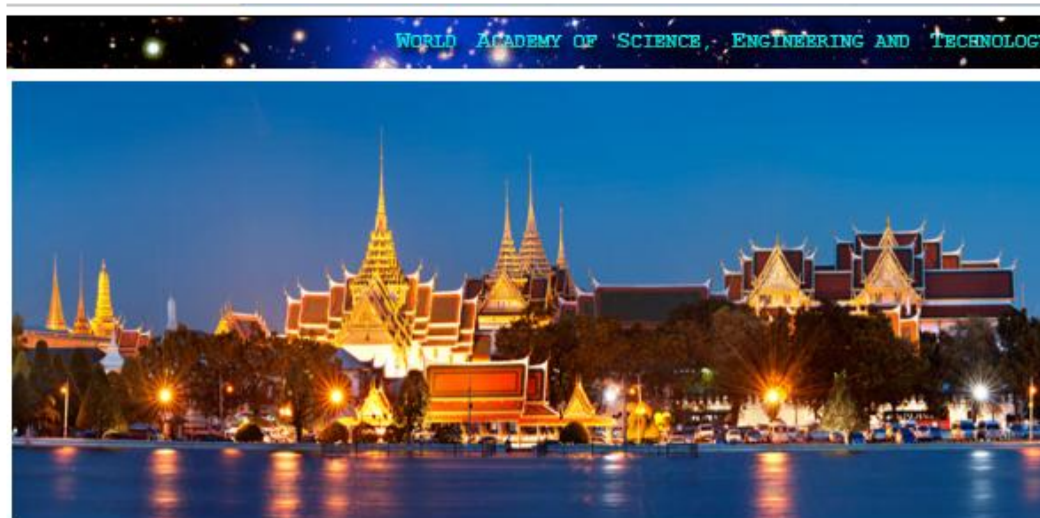

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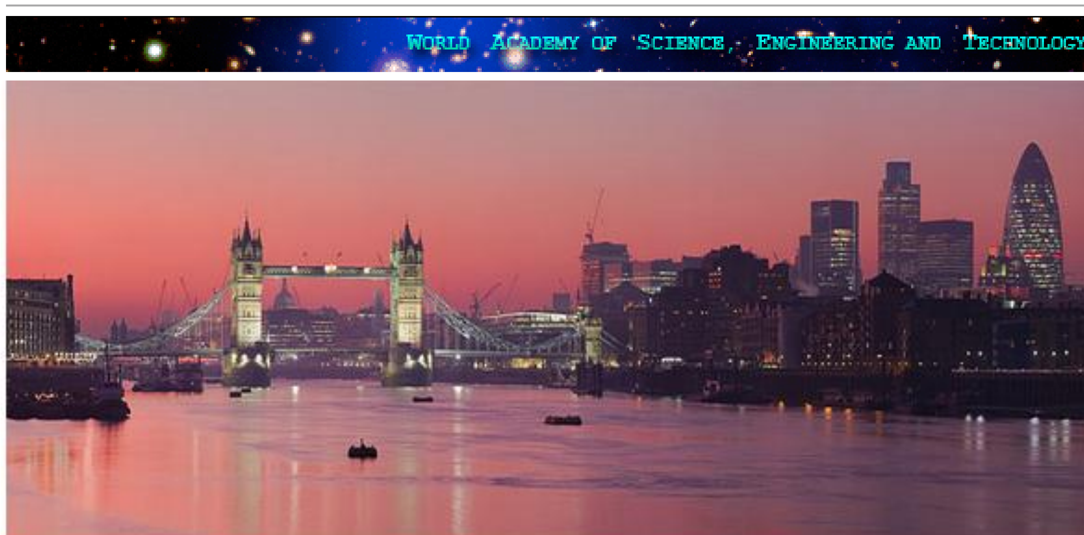

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