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Full Length Research Paper

Application of rep-PCR as a molecular tool for the genetic diversity assessment of *Jatropha curcas*

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***Jatropha curcas* L., a non-edible Euphorbiaceae oil-rich crop cultivated in subtropical/tropical countries, has gained global attention as a promising renewable resource for biodiesel production. Rep-polymerase chain reaction (PCR) was used to investigate the genetic diversity of 15 populations of *J. curcas* L. Distinct populations of the plant growing wildly in Mauritius were characterised using three molecular markers random-amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (REP), and BOX. Furthermore, to confirm that the amplicons obtained with rep-PCR were derived from mitochondrial genomes, six randomly chosen bands were cloned and sequenced to demonstrate that the amplified products were mitochondrial genome-specific. The average polymorphism information content (PIC) values were 0.329 and the average percentage of polymorphic loci obtained were 89.28 for BOX primer, followed by RAPD (83.41), and REP (55.81) among the different populations with the percentage polymorphic loci ranging from 13.95 to 100. The homology recorded clearly indicated that the amplified products were mitochondrial genome-specific. Rep-PCR provides a quick and cheap method to study diversity at the mitochondrial level in plants.**

Key words: Genetic diversity, *Jatropha curcas,* rep-polymerase chain reaction (PCR).

INTRODUCTION

Jatropha curcas L., an economical non-edible energy crop of the family Euphorbiaceae, has received great attention in recent years for its utilization in biodiesel production, rehabilitation of wasteland, and rural development (Kumar and Sharma, 2008; Koh and Ghazi, 2011; Pandey et al., 2012). However, despite enormous and up-to-date research made to develop *J. curcas* as an energy profitable crop, the absence of improved cultivars and lack of agronomic knowledge refrain the full exploitation of the plant's potential. Knowledge in genetic divergence between *J. curcas* populations around the world is vital for the selection of parent plants aiming at the breeding and selection of progenies with superior traits of interest as well as for the maintenance of genetic diversity in improvement programmes and germplasm banks (Sun et al., 2008).

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Several studies have conceded that molecular markers are fundamental technological instruments that help in improving selection and gaining more insight about the divergence of the phenotypic level among the different populations. Amplified fragment length polymorphism (AFLP), random-amplified polymorphic DNA (RAPD), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are molecular markers that have been used in several studies to assess genome-wide variability of *J. curcas* (Basha and Sujatha, 2009, Pamidimarri et al., 2009; Sunil et al., 2011). Comparative studies have shown a very high genetic uniformity even among accessions from different continents; however, the only genetic variability in *J. curcas* was observed in Mexicans accessions (Basha et al., 2009; Ambrosi et al., 2010; Maghuly et al., 2014). Basha and Sujatha (2009) evaluated 42 *J. curcas* accessions from different regions in India. These authors used random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers to determine the genetic diversity and reported on the immediate need to improve the genetic base of the Indian *J. curcas*.

Organellar genomes, chloroplast DNA (cpDNA), and mitochondrial DNA (mtDNA) have remarkably been accepted in recent years as markers to assess maternal/or paternal gene flow mainly explained by their uniparental mode of inheritance (Grivet and Petit, 2003). Repetitive sequence based polymerase chain reaction (rep-PCR) technique has been devised for the characterisation of bacteria and also widely employed to distinguish species, strains, and serotypes among others. The technique devised by Lupiski and Weinstock (1992) used three specific primers, designated BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) designed to match the conserved sequences distributed in diverse bacterial genomes. REP sequences also known as elements were first described in *Escherichia coli* and *Salmonella typhimurium* operons. These sequences have the ability to form stable stem-loop structures which has a regulatory role in transcriptional termination, mRNA stability, and chromosomal organization in bacteria (Versalovic et al., 1991). Other related families of repetitive elements, such as ERIC and BOX sequences, have been exploited in the molecular identification of bacteria pathogenic to plants. Three types of PCR (known as Rep-PCR) based on these elements have been favoured mainly because it was quick and more costly-effective than with other methods, such as AFLP and RFLP. These primers amplify genomic regions located between repetitive sequences and have proven extremely useful in the study of microbial diversity. Rep-PCR application in plant was first reported by Dwivedi et al. (2005) where this technique was used for the determination of the different cytoplasmic male sterility (CMS) lines of *Brassica juncea* and for identifying mitochondrial genome diversity in safflower (*Carthamus tinctorius* L.) as well as their wild relatives.

This study was undertaken to evaluate the genetic diversity of *J. curcas* from the different regions in the subtropical Island of Mauritius. Rep-PCR as an innovative and potential tool for studying plant's diversity was used to access the *Jatropha* intra-populational variance using RAPD, BOX and REP molecular markers.

MATERIALS AND METHODS

The samples were collected from 15 areas based on their geographical locations. Fresh leaves of wildly distributed plants were collected at 10 diffrenet places in the same location. Initially, 150 samples of fresh leaves of the wildly distributed *J. curcas* were used for the research work (Figure 1).

Isolation and quantification of genomic DNA

Fresh, young and tender leaves were selected and frozen at -80°C. Genomic DNA was extracted from the leaves crushed to a fine powder by cetyltrimethylammonium bromide (CTAB) method. The leaf tissues were ground with a mortar and pestle to a fine powder using liquid nitrogen. Five grams of the leaves powder were homogenized in 20 ml of extraction buffer (2% w/v) CTAB, 20 mM ethylenediaminetetraacetic acid (EDTA), 2% (v/v) polyvinyl pyrrolidone (PVP), 1.4 M NaCl, 100 mM Tris–HCl (pH 8.0) and 1% (v/v) β-mercaptoethanol and were incubated at 65°C for 45 min. The supernatant was treated with RNase A (10 mg/µl), incubated at 37°C for 30 min and twice extracted with chloroform:isoamylalcohol (24:1 v/v). The DNA was precipitated with isopropanol and washed twice with 70% (v/v) ethanol. The pelleted DNA was air dried and resuspended in 500 µl of sterile millipore water and stored overnight at -20°C. The purity of the extracted DNA was determined by taking the ratio of the absorbance at 260 and 280 nm.

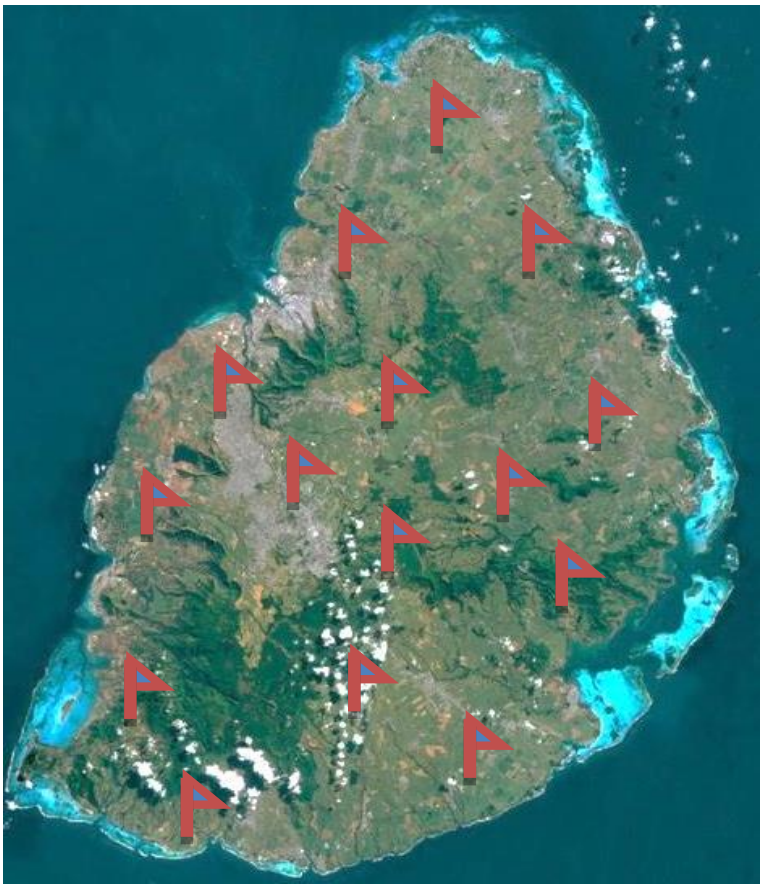
PCR Protocols

RAPD

All the PCR reactions were carried out in 25 µl volumes containing 50 ng of template DNA, 200 mM of each of the four dNTPs, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase, and 10 pmol of primer of RAPD primers. The reaction programmes were set at 94°C for 4 min followed by 40 cycles of 92°C for 30 s, 1 min at annealing temperature, 2 min elongation at 72°C and a final extension at 72°C for 7 min. After completion of the amplification, 2.5 ml 10X blue dye was added to the samples, and the amplified DNA was analysed on 2% agarose gel in 5X TBE buffer at 70 V for 4 h. Out of 40 RAPD primers selected, only the following 11 primers were successfully amplified: Opb 3 catccccctg, Opc 16 cacactccag, Opd 3 gtcgccgtca, Ope 2 ggtgcgggaa, Opf7 ggagtactgg, Oph 9 tgtagctggg. Oph 12 acgcgcatgt, Opi 20 aaagtgcggg, Opj 5 ctccatgggg, Opo 9 tcccacgcaa, and Opp 3 ctgatacgcc

Rep-PCR

Amplification was carried out in a 20 µl reaction mixture consisting of 50 ng/µl genomic DNA, 10X PCR reaction buffer containing 15 mM $MgCl_2$, 10 pM primer, 2.5 mM of each dNTP and 3 U/µl of Taq DNA polymerase (Genei, Bangalore, India). Amplification was performed in a thermal cycler (Eppendorf, Germany) PCR machine.



North Samples codes	
Pamplemousses	N11-N110
Triolet	N21-N210
Point aux Piments	N31-N310
South	
Souillac	S11-S110
Chemin Grenier	S21-S210
Mahebourg	S31-S310
Central	
Curepipe	C11-C110
Rose Belle	C21-C210
Floreal	C31-C310
East	
Centre de Flacq	E11-E110
Camp de Masque Pave	E21-E210
Saint Julien D'Hotman	E31-E310
West	
Flic en Flac	W11-W110
Tamarin	W21-W210
Bamboos	W31-W310

Figure 1. Samples collection location and codes around Mauritius Island.

The PCR conditions were 94°C for 3 min, followed by 45 cycles of DNA amplification 20 s at 92°C, 1 min at 52°C for BOXA1R primer and 1 min at 38°C for REP primers and 8 min at 68°C and 15 min incubation at 68°C, respectively.

(1) BoxAIR: CTACGGCAAGGCGACGCTGACG,
(2) REP1R:IIIICGICGICATCIGGC/REP1I: ICGICTTATCIGGCCTAC

All PCR reactions were carried out in triplicate. The PCR products were run on 2% (w/v) agarose gel for 7 to 8 h at constant voltage (2 V/cm). To ensure reproducibility and representativeness of the experiment, 10 PCR runs were carried out.

Cloning

To confirm that the amplicons obtained from rep-PCR were of mitochondrial genomes, six representative amplicons obtained using the two (BOX and REP) primers were extracted using a gel extraction kit (Qiagen, Hilden, Germany) and cloned into the PCR cloning vector, pMiniT Vector (NEB PCR Cloning Kit, New England Biolads, UK). The amplicons cloned ranged from 575 bp to 1 kb in length. All the clones were sequenced at Inquaba (Pretoria, South Africa) using the Forward Primer: 5´ ACCTGCCAACCAAAGCGAGAAC 3´ and Reverse Primer: 5´ TCAGGGTTATTGTCTCATGAGCG 3´ available in the vector. The sequences obtained were subjected to basic local alignment search tool (BLAST) analysis to determine their identity.

Data scoring and statistics analysis

For scoring and analysis of data from the three molecular markers (RAPD, BOX and REP markers), bands which were clear, unambiguous and reproducible were scored and data scoring was carried out using a binary number system for '1' as presence and '0' as absence of fragment (band) for primers. The allele frequency of the populations and the basic statistics were generated for the different generated scored profiles using the POPGEN32 software and the difference between the populations were determined using the analysis of molecular variance (AMOVA- GenAIEx version 6.5).

Phylograms were constructed using the maximum parsimony method. Furthermore, to test the robustness of the phylo, the indices were bootstrapped 10000 times using PAUP version 4.0b (Swofford, 2002). The information content of each markers was computed as $P_iC_i = 2\ f_i(1-f_i)$; where, f_i is the frequency of the amplified allele (band present) and $1- f_i$ is the frequency of the null allele.

RESULTS

The dendrogram generated based on the maximum parsimony cluster analysis for the three markers separated the populations in different number of clusters: RAPD (14), BOX (6), and REP (11). 46% of the populations formed the largest cluster for REP primer

followed by BOX primer (36.7%). For REP marker, the cluster with low genetic diversity grouped most of individuals collected in three distinct regions (North 70%, South 66.7%, and West 53.33%) and the remaining clusters with the higher genetic diversity were composed with basically populations collected in Central and East, but it also had samples collected in north, south and west, where it is possible to select individuals to be included in breeding programs. Most of the clusters for the BOX and RAPD showed high genetic variation (Figure 2).

Genetic variation among the three primers used revealed that the BOX showed the lowest variation (0.051), whereas REP primer had the highest genetic variation (0.429) ($p < 0.001$) which represents high differentiation among population (Table 1) as compared to RAPD primer (0.079). REP primer analysis of molecular variance (AMOVA) showed that 42.89% of the total variation corresponds to those between populations, intra-populational, and the remaining to variation among populations. The dendrogram generated based on the unweighted pair group method with arithmetic (UPGMA) cluster analysis for the three markers separated the populations in different number of clusters: RAPD (4), BOX (6), and REP (9). 71.33% of the populations formed the largest cluster for BOX primer followed by RAPD primer (60.67%) (Figure 2).

A total of 140 well-defined and visible bands for the three primers were scored on agarose gel for the molecular analysis. The minimum and maximum numbers of polymorphic bands observed for each primer were 41 and 56, respectively. The highest polymorphism information content (PIC) value (0.3712) was obtained for BOX primer (Table 2). For the computation and description of the genetic variation among the fifteen sampled regions from allele frequencies, Hardy Weinberg equilibrium was assumed. For the fifteen different regions, the number of alleles averaged to 1.5 and 100% total polymorphic loci. All bands were polymorphic, the percentage polymorphic loci ranged from 13.95 to 100 for the primers (Table 3). The lowest percentage of polymorphic loci was for REP primer, whereas BOX had the highest polymorphic percentage. Considering all five populations, a 100% polymorphic loci were recorded for BOX primer. Populations from S3 presented the lowest Shannon diversity index overall (REP: 0.06, BOX: 0.277, RAPD: 0.368), respectively. The highest number of locus per allele was observed for the primer BOX and the percentage of polymorphic loci was the highest. Population from E2 and E3 presented the highest percentage of polymorphic loci (100%) using BOX primer, followed 55.81 and 83.7% with REP primer (Table 3).

To further confirm the amplified regions are of mitochondrial origin, four sequences were randomly cloned and sequences. The blast results showed that the sequences, coded for proteins that were located in the mitochondrial region. The homology of the second REP primer sequence result was in the chloroplast region, which again maybe due to horizontal gene transfer as mitochondrial genomes known to acquire sequences from chloroplast (Figure 3).

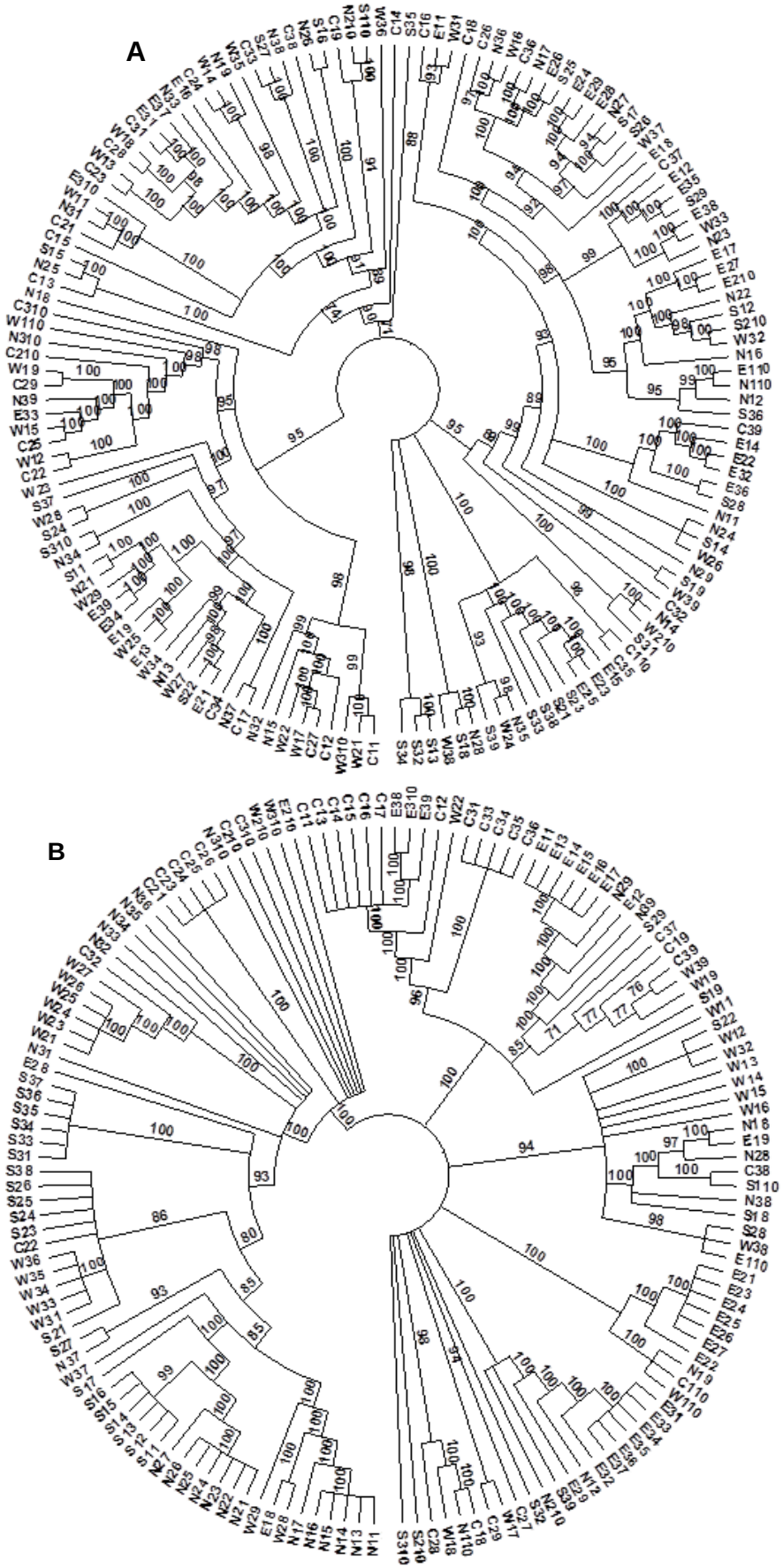
DISCUSSION

Genetic variation among *J. curcas* population is a pre-requisite for commercialising the plant. Intensive selection programme among the plant population represents a crucial part for the development of new viable economical cultivars. Molecular markers play a fundamental role in the study of genetic variability in plants. There are several DNA markers such as RAPD, SSR, ISSR, and AFLP that have been used for the fingerprinting of plant germplasm (Shen et al., 2010). Diversity in the *J. curcas* has been investigated previously based onagromorphological traits, biochemical traits, molecular markers including isozymes. Genetic variability of the plant still needs to be established as different marker systems previously used showed high degree of homozygosity (Heller, 1996).

This study demonstrates that REP and BOX primers are convenient and inexpensive solutions for screening wild plant species as compared to RFLP or RAPD techniques. RFLP requires high technical and resource demands (Fukunaga and Kato, 2003), whereas RAPD requires a large number of random primers to be screened to identify the polymorphic ones. Therefore, PCR-based method that targets the varied interspersed repeat sequences found in mitochondrial genomes has an immense importance in assessing chondriome diversity.

The markers generally applied in prokaryotes were successfully applied in eukaryotes and have equally proved to be very effective. The degree of polymorphism was comparable to primers generally employed for diversity studies. The PIC values ranged from 0.3067 to 0.3712 and were higher than in SSR and EST-SSR markers (average 0.216 ± 0.078 standard deviation) with respect to those in SNP markers (average 0.272 ± 0.108 standard deviation) recorded. The results are also in accordance with the findings of Tatikonda et al. (2009) and Grativol et al. (2011) showing PIC values ranging from 0.20 to 0.34 in *J. curcas*; although for BOX primer, the PIC was higher than what the authors obtained.

Furthermore, the level of polymorphism found in our work (100%) was superior to those already reported previously. Pamidimarri et al. (2009) used RAPD and AFLP for the analysis of species of *Jatropha* and the mean percentage of polymorphism was 68.48 and 71.33%, respectively, and determined 69.57% polymorphisms in Indian selected germplasm. *J. curcas* populations of Chiapas-Mexico (Medina et al., 2013; Yi et al., 2010) produced 52 useful markers with 81.18% polymorphism, 88% in elite germplasm collection of *J.*


A
B

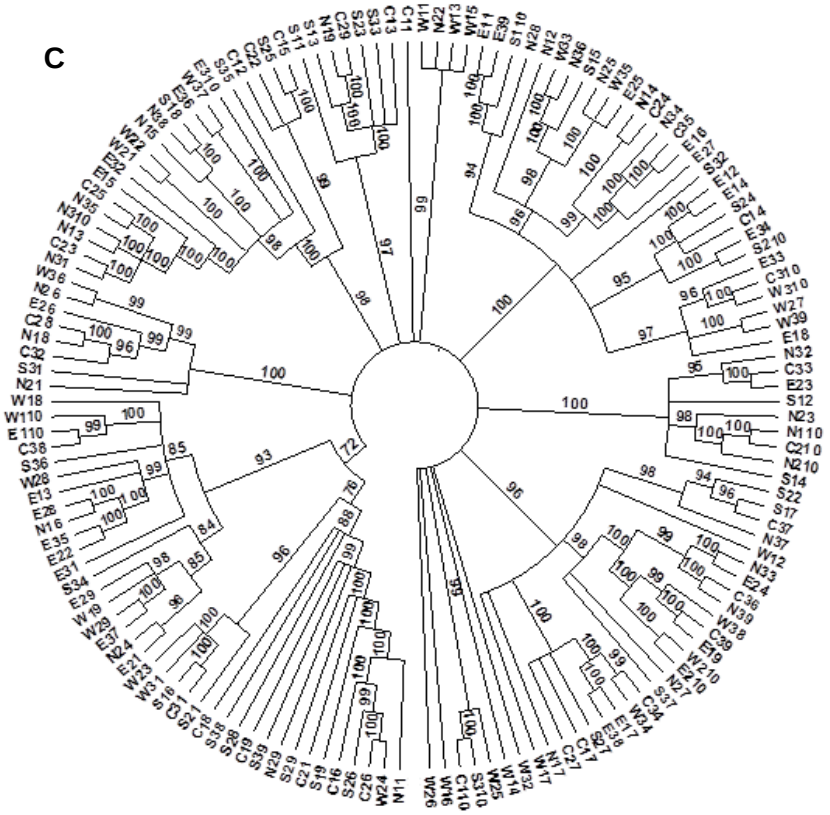


Figure 2. Relationships among the populations of *J. curcas* L. in the 15 different regions based on the maximum parsimony method for **A)** BOX, **B)** REP and **C)** RAPD markers (Bootstrap=10000 using PAUP Version 4.0b).

Table 1. AMOVA between and within population for RAPD, REP, and BOX Primers.

Source	df	SS	MS	Variance components	% of total variation	p	PHIst
BOX							
Between population	14	211.933	15.138	0.530	5.107	<0.001	0.051
Within population	135	1328.600	9.841				
REP							
Between population	14	425.533	30.395	2.682	42.89	<0.001	0.429
Within population	135	482.200	3.572				
RAPD							
Between population	14	151.413	10.815	0.500	7.929	>0.001	0.079
Within Population	135	784.500	5.811				

df: Degree of freedom; SS: sum of squares; MS: sum of mean squares; p: level of significant for the estimate of genetic variation based on 1000 permutations. PHIst statistic: Genetic variation (Wright statistics). The analyses were done using GenAlEx 6.5.

Table 2. Markers selected for *J. curcas* molecular analysis.

Marker	Total number of bands	Polymorphic bands	Polymorphism (%)	PIC value
Box	56	56	100	0.3712
RAPD	41	41	100	0.3083
REP	43	43	100	0.3067

PIC: Polymorphism information content.

Table 3. Descriptive statistics of three markers for the 15 different regions in Mauritius.

Population	na*			h*			I*			P*		
	REP	BOX	RAPD	REP	BOX	RAPD	REP	BOX	RAPD	REP	BOX	RAPD
Cl	1.6152 (0.4822)	1.7500 (0.4369)	1.7561 (0.4348)	0.1856 (0.1574)	0.2079 (0.1475)	0.2254 (0.1664)	0.2953 (0.2372)	0.3327 (0.2187)	0.3525 (0.2369)	65.12	75	75.61
C2	1.3488 (0.4822)	1.9464 (0.2272)	1.8780 (0.3313)	0.0809 (0.1282)	0.3571 (0.1361)	0.2649 (0.1381)	0.1348 (0.2004)	0.5288 (0.1759)	0.4151 (0.1929)	34.88	94.64	87.80
C3	1.5349 (0.5047)	1.8393 (0.3706)	1.8730 (0.3313)	0.1330 (0.1459)	0.2911 (0.1641)	0.2268 (0.1260)	0.2182 (0.2242)	0.4404 (0.2251)	0.3692 (0.1772)	53.49	83.93	87.80
E1	1.7674 (0.4275)	1.9643 (0.1873)	1.8730 (0.3313)	0.2247 (0.1561)	0.3568 (0.1133)	0.2571 (0.1459)	0.3541 (0.2264)	0.5328 (0.1459)	0.4043 (0.1990)	76.74	96.43	87.80
E2	1.5581 (0.5025)	2.0000 (0.00)	1.7805 (0.4191)	0.1437 (0.1515)	0.4493 (0.0632)	0.2537 (0.1738)	0.2335 (0.2304)	0.6398 (0.0696)	0.3885 (0.2435)	55.81	100	78.05
E3	1.8372 (0.3735)	2.0000 (0.00)	1.8537 (0.3578)	0.2349 (0.1418)	0.4411 (0.0707)	0.2551 (0.1423)	0.3742 (0.2202)	0.6309 (0.0773)	0.4008 (0.2013)	83.72	100	85.37
N1	1.7442 (0.4415)	1.8571 (0.3531)	1.8049 (0.4012)	0.2009 (0.1528)	0.2807 (0.1469)	0.2746 (0.1696)	0.3224 (0.2225)	0.4316 (0.2071)	0.4166 (0.2386)	74.42	85.71	80.49
N2	1.7674 (0.4275)	1.8571 (0.3531)	1.9512 (0.2181)	0.2205 (0.1458)	0.2296 (0.1355)	0.2839 (0.1264)	0.3505 (0.2171)	0.3698 (0.1913)	0.4449 (0.1638)	76.74	85.71	95.12
N3	1.4651 (0.5047)	1.9107 (0.2877)	1.8049 (0.4012)	0.1191 (0.1459)	0.3279 (0.1496)	0.2468 (0.1579)	0.1941 (0.2259)	0.4908 (0.1991)	0.3845 (0.2243)	46.51	91.07	80.49
S1	1.5581 (0.5025)	1.9107 (0.2877)	1.7073 (0.4606)	0.1488 (0.1604)	0.2600 (0.1274)	0.1907 (0.1587)	0.2390 (0.2391)	0.4127 (0.1747)	0.3056 (0.2305)	55.81	91.07	70.73
S2	1.3953 (0.4947)	2.0000 (0.00)	1.9024 (0.3004)	0.1014 (0.1446)	0.4204 (0.0918)	0.2976 (0.1431)	0.1647 (0.2200)	0.6073 (0.1036)	0.4556 (0.1939)	39.53	100	90.24
S3	1.1395 (0.3506)	1.6607 (0.4778)	1.8293 (0.3809)	0.0377 (0.1064)	0.1707 (0.1468)	0.2312 (0.1440)	0.0600 (0.1614)	0.2778 (0.2224)	0.3688 (0.2048)	13.95	66.07	82.93
W1	1.4651 (0.5047)	1.9821 (0.1336)	1.6585 (0.4801)	0.1140 (0.1431)	0.3504 (0.1283)	0.1927 (0.1675)	0.1874 (0.2205)	0.5248 (0.1553)	0.3033 (0.2461)	46.51	98.21	65.85
W2	1.6977 (0.4647)	1.9464 (0.2272)	1.9268 (0.2637)	0.1879 (0.1532)	0.3555 (0.1333)	0.3376 (0.1487)	0.3022 (0.2271)	0.5268 (0.1733)	0.5034 (0.1937)	69.77	94.64	92.68
W3	1.4419 (0.5025)	1.7679 (0.4260)	1.9024 (0.3004)	0.1098 (0.1437)	0.2471 (0.1666)	0.2888 (0.1457)	0.1798 (0.2213)	0.3806 (0.2387)	0.4447 (0.1956)	44.19	76.79	90.24
									Average	55.81	89.28	83.41

Mean sample size=10; Number of observed alleles per locus (na); Nei's gene diversity (h); Shannon's Information (I) and percentage of polymorphic loci (P). The Hardy-Weinberg equilibrium was assumed and the parameters were compiled using Popgen32. Figures in brackets are ± standard deviation.

curcas from India, and 26.99% using seeds from cultivated populations in China (Ganesh et al., 2008).

In this study, rep-PCR was used to delve intraspecific diversity within *J. curcas* populations around Mauritius. The basis was to use a multi loci primer which targets the whole genome, such as RAPD markers, then compare the diversity obtained to only mitochondrial repeat markers BOX and REP. Diversity at mitochondrial level obtained from only BOX and REP proved to be very informative as compared to RAPD. Although BOX and REP target diversity only in the mitochondria, the genetic variation among the three primers used revealed that BOX showed the lowest variation (0.074), whereas REP primer had the highest genetic variation (0.429) ($p < 0.001$) which represents high differentiation among population (Table 1) as compared to RAPD primer(0.051). Hence, the ten percent of mitochondrial repeats are as informative and useful in diversity study as a genome based primer (Dyall et al., 2004).

Conclusions

Rep-PCR could be used effectively to study the variability in genetic diversity. Sequencing of randomly chosen PCR products demonstrated clearly that the PCR products obtained were derived from plastid genomes. Thus, the rep-PCR technique seems to combine the advantages of economy and adequate resolution for distinguishing different chondriome. Furthermore, it has added advantages over RFLP technique, as it does not require high amount of good quality DNA. The same set of primers could also be used to analyse diversity in chloroplast genomes, as plastids are also derived from ancient prokaryotic endosymbionts and their genomes show similarity to prokaryotes. This diversity information can represent a great resource for crosses and breeding programmes.

Conflict of Interests

The authors have not declared any conflict of

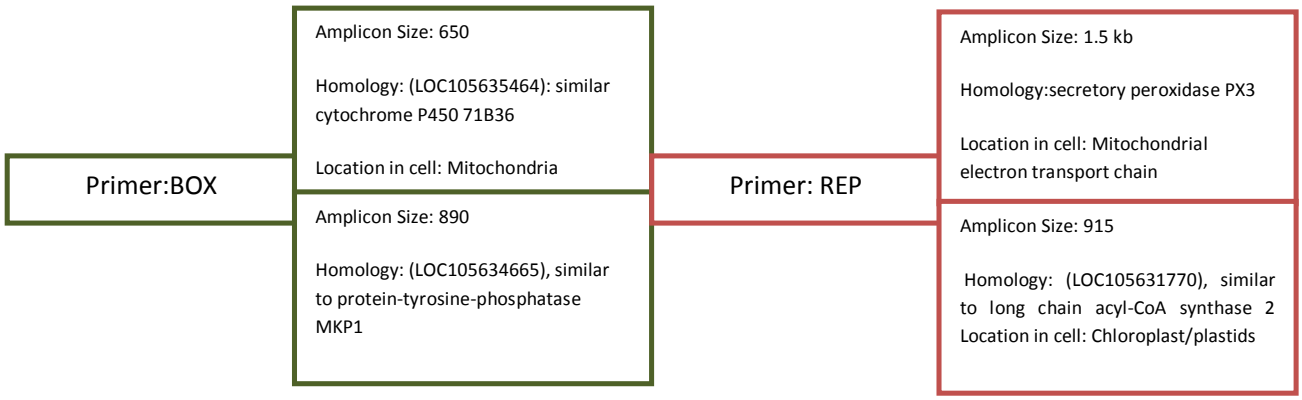


Figure 3. Blast results of clone sequences from both BOX and REP primers.

interests.

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Abbreviations

PCR, Polymerase chain reaction; **RAPD,** random-amplified polymorphic DNA; **REP**, repetitive extragenic palindromic; **PIC,** polymorphism information content.

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Full Length Research Paper

High genetic variation of *Portunus pelagicus* from Makassar Straits revealed by RAPD markers and mitochondrial 16S rRNA sequences

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The blue swimmer crab, *Portunus pelagicus*, is presently managed as a single stock. In this study, random amplified polymorphic DNA (RAPD) markers and 16S rRNA sequences were used to understand the genetic diversity and parentage of *P. pelagicus* from Makassar Strait. A total of 150 samples were collected from the sea, grouped by gender and morphological features. DNA genome was performed at approximately 23130 base pairs (bp) and mitochondrial 16S rRNA sequences in the 500 to 600 bp. The percentage of polymorphic band using OPA-5, OPA-11, and OPA-17 primers was high (44.4 to 100%). Genetic identity ranged between 0.7266 and 0.9050 and genetic distance between 0.1008 and 0.3214. Alignment of 16S rRNA sequences shows 96 to 99% homology with *P. pelagicus* available in GenBank. Both RAPD markers and 16S rRNA sequences indicate that there was a high genetic variation observed among population, which formed two clusters. Specific unique bands found at 450 and 600 bp in OPA-11 gives an indication of hybridization among the population. The variation of white spot pattern on the carapace can be used as indicators of differentiation in population and parentage of *P. pelagicus*. Therefore, farmers or hatchery operators can continue to use the population as sources of natural broodstock.

Key words: *Portunus pelagicus*, random amplified polymorphic DNA (RAPD), 16S rRNA, genetic, aquaculture.

INTRODUCTION

The blue swimmer crab (*Portunus pelagicus* is Linnaeus, 1758) is the most commercially important marine species in Indonesia and several countries in the world. This crab is extracted from the sea using traps and gill nets to meet demand of the world market. *P. pelagicus* spread throughout the Indian and West Pacific Oceans: From Japan and Philippines throughout Southeast and East Asiato Indonesia, the East of Australia, and Fidji Islands, and westward to the Red Sea and East Africa. This species occurs also in the

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Mediterranean Sea along the coast of Egypt, Israel, Lebanon, Turkey, the Syrian Arab Republic, Cyprus and the East Southern Coast of Sicily. *P. pelagicus* occupies sandy and sand-muddy in shallow waters between 10 and 50 m depth, including areas near reefs, mangroves, sea grass, and algal beds. The juvenile is commonly occurring in intertidal shallower areas (FAO, 2014).

In Indonesia, this crabs spread almost throughout the coastal island. Several key indicators show that *P. pelagicus* population is in crisis due to overexploitation. Kunsook et al. (2014) reported an increase in fishing mortality to 4.14. The exploitation rate was higher (0.71) than the optimal value (0.38). The size of the mature females has also decreased from 8.10±0.39 cm to 7.52±1.14 cm. Mehanna et al. (2013) reported along the Oman coasts on both Arabian and Oman Seas, the yield per recruit showed that the crab stock is being exploited beyond its maximum biological limit, but the increase of fishing mortality to the level which gives the maximum Y/R (83% of its current value) will be accompanied with a negligible increase in Y/R (2.7%) and a considerable decrease in both biomass per recruit (21.1%) and spawning stock biomass (37.6%). Harris et al. (2014) also reported that since July 2011 the relative abundance of all size classes of the crabs in Shark Bay declined significantly.

The decline in catches of the crabs was also reported from Cockburn Sound, Western Australia since 2000 resulted in closure of the crab fishery in December 2006 (Johnston et al., 2011). Similar phenomenon also occur in Indonesia (personal observation), therefore, Indonesia government recently issued a regulation to protect the crabs population. Aquaculture and stock enhancement is a prospect to solve the problem. Aquaculture refers to the breeding, rearing, and harvesting of aquatic species for food and other human uses, while marine stock enhancement is a set of management approaches involving the release of cultured organisms to enhance or restore fisheries (Lorenzen et al., 2010). Hatchery-produced stocks are used to replenish or supplement wild stocks (Waples and Drake, 2012). Enhancement of wild stocks by release of hatchery-reared seed is one method by which the yield from the hatchery can be improved (Roberts et al., 2007; Altamirano, 2010). But there are important question on how increase in global aquaculture production without treating sustainability of natural populations and the ecosystems for long-term viability. Wang et al. (2012) reported that the cultured croaker had significantly reduced genetic diversity in contrast to the wild populations. These changes may be caused by founder's effects, artificial selection, and random genetic drift.

Genetic status is an essential information in fisheries management through stock enhancement or cultivation. Since *P. pelagicus* is managed as a single stock, but there were a lot of evidence leading to variations in species. According to Lai et al. (2010), *P. pelagicus* is a species complex consisting of four species. Klinbunga et al. (2010) also found that the populations of *P. pelagicus* from the same geographic location of Thailand waters have high genetic diversity. Sienas et al. (2014) estimated that *P. pelagicus* in Philippine waters are cryptic species and consists of at least two species. In Makassar Strait around South Sulawesi Indonesia, *P. pelagicus* found in some different morphologically features especially in color and patterns of white spots in its carapace (personal observation) when compared with *P. pelagicus* reported by Lai et al. (2010). This raises the suspicion that *P. pelagicus* of Makassar Strait may differ, even though Lai et al. (2010) also took samples from Indonesia waters (Sumatra: Padang; West Nusa Tenggara: Lombok; Sulawesi: Manado). The application of DNA markers has allowed rapid progress in aquaculture investigation of genetic variability and inbreeding, parentage assignment, species and strain identification, and the construction of high-resolution genetic linkage maps for aquaculture species (Liu and Cordes, 2004). Random amplified polymorphic DNA (RAPD) analysis has been described as a simple and easy method to detect polymorphisms based on amplification of DNA segment with single primers of arbitrary nucleotide sequences (Williams et al., 1990; Welsh and McClelland, 1990). Suresh et al. (2013) used RAPD markers to study the genetic structure of the population of *Mugil cephalus,* while de Freitas and Galetti (2005) evaluated the genetic diversity of a commercial broodstock line of *Litopenaeus vannamei* shrimp. Klinbunga et al. (2010) suggested that the RAPD technique is simpler and more cost-effective than amplified fragment length polymorphism (AFLP) analysis for monitoring levels of genetic diversity of *P. pelagicus*.

Mitochondrial DNA sequences have also been widely used to study genetic variability and relationships in many crustacean groups (Khedkar et al., 2013). The mtDNA localizes to the mitochondrial matrix. Besides protein coding genes, mtDNA also codes for 22 transfer RNAs (tRNAs) and two ribosomal RNAs (12S and16S rRNAs) (Moraes et al., 2002). The rRNA gene in all cells is the most conserved (least variable). This means that sequences from distantly related organism can be precisely aligned, making the true differences easy to measure. An et al. (2005) also reported that the 16S rRNA sequences more conserved than cytochrome c oxidase subunit I (COI) sequences. The important question about genetic variability of *P. pelagicus* in Makassar Strait has not been identified. In this study, specimens from Makassar Strait were described and determine the relationship between morphological appearance (color and spot pattern in carapace) and genetic variation. This information will provide valuable input to the protection, genetic enhancement, and the cultivation of this species in the future.

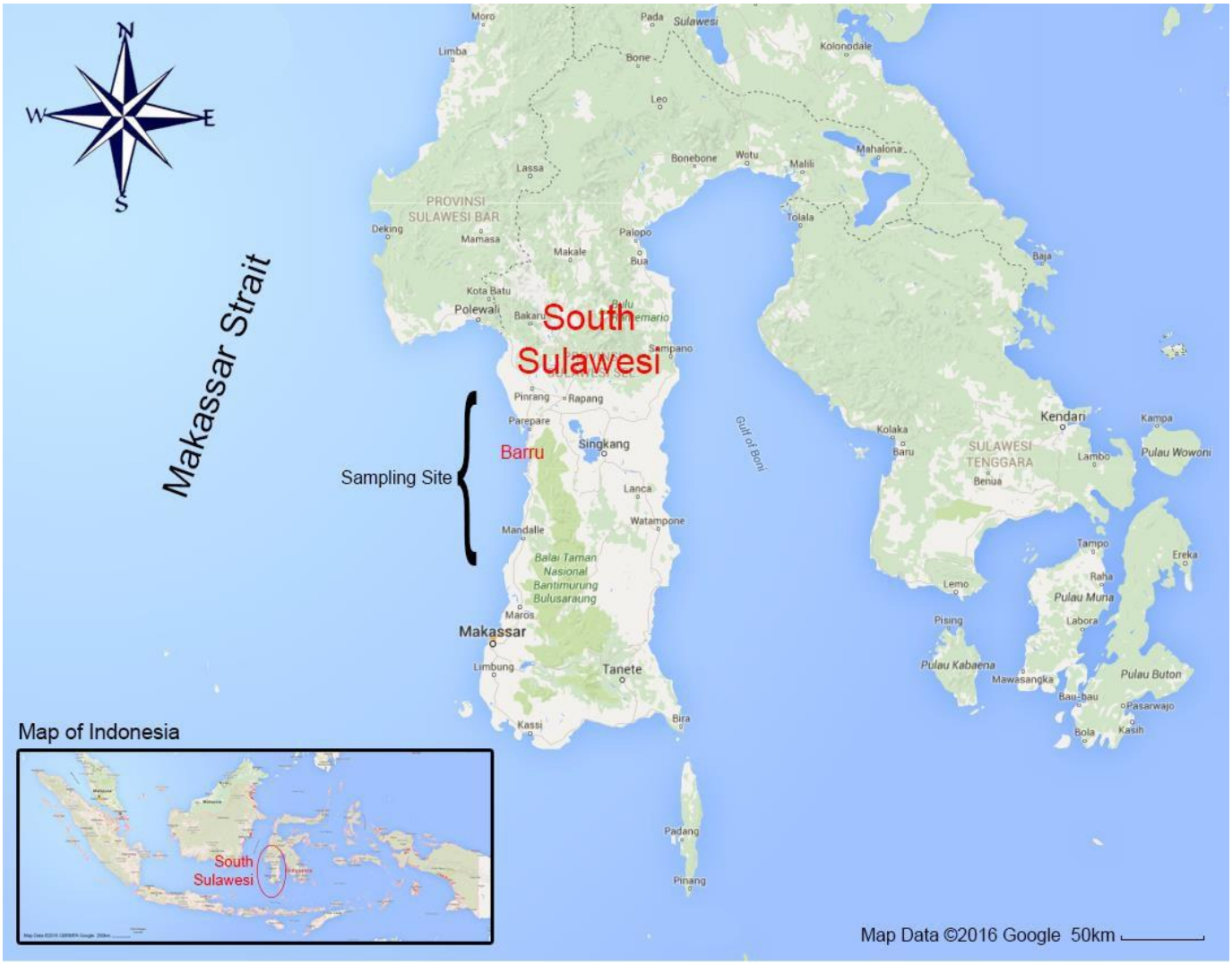

Figure 1. Sampling site of *Portunus pelagicus* specimens in Makassar Strait around. The Barru Regency South Sulawesi.

MATERIALS AND METHODS

Sample collection

The sample of *P. pelagicus* was obtained from fisherman that caught it in the Makassar Strait, South Sulawesi, Indonesia (Figure 1) using traps. 150 samples were collected during March 2013 to May 2014. The samples were grouped by gender and morphological variation (white spot pattern on carapace) (Figure 2). There were 30 male and 30 female crabs randomly selected for DNA analysis. The selected crabs were anesthetized using cold water (8°C) before releasing their first pereiopod (claw) by autotomy. Muscle of the claw was removed from each crab taken as much as 50 mg, preserved using 250 µl TNES-Urea buffer (Tris for 200 ml; 2 ml of 1 M pH 7.5; final concentration: 10 mM NaCl; for 200 ml: 5 ml of 5 M; final concentration: 125 mM ethylenediaminetetraacetic acid (EDTA)-2Na; for 200 ml: 2 ml of 0.5 M pH 7.5; final concentration: 10 mM sodium dodecyl sulfate (SDS); for 200 ml: 10 ml of 10%; final concentration: 0.5% Urea; for 200 ml: 48.05 g; final concentration: 4 M) (Asahida et al., 1996) and stored at room temperature until extraction.

DNA extraction

DNA was extracted based on the phenol-chloroform method described by Parenrengi et al. (2001) as follows: (1) Digestion buffer (0.5 M NaCl, 0.001 M EDTA, 1% SDS, 0.8% Triton-X, and 0.1 Tris-HCl at pH 9.0) were added into 1.5 ml microcentrifuge tube containing 50 mg crab muscle and then 40 µl SDS 10% and 40 µl Proteinase K (20 mg/ml) were added. The tube was shaken gently and incubated at 55°C for 1 to 3 h; (2) the sample was treated with 25 µl of RNase (20 mg/ml) and was left at room temperature for 15 to 30 min; (3) the samples were treated with 500 to 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and gently homogenized. The sample was left at room temperature for 10 min before centrifugation at 13,000 rpm for 4 min; (4) the top layer of aqueous were removed and dispersed into the new microcentrifuge tube. The step of adding phenol:chloroform:isoamyl alcohol were repeated three times; (5) the samples were treated with 500 µl of chloroform:isoamyl alcohol (24:1) and were centrifuged at 13,000 rpm for 2 min; (6) The upper aqueous layer was mixed with 1 ml of ice-cold absolute ethanol by rapid inversion of the tubes several times.

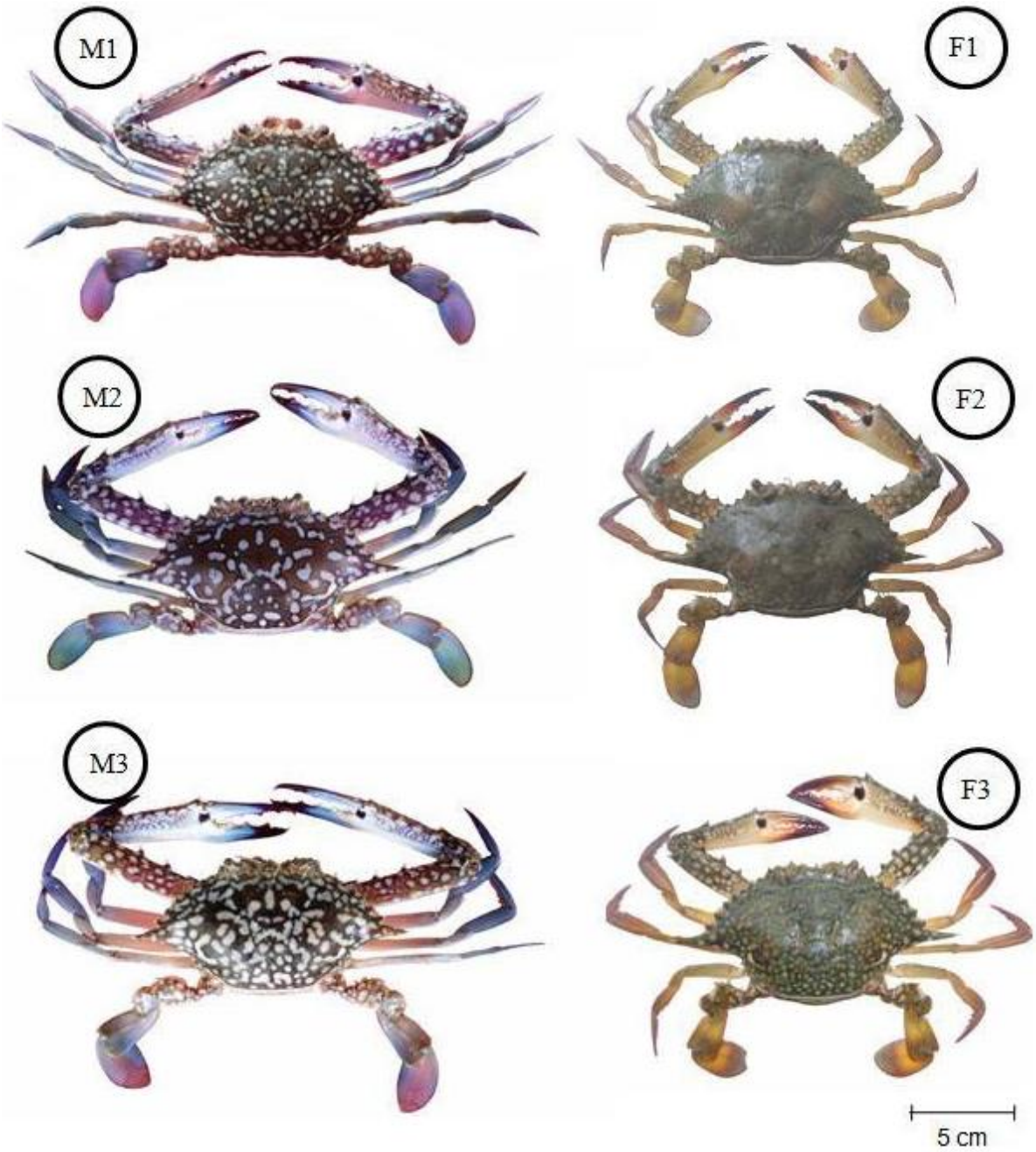

Figure 2. Colors and white spot pattern of *Portunus pelagicus* (males and females) from Makassar Strait. M, Male; F, female; M1, body color is light blue, white spot pattern on carapace is small and fills the entire carapace; M2, Body color is purple, large white spots scatter on carapace with medium density; M3, body color is purple greenish, pattern of white spots on carapace are rarely; F1, body color is greenish without spot; F2, body color is brownish, dark spots each one at the left and right of the carapace; F3, body color is light greenish, faintly visible white spots on the carapace.

Then, centrifuged at 6,000 rpm for 30 min; (7) the precipitated DNA were collected at the bottom tubes as a white pellet and washed with 500 µl of 70% of ethanol and then centrifuge at 6,000 rpm for 15 min; (8) the DNA was allowed to dry at room temperature for 20 min and resuspended with 50 µl sterile distilled water (SDW) for at least 24 h at room temperature to fully dissolved before proceeding to the next step. The samples were purified by electrophoresis in a 0.8% agarose. This DNA genome samples was kept in -20°C to avoid DNA degradation.

RAPD-PCR

Seven RAPD primers: OPA-1 (5'-cag gcc ctt c-3'), OPA-5 (5'-

agg ggt ctt g–3'), OPA-9 (5'-ggg taa cgc c-3'), OPA-10 (5'-gtg tcg ccg t-3'), OPA-11 (5'-caa tcg ccg t–3'), OPA-17 (5'-gac cgc ttg t–3'), and OPA-18 (5'-agg tga ccg t-3') were screened for the amplification success against genomic DNA of representative individual of *P. pelagicus* from each morphological variation and gender. Three primers, OPA-5, OPA-11, and OPA-17 were selected for genetic variation analysis of *P. pelagicus*. Polymerase chain reaction (PCR) was performed in a 25 µl reaction volume containing 1 µl primer (50 pmol/µl), 3 µl DNA template, and 21 µl water free RNAse. PCR reaction using kit PureTaq Ready-To-Go Beads (GE Healthcare, USA) contained 2.5 unit Taq Polimerase; 10 mM Tris-HCl, pH 9; 50 mM KCl; 1.5 mM $MgCl_2$; and 200 µM dNTP-mix and 50 ρmol of each primer. The amplification profiles consisted of predenaturation at 94°C for 2 min followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 30 s and extension at 72°C for 60 s. The final extension was carried out at 72°C for 2 min. Three microliters of the amplification reaction was electrophoresed through 2.0% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

16S rRNA-PCR and sequencing

Amplification of 16S rRNA was carried out using the following primers: forward 16S rRNA-F: 5'- cgc ctg ttt aac aaa aac at -3' and reverse 16S rRNA-R: 5'- ccg gtc tga act cag atc atg t -3'. DNA amplification was performed using PCR System 2700 GeneAmp (Applied Biosystems, USA). PCR reaction using kit PureTaq Ready-To-Go Beads (GE Healthcare, USA) contained 2.5 unit Taq Polimerase; 10 mM Tris-HCl, pH 9; 50 mM KCl; 1.5 mM $MgCl_2$; and 200 µM dNTP-mix and 50 ρmol of each primer. Sterile distilled water (SDW) was added until 25 µl final volume.

Amplification mtDNA was performed using PCR GeneAmp PCR System 2400 (Applied Biosystems, USA) as 1 cycle at 98°C for 1 min; 30 cycle at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and final extension at 72°C for 5 min. PCR products were purified by electrophoresis in a 1.0% agarose gel using 1x TAE buffer. The gel was stained with ethidium bromide, and the desired DNA band was cut and eluted using kit QIAquick Purification (Qiagen, USA). DNA concentration was measured with Spectrophotometer Bio-Spectro (Shimazu, Japan).

PCR for the 16S rRNA sequencing was done using primers and special reagents for DNA sequences (Big Dye). PCR volume of 10 ml consists of 1 to 1.5 DNA, Big Dye 2 ml, 6 ml H_2O, and 1 ml of primer. PCR cycle consists of 3 stages: 95°C for 15 min, 43°C for 15 min, and 60°C for 240 min. PCR products were purified and denatured, then sequenced with an automated tool ABI Prism 3103-Avant Genetic Analyzer (Applied Biosystems, USA). Sequencing was viewed manually by navigator sequence program (Applied Biosystems). The partial mitochondrial 16S rRNA genes were sequenced from four individuals for each of the male crab morphological variation.

Data analysis

RAPD bands were treated as dominant markers. The percentage of monomorphic (>95% of investigated specimens) and polymorphic (<95% of investigated specimens) bands was estimated for each morphological variation and gender. Unbiased Nei's genetic distance between gender and morphological variation was determined. Population genetic parameters (similarity index and genetic distance) were analyzed with Tools for Population Genetic Analyses version 1.3 (TFPGA Ver. 1.3) and genetic distance clusters using Unweighted Pair Group Method of Aritmethic (UPGMA) program. The data of mt-DNA sequencing were analyzed using Genetyx Version 7 program and Basic Local Alignment Search Tool (BLAST). Genetyx program was used to determine nucleotide variation and genetic distances between individual of crab. BLAST program online, especially for BLAST-N was used to determine the similarity (similarity index) of 16S rRNA sequences and relationship among several species of crustaceans which are available in the GenBank.

RESULTS

Random amplified polymorphic DNA (RAPD)

DNA genome of *P. pelagicus* sample was performed at approximately 23130 base pairs (bp). Among seven RAPD primers that were screened, three of them (OPA-5, OPA-11, OPA-17) were successfully amplified genomic DNA of 60 individual of *P. pelagicus*. 162 RAPD fragments ranging from 200 to 1200 bp in length were generated (Table 1). High genetic polymorphism was observed in all population.

The percentage of polymorphic bands for each primer across all population samples was 44.4 to 100%. In the male, the highest percentage of polymorphism was found in M3 (84%) and the lowest in M1 (71%), while in the female, the highest level of polymorphism (80%) was exhibited by the F3 whereas the lowest (73%) was exhibited by the F1 population. The highest and lowest number of RAPD bands was detected for primers OPA-17 (85.45%) and OPA-11 (68.96%) respectively.

Polymorphic bands are essential in identification in two ways; first in generating patterns of banding that are unique to individual species and second in exhibiting or lacking unique band (s) (marker bands) that distinguish an individual from the rest of the population. In this study, specific unique bands were found among population (Figure 3). These fragments were considered as potential species-specific marker for *P. pelagicus*. There are indication that the M2 population was a hybrid between M1 and M3 based on a specific unique band at 450 and 600 bp in OPA-11. Large genetic distances between pairs of population samples were observed.

Similarity index ranged from 0.7266 to 0.9050 and genetic distances from 0.1008 to 0.3214 (Table 2). In the male, the lowest similarity index values obtained were between M1 and M3 population and the highest were among M2 and M3 population, namely 0.9050 and 0.7266, respectively. In the females, the lowest similarity index was between F3 and F2 populations, whereas the highest obtained were between F1 and F3 populations, namely, 0.9011 and 0.7461, respectively. These values indirectly reflected degrees of differentiation in the blue swimming crab population of Makassar Strait. UPGMA dendogram among *P. pelagicus* populations using Nei's genetic distance obtained two main clusters. The first cluster consists of a population of M2, M3, and F2, while the second cluster consists of population M1, F1, and F3 (Figure 4).

The dendogram also explained the parentage of the

Table 1. Pattern of polymorphism between 60 samples of *P. pelagicus*.

Population	Primer	Total of fragment	Total of polymorpic bands	Polymorphism (%)	Fragment Size
	OPA-5	9	6	66.6	250-900
M1	OPA-11	12	8	66.6	200-1100
	OPA-17	10	8	80	200-1200
	OPA-5	8	7	87.5	250-900
M2	OPA-11	9	6	66.6	300-1200
	OPA-17	8	7	87.5	250-1000
	OPA-5	8	8	100	250-900
M3	OPA-11	9	8	88.8	300-1200
	OPA-17	8	5	62.5	300-1100
	OPA-5	8	4	50	250-800
F1	OPA-11	8	5	62.5	200-1200
	OPA-17	10	10	100	200-1200
	OPA-5	9	7	77.7	250-900
F2	OPA-11	9	4	44.4	300-1200
	OPA-17	12	12	100	250-1200
	OPA-5	7	6	85.7	250-900
F3	OPA-11	11	9	81.8	200-1200
	OPA-17	7	5	71.4	250-900

population, that M1 is paired with F1 and F3, while M2 and M3 are paired with F2.

Mitochondrial 16S rRNA

Isolation of mitochondrial 16S rRNA of 12 specimen of *P. pelagicus* representing three different male populations morphologically showed a single band at about 500 to 600 bp. The partial of 16S rRNA gene alignment was 556 bases long, including insertions and deletions. BLAST nucleotide analysis results indicate that the samples have a high similarity (identity) with *P. pelagicus* found in Genbank (Accession number: DQ062734.1, FM208750.1, FJ152161.1, KF220520.1, KF220521.1, KF220519.1, FJ812329.1, DQ388052.1) that is 96 to 99%, while with *Portunus trituberculatus* ranged between 94 and 95% (Accession number: FJ919807.1, AB093006.1, GQ180777.1, DQ062735.1, GU321227.1, GU321228.1, AY303612.1, AY264913.1). The lower similarity was found with *Parablennius sanguinolentus* that is 93 to 94% (Accession number: KF220524.1, KF220522.1, KF220525.1, KF220526.1, KF220523.1). This BLAST analysis results also provide information that there is a 3% intra-species diversity of *P. pelagicus* registered in the genbank.

Figure 5 shows the relationships among the *P. pelagicus* population inferred from their partial 16S rRNA sequences. Two clusters were formed, of which cluster 1 showed a higher variation in the appeal cluster 2. This clustering gives a strong indication that there is a high variation among *P. pelagicus* from Makassar Strait and may consists of two species or strain.

Morphological performance

Morphological performance of *P. pelagicus* from Makassar Strait was different from *P. pelagicus* shown by Lai et al. (2010). They reported that the blue swimming crab found in Indonesia is *P. pelagicus* with morphological characteristics are as the following.

Carapace colour is a dark blue-green with purple-blue chelipeds. Both males and females possess white spots on the carapace, often merging into broad almost banded reticulations, in particular on the posterior and branchial regions. While *P. pelagicus* found in Makassar Strait possess different white spot pattern (Figure 2).

This distinction reinforces the notion that *P. pelagicus* was found in Makassar Strait in contrast to those

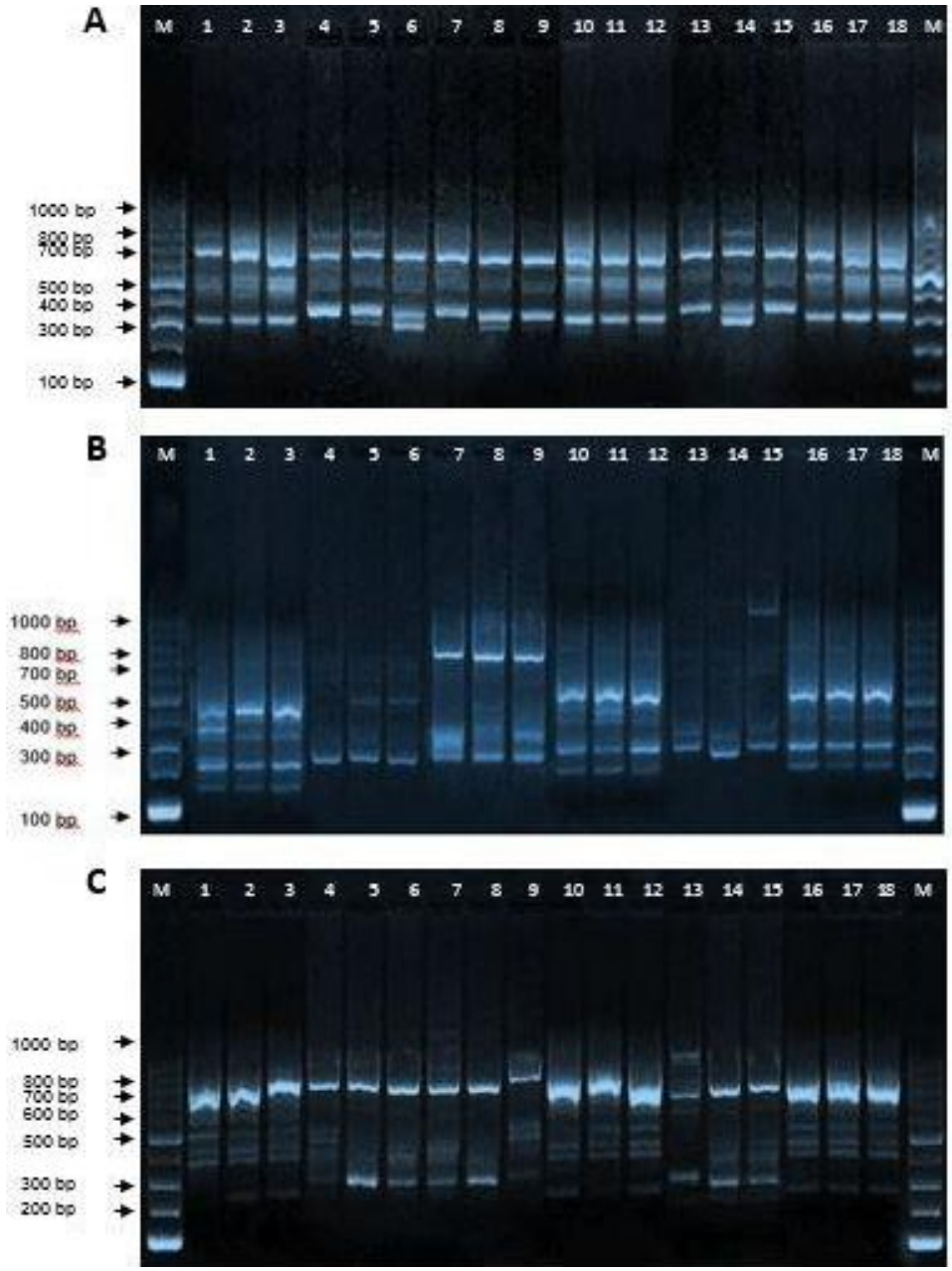

Figure 3. Random amplified polymorphic DNA fragment pattern generated of *Portunus pelagicus* from Makassar Strait using OPA-5. **(A)**, OPA-11. **(B),** OPA-17. **(C)** primer. M=Marker; 1-3 = M1; 4-6 = M2; 7-9 = M3; 10-12 = F1; 13-15 = F2; 16-18 = F3.

Table 2. Nei's genetic distance (below diagonal) and identity (above diagonal) among population of *Portunus pelagicus* from Makassar Strait.

Population	M1	M2	M3	F1	F2	F3
M1	-	0.7569	0.7266	0.7861	0.7857	0.7924
M2	0.2785	-	0.9050	0.7922	0.9041	0.8254
M3	0.3193	0.0998	-	0.7252	0.8472	0.8166
F1	0.2407	0.2329	0.3214	-	0.7774	0.9011
F2	0.2412	0.1008	0.1658	0.2517	-	0.7461
F3	0.2327	0.1919	0.2026	0.1042	0.2929	-

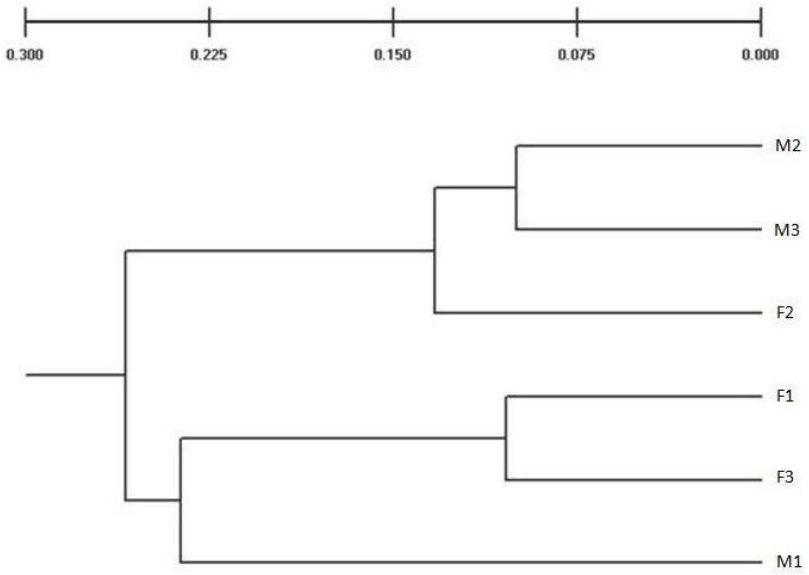

Figure 4. The UPGMA dendogram using Nei's genetic distance. M: Male; F: Female.

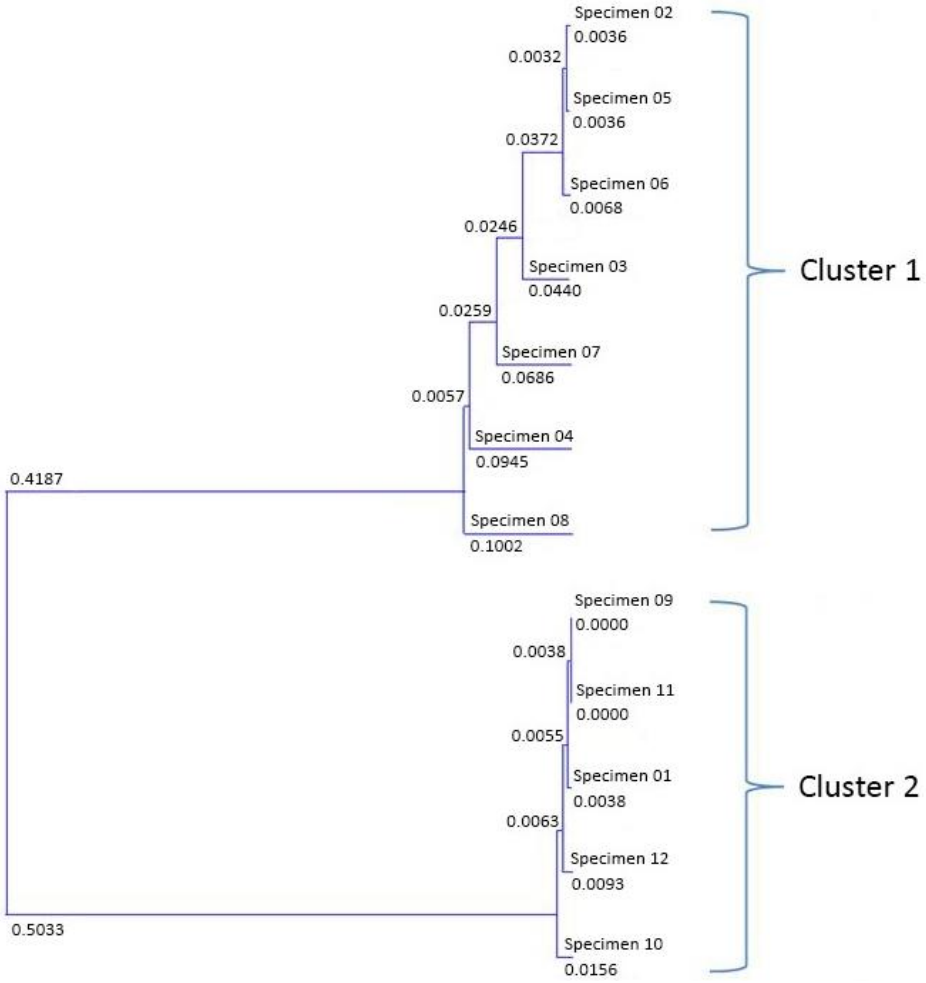

Figure 5. Phylogenetic relationship among 12 specimens of *P. pelagicus* population from Makassar Strait based on 16S rRNA sequences.

reported by Lai et al. (2010). The results also explained that the differences in the white spot pattern in the carapace of *P. pelagicus* are influenced by genetic. Thus, the variation of white spot pattern on the carapace can be used as indicators of differentiation in population of *P. pelagicus*.

DISCUSSION

The high degree of polymorphism and genetic distances on the population studied showed that the genetic diversity of *P. pelagicus* live in Makassar Strait is quite high. Similar result was reported by Suresh et al. (2013) that genetic distance of *M. cephalus* from Gujarat, Maharashtra, Andhra Pradesh and Tamil Nadu in India varied from 0.3717 ± 0.1460 (Gujarat population) to 0.5316 ± 0.1720 (Maharashtra population). A dendrogram based on Nei's genetic distance also showed two clusters. The Maharashtra and Gujarat populations appear in one cluster, while the Tamil Nadu and Andhra Pradesh populations formed the other cluster. A high degree of polymorphism suggested a high degree of genetic variability between the samples (Prasad, 2014). According to Amavet et al. (2007), the presence or the absence of the polymorphism in PCR-RAPD profile is either caused by nucleotide sequence divergence in primer sites or by insertions or deletions in the amplified segment of the template DNA.

The indication of hybridization is even more interesting among the population through the result of unique RAPD bands analysis. These population-specific unique bands can be used to detect any possible mixing of these populations, especially during selective breeding programs (Ferguson et al., 1995). Lai et al. (2010) also reported a phenomenon of natural hybridization between *P. pelagicus* and *Phrynocephalus reticulatus* in the Bay of Bengal.

The result of a combination of RAPD markers and 16S rRNA sequence was mutually reinforcing and complementary that the *P. pelagicus* have a high genetic variation. This indication was reinforced by the formation of two clusters based on RAPD as well as 16S rRNA sequences. The 16S rRNA is one region in mitochondrial genome. The mtDNA in animals is maternal inheritance (Rawson and Hilbish, 1995; Castro et al., 1998; Miller et al., 2005). Miller et al. (2005) suggests that characteristics of mitochondrial genome are maternal inheritance, lack of intermolecular recom-bination, and relatively rapid mutation rate.

Some studies have indicated that the sequence of 16S rRNA accumulates mutations more rapidly than the nuclear rDNA genes and can infer relationships beneath the family level within insects (Simon et al., 1994). Therefore, groups of animals that were descended from the same maternal line have a high similarity index unless there has been a mutation or gene flow. In this study, mutations were shown in the results of 16S rRNA sequencing, because the sequence of 16S rRNA is precise to study the mutation rate of the population as a result of aquaculture and stock enhancement.

A better understanding of population genetic structure is important to the effective fisheries management and conservation of genetic resources in exploited marine organism (Bert et al., 2007). Stock enhancement and cultivation can reduce the probability. Admixed populations will undergo damaging genetic alteration in the event of a decrease in genetic variation, fitness, and effective population size, because maintaining the genetic diversity of admixed populations and their wild-population components first requires managing both the genetic variability (e.g., numbers of alleles) and the genetic composition (frequencies of alleles) in the broodstocks and the broods (Bert et al., 2007). According to Waples et al. (2012), the major genetic risks of aquaculture include loss of genetic diversity within and among the populations and loss of fitness caused by the use of low genetic diversity and small numbers of brood stock. This situation can lead to inbreeding which in turn has implications for the quality of fry, a symmetrical survival of 'families' causing declines in stock quality.

However, this study failed to determine whether the two clusters found were distinct species or a sub species based on 16S rRNA sequences, because the data available on GenBank is only for *P. pelagicus* as a single species. Using COI sequences may help to identify the species complex of *P. pelagicus* in the Makassar Strait. The use of mitochondrial COI gene region in identifying the species *P. pelagicus* have been done by Lai et al. (2010) and Sienas et al. (2014).

Lai et al. (2010) reported, based on morphometric and mt DNA (COI) analysis that *P. pelagicus* in the world is a species complex consisting of four species, namely, *P. pelagicus* (Linnaeus, 1758), *P. reticulatus* (Herbst, 1799), *Petrolisthes armatus* (Milne-Edwards, 1861) and *Portunus segnis* (Forskal, 1775). The species complex is distributed in different geographic locations. *P. pelagicus* is widespread across Southeast and East Asia and is sympatric with *P. armatus* in the Northern Territory, Northern Australia. *P. armatus* is found around most of Australia and East to New Caledonia.

P. reticulatus occurs in the Eastern Indian Ocean. *P. segnis* appears to be confined to the Western Indian Ocean from Pakistan to South Africa, and is a Lessepsian migrant into the Mediterranean from the Red Sea. While Sienas et al. (2014) reported that *P. pelagicus* occuring across the entire Philippine archipelago potential consists of 2 species. Li et al. (2009) also used COI gene to study historical events of chinese shrimp (*Feneropenaeus chinensis*) in the Yellow Sea and Bohai Sea.

The conclusion of this study was the high genetic variation of *P. pelagicus* from Makassar Straits. This study suggests that farmers or hatchery operators can continue to use the population as sources of natural broodstock. Farmers can easily identify the genetic differences of the crab population by color and spot pattern in carapace. Morphologically, male and female were also distinguished and that is where they mate. This information also will be helpful in developing superior strain for aquaculture through selective breeding and formulating stock specific management for conservation of the species.

Conflict of Interests

The authors have not declared any conflicts of interest.

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Abbreviations

RAPD, Random amplified polymorphic DNA; **OPA,** operon teknologi kit A; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **16S rRNA,** ribosomal RNA region 16S; **COI,** cytochrome oxidase sub unit I; **TFPGA,** tools for population genetic analyses; **UPGMA,** unweighted pair group method of arithmetic.

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Full Length Research Paper

DNA-based identification of *Lentinula edodes* strains with species-specific primers

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***Lentinula edodes* is among the five globally cultivated edible mushrooms, which are wood decaying spore bearing Basidiomycetes possessing separate hyphae. Specific identification of this fungus from others in the division Basidiomycota using specific primers enables a fast and accurate detection through polymerase chain reaction (PCR). As a prelude to additional nutritional and sequence characterization research, we have developed a species-specific PCR assay for this fungus after screening four primer-pairs and two universal primer pairs. The primer-pair LE1F/R was specific in amplifications of ATCC-defined *L. edodes* strains and did not amplify DNA from six medicinally and nutritionally important fungal reference strains, Oyster (*Pleurotus ostreatus*), Maitake (*Grifola frondosa*), Enoki (*Flammulina velutipes)*, Baby bella (*Agaricus bisporus)*, Porcini (*Boletus edulis*), and Chanterelle (*Cantharellus cibarius*). However, amplifications using the universal primers were positive for all six strains. This assay will therefore serve to validate morphology-based-identifications of *L. edodes* strains.**

Key words: *Lentinula edodes*, LE1F/R, species-specific primers.

INTRODUCTION

Lentinula edodes is a saprophyte, and is often referred to as white-rot fungi, because of its capability in degrading cellulose, lignin, and other plant biomass macromolecules enzymatically (Sabotic et al., 2006). This mushroom also ranks second in production (~2 million tons) as food and for medicinal purposes next to *Agaricus bisporus* (Chang and Buswell, 1999; Chiu et al., 1999). The most widely used common name, “Shiitake” was derived from two words, “shii”, the Japanese Chinquapin tree, *Castanopsis cuspidata* (Thunb.) Schottky and “take”, which means mushroom in Japanese. Although, Shiitake grows throughout East and Southeast Asia, this mushroom is

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widely distributed and cultivated in China, Japan, Korea, Vietnam, Thailand, Burma, North Borneo, Philippines, Taiwan, and Papua New Guinea (Stamets, 2000; Wasser and Weis, 1997).

Shiitake mushroom cultivation is gaining importance in Europe, North America and especially in Africa where greater interest is there in expanding consumption of plant protein and diet diversification mainly because of its nutritional and medicinal properties. This is because of possessing the ability to thrive well in warm and moist climatic regions, and also having a wide host range which includes woody deciduous trees such as: alder, beech, chestnut, shii or chinquapin, hornbeam, ironwood, maple, mulberry, oak, poplar, sweet gum, and others. These species are primarily used in cultivation of shiitake mushroom (Wasser and Weis, 1997).

Lentinula species used for human consumption or as herbal medicine are known to have pharmacological properties. The polysaccharide lentinan, shiitake mushroom mycelium, and culture media extracts (LEM, LAP and KS-2) have been used in traditional herbal medicine for the treatment of diseases associated with liver, heart, tumor, cancer, and immune system (Wasser and Weis, 1997; Gordon et al., 1998). There is an increasing demand for shiitake mushroom also due to its flavor-enhancing compound lenthionine and its high protein, low fat content and unique dietary fiber (Maga, 1981; Yasumoto et al., 1976). A comparative analysis of elemental composition of this and other mushrooms has also been a focus in our laboratory (George et al., 2014).

Despite the global importance for this mushroom, there are very limited studies in molecular identification of *L. edodes*. *L. edodes* is identified based on morphological characteristics, such as the size, shape, form, gills, basidium, and basidiospores. The past taxonomic studies were based mainly on morphological features (Alexopoulos et al., 1996; Hibbett and Donoghue, 1996). Identification of species belonging to Basidiomycota can be achieved by amplification of 18S rRNA genes using designed conserved PCR primers (Swann and Taylor, 1993, 1995; Park et al., 2004; Rajesh et al., 2014). However, such specific primer pairs are not readily available for *L. edodes*. Basidiomycota comprises 3 subphyla, 16 classes, 52 orders, 177 families, 1,589 genera, and 31,515 species.

Primers available amplify partial regions of the 18S rRNA gene and the sequences were collected from four major fungal phyla namely, Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (Smith et al., 1999; Borneman and Hartin, 2000). Designed primers have been utilized in amplifying fungal rDNA from species of different taxonomic groups (White et al., 1990), however, only few of these primers have been found not to co-amplify DNA from taxonomically similar sources. Evaluation of genetic diversity in *L. edodes* strains has also been achieved through RAPD, ISSR and SRAP markers (Fu et al., 2010).

Previous studies characterized *L. edodes* strains through restriction fragment length polymorphisms (RFLP) of the ribosomal RNA internal transcribed spacer (rRNA-ITS) regions (Sharma, 2014; Mallick and Sikdar, 2015). However, designing specific primers for molecular characterization of *L. edodes* from a more conserved rDNA region will enable a rapid and accurate detection and identification of this mushroom through PCR. Therefore, this study aimed at designing specific primers by utilizing 18S rDNA sequences for characterizing *L. edodes* strains from other fungi.

MATERIALS AND METHODS

Collection of mycelial tissue

Eleven (11) strains of shiitake, namely LE005 (ATTC #28759), LE006 (ATTC# 28760), LE 008 (ATTC# 48857), LE6 (ATTC# 48855), LE010 (ATTC# 38164), LE014, (ATTC# 38168), LE015 (ATTC# 38169), LE020 (ATTC# 38174), LE025 (ATTC# 42253), LE37 (ATTC# 48177), and LE38 (ATTC# 48564), obtained from the American Type Culture Collection (ATCC, Washington, D.C.), were maintained at Alabama A&M University by sub-culturing the mycelium on PDA media every two to three months.

Preparation of the media

The *L. edodes* strains were grown on Czapek Solution Agar (Difco) (BD Diagnostics, MD, USA), which is a minimal organic media with the aim of avoiding contamination of DNA from gels derived from living organisms. This medium included 30 g sucrose, 3.0 g sodium nitrate, 1.0 g dipotassium phosphate (dibasic), 0.5 g magnesium sulphate, 0.5 g potassium chloride and 0.010 g ferrous sulphate. The final pH at 25°C was adjusted to 7.3 (+/- 0.2). The medium was autoclaved at 121°C for 15 min and partially cooled then poured into Petri plates under a sterilized laminar hood. Nitrocellulose membranes were placed on the solidified media prior to the placement of the mycelial inoculum for easy removal of the mycelia for DNA extraction and to physically separate the mycelia from the agar. The plates were kept at room temperature for 21 days for optimal growth.

PCR primer design

The 18S rRNA gene sequences of fungi *L. edodes* were retrieved from the NCBI GenBank databases [http://www.ncbi.nlm.nih.gov/Entrez]. Three strains with the longest base pair sequences were selected for primer design. These were LE 217 (Accession # FJ379282.1), GL 51 (Accession # FJ379280.1) and Cro4 (Accession # FJ379277.1). These had sequence lengths of 1648 bp, 1648 bp, and 1728 bp, respectively. The CLUSTALW program was employed in generating consensus sequence using three strains of *L. edodes* (Table 1). The primer sequences selected in this study were synthesized at MWG Biotech (Huntsville, AL, USA). After Multiple Sequence Alignment (MSA) and visualization of the conserved region, five primer pairs that covered total length of consensus were designed for further analysis (Table 1). The primers were designed using the primer designing tool primer3 and Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Four primer pairs selected (based on the primer length, GC content, T_m and fragment size) and two universal fungal primer pairs selected in this study were presented with the location on the consensus

Table 1. Sequences of primers designed with their respective sites on consensus 18S rDNA gene obtained from related species.

Primer	Sequence (5' → 3')	Primer length	Amplicon size	Amplified between	Location on consensus
LE 1F	5'-CCGGCGTGCCCTTTATTGGTGT-3'	22bp	~680 bp	640	634-655
LE 1R	5'-TAAGAAGCCGGCGACCATCCGA-3'	22bp	~680 bp	1320	342-363
LE 2F	5'-GCTCGCCGCTCACTTGGTGATT-3'	22bp	~500 bp	160	157-178
LE 2R	5'-ACACCAATAAAGGGCACGCCGG-3'	22bp	~500 bp	660	1001-1022
LE 5F	5'-GCGCGCAAATTACCCAATCCCG-3'	22bp	~300 bp	360	359-380
LE 5R	5'-AATAAAGGGCACGCCGGCTCAC-3'	22bp	~300 bp	660	1006-1027
LE 8F	5'-TCGGATGGTCGCCGGCTTCTTA-3'	22bp	~300 bp	1290	1293-1314
LE 8R	5'-TAGCGACGGGCGGTGTGTACAA-3'	22bp	~300 bp	1590	74-95
NS 3	5'-GCAAGTCTGGTGCCAGCAGCC-3'	21bp	~620 bp	480	482-502
NS 4	5'-CTTCCGTCAATTCCTTTAAG-3'	20bp	~620 bp	1100	571-590
NS 5	5'-AACTTAAAGGAATTGACGGAAG-3'	22bp	~270 bp	1050	1064-1085
NS 6	5'-GCATCACAGACCTGTTATTGCCTC-3'	24bp	~270 bp	1320	285-308

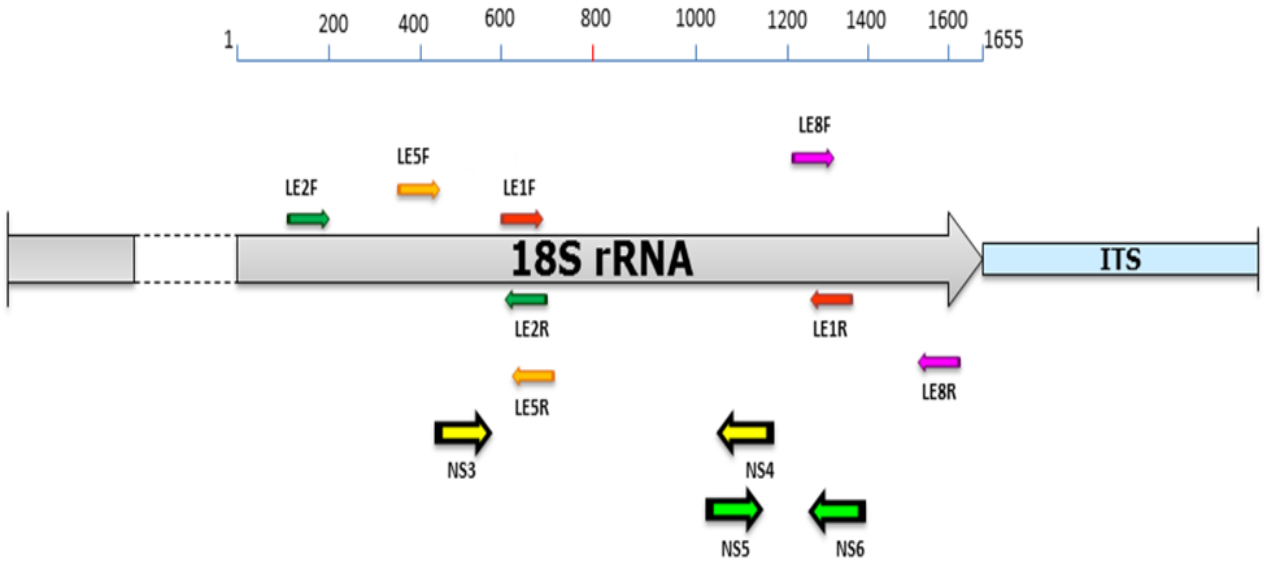

Figure 1. Schematic representation of the *L. edodes* 18S rRNA gene (consensus) with primer binding locations. Smaller arrows represent the binding positions of specific primers and larger arrows represent the binding positions of fungal universal primers. The extended region on left indicates the tandem nature of 18S rRNA and on the right is the internal transcribed spacer (ITS) region.

(Figure 1). The sensitivity and specificity of the primers designed were further tested.

DNA extraction and from *L. edodes* strains and six other fungal strains

DNA was extracted from eleven (11) strains of *L. edodes*, namely, LE05, LE015, LE005, LE006, LE010, LE014, LE020, LE025, LE37, LE38, and LE8. Additionally, six medicinally important commercial mushrooms, Oyster (*Pleurotus ostreatus*), Maitake (*Grifola frondosa*), Enoki (*Flammulina velutipes)*, Baby bella (*A. bisporus)*, Porcini (*Boletus edulis*), and Chanterelle (*Cantharellus cibarius*) were selected as reference strains and used in DNA isolations. Fungal mycelia (500 mg) was ground in a mortar and pestle and DNA was extracted from the mycelia using a DNeasy plant maxi kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. The quantity of the DNA was checked using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification

Two (2) µl of LE 015 and LE 6 DNA (32.27 and 25.92 ng/µl) were amplified with each primer pair (10 µm), Go Taq Master Mix (25 µl; Promega, Madison, WI, USA), in a 50 µl of reaction buffer using a

Table 2. *Lentinula edodes* strains amplified with 6 primer-pairs.

Strain	LE1F/R	LE2F/R	LE5F/R	LE8F/R	NS3/NS4	NS5/NS6
LE005	+	+	+	+	+	+
LE006	+	+	+	+	+	+
LE010	+	+	+	+	+	+
LE014	+	+	+	+	+	+
LE020	+	+	+	+	+	+
LE025	+	+	+	+	+	+
LE037	+	+	+	+	+	+
LE038	+	+	+	+	+	+
LE08	+	+	+	+	+	+
LE06	+	+	+	+	+	+
LE15	+	+	+	+	+	+

+ indicates presence of amplified product.

DNA Peltier Thermal cycler (MJ Research Inc, Watertown, MA, USA). The PCR conditions were: initial denaturation of DNA at 95°C for 5:00 min and then 29 cycles of three-step PCR amplifications consisting of denaturation at 95°C for 0:30 s, primer annealing at 60°C (for LE1F/1R, LE2F/2R, LE5F/5R, LE8F/8R) or 52°C (for NS3/NS4) or 56°C (for NS5/NS6) for 0:30 s and final extension at 72°C for 7:00 min.

Gel electrophoresis

Each PCR product (10 µl) was mixed with 2 µl of the blue orange 6x loading dye and these were run on a 1% (w/v) agarose gel along with 7 µl of 100 bp molecular marker. The gel was electrophoresed with 1x TBE buffer and run at 60 V for 150 min, stained with ethidium bromide and visualized under UV light using a Gel Doc XR + System (BIO- RAD, CA, U.S.A).

In-silico analysis

In-silico analysis was performed on the various primer pairs designed using Primer Blast algorithm, limiting the search to i) Non-redundant and ii) Organism-specific database (*L. edodes*) in GenBank.

RESULTS

Sensitivity and specificity evaluation of 18S primers in *L. edodes*

The PCR sensitivity of the six primer pairs was tested by amplifying the DNA of LE06 and LE15 at varied concentrations (0.5X, 1.0X and 2.0X). The estimated amplicon size for each primer pair was determined by agarose gel electrophoresis at all concentrations. To determine the specificity of primers within the *L. edodes* species, six primer pairs reported in this study were tested to successfully amplify the DNA from eleven different strains of fungi (Table 2). The sizes of the fragments for the primers designed were in agreement with those obtained by PCR. The primer pairs, LE1F/LE1R, LE2F/LE2R, and LE5F/LE5R amplified the 18s rDNA fragments of ~600 bp, ~500 bp and ~300 bp, respectively (Figure 2). Similarly, the primer pair, LE8F/LE8R amplified a product of ~300 bp (Figure 3). The universal primer pairs, NS3/4 and NS5/6 generated specific bands at 620 bp and 270 bp, respectively (Figure 4).

To determine the specificity of primers within the Basidiomycota family, six medicinally important fungal reference strains were selected randomly and amplified with six primer pairs along with the *L. edodes* strain, LE15 (Table 3). Four mushroom species (*P. ostreatus*, *F. velutipes*, *A. bisporus*, and *C. cibarius*) amplified with all primer pairs except with LE1F/R. However, amplification of *G. frondosa* was negative with both the primer pairs, LE1F/R and LE2F/R. *B. edulis* on the contrary amplified only with the universal primer pairs (NS3/NS4 and NS5/NS6). This screening helped in identification of LE1F/R as species-specific primer pair for *L. edodes*.

The specific nature of the primer pair (LE1F/R) was further validated by amplifying the DNA isolated from randomly selected organisms (*Escherichia coli* DH10, *Rotylenchulus reniformis*, *Gossypium hirsutum*, and Human) outside the Basidiomycota. The primer pair LE1F/LE1R did not amplify with any of these four organisms (Figure 5).

In-silico analysis of LE1F/R to GenBank non-redundant database shows hits to uncultured eukaryote 18S rRNA gene, however, there were three nucleotide base differences in relation to the reverse primer. The topmost hit obtained with accession number LN581510.1 had base differences at positions 700, 706, and 708 respectively in the reverse primer sequence. However when the search was limited to *Lentinula*, hits to *L. lateritia* were obtained. A blast2 algorithm used in comparison of a sequence each from *L. edodes* strain (KM01546.1) and *L. lateritia* strain (HM347336.1) showed

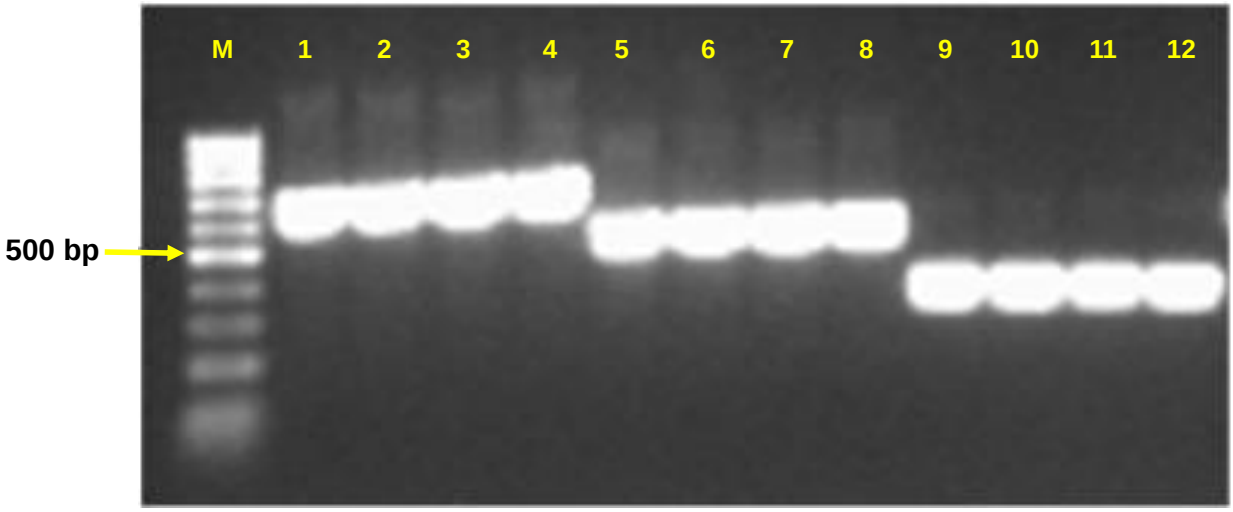

Figure 2. Gel electrophoresis of PCR-amplified DNA with primers LE1F/LE1R, LE2F/LE2R, and LE5F/LE5R. M, 100bp ladder; Lanes 1-4, amplified LE 15 (lanes 1&2) and LE 6 (lanes 3&4) DNA with LE 1F/1R primers; Lanes 4-8, amplified LE 15 (lanes 5&6) and LE 6 (lanes 7 &8) DNA with LE 2F/2R primers; and Lanes 9-12, amplified LE 15 (lanes 9&10) and LE 6 DNA (lanes 11 &12) with LE5F/5R primers.

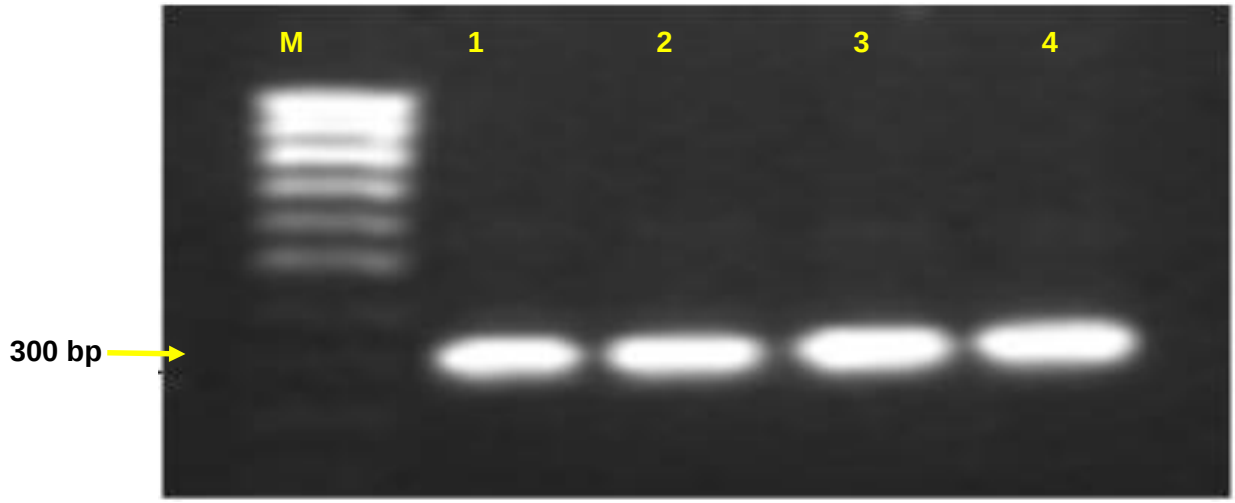

Figure 3. Gel electrophoresis of PCR-amplified DNA with primer LE8F/LE8R. Lane M, 100 bp ladder; Lanes 1-4 amplified LE 15 (lanes 1&2) and LE 6 (lanes 3&4) DNA with LE8F/8R primers.

99% identity, maximum score of 3,095, and a query cover of 96%.

DISCUSSION

In this study, an optimized, inexpensive and rapid method for the molecular identification of medicinally important mushroom, *L. edodes* based on amplification of 18S rDNA is presented. The 18S rRNA is a portion of smaller sub-unit (SSU) of ribosome and highly conserved among species, and can therefore be used for designing species-specific primers in distinguishing closely related taxa (Vanittanakom et al., 2002). Molecular screening in conjunction with morphological identification has aided in classification of various fungal species (Luo et al., 2002; Bu et al., 2005). The ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions of rDNA have been extensively used in identification of species and in resolution of phylogenetic classification of different organisms belonging to the same species or in distant taxa of plants, animals, and fungi (Hillis and Dixon, 1991; Hibbett, 1992; Bruns et al., 1992). Restriction fragment length polymorphisms (RFLP) of the ribosomal RNA

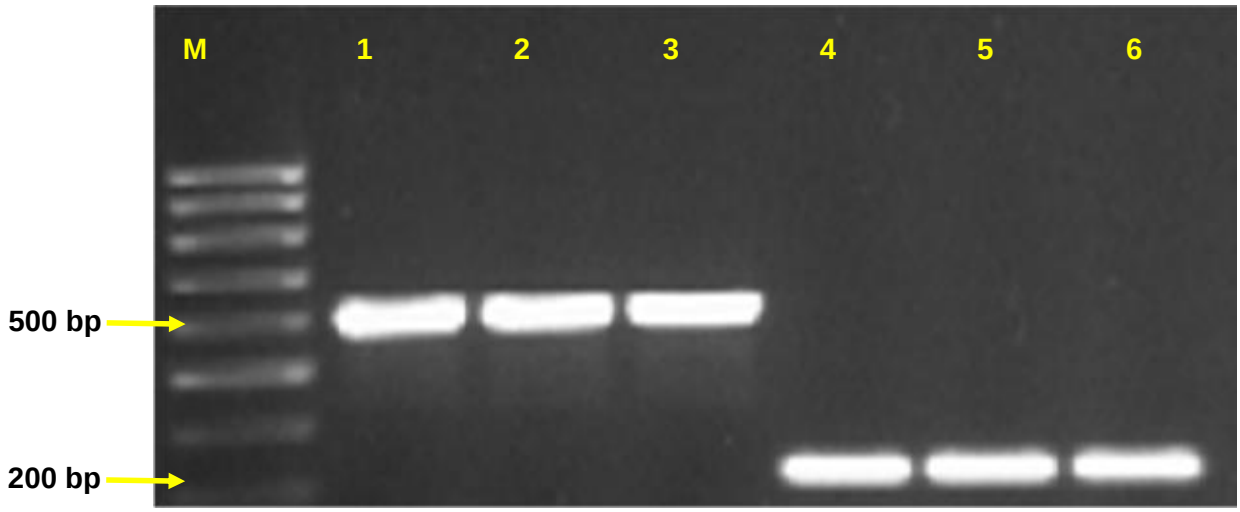

Figure 4. Gel electrophoresis of PCR-amplified DNA with primers NS3/NS4 and NS5/NS6. M, 100bp ladder; Lanes 1- 3, amplified LE 15 (lane 1) and LE 6 (lanes 2&3) DNA with primers NS3/NS4; and Lanes 4-6, amplified LE 15 (lane 4), and LE 6 (lanes 5&6) DNA with NS5/NS6 primers.

Table 3. Six fungal strains amplified with 6 primer-pairs.

Strain	LE1F/R	LE2F/R	LE5F/R	LE8F/R	NS3/NS4	NS5/NS6
Pleurotus ostreatus	-	+	+	+	+	+
Grifola frondosa	-	-	+	+	+	+
Flammulina velutipes	-	+	+	+	+	+
Agaricus bisporus	-	+	+	+	+	+
Boletus edulis	-	-	-	-	+	+
Cantharellus cibarius	-	+	+	+	+	+
Positive control (LE15)	+	+	+	+	+	+
Negative control (ddH_20)	-	-	-	-	-	-

+ indicates presence of amplified product, - indicates un-amplified product.

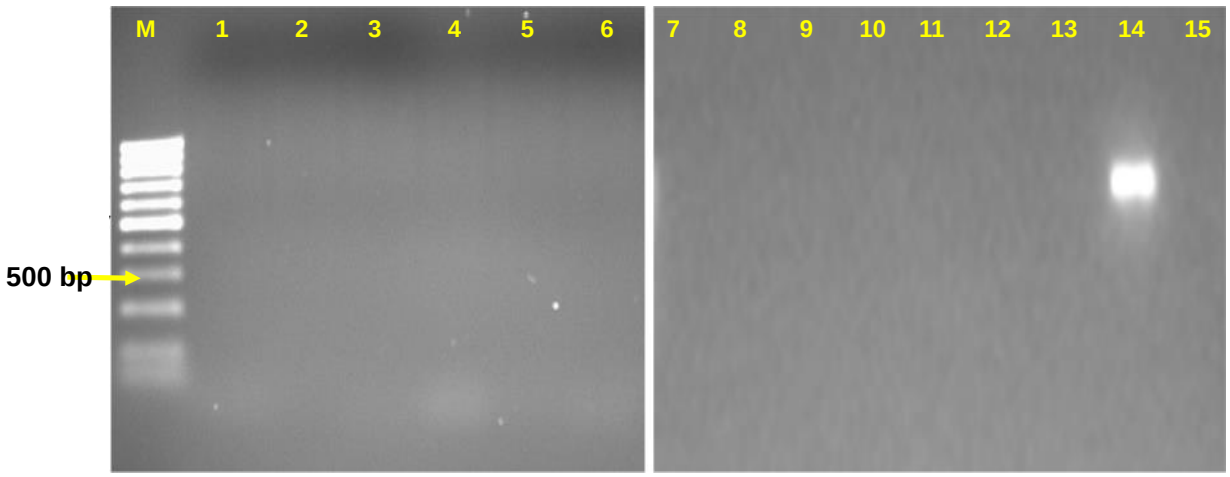

Figure 5. Specificity of LE1F/R primer pair was tested by amplifying DNA from various organisms: 1. *E. coli* DH10; 2. Reniform nematode; 3. Cotton TM-1; 4. Human; 5. Negative control-1 (amplified without LE6 DNA); 6. Negative control-2 (ddH20), 7. *Pleurotus ostreatus*; 8. *Grifola frondosa*; 9. *Flammulina velutipes*; 10. *Agaricus bisporus*; 11. *Boletus edulis*; 12. *Auricularia auricula-judae*; 13. *Cantharellus cibarius*; 14. Positive control (amplified with LE6 DNA); 15. Negative control-2 (ddH20).

internal transcribed spacer (rRNA-ITS) regions have been used to characterize species of *L. edodes* (Avin et al., 2012; Sharma et al., 2014; Mallick and Sikdar, 2015). Restriction digestion of the 18S rDNA has been investigated in 18 strains of *Lentinus*, *Neolentinus*, *Pleurotus*, and *L. edodes*. Amplified products digested with 10 restriction enzymes showed all strains of *L. edodes* could be easily distinguished from *Lentinus*, *Neolentinus*, and *Pleurotus* (Molina et al., 1992).

Recently, molecular characterization and phylogeny of seven *L. edodes* strains have been reported using random amplified polymorphic DNA (RAPD) and ITS sequencing (Sharma et al., 2014). They identified fifteen primers that amplify DNA from seven different strains. However, the fragment sizes of the primers for ITS regions (1-4) varied significantly between the strains. In our study, the products amplified by the primers LE1F/R, LE2F/R, LE5F/R, and LE8F/R for 18S rDNA region were uniform across the *L. edodes* strains. The sensitivity of the primers designed was tested at varied concentrations while the specificity was tested at three different levels: i) within the *L. edodes* strains, ii) within the Basidiomycota and iii) outside the Basidiomycota. Universal primer pairs NS3/NS4 and NS5/NS6 were further used to validate the amplifications associated with fungal origins.

The strains, LE005, LE006, LE010, LE014, LE020, LE025, LE037, LE038, and LE8 were positive to PCR assay with the four newly designed primer pairs and universal fungal primer pairs. The primer pair LE1F/LE1R did not amplify with any of the other important reference mushroom species selected from Basidiomycota, this suggests that it was specific to *Lentinula* spp. However, this primer pair had a hit to *L. lateritia* through *in-silico* analysis. Further analysis revealed a 99% sequence identity between *L. edodes* and *L. lateritia* isolates. Shiitake comprises three morphological species: *L. edodes* (continental and northeast Asia), *L. lateritia* (tropical Asia and Australasia), and *L. novaezelandieae* (New Zealand) (Pegler, 1983). Mating compatibility studies have revealed morphological species inter-fertility among the three species, thus these three species of shiitake according to investigators, should be classified as a single species (Shimomura et al., 1992).

Furthermore, ribosomal DNA restriction fragment length polymorphism analysis (RFLP) (Nicholson et al., 1997) and phylogenetic analysis of the ITS1 region of rDNA (David et al., 1998) reveals a low level of sequence divergence among the three morphological species. Therefore the high sequence similarity between *L. edodes* in our study and *L. lateritia* isolates confirms results of previous studies.

The specificity of LE1F/LE1R tested with representative organisms from three kingdoms of life, (protists, animals, and plants) (Scamardella, 1999), revealed absence of amplification, thus supporting the specificity of these primer pair.

This study has demonstrated the efficacy of a specific primer pair (LE1F/R) in detecting *L. edodes* strains from other taxa. This PCR based assay is rapid, highly specific, and sensitive for molecular identification of *L. edodes*, from other related Basidiomycetes. Furthermore, this assay will serve to validate morphological based-identifications of *L. edodes* strains.

Conflict of Interests

The authors have not declared any conflicts of interest.

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Full Length Research Paper

Ascorbic acid content in leaves of Nightshade *(Solanum spp.)* and spider plant (*Cleome gynandra*) varieties grown under different fertilizer regimes in Western Kenya

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Vitamin C is an important micronutrient because of its antioxidant and health promoting properties. With the introduction and commercialization of improved African indigenous plants, few studies have examined the impact of leaf age or the nutrient status of the plants by fertilizer. This study sought to determine amounts of vitamin C using redox titration in mature and immature leaves of spider plant (*Cleome gynandra*) and black nightshade (*Solanum ssp*) grown in fields and subjected to various sources of fertilizers which were chicken manure to provide an organic source, Mavuno fertilizer to provide a conventional synthetic source and no fertilizer to serve as a control. Chicken manure led to the highest (167 mg/100 g) vitamin C content which was however not statistically significant from Mavuno fertilizer (150 mg/100 g) at P≤ 0.05 in the nightshade variety. The highest vitamin C with no fertilizer application was 105/100 g and 79 mg/100 g in SS-49 and UG-SF varieties respectively. Moreover, vitamin C content was highest in mature leaves than in immature ones whatever the kind of fertilization treatment applied. By recognizing the impact of leaf age and importance of providing adequate fertilization, farmers can produce higher yielding and more nutritious leafy greens.

Key words: Leaf age, vitamin C, fertilizers, plant nutrition, spider plant, nightshade

INTRODUCTION

Sub-Saharan countries are endowed with a variety of nutrient dense African Indigenous vegetables (AIVs) which are traditional vegetables with edible shoots, flowers and young leaves (Oji, 2009). In Kenya the most

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consumed of them is black nightshade, spider plant, and amaranthus (Kimiywe et al., 2007). Five black nightshade species reported by Maundu et al. (1999) to be common to Kenyans include *Solanum physalifolium, Solanum scarbrum, Solanum american, Solanum nigrum and Solanum villosum.* Of the five species*, S. nigrum* is the most popular one (Ondieki et al., 2011). African Nightshade *(S. nigrum)* is a highly valued indigenous vegetable and it is consumed for its flavorful and perceived healthy benefits. For instance, black nightshade leaves are consumed to manage diabetes, high blood pressure, anaemia, peptic ulcers, colds, coughs and sight problems (Kimiywe et al., 2007; Keding et al., 2007). Spider plant (*Cleome gynandra*) also called cat whiskers, is consumed as a side dish, herb or as a tasty relish (Chweya and Mnazwa, 1997).

However, hunger, micronutrients deficiencies and children malnutrition remain perennial problems in African developing countries and this due, in part, to low vegetable consumption. Moreover, World Health Organization (2005) data reveals that fruits and vegetable consumption in Sub-Saharan Africa (SSA) is below the recommended 400 g/day and Standing Committee on Nutrition (SCN, 2010) estimate that 60% of children in the African continent suffer from iron deficiency aneamia. In Kenya, fruits and vegetable consumption stands at 85.1 kg/person/annually (Afari-Sefa et al., 2012) and nearly 1.3 million persons are food insecure (Loewenberg, 2014).

African Indigenous vegetables production and consumption can be used to alleviate these food insecurity and micronutrient deficiencies (Mavengahama, 2013). In fact, some wild AIVs are capable of thriving during harsh climatic conditions while some food crops are incapable and AIVs also mature early, usually within 3 to 4 weeks and can be used to fight hunger before food crops maturation (Mavengahama, 2013). However, AIVs consumption declined in Kenya with the introduction of exotic vegetables that are less bitter hence preferred by younger generations. There is also shortage of knowledge regarding the nutritional importance of AIVs and the perception that AIVs are inferior compared to other foods. However, they, especially African nightshade and spider plant, contain micronutrients such as iron, protein, vitamin C, carotene, magnesium, calcium, fiber, flavonoids, terpenoids and phenols (Mibei et al., 2012; Yang and Keding, 2009). These micronutrients are essential for plant growth and human being, especially vitamin C which are synthesized only by plant but useful for human organism.

Indeed, vitamin C, also called ascorbic acid has many vital roles in many processes in the human body. It is a cofactor in many enzymatic reactions, acts as an antioxidant, maintains the flexibility of blood vessels improves blood circulation and facilitates iron absorption in the human body (Shokunbi et al., 2011; Lee and Kader, 2000).

These important roles played by ascorbate have necessitated the need to preserve and increase the amounts of the vitamin in fruits and vegetables. In fact, there are many factors that affect vitamin C in fruits and vegetables. Among them include oxygen, temperature and water (Mhina and Lyimo, 2013). So, ascorbic acid is easily oxidized in the presence of oxygen and moisture to dehydro ascorbic acid and further to diketogluconic acid which is irreversible and inactive (Kiremire et al., 2010). Prolonged exposure of ascorbic acid to high temperatures also converts it to diketogluconic acid which is physiologically inactive (Kiremire et al., 2010). Other factors that lead to vitamin C loss are trimming, extended storage time, chilling injury and physical damage (Lee and Kader, 2000).

To increase vitamin C in leafy vegetables, horticulturalists have resorted to use of both inorganic and organic fertilizers. For example, Gendy et al. (2012), in their study, used cattle manure and biofertilizers application to increase vitamin C amount and compared fresh yields of vegetables to when no fertilizers were used. Similarly, application of chicken manure (60 t/h) increased vegetable heights, market yield and leaf number (Masarirambi et al., 2012a). In another study, analysis of chicken manure revealed that it was higher in potassium and phosphorous than cattle manure and led to increased plant yield compared to inorganic/ chemical fertilizers (Masarirambi et al., 2012b). Nonetheless, increasing the overall amount of vitamin C by application of fertilizers does not reveal the vitamin's accumulation levels in the leaves. The study sought to establish whether different fertilizer sources impacts vitamin C accumulation in the edible leaves of nightshade and spider plant.

MATERIALS AND METHODS

Study area description

Field experiments were conducted at the University of Eldoret Agricultural Research Farm. The farm is located 9 km from Eldoret. The altitude of University of Eldoret is 2120 m above sea level while the latitude is 0° 34'N and the longitude is 35° 18'E. Eldoret receives an annual rainfall between 900 to 1300 mm. The mean range of temperature is 10 to 25°C in July and January respectively (Jaetzold and Schmidt, 1983). Soil fertility is low with a pH below 5.5. The soil class of Eldoret town is rhodic Ferralsols (FAO, 1994).

Experimental designs

Soil pH was determined as before and after planting using a procedure by Okalebo et al. (2002). The soil pH was adjusted to 5.5 from 4.90 before planting by liming. Lime was applied at the rate of 200 g per 8 m plot. Land was cultivated and soil prepared to fine tilth for direct sowing and then raised 10 cm above the ground. Spider plant (Figure 1) and nightshade (Figure 2) were planted using a spacing of 60 cm and 45 cm on 19th of December 2013 and harvested manually on 10th February 2014 at 9.00 am. Field experiments were conducted as a randomized complete block design with three replications.

Seeds were planted through direct seeding method. The land was

Figure 1. (a) Mature and (b) immature leaves used for vitamin C analysis in fields grown at the University of Eldoret, Eldoret, and western Kenya.

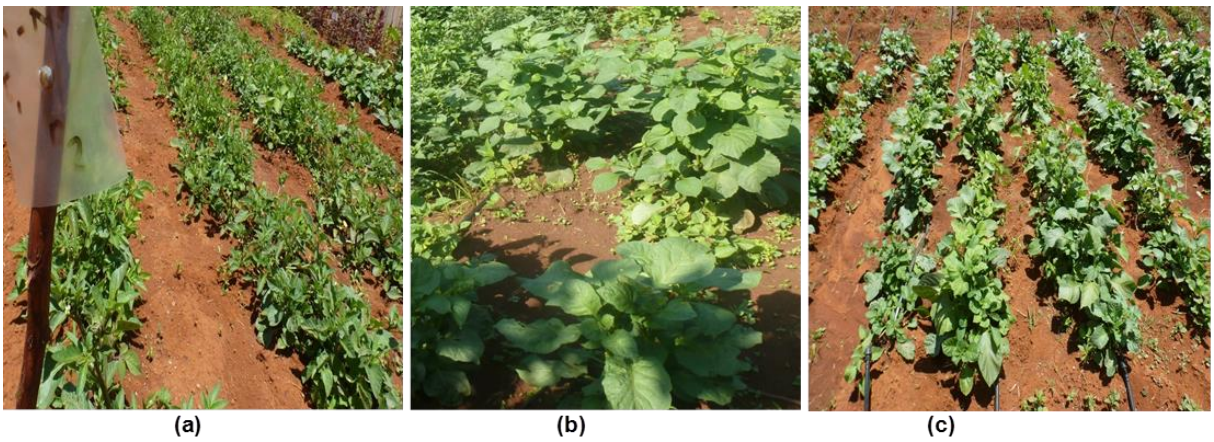

Figure 2. Pictures of three nightshade varieties. **(a)** Nightshade (local variety). **(b)** BG-16 variety. **(c)** SS- 49 variety grown at the University of Eldoret, Eldoret, and western Kenya.

subdivided into small plots of 4 m × 2 m in the main blocks. The subplots were separated by a margin of 0.5 m and 1 m within replicate blocks. Each block was separated by 1.5 m. Three varieties of black night shade (Figure 3) and spider plant species were planted using Mavuno fertilizer (a commercial fertilizer in Kenya), chicken manure and a control where (no fertilizer/manure was applied) at the rates of 0.4 g, 4.2 kg and 0.0 kg per 8 m^2 plot, respectively. Mavuno fertilizers contains 11 essential plant nutrients: primary nutrients are nitrogen (N), phosphrous (P), potassium (K); and other micronutrients such as calcium (Ca), sulphur (S), magnesium (Mg), zinc (Zn), copper (Cu), molybdenum (Mo), boron (Bo) and manganese (Mn). Plants that did not germinate were replanted after two weeks and weeding and harvesting was done manually. The experimental plots had drip irrigation installed to prevent the plants from suffering from water stress. The first harvesting was done 7 weeks and 4 days after planting on the 10th February 2014 at 9.00 am. Second harvesting was done at the pre-flowering stage 9 weeks after planting for spider plant and 10 weeks 4 days for Nightshade between 8.00 am and 9.00 am. Tissues from the second harvest were used for vitamin C analysis. Harvesting analysis was done at the pre-flowering stage during the second harvest. Harvesting was done by uprooting the whole plant above the soil surface. Ten to 15 plants were sampled in each plot to make a composite sample by uprooting the whole plant and

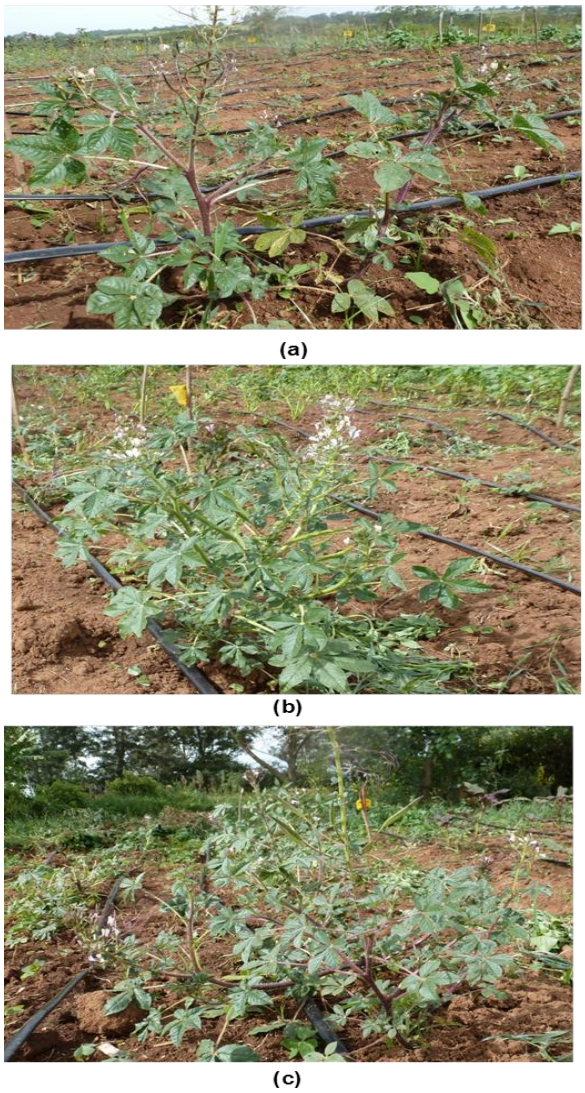

Figure 3. Pictures showing various photos of spider plant. **(a)** UG-SF-17. **(b)** local variety. **(c)** ML-SF-29 used in fields grown at the University of Eldoret, Eldoret, and western Kenya.

keeping in a cool box before being transported to the Chemistry Laboratory at the University of Eldoret. While in the lab, the petioles of the leaves were harvested manually from the plant stem. The dark green leaves located in the branch position were grouped as mature while the light green leaves from the apex were considered immature leaves (Figure 4).

The experimental model is, $yij = \mu + Ti + \beta j + \varepsilon ij.$

Where μ= population mean, Ti is the effect due to treatments (varieties and fertilizers), βj= is the effect due replicates and εij is the error that results from treatments and replicate interaction, yij= is vitamin C concentration in the leaves.

Vitamin C analysis

Vitamin C was analyzed as described by College of Science (2011) at the University of Canterbury. First, 100 g of fresh leaves were manually ground using mortar and pestle. The ground vegetable pulp was then strained in cheese cloth to obtain the vegetable juice. The extracted sample was diluted with distilled water and 0.2 g of potassium iodide was carefully weighed into a beaker of 100 ml. Next, 1.3 g of iodine was added into the same beaker and swirled to dissolve the iodine solution where it was then transferred into in to a one litre volumetric flask. This solution was then made to a litre using distilled water. Next, 20 ml of the sample solution was pipetted into a 250 ml conical flask and 150 ml of distilled water was added to the conical flask. Finally, 2 drops of starch solution previously made was added into the flask. Titration was done using 0.005 moles per litre solution of potassium iodine until a dark blue black color of starch-iodine complex was formed. Leaves from each plot were analyzed in triplicates and vitamin C results were expressed in mg/100 g.

$$I_2 \longrightarrow 2I^- + \text{dehydro ascorbic acid}$$

Data analysis

Data analysis was done using Portable SAS version 9.3. The effects of different fertilizer application on vitamin C were done at P≤0.05 using the general linear model (GLM) procedure. Treatments means of vitamin C were separated by least significance difference (*lsd*) at P≤ 0. 05 and the interactions of varieties and fertilizers were generated in ANOVA.

RESULTS

Mean vitamin C of three nightshades and spider plant varieties under different fertilizers are presented in Table 1. In general, leaves of both nightshade and spider plant from control plots which received no fertilizer application had significantly (P≤ 0.05) lower vitamin C content compared to those from plots which received either chicken manure or mavuno fertilizer. Local night shade variety had the least vitamin C content compared to two improved varieties regardless of the type of fertilizer applied. This can be attributed to the inherent genetic differences.

Table 2 presents vitamin C amounts in spider plant varieties. Unlike in nightshade, there were no significant varietal differences in vitamin C contents in all the three spider plant varieties tested.

In all varieties of nightshade plant studied, mature leaves had significantly ((P≤0.05) higher vitamin C than immature leaves (Figure 5).

Similarly, Vitamin C concentration in the three spider plant varieties was also found to be significantly higher in the mature leaves than in immature ones (Figure 6). The vitamin C content in immature leaves of ML-SF-29 was slightly lower compared to UG-SF-17 and local night shade variety. Mature leaves had approximately one and a half times more vitamin C than immature leaves.

DISCUSSION

Application of the organic fertilizer source, chicken

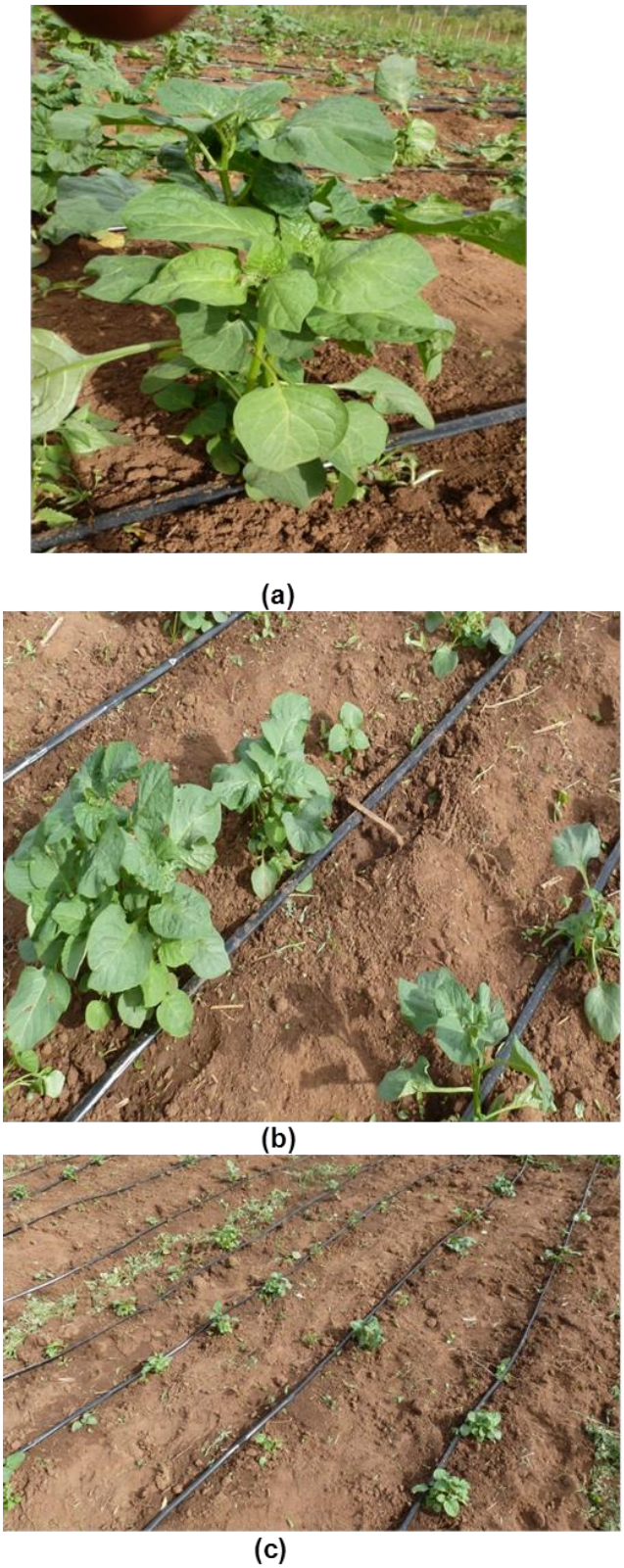

Figure 4. Pictures of black nightshade plants- BG-16 grown (a) with chicken manure (b) with mavuno fertilizer and (c) without fertilizer grown under inorganic and organic fertilizer sources at the University of Eldoret Research Farm, Western Kenya.

manure led to higher vitamin C content in the leaves but was not statistically different from Mavuno fertilizer in most of the varieties studied. Plants grown with chicken manure were observed to have bigger leaves and higher vigour than those grown under Mavuno fertilizer and in plots that had no fertilizer. This can be attributed to higher nutrient density of chicken manure and its ability to hold more water and nutrients as well as serve as a slower release of nutrients. Also, the soil pH was amended to about 5.5 from 4.9. At soil pH of 5.5 nutrient uptake for plant growth is enhanced. Application of either Mavuno fertilizer or chicken manure application led to increase vitamin C in the harvested plant tissues. In a separate study, Gendy et al. (2012) also found that application of cattle manure and biofertilizers at 30 m^3 per fed increased vitamin C amounts in Roselle (hibiscus) plants. Improved root development from the application of manure was reported to have increased nutrient uptake from soil for plant growth and development (Gendy et al., 2012).

From this study, the increased vitamin C in the three nightshade and spider plant varieties can be attributed to interaction effects between variety. Materechera and Seeiso (2012) have also reported that interactions of fertilizers and plants increased plant height unlike where no manure/fertilizer application is done.

The RDA for lactating and pregnant mothers in the USA is 85 and 120 mg respectively (National Institute of Health, 2013). Further, the RDA for an adult female and male above 19 years is 75 and 90 mg respectively. Results from this study suggest that consumption of adequate amounts of improved varieties of nightshade and spider plant has the potential to meet vitamin C for all the age groups because they can provide up to 167 and 136 mg respectively. These leafy vegetables can meet vitamin C demands for such age groups.

Another significant finding of this study is that the amount of vitamin C was higher in mature than immature leaves. This is because the immature leaves are physiologically active than mature ones. The young leaves have more demand for vitamin C and cannot accumulate enough vitamin C to meet their physiological processes. In contrast, mature leaves have higher capability to synthesize ascorbic acid but their utilization rates are lower. This difference in requirement between the mature and immature leaves for ascorbate is the reason for ascorbic acid translocation from the source of manufacture to the immature leaves that have high demand for the vitamin. Nutrients are always stored in the mature leaves and are translocated to the young ones for growth and development. Young leaves have not formed storage organs for nutrients storage. Consequently, nutrient translocation is greater in young than in old leaves. In the mature leaves, vitamin C exists in its active form but is converted into the inactive dehydroascorbic acid (DHAA) in immature leaves, a process that is reversible. Furthermore, Lee and Kader (2000) found out that ascorbic acid oxidase is found in

Table 1. Fertilizer effects on vitamin C (mg/100 g) content of nightshade plant varieties, field grown at the University of Eldoret Research Farm, Eldoret, Kenya.

Fertilizer	Nightshade varieties		
	SS-49	***BG-16***	***LV-NS[1]***
No fertilizer (F0)	105.3[d]	102.0[d]	96.3[d]
Mavuno fertilizer (F1)	150.0[b]	149.7[b]	120.67[c]
Chicken manure (F2)	160.3[ab]	167.0[a]	125.0[c]

*R^2=0.94, C.V= 6.23; LV-NS[1] = refers to local variety of nightshade; BG-16 = refers to improved varieties of nightshade; SS-49 = refers to improved varieties of nightshade. Means with the same letter within the same vegetable species do not differ at P≤ 0.05.

Table 2. Fertilizer effects on vitamin C (mg/100 g) content of spider plant varieties, field grown at the University of Eldoret Research Farm, Eldoret, Kenya.

Fertilizer	Spider plant varieties		
	UG-SF	**ML-SF-29**	**LV-SP[1]**
No fertilizer (F0)	79.7[c]	65.7[c]	68.6[c]
Mavuno fertilizer (F1)	125.7[b]	119.7[b]	135.0[ab]
Chicken manure (F2)	136.0[a]	130.7[b]	134.3[b]

*R^2=0.86; C.V=12.25; LV-SP[1]= refers to local variety of spider plant; UG-SF = refers to improved spider plant variety; ML-SF-29 = improved spider plant variety. Means with the same letter within the same vegetable species do not differ at P≤ 0.05.

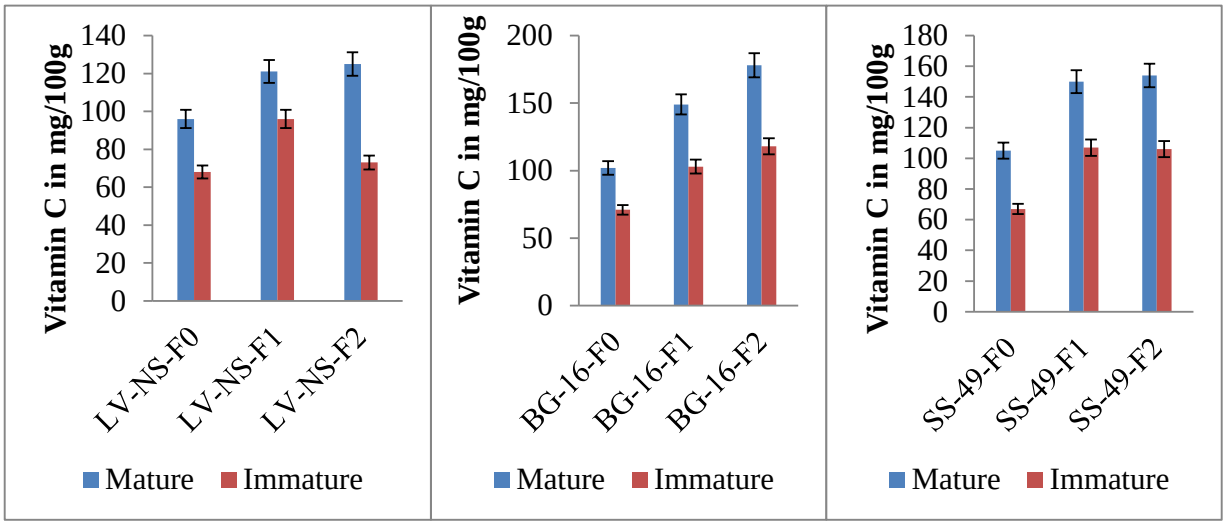

Figure 5. Vitamin C concentration in mature and immature leaves of the three night shade varieties in fields grown at the University of Eldoret Research Farm, Eldoret, western Kenya. F0 = refers to where no fertilizer/manure was used; F1 = mavuno fertilizer; F3 = chicken manure; LV-NS = local variety of nightshade; BG-16 = refers to improved varieties of nightshade; SS-49 = refers to improved varieties of nightshade.

plant parts that are growing rapidly. Ascorbic acid oxidase (AAO) oxidizes vitamin C to dehydroascorbic acid. This is also a possible reason why there was more vitamin C in the mature than immature leaves. Franceschi and Tarlyn (2002) also reported that mature leaves of a plant have a higher capability to synthesize ascorbic acid than flowers and shoot tips. They estimated that mature leaves could easily make vitamin C from its GAL-L precursor than immature ones. These findings are in agreements with that of Rosli et al. (2013) where he

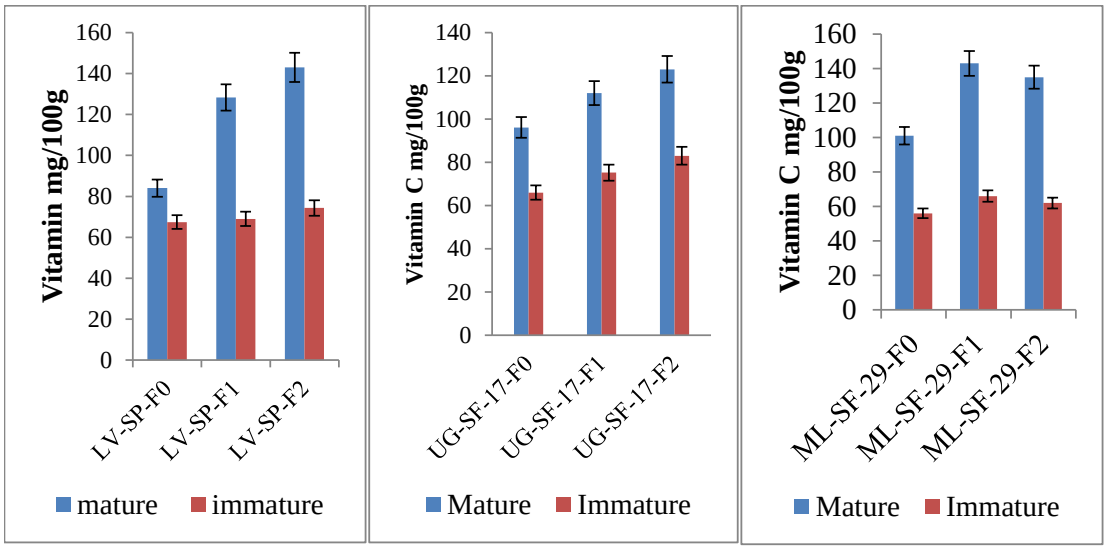

Figure 6. Vitamin C concentration in mature and immature leaves of three spider plant varieties in fields grown at the University of Eldoret, Eldoret, western Kenya. LV-SP= refers to local variety of spider plant; F0 = no fertilizer/manure application; F1 = mavuno fertilizer; F2 = chicken manure; ML-SF-29 = refers to improved spider plant variety; UG-SF-17 = refers to improved variety of spider plant.

observed that mature leaves had more vitamin C than immature ones. However, young leaves have better acceptability and eating quality. In order to optimize vitamin C intake, consumers should thus be sensitized to consume mature leaves so as to benefit more from the leaves. This information is relevant for capacity building purposes for both the producers and consumers so that they are both aware of critical harvesting stages, and the various parts targeted for harvesting in order to maximize nutrient intake from the vegetables. This can help prevent the common harvesting practice of uprooting young plants, particularly spider plant just a few weeks after emergence for consumption even before they form branches.

There was no difference in vitamin C in immature leaves attributed to either inorganic or organic fertilizers. This may be due to similarity in physiological functions of immature leaves across the varieties. Kipkosgei et al. (2003) demonstrated that regardless of the type fertilizer used to increase yield, vitamin C content does not vary in immature leaves.

Conclusions and recommendations

Black nightshade and spider plant are important components of diets of most households in Kenya. Therefore, it is good to increase nutrient composition of these vegetables as an attempt to expand intake of nutrient from the vegetables. The major findings of this study can therefore be summarized as: (1) the common AIVs, namely nightshade and spider plant can contribute to almost the entire recommended daily amounts of vitamin C to all age groups, including pregnant and lactating women, as long as other factors such as postharvest handling, storage and cooking methods are carefully selected to minimize losses; (2) improved varieties of nightshade used in this study are more superior than their local counterparts in terms of vitamin C content; (3) improving soil fertility is key to enhancing vitamin C content of these vegetables. As much as spider plant and black nightshade are good sources of ascorbic acid, vitamin C amounts depend on vegetable maturity, fertilizer application and post-harvest handling procedures. Therefore care needs to be taken during and after harvest, and during preparation to ensure maximum retention of vitamin C is attained. Further studies need to be done on how fertilizer application influences other vitamins and antioxidants in plant tissues. Farmers should also be sensitized on appropriate harvesting practices that target plant parts with higher vitamin C amounts.

Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Production of laccase without inducer by *Chaetomium* species isolated from Chettaba forest situated in the East of Algeria

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A positive laccase strain which showed a positive reaction with guaïacol was isolated from Chettaba Forest, which is situated in Constantine, the East of Algeria. It was identified as *Chaetomium* species (Ref 051A) according to the morphological and ribosomal internal transcribed spacer (ITS) DNA genomic sequence analysis. Laccase activity was determined by using 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS) as a substrate. Its highest activity was achieved by using potato dextrose broth (PDB) as a culture medium. Metal ion $CuSO_4$ had no positive effect on laccase production. The laccase activity obtained in submerged fermentation (20 L) was higher than that produced in Erlenmeyer flask (500 ml). The study of pH and temperature effects showed that pH optimum was 5 and 6, and temperature was 35°C. Laccase produced by *Chaetomium* spp. can be used in industrial production.

Key words: *Chaetomium* species, guaïcol, laccase, 2,2-azinobis(3- ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS), submerged fermentation.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a part of a broad group of enzymes called polyphenol oxidases containing copper atoms in the catalytic centre and are usually called multicopper oxidases (Khushal et al., 2010), which are able to oxidise a variety of organic and inorganic compounds, including mono-, di-, and polyphenols, aromatic amines, carboxylic acids, and non-phenolic substrates (Zhixin et al., 2010). Fungal laccases play an important role in plant pathogenesis, pigment production and degradation of lignocellulosic materials (Thurston, 1994; Gianfreda et al., 1999; Sunil et al., 2011). Laccases are widely distributed in higher plants, fungi and insects, but recently, it was found in some bacteria, such as *Streptomyces lavendulae*, *Streptomyces cyaneus*, and *Marinomonas mediterranea* (Thakker et al., 1992; Arias et al., 2003; Jimenez-Juarez et al., 2005). Up to now, fungal laccases were mainly isolated and characterised from ligninolytic basidiomycetes (Baldrian, 2006). A smaller number of laccases was characterised from other fungi, such as

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Ascomycetes and imperfect fungi (Junghanns et al., 2009).

Laccase is important because it oxidizes both the toxic and nontoxic substrates; this enzyme is very specific, ecologically sustainable and a proficient catalyst. It plays an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors (Couto and Herrera, 2006; Faccelo and Cruz, 2008; Shraddha et al., 2011). Recently, laccases have been efficiently applied to nanobiotechnology due to their ability to catalyse electron transfer reactions without additional cofactor (Majolagbe et al., 2012). However, a serious problem often encountered with industrial exploitation of fungal laccases is the low production level by the native hosts. This problem may be overcome by heterologous production in fungal hosts capable of producing high amounts of extracellular enzymes (Sunil et al., 2011). Submerged mode of fermentation has been used intensely for the production of laccase (Shraddha et al., 2011).

The aim of the present paper was to screen and isolate laccase producing fungi from Chettaba Forest's soil samples. The objective of this study was to produce laccase for large scale from the isolated wild strain and its partial characterization.

MATERIALS AND METHODS

Isolation and purification of fungal strains

Soil's sample of botany pine was collected in sterile plastic bags from Chettaba Forest which is situated in Constantine, in the East of Algeria. The isolation of fungal strains was done by employing standard serial dilution method. Soil samples (1 g) were added into 9 ml of physiological water and a serial dilution (10^{-1} to 10^{-6}) was prepared, respectively, then 0.1 ml of each dilution was distributed on potato dextrose agar (PDA) screening medium and incubated at 28°C until apparition of colonies. The fresh fruiting body of each fungus was split by a spatula; a small part of basidiocarp was then picked up and put on PDA Petri dish for 7 days. The PDA screening medium contained potato 20%, glucose 2.0%, and agar 1.5% (Zhixin et al., 2010).

Selection of strains producing laccase

The selection of fungal strains producing laccase was done by following the methods of Budolla et al. (2008) and Thakur and Gupte (2014) with minor modifications. Isolated strains were inoculated on plates containing Olga medium, supplemented with 0.01% guaïacol and incubated at 30°C for 7 days. Laccase activity was visualized on plates by forming reddish brown zones in the medium, caused by laccase which catalyses the oxidative polymerization of guaiacol. The selected strain was identified by Mycotec of Louvain la Neuve (Belgium).

Production of laccase in different media of culture

The method of Das et al. (1997) was used with some modifications. Five discs of potent strain selected previously with 1 cm diameter were inoculated into 500 ml Erlenmeyer flasks, containing 250 ml of three different liquid mediums: Olga g/L (Peptone 3; Glucose 10; KH_2PO_4 0.6; $ZnSO_4$ 0.001; K_2HPO_4 0.4; $FeSO_4$ 0.005; $MnSO_4$ 0.05; $MgSO_4$ 0.5); potato dextrose broth (PDB g/L: Glucose 20; potato extract 200), and ME g/L (Malt extract 20; Peptone 3) at pH 5.5, and kept in incubator shaker at 100 rpm, at 30°C until optimum laccase activity is obtained.

Enzyme essay

After 12 days, fungal mycelium was separated from the broth by filtering it through Whatman No1 filter paper, and then the filtrate was centrifuged at 10000 rpm for 30 min at 4°C.

The culture supernatant was collected and protein concentration was estimated following Folin's method as modified by Lowry et al. (1951) using bovine serum albumin as standard. The colour was changed into blue one, which was measured at 660 nm against the blank (Majolagbe et al., 2012).

Laccase activity was determined spectrophotometrically as described previously by Sunil et al. (2011) with minor modification.

The assay of laccase was based on the oxidation of 2,2-azinobis(3- ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS) (Sigma) (A420 (ε420, 3.6 × 104 $M^{-1}cm^{-1}$). 100 µl of enzymatic extract was incubated in 900 µl of tartrate acid solution adjusted at pH 4.5 with NaOH 10 N, which contained 10 µl of 10 mM ABTS. The reaction mixture was stopped by adding 50 µl of 50% (w/v) trichloroacetic acid (TCA). Oxidation of ABTS was monitored by spectrophotometer at 420 nm. One unit was defined as the amount of the laccase that oxidized 1 µmol of ABTS substrate per minute.

Effect of metal $CuSO_4$ on laccase activity

It was done by following Zouari et al. (2006) method with minor modification. For laccase production and induction studies, 5 discs of potent strain with 1 cm in diameters were inoculated into 500 ml Erlenmeyer flasks, containing 250 ml of PDB as culture medium, which was supplemented with $CuSO_4\,5H_2O$ (0 - 600 µM) as inducer of laccase.

Production of laccase in submerged medium on fermenter (20 L)

Laccase production was done on fermenter of 20 L. Potent strain was cultivated on PDB, the pH was adjusted and maintained at 5.8 and temperature at 30°C, with agitation of 120 rpm and 100% of dissolved oxygen. At the end of fermentation, the culture was first filtered and centrifuged at 10000 rpm for 30 min at 4°C. The supernatants obtained were stored at 4°C and used as crude enzyme extracts. Laccase's concentration was measured spectrophotometrically each day as explained previously.

Partial characterization of laccase

The optimum of temperature and pH of laccase was determined by following Xia et al. (2014) with minor modification.

The effect of pH on laccase activity was studied by recording the absorbance of enzyme extract catalysed reaction at room temperature after 15 min of incubation using reaction mixture dissolved in buffers over pH range 2.0 to 8.0. Different pH gradients were obtained using sodium acetate buffer (3.0 to 6.0) and sodium phosphate buffer (6.0 to 8.0).

The effect of temperature on laccase activity was determined by recording the absorbance of enzyme extract using reaction mixture

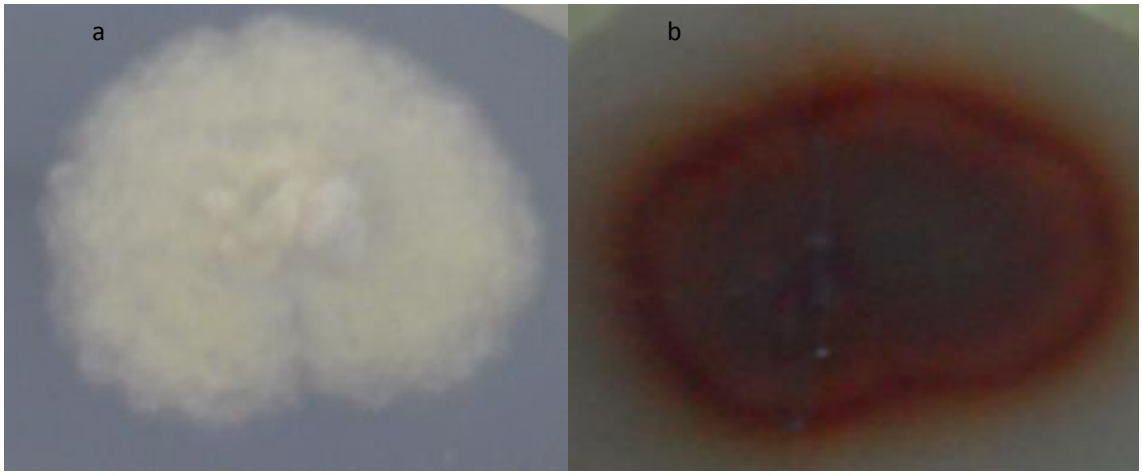

Figure 1. Macroscopic aspect (a) before and (b) after reaction with guaiacol.

incubated for 15 min at pH 5.5 and at temperatures ranging from 15 to 85°C.

The absorbance was recorded spectrophotometrically at 420 nm. The enzyme essay was done as explained previously with ABTS as substrate and the highest laccase activity was taken as 100%.

RESULTS

Isolation and selection of fungal strains

Twelve strains were isolated and purified from samples taken from Chettaba Forest, situated in Constantine, in the East of Algeria.

After 7 days of incubation, 1 fungal strain (P4) had a positive reaction with guaïcol indicating laccase producer (Figure 1). Laccase activity was visualized on plates by forming reddish brown zone in the medium.

The selected strain (P4) did not give spores; this makes the strain identification difficult. However, according to the morphological and ribosomal internal transcribed spacer (ITS) DNA genomic sequence analysis effected by Louvain la neuvemycotec (Belgium); the genus only was known but not the species. It was identified as *Chaetomium* species (Ref 051A).

Production of laccase in different media of culture

Chaetomium spp. was grown and tested on three different mediums; the optimum of laccase activity was obtained after 12 days of incubation, on PDB medium, with 550 U/L of laccase concentration. The use of ME as a medium for the production of laccase gave an important activity when compared with the Olga medium (Figure 2).

Effect of metal $CuSO_4$ on laccase activity

The effect of different $CuSO_4$ concentrations on

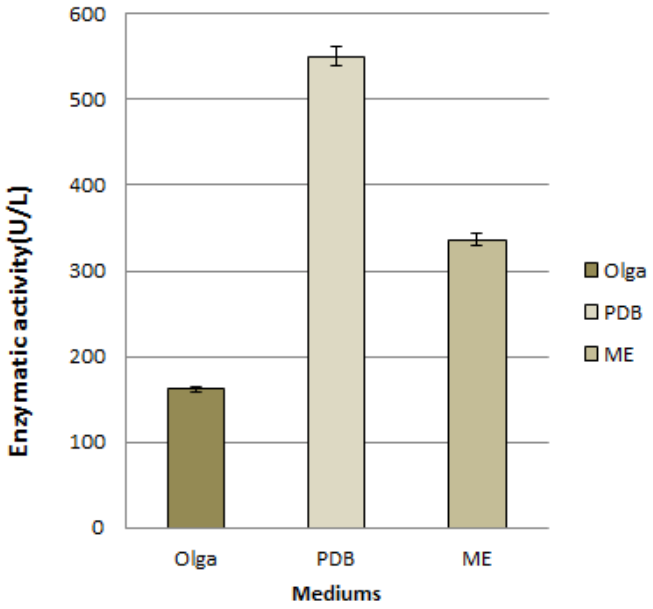

Figure 2. Laccase activity in different meda.

Chaetomium spp. laccase production is as shown in Figure 3. Results show that laccase activity decreases when $CuSO_4$ was added. The maximum laccase activity was obtained without adding any concentration of $CuSO_4$.

Laccase production in submerged fermentation (20 L)

Laccase production increased greatly from the 7th day of incubation and reached the highest laccase activity of 986.63 U/L and protein content of 170 µg/µl at the 12th day (Figure 4); thereafter, the laccase yield dropped rapidly.

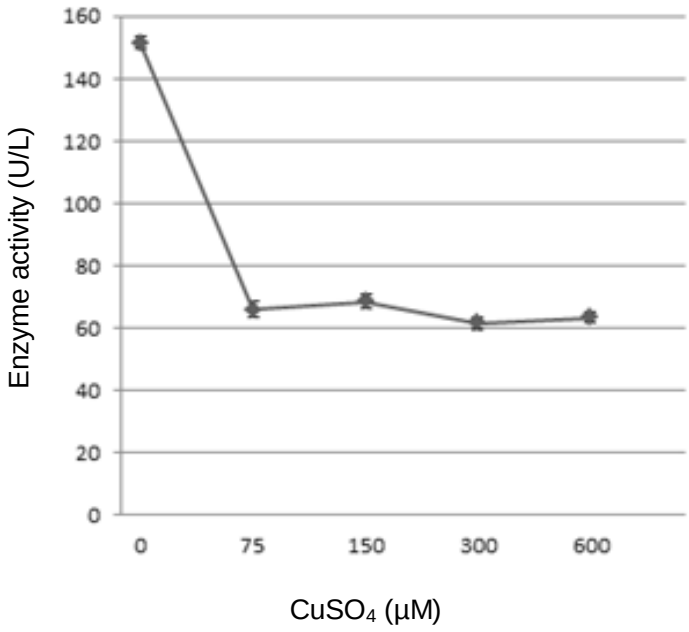

Figure 3. Effect of different concentrations of $CuSO_4$ on laccase activity.

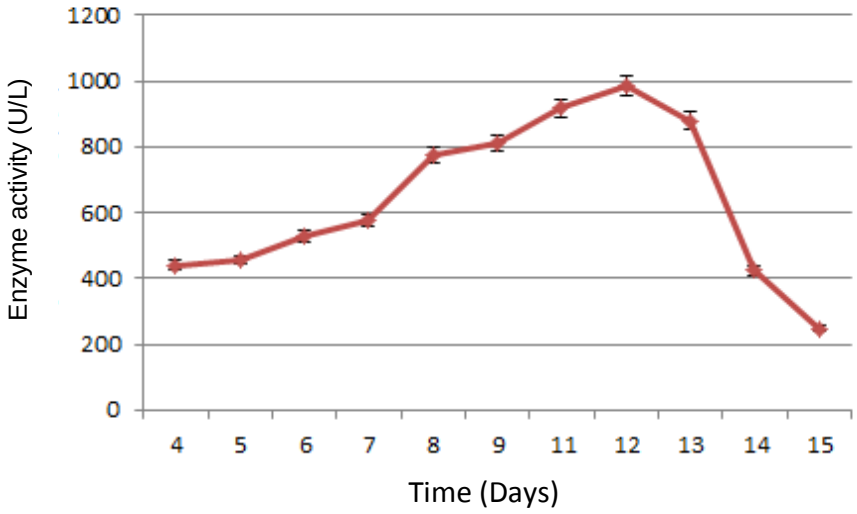

Figure 4. Time course of extracellular laccase activity.

Partial characterization of laccase

The effects of pH and temperature on laccase activity are as shown in Figures 5 and 6. High laccase activity was obtained over a pH range of 5.0 to 6.0. The optimal temperature for laccase activity was 35°C.

DISCUSSION

Laccases were first described in 1883 from the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1983). Since then, several laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and industrial applications (Thurston, 1994; Gianfreda et al., 1999; Xu et al., 2000; Sunil et al., 2011). In this study, a *Chaetomium* spp. (Ref051A) was isolated from Chettaba forest which is situated in Constantine, in the East of Algeria. This strain is able to secret laccase in submerged medium.

El zayat (2008) showed the ability of *Chaetomium globosum* to produce appreciable amounts of laccase

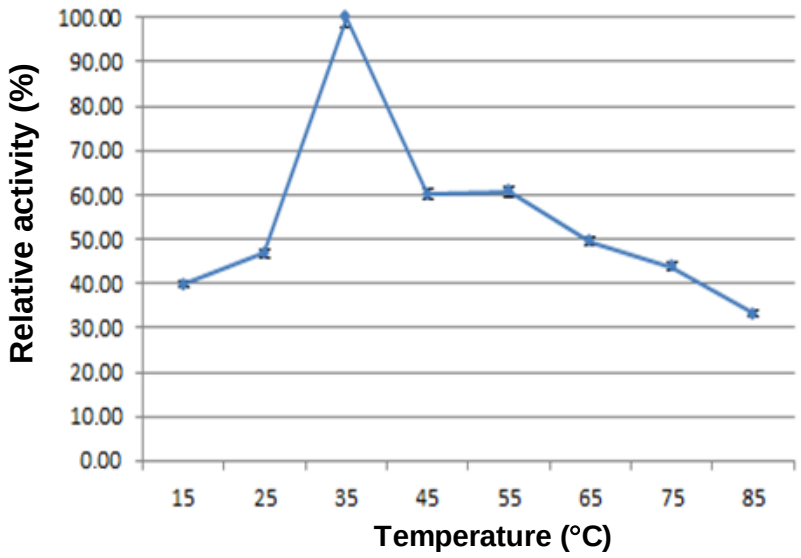

Figure 5. pH optimum with ABTS as substrate.

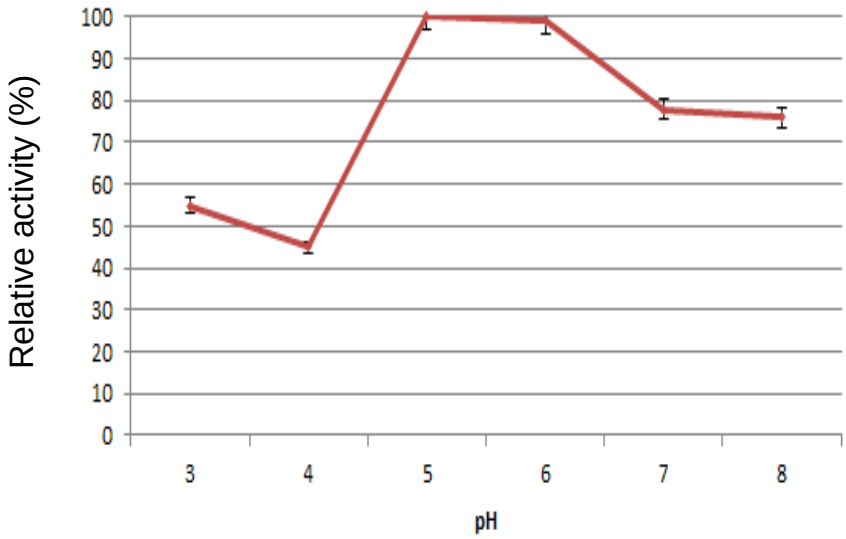

Figure 6. Temperature optimum with ABTS as substrate.

extracellularly. However, Qasemian et al. (2012) confirmed the capacity of *Chaetomium* spp., isolated from a Mediterranean coastal area, to produce an halotolerant laccase.

One of the parameters widely used in the detection of ligninolytic enzymes is the chromogen. In the present study, guaiacol was used as a chromogen. The reddish brown zone surrounding the mycelia of the culture on the plate supplemented with guaiacol was an indication of Bevandamm's reaction (Thakur and Gupte, 2014). Out of the 12 isolates, one isolate showed a positive Bevandamm's reaction for laccase activity. Similar results have also been reported by Viswanath et al. (2008), Patel et al. (2009), Gao et al. (2011) and Thakur and Gupte (2014).

Our results show that the optimum of laccase activity is done by using PDB as a culture medium when compared with Olga and ME, which is similar with the results obtained by Rosales et al. (2002). It can be explained that the extract of potato is used as inducer of production of laccase. However, Buddolla et al. (2008) confirmed that the maximum laccase activity was obtained by using Olga as a culture medium.

The activity of laccase obtained from the isolated strain

without inducer ($CuSO_4$) is more important than that acquired with inducer. Similarly, Sunil et al. (2011) obtained a higher activity without inducer (Tannic acid), though Zouari et al. (2006) showed that the addition of 300 µg of $CuSO_4$ allows the optimum laccase activity to be obtained.

Extracellular laccase activity reached its maximum on the 12^{th} day with 986.63 U/L without inducer, which corroborate with the study of Cordi et al. (2007), but with 40.77 U/L. Dissimilarly, Sunil et al. (2011) showed that the maximum laccase activity was obtained after the 19th day. Therefore, Buddolla et al. (2008) obtained 600 U/L of laccase activity after the 4th day of incubation using PDB as a culture medium.

The effect of temperature is limited in case of laccase production. The optimal temperature differs greatly from one strain to another (Shraddha et al., 2011). Laccase examined in this study had an optimal temperature at 35°C. Generally, the optimal temperature for fungal laccase activity ranged from 30 to 60°C (Nishizawa et al., 1995; Youn et al., 1995).

The optimal of laccase activity was obtained at pH 5 and 6. Generally, the growth of the fungi is ideal at low pH (Sunil et al., 2011). Zhixin et al. (2010) obtained the maximum activity at pH 4.4, which is approximately similar to that realized by Khushal et al. (2010).

Conclusion

The reddish brown zone surrounding the mycelia of the culture on the plate supplemented with guaiacol was an indication of the presence of laccase activity produced by *Chaetomium* spp. The results show that PDB is the suitable medium for laccase production. Partial characterization displays that the maximum laccase activity is obtained over pH range of 5.0 to 6.0 and at 35°C. The laccase activity obtained in submerged fermentation (20 L) is higher than that produced in Erlenmeyer flask (500 ml), which revealed that this strain can be used in industrial production.

Conflict of interests

The authors have not declared any conflict of interest.

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