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Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Table of Contents: Volume 14 Number 29, 22 July, 2015

ARTICLES

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Mini Review

Rhodanese is a possible enzyme marker for cyanide environmental stress on aquatic life

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Rhodanese is a cyanide detoxifying enzyme. The role of man through his anthropogenic activities in and around water bodies have increased in recent times. These have led to constant exposure of water body to cyanide and cyanide compounds with increase to loss of many aquatic lives. There are limited methods employed in quick detection of cyanide in water. The aim of this paper was to present rhodanese, an enzyme, as a possible marker for detecting and monitoring water pollution as a result of environmental stress from anthropogenic activities and constant climatic changes.

Key words: Rhodanese, aquatic life, water body, cyanide, toxicity.

INTRODUCTION

Fish and aquatic invertebrates are particularly sensitive to cyanide exposure. Free cyanide was reported to be the primary toxic agent in the aquatic environment. Environmentally relevant exposures to cyanide ions can cause stress, increase in mortality and place an appreciable metabolic load on fishes and other aquatic organisms (Eisler, 1991). It is now accepted that some human-induced climate change is unavoidable. Potential impacts on water supply have received much attention (Whitehead et al., 2009; Oyedeji et al., 2013), projected changes in air temperature and rainfall could affect river flows and, hence, there is mobility and dilution of contaminants (Whitehead et al., 2009). Increased water temperatures will also affect chemical reaction kinetics and freshwater ecological status. It has also been reported that increase flow of water will lead to changes in sediment loads that will further result to invasion by alien species stimulating the potential for toxic algal blooms and reducing dissolved oxygen levels (Whitehead et al., 2009). The bacteria rhodaneses have potential for effective remedia-tion of cyanide-polluted environments, ultimately leading to improvement of fish and other aquatic organisms in receiving water bodies (Oyedeji et al., 2013).

Anthropogenic sources of cyanide in the environment include certain industrial processes, laboratories, fumigation operations, cyanogenic drugs, fires, cigarette smoking, and chemical warfare. Although, cyanide is ubiquitous in the environment, levels tend to be elevated

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in the vicinity of metal processing operations, electroplaters, gold-mining facilities, oil refineries, power plants, and solid waste combustion (Eisler, 1991). Many chemical forms of cyanide are present in the environment, including free cyanide, metallocyanide complexes, and synthetic organocyanides, also known as nitriles. But only free cyanide (that is, the sum of molecular hydrogen cyanide, HCN, and the cyanide anion, CN-) is the primary toxic agent, regardless of origin (Smith et al., 1978; Eisler, 1991).

Fish are the most sensitive aquatic organisms tested. Adverse effects on swimming and reproduction were observed between 5 and 7.2 µg free cyanide per liter; lethal effects usually occurred between 20 and 76 µg/L (Smith et al., 1978; Billard and Roubaud 1985). Biocidal properties of cyanide in aquatic environments were significantly modified by water pH, temperature, and oxygen content; life stage, condition, and species assayed; previous exposure to cyanides; presence of other chemicals; and initial dose tested. Natural sources of cyanide include various species of bacteria, algae, fungi, and higher plants that form and excrete cyanide (Way, 1984). Activities that will increase the concentration of bacteria and other forms of microorganism will affect the water quality that invariably affects the aquatic life. While, the activities around reservoirs, rivers, sea and ocean have been described as those that enhance increase in environmental pollution especially the use of fertilizers and other chemicals by farmers (Okonji et al., 2010), effluent from industries and poor sewage disposal by the locals and also the climatic changes that brings along with it, increase in rain fall and melting of ice which has a concomitant increase in water volume (Okonji et al., 2010). The use of cyanide compounds by the mining industry, coupled with limitations in current analysis and monitoring of these compounds, raises serious concerns regarding public safety and environmental protection at mine sites using cyanide processing (Flynn and Haslem, 1995). There is need for continuous monitoring of pollutants such as cyanides in fresh and marine waters.

CYANIDE

Cyanide is highly toxic and its ineffective detoxification may result in inhibition of respiration (through iron complexation in cytochrome oxidase), ATP, subcutaneous hemorrhaging, liver necrosis, and hepatic damage in aquatic organisms (Eisler, 1991; Okonji et al., 2011). Cyanides are produced by certain bacteria, fungi, and algae and are found in a number of plants (Eisler, 1991; U.S. Department of Health and Human Services, 2006). The principal detoxification pathway of cyanide is catalysed by a liver mitochondrial enzyme, rhodanese.

RHODANESE

The enzyme rhodanese is ubiquitous in nature (Sorbo, 1951; Aminlari et al., 2002; Okonji et al., 2010). The enzyme is known to be responsible for the biotransformation of cyanide to thiocyanate (Sorbo, 1953; Jarabk and Westley, 1974; Lee et al., 1995). The overall reaction can be described according to the following scheme:

$$E + S_2O_3^{2-} \rightarrow ES + SO_3^{2-}$$

$$ES + CN^- \rightarrow E + SCN^-$$

The reaction proceeds by way of double displacement mechanism in which covalent enzyme-sulphur intermediate is formed (Westley, 1981). Rhodanese shows activity in all living organisms, from bacteria to man. It has been studied from variety of sources, which include bacteria, yeast, plants, and animals (Himwich and Saunders, 1948; Jarabak and Westley, 1974; Anosike and Ugochukwu, 1981; Lee et al., 1995; Agboola and Okonji, 2004). The activities of rhodanese enzyme have been detected in different species of fish (Okonji et al., 2010).

ANALYTICAL METHODS

There are very limited numbers of toxicological studies suitable for use in deriving a guideline value. Cyanide can be determined in water by both titrimetric and photometric techniques, methods that are described as been cumbersome. The determination of rhodanese follows spectrophotometric determination of cyanide present in experimental sample. The process is cheap and very fast to achieve. The assay follows the modification of the method described by Sorbo (1953), though; many scientists have come up with different methods with little variations in all the methods described (Lee et al., 1995; Nagahara et al., 1995; Agboola and Okonji, 2004).

PERSPECTIVES

Mining and regulatory documents often state that cyanide in water rapidly breaks down—in the presence of sunlight into largely harmless substances, such as carbon dioxide and nitrate or ammonia. However, cyanide also tends to react readily with many other chemical elements, and is known to form, at a minimum, hundreds of different compounds. Many of these breakdown compounds, while generally less toxic than the original cyanide, are known to be toxic to aquatic organisms. In addition, they may persist in the environment for long periods of time, and there is evidence that some forms of these compounds can be accumulated in plant (Eisler, 1991) and fish tissues (Heming and Blumhagen, 1989). Discussions on toxicity of cyanide and cyanide compounds focuses on fish, since they are the most sensitive species studied (Eisler, 1991), and are impacted by relatively low cyanide concentrations. For example, fish are killed by cyanide concentrations in the microgram per liter range, whereas

bird and mammal deaths generally result from cyanide concentrations in the milligram per liter range (Eisler, 1991). Acute toxicity is described as those concentrations of cyanide that lead to the death of more than 50% of the test population within 96 h (Ingles and Scott, 1987); chronic exposure can be lethal. Chronic toxicity describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer time period. In addition, chronic cyanide exposure may affect reproduction, physiology, and levels of activity of many fish species, and may render the fishery resource non-viable (Leduc, 1984; Ruby et al., 1986). Whether the toxic effects of HCN are cumulative, is apparently not known.

CONCLUSION

There are limited methods employed in quick detection of cyanide in water. The use of enzyme in environmental studies is becoming popular especially in area of toxicology. Rhodanese assay is a rather cheap and fast method of determining the presence of cyanide in polluted aquatic environment.

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Full Length Research Paper

Authenticating the origin of different shrimp products on the Tunisian markets by PCR/RFLP method

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This study describes a polymerase chain reaction using restriction fragment length polymorphism (PCR-RFLP) assay based on the 16S rRNA mitochondrial gene to identify commercial food products of wide range of *Penaeidae* and *Pandalidae* shrimp species commercialised in the Tunisian market. Phylogenetic analyses on 16S rRNA mitochondrial gene were used to study the relationships among the considered species. *Penaeidae* shrimp species was easily differentiated and confirmed by direct sequencing, showing a genetic distance of 0.34 with respect to *Pandalidae* species. A rapid and reliable PCR method using restriction fragment length polymorphism (RFLP) with three restriction enzymes (HpyCH4III/ MboI / AluI) was optimized for unambiguous differentiation of shrimp from 19 commercial market samples (raw and processed products). Results showed that the restriction fragment length polymorphism technique can be used to identify Tunisian shrimp species and thus to control not only commercial fraud but also efficient restocking program.

Key words: Shrimps, species identification, 16S rRNA mtDNA, PCR-RFLP.

INTRODUCTION

In recent decades, there has been a tendency towards increased globalisation of the fishery trade and a diversification of seafood in term of species and products, with prawns and shrimps being among the most internationally commercialised aquatic product (FAO, 2012). The shrimps of Tunisia belong mainly to the Penaeidae family; with a minority of Pandalidae of the decapod groups (Santos and Chaouchi, 2002). Penaeidae species, such as the caramote prawn *Penaeus kerathurus* and the deepwater rose *Parapenaeus longirosttris* are highly prized shellfish products with considerable commercial value. For instance *P. kerathurus* gained important market niche in some European countries (unpublished data), at the same time it is an overfished species submitted to fishing regulation (Ben Mariem, 1993). Such controversial

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Table 1. Shrimp species considered in this study, scientific names, commercial name, origin, 3-alpha codes, 16S rRNA sequence GenBank accession numbers.

Scientific name	Commercial name	Origin	FAO species code	16S rRNA accession numbers NCBI
Penaeus kerathurus	Caromate prawn	Tunisia	TGS	JX089981
Parapenaeus longirostris	Deepwater rose shrimp	Tunisia	DPS	JX089984
Metapenaeus monoceros	Speckled shrimp	Tunisia	MPN	JX089983
Plesionika heterocarpus	Shrimp	Tunisia	LKO	JX089982
Plesionika edwardsii	Shrimp	Tunisia	LKW	JX089986
Litopenaeus vannamei	White Leg shrimp	Dubaii	PNV	JX089979
Penaeus monodon	Black Tiger shrimp	Dubaii	GIT	JX089985
Pandalus borealis	Shrimp	Groenland	PRA	JX089980

situation may lead to adulteration to the consumers, but can also reduce the efficiency of management programs that help protecting over exploited species such as the Mediterranean *P. kerathurus*.

Adulteration occurs when the usual identifying characteristics are removed by processing and/or when accurate labeling is missed. It is therefore of prime importance to develop a common system for all fishery trading countries to control frauds when species substitutions occur. Such system should be recognised internationally and should include a data base through method diversification across species and laboratories for two main reasons. Firstly to exchange homologous data and secondly; to develop alternative but reliable method for species identification overcoming the lack of expensive product or consumable needed for such analysis especially in developing countries. Biochemical and molecular tools provide valuable support for the rapid and accurate identification of morphologically indistinct processed species.

Most of the genetic approaches for species identification are based on the amplification of a region of mitochondrial (mtDNA) DNA by polymerase chain reaction (PCR) (Maggioni et al., 2001; Khamnamtong et al., 2005; Calo-Mata et al., 2009; Pascoal et al., 2011; Benedict et al., 2013; Armani et al., 2015). The aim of this study was to develope a method that is quick and reliable molecular tool for the authentication of commercial shrimps in Tunisian markets. Therefore 19 commercial shrimp/prawn products collected from various markets were analyzed using PCR–RFLP of a 400-bp fragment in the mitochondrial 16S rRNA region.

MATERIALS AND METHODS

Commercial food products

The fresh specimens used in this study were caught along the Tunisian coast and identified morphologically by researchers using the FAO species identification keys. Shrimp-type products were purchased at local supermarkets or directly from Tunisian seafood companies. The scientific names are given in Table 1. Frozen products and pre-cooked products (Table 2) were purchased from local supermarkets. All samples (19) were frozen upon arrival in the laboratory.

DNA extraction

Total genomic DNA was individually extracted from muscle of shrimp and was performed following optimisation of the method (unpublished data): For this study, the optimisation concerned the concentrations of the Tris–HCl, the EDTA and the SDS, the duration of the tissue incubation and DNA precipitation. The optimal conditions were used and the result, in term of DNA concentration and purity, were compared to seven published methods including the phenol–chloroform-based approaches, the Triton and CTAB methods (with various modifications) and the use of commercial kit such as Chelex, kit fasta DNA, DNA extraction by DNeasy Tissue Kit. The tissue was chopped into small pieces and homogenised in an appropriate volume of the extraction solution (10 mM Tris–HCl, pH 8, 100 mM EDTA, 1% SDS, and 5 mg/ml proteinase K). The homogenate was incubated at 55°C overnight. DNA was extracted twice using phenol/chloroform/isoamyl alcohol (25:24:1) and recovered by ethanol precipitation. DNA concentrations were spectrophotometrically determined at using a NanoDrop; 1000 Spectrophotometer at the absorbance of 260 (A260) and 280 nm (A280). The purity of extracted DNA was determined by using A260/A280 ratio and later tested by PCR amplification.

Primer design

Using Mega software, a set of primers were originally designed for this study and were used for the amplification of mitochondrial 16S rRNA gene. Fifty crustacean shrimp 16S rRNA gene sequences were obtained from GenBank; nucleotide sequence alignment in the shrimp species help in identifying the specific fragments of the 16S rRNA mitochondrial region that lead to the design of the primers. In addition, 16S rRNA gene sequences of shrimp species purchased for this study were determined by a direct sequencing analysis of the amplified products using the sequencing primer pairs, namely:

PNF1 (forward primer 5'-GCGGTATTTTGACCGTGCGAAGG-3')
PNF2 (forward primer 5'-GCGAAGGTAGCATAATCATTAGTC-3')
PNR1reverse primer 5'-CAAAGAAGATTACGCTGTTATCCC-3')
PNR2 reverse primer 5'-GATTACGCTGTTACCCTAAAG-3')
PNR3 reverse primer 5'-CCTTAATTCAACATCGAGGTCGC-3').

Six set primer pairs based on the highly conserved sequence among the target species, PNF1/PNF2 and PNR1/PNR2 /PNR3 for detecting shrimp, were designed to amplify the 16S rRNA gene. For

Table 2. Authenticity determination of prawn and shrimp species in commercial frozen, pre-cooked products.

Sample	16S rRNA Accession numbers NCBI	Product type	Processing	Declared species	Detected species
FP1	JX196945	Frozen shrimps	Freezing	*P. kerathurus*	*P. kerathurus*
FP2	JX196948	Frozen shrimps	Freezing	*Par. longirostris*	*P. longirostris*
FP3	JX196950	Frozen shrimps	Freezing	*Par. longirostris*	*P. heterocarpus*
FP4	JX196951	Frozen shrimps	Freezing	*Par. longirostris*	*P. edwardsii*
FP5	JX196952	Frozen shrimps	Freezing	*M. monoceros*	*M. monoceros*
PP1	JX196946	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. kerathurus*
PP2	JX196947	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. kerathurus*
PP3	JX196949	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. longirostris*
PP4	JX089985	Cooked shrimps	Peeling + Boiling + Freezing	Black tiger	*P. monodon*
PP5	JX089979	Premium peeled shrimps	Peeling + Freezing	Shrimp	*L. vannamei*
PP6	JX196955	Breaded shrimps	Peeling + Frying + Freezing	Shrimp	*L. vannamei*
PP7	JX089980	Cooked shrimps	Peeling + Boiling + Freezing	Northern Shrimp	*P. borealis*
PP8	JX196953	Cooked shrimps	Peeling + Boiling + Freezing	Shrimp	*M. monoceros*
PP9	JX196954	Cooked shrimps	Peeling + Boiling + Freezing	Shrimp	*M. monoceros*

FP, Frozen products; PP, Pre-cooked product.

all species, one set of primer pairs PNF1/PNR3 were used in the PCR amplifications to obtain 400 bp DNA fragments of 16S rRNA.

PCR amplification

PCR amplifications were carried out using Bio-Rad MyCycler (Bio-Rad). The reactions were set up in volumes of 50 µL containing PCR buffer (75 mM Tris-HCl, pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$); 400 µM dNTP mix; 0.2 µM each primer; 2.5 units of UptiTherm DNA polymerase (Uptima- Interchim); and 100 ng of template DNA (PCI extracts). Amplification conditions were as follows: a previous an initial denaturation step of 94°C for 3 min, followed by 35 cycles consisting of denaturation (30 s at 9°C), annealing (30 s at 55°C) and extension (40 s at 72°C), and the final extension for 5 min at 72°C was also included.

Cleanup and sequencing of PCR products

Before sequencing, double-stranded PCR products were purified by filtration through a DNA Clean and Concentrator-25 of ZYMO RESEARCH CORP according to the manufacturer's protocol.

PCR fragments were used for direct cycle sequencing with the dye terminator cycle sequencing kit (Beckman). Sequencing analysis was performed with a Beckman Coulter CEQ 8000 DNA sequencer in both directions with the primers used for PCR amplification.

Sequence alignment, genetic distances, and phylogenetic analyses

The DNA sequences were edited with BioEdit software (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.1. (Tamura et al., 2011). Nucleotide divergences were computed using the Tamura-Nei model, (Tamura et al., 1993) which takes substitutional rate biases and the inequality of base frequencies into account. Phylogenetic trees were constructed using the neighbor-joining (NJ) method, (Saitou and Nei, 1987) and the robustness of topology nodes was tested by the bootstrap method with 1500 replicates. The new sequences were deposited in the NCBI GenBank.

RFLP analysis

The sequences of 400 bp fragments of the tested species from GenBank database were aligned using the Mega software to detect the presence of one or more restriction sites that could be characteristic of each species. On the basis of such analysis some restriction enzymes were selected for carrying out the RFLP analysis: HpyCH4III, MboI and AluI. (Fermentas Int., Burlington, Ontario, Canada).

The endonucleases with the position of restriction sites and the length of expected fragments in each of the tested species are reported in (Table 4). For each sample, 5-10 µL of the PCR reaction containing amplified DNA was digested overnight at 37°C with 2 U of each endonuclease in a final volume of 20 µL. The resulting fragments were separated by electrophoresis on a 2% agarose gel, the sizes of the resulting DNA fragments were estimated by comparison with a commercial 100-bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS AND DISCUSSION

PCR amplification of prawn and shrimp mtDNA from commercial food products

Although DNA exhibits fairly high thermal stability, it is well known that intense heat may cause severe DNA degradation. A direct relationship between heat treatment and the intensity of DNA fragmentation has been reported (Chapela et al., 2007; Besbes et al., 2011). Other processing conditions, including overpressure conditions and the presence of additives that may inhibit DNA polymerase have also been reported as important cause for the lack of amplification (Pascoal et al., 2008;

2011; Besbes et al., 2011). For PCR amplification, the melting temperature (Tm) correlates with how easily a double stranded DNA complex is formed. The Tm is affected by a number of factors such as concentration of DNA, concentration of ions in the solution (specially Mg^+ and K^+), nucleotide sequences (that is, nucleotide pair 'A-T' has a weaker bond than the nucleotide pair 'G-C') and the length of the DNA fragment (Liedl and Simmel, 2007).

Several studies revealed the usefulness of a 1.38 kb mitochondrial region that comprises fragments of the 16S rRNA and 12S rRNA genes and the entire tRNAVal region for phylogenetic analysis of penaeid shrimps (Gutiérrez-Millan et al., 2002; Rasmussen et al., 2008). Moreover, the mitochondrial gene coding for 16S rRNA has been reported to be a good candidate for authentication of fish and seafood due to its acceptable length, mutation rate, and availability of sequence information in databases. Other studies described Pascoal et al., 2011). In this study, we amplified and sequenced a mitochondrial marker corresponding to 16S rRNA gene in an effort to improve fraud detection of shrimp for the first time in Tunisia, and for its validation in commercial products.

The analysis of the sequences obtained revealed that most of the fragments are very conserved between the species studied, with very low interspecific and intraspecific variability found in 16s rDNA located in the mtDNA. Although a lower evolution rate has been described for this fragment, this rate depends on the species groups, with some reports showing its usefulness for population studies (Chapela et al., 2002).

In this study a total of 19 different commercial food products containing or consisting of shrimps were subjected to DNA extraction and purification. The primers were designed to anneal the 16S rDNA region; both primers have equal length (23 nucleotides), GC content (56.52 and 47.82%) and Tm (72°C and 68°C), low self-annealing, and low complementarities between them. DNA amplification was successful in all primers and for all samples analyzed, showing that the designed primers are useful to amplify mitochondrial DNA, not only in the species whose sequences were employed to design them, but also in other species of crustaceans belonging to families *Penaeidae* and *Pandalidae*.

Accordingly, the size of the molecular target (400 bp) proved to be accurate to achieve PCR amplification even in processed products subjected to a variety of technological treatments such as boiling, frying or freezing. Such procedure was also effective in food including shrimp or prawn as ingredients.

Species identification of commercial shrimp food product

It is well recognized that, identification of unknown sample can be performed if the unknown sequence is introduced in the estimation of genetic distance among a set of reference sequences. The unknown sequence will show the lowest distance value with the species groups to which it belongs (Chapela et al., 2002). In this study, the correct labelling for the different processed shrimp-products found in the local Tunisian market was only for species with the common name deepwater pink shrimp or Caromate shrimp. The others imported products were labelled shrimp or did not have any genera specification on their labels, and were phenotypically indistinguishable due to processing. In six of the processed products, the included Decapoda crustacean species were identified as ''prawns" or ''shrimps" (Table 2). Consequently, the DNA amplification products were sequenced and aligned against the 16S rRNA sequences of shrimp obtained from the NCBI GenBank (Table 1). The new sequences, which were equally deposited in the NCBI GenBank, were aligned against the other *Penaeidae* and *Pandalidae* species sequences allowing the construction of a neighbour-joining tree (NJ) (Figure 1). Thus the constructed phylogram showed two distinct family clades, for the two families *Penaeidae* and *Pandalidae*, each associated with each reference species. The genus *Penaeus*, *Litopenaeus*, *Parapenaeus* and *Metapenaeus* are grouped in the clade *Penaeidae* and the species *Plesionika* and *Pandalus* are grouped in the clade *Pandalidae*.

The species exhibiting the highest values of intra-specific homology were *P. kerathurus* (100%), *M. monoceros* (100%), *P. longirostris* (97%), *L. vannamei* (96%), *P. monodon* (75%), *P. heterocarpus* (100%), *P. edwardsii* (100%) and *P. borealis* (99%).

The nucleotide sequences determined for the prawn specimens present in the commercial peeled and frozen products were compared (Figure 1). Fresh *P. kerathurus* (F), *P. kerathurus* food products (FP1, PP1, PP2) and the *P. kerathurus* reference were grouped in the same cluster with a bootstrap value at the nodes of 100%. The shrimp species names in commercial pre-cooked products (PP6, PP5) were not indicated on packages. The genetic identification revealed that species were grouped in the same cluster of *Litopenaeus vannamei* with a bootstrap values at the nodes of 96 and 91% respectively. The commercial pre-cooked products (PP8, PP9), which were not indicated on labels, were grouped in the same cluster of *Metapenaeus monoceros* with a bootstrap values at the nodes of 100%.

The clade of *Pandalidae* grouped PP7 in the same cluster of *Pandalus borealis* with a bootstrap value at the nodes of 99%, and (FP3, FP4) were grouped of *Plesionika heterocarpus* and *P.edwardsii* respectively with a bootstrap values at the nodes of 100%. DNA sequencing and nucleotide analysis confirmed that sample PP5, PP6 (not declared species) were *L. vannamei*, samples PP8, PP9 were *M.monoceros*, and samples FP3, FP4 were *plesionika* species, thus confirming mislabeling of these samples.

Species divergence is an another tool to evaluate

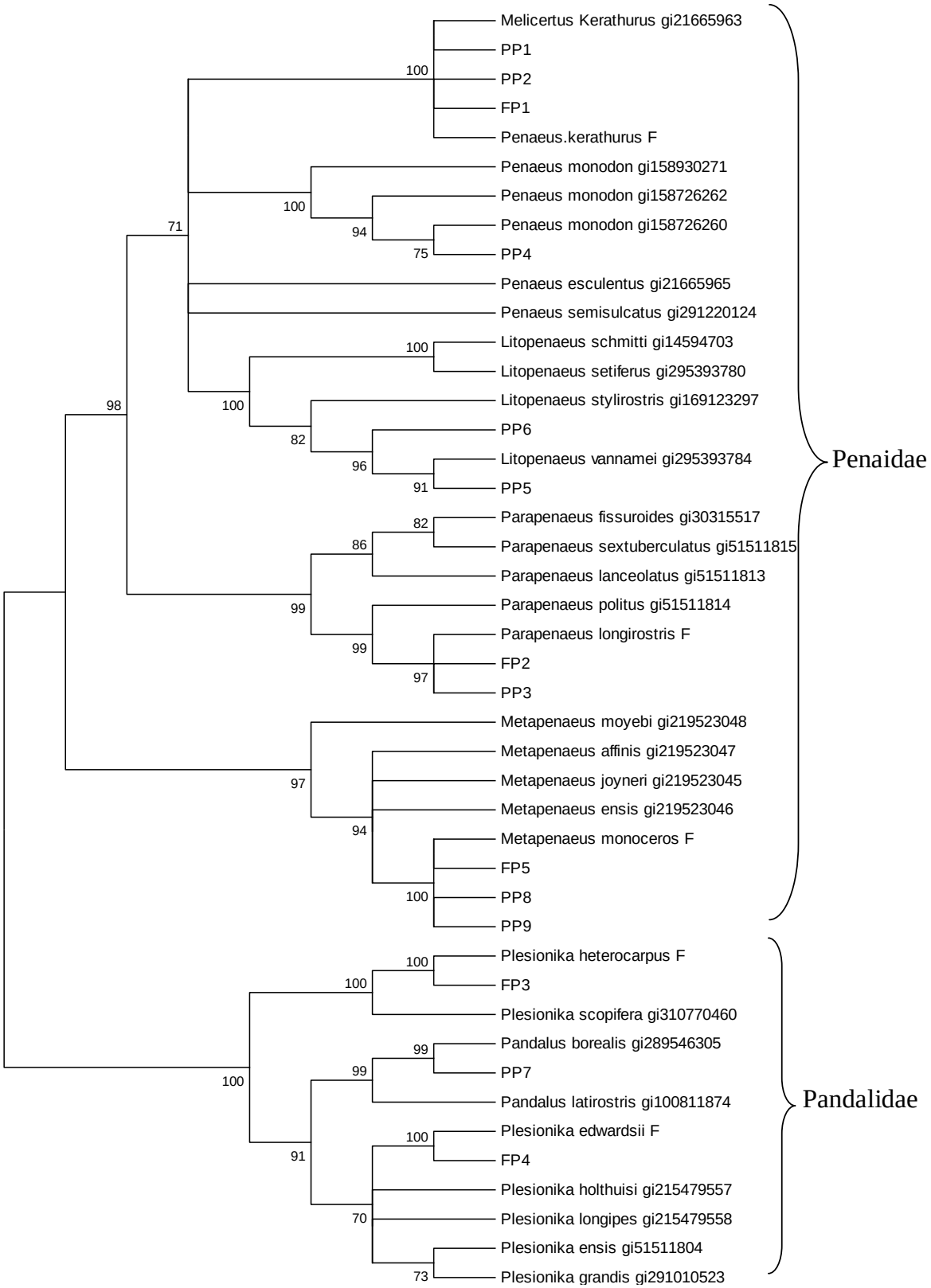

Figure 1. Phylogenetic tree obtained from sequence divergence of a 16S rRNA mitochondrial gene for Nineteen sequences of Penaidae and Pandalidae Shrimp Species. NJ tree inferred from TN distances between sequences of partial 16S rRNA gene in 16 samples of Penaidae and 3 Panadalidae species and the sequenced gene referenced in the NCBI GenBank; Numbers denote bootstrap percentages based on 1500 replications from neighbor-joining analysis.

Table 3. Pairwise sequence divergences for the 16S rRNA mitochondrial gene for *Penaeidae* and *Pandalidae* shrimp estimated by the Tamura and Nei method.

Genera	F1	F2	F3	F4	F5	PP1	PP2	FP1	FP2	PP3	PP8	FP5	PP9	PP5	PP6	FP3	FP4	PP4
P. kerathurus-F1																		
Par. longirostris-F2	0.11																	
Met. monoceros-F3	0.17	0.13																
Pl. heterocarpus-F4	0.34	0.31	0.33															
Pl. edwardsii-F5	0.33	0.32	0.29	0.31														
P. kerathurus-PP1	0.01	0.11	0.17	0.34	0.33													
P. kerathurus-PP2	0.01	0.11	0.17	0.34	0.33	0.01												
P. keratherus-FP1	0.01	0.11	0.17	0.34	0.33	0.01	0.01											
Par. longirostris-FP2	0.11	0.00	0.13	0.31	0.32	0.11	0.11	0.11										
Par. longirostris-PP3	0.11	0.00	0.13	0.31	0.32	0.11	0.11	0.11	0.01									
Met. monoceros-PP8	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13								
Met. monoceros-FP5	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13	0.01							
Met. monoceros-PP9	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13	0.01	0.01						
Lit. vannamei-PP5	0.14	0.12	0.17	0.40	0.33	0.14	0.14	0.14	0.12	0.12	0.17	0.17	0.17					
Lit. vannamei-PP6	0.13	0.12	0.17	0.40	0.33	0.13	0.13	0.13	0.12	0.12	0.17	0.17	0.17	0.01				
Pl. heterocarpus-FP3	0.34	0.31	0.33	0.01	0.31	0.34	0.34	0.34	0.31	0.31	0.33	0.33	0.33	0.40	0.40			
Pl. edwardsii-FP4	0.33	0.32	0.29	0.31	0.01	0.33	0.33	0.33	0.32	0.32	0.29	0.29	0.29	0.33	0.33	0.31		
P. monodon-PP4	0.11	0.13	0.18	0.37	0.31	0.11	0.11	0.11	0.13	0.13	0.18	0.18	0.18	0.13	0.13	0.37	0.31	
Pd. borealis-PP7	0.32	0.27	0.28	0.31	0.23	0.32	0.32	0.32	0.27	0.27	0.28	0.28	0.28	0.34	0.33	0.31	0.23	0.30

similarity of an unknown sequence with a pool of reference samples. In this study, the genetic distances of *Penaeidae* was easily differentiated from the studied *Pandalidae* species (0.34) (Table 3). The genetic distance measured between *P. kerathurus* and the *P. kerathurus* processed product was 0.01 and with *P. longirostris* species was 0.11 and with *M. Monoceros* was 0.17 and with Plesionika species was 0.34.

The identification of the shrimp species and the different processed food products could be easily achieved by PCR-RFLP analysis. The differentiation of the shrimp could be achieved by cleaving the different species on two groups (Pink and White shrimp) according to morphological similarity (and to the fact that their industrial processing often removes their external shell). On one hand the Pink shrimps groups composed of *Parapenaeus longirostris*, *Pandalus borealis*, *Plesionika edwardsii* and *Plesionika heterocarpus* was discriminated and cleaved with HpyCH4III (Table 4 and Figure 2A). The white prawn groups composed of *Litopenaeus vannamei*, *Penaeus kerathurus*, *Metapenaeus monoceros* and *Penaeus monodon* were discriminated and cleaved with MboI (Figure 2B). However, *Litopenaeus vannamei*, *P. kerathurus* displayed the same profile, and were discriminated with AluI (Figure 2C). Species identification by sequencing and PCR-RFLP revealed that among the food products analysed, three were not correctly labelled (Table 2).

Concerning species substitution, some instances were likely unintentional probably because of phenotypic similarities. This is the case of *P. longirostris* which was confounded with *P. ldwardsii* and *P. leterocarpus* species (distributed in Tunisia waters) because they are of very similar phenotype and caught together in mixed fisheries. Moreover, endonuclease HpyCH4III also allowed the differentiation of *P. longirostris* species from *Plesionika* species, since the 400 bp PCR product exhibits a sequence recognized by HpyCH4III in the case of *P. longirostris* thus producing five restriction fragments of (380 bp, 261 bp, 176 bp, 127 bp and 61 bp) (Table 4). Other detected cases of incorrect labelling involved the complete substitution of *P. keratherus* by the pink prawn *P. longirostris* PP3 (Table 3). *P. longirostris* of lower commercial value than *P. keratherus* was easily distinguished with the restriction enzymes HpyCH4III (Table 4).

The risk of miss-identification would be the similarity in external morphological feature such as the case of the shrimp species having the same colors of the carapace (*P. longirostris*, *Pandalus borealis*, *P. edwardsii* and *P. heterocarpus*). In this study, such species were easily distinguished with the restriction enzymes HpyCH4III. Other cases of incorrect labelling involved the possibility of substitution of *P. keratherus* with imported species such as *L. vannamei* and *P. monodon*. These species were also, easily distinguished with the restriction enzymes MboI. Hence, the molecular method used in this work was revealed as a valuable tool to circumvent these problems, and was successful for the identification of a large number of crustacean products found in the local market.

Table 4. RFLP specific patterns (bp) for the *Penaeidae* and *Pandalidae* shrimp species considered as references in this study.

Shrimp species	Restriction enzyme		
	HpyCH4III	**MboI**	**AluI**
P.longirostris-F2	380+261+176+127+61		
P.longirostris-FP2	380+261+176+127+61		
P.longirostris-PP3	380+261+176+127+61		
P.borealis-PP7	235+121		
P.heterocarpus-F4	209+86+61		
P.heterocarpus-FP3	209+86+61		
P.edwardsii-F5	147+121+87		
P.edwardsii-FP4	147+121+87		
P.kerathurus-F1		276+86	222+81+69
P.keratherus-FP1		276+86	222+81+69
P.kerathurus-PP1		276+86	222+81+69
P.kerathurus-PP2		276+86	222+81+69
M.monoceros-F3		167+110+86	
M.monoceros-FP5		167+110+86	
M.monoceros-PP8		167+110+86	
M.monoceros-PP9		167+110+86	
L.vannamei-PP5		277+72+14	223+140
L.vannamei-PP6		277+72+14	223+140
P.monodon-PP4		183+93+86	

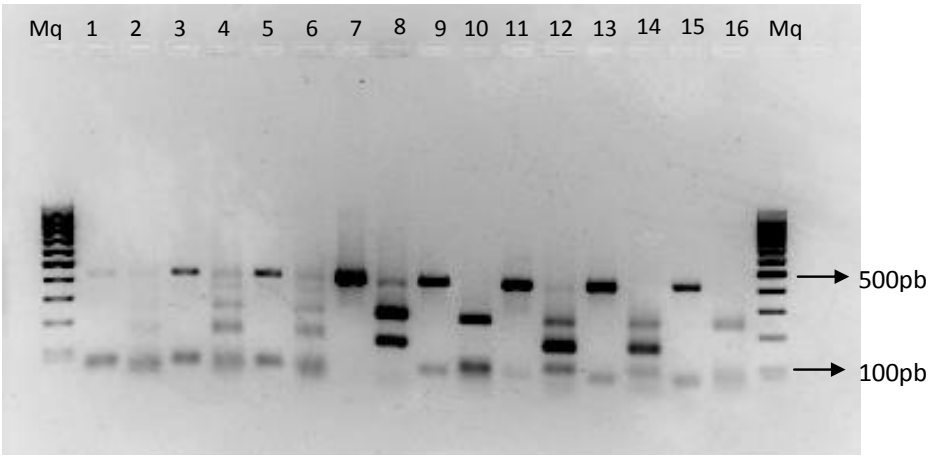

Figure 2A. Example of PCR-RFLP identification on 2% agarose gel of pink prawn fresh and food product with the restriction by HpyCH4III of 16S rRNA mitochondrial gene **(A)** from left to right, Mq:molecular weight marker (100pb DNA ladder, Protech Technology), DNA fragment products obtained with *P.longirostris* F2, FP2, PP3 (lane 2, 4, 6), *P.borealis*-PP7(lane 8), *P.heterocarpus*-F4 (lane 10), *P. edwardsii*-F5 (lane 12), *P.edwardsii*-FP4(lane 14), *P.heterocarpus*-FP3 (lane 16), and Ctrl: Non-digested fragment *P. longirostris* F2, FP2, PP3 (lane 1, 3, 5), *P.borealis*-PP7(lane 7), *P. heterocarpus*-F4 (lane 9), *P.edwardsii*-F5 (lane 11), *P.edwardsii*-FP4(lane 13), *P.heterocarpus*-FP3 (lane 15).

This study demonstrates that PCR-RFLP method, aimed at the molecular analysis of a 400 bp mtDNA fragment on the basis of the use of novel PNF1/PNR3 primers targeted to conserved regions of the 16S rRNA

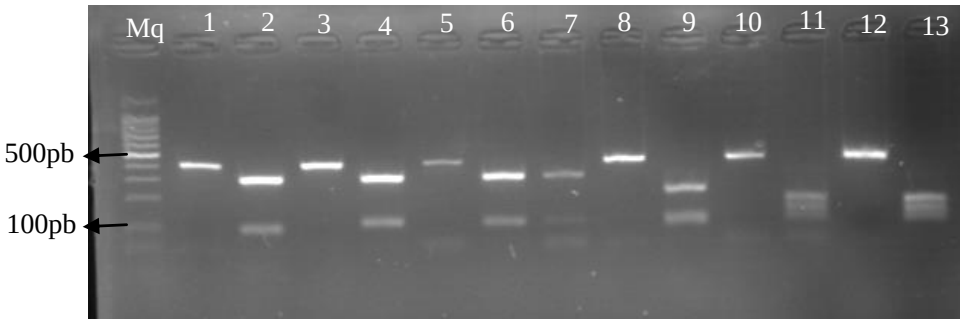

Figure 2B. white shrimp fresh and food product with the restriction by MboI of 16S rRNA mitochondrial gene from left to right, Mq : molecular weight marker (100pb DNA ladder, Protech Technology), DNA fragment products obtained with *L.vannamei*-PP5 (lane 2), *P.kerathurus*-F1, PP1, PP2 (lane 4,6,7), *P.monodon*-PP4 (lane 9), *M.monoceros*-F3, PP8 (lane 11,13), and Ctrl: Non-digested fragment *L.vannamei*-PP5 (lane 1), *P.kerathurus*-F1, PP1, PP2 (lane 3, 5), *P.monodon*-PP4 (lane 8), *M.monoceros*-F3, PP8 (lane 10,12).

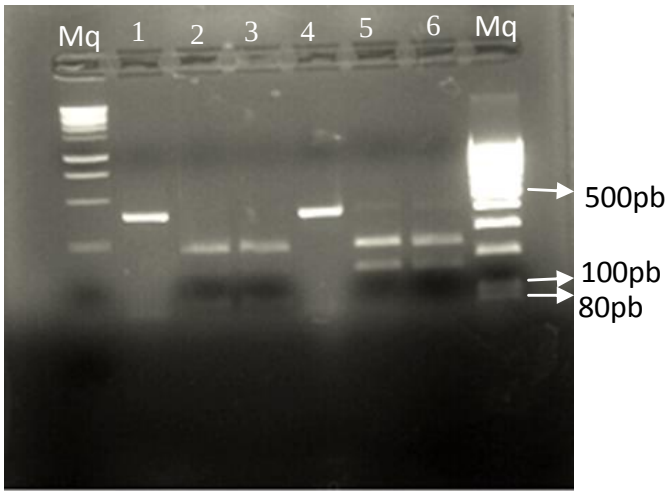

Figure 2C. shrimp fresh and food product with the restriction by AluI of 16S rRNA mitochondrial gene from left to right, Mq (1): molecular weight marker (1 Kb DNA ladder), Mq (2): molecular weight marker (100 pb DNA ladder, Protech Technology) DNA fragment products obtained with *P.kerathurus*-F1, PP1(lane 2,3), *L.vannamei*-PP5, PP6 (lane 5,6), and Ctrl: Non-digested fragment *P.kerathurus* (lane 1), *L.vannamei* (lane 4), (n=3 in each case).

represents an additional data to previous reported methods targeting other regions of the mtDNA. The diversification of method is necessary as molecular tools are not always favourable to adaptation by multiples sectors or countries; these tools are however valuable to the authorities for the rapid and accurate identification of morphologically indistinct species of commercial interest. Such need is more obvious with the increased international shrimp/ prawns trade where control should be reinforced to mitigate imports of product with lower unit value such as cultured shrimps, but also exports of overexploited species such as *P. kerathurus* outside the fishing seasons.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Diversity analysis of the immunoglobulin M heavy chain gene in Nile tilapia, *Oreochromis niloticus* (Linnaeus)

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A full-length cDNA encoding the immunoglobulin (IgM) heavy chain gene of Nile tilapia was successfully cloned using the 5' and 3' RACE techniques. The complete cDNA of the Nile tilapia IgM heavy chain gene is 1,921 bp in length and has an open reading frame (ORF) of 1,740 bp, which corresponds to 580 amino acid residues. The deduced amino acid sequence of the Nile tilapia IgM heavy chain includes a typical secretory IgM heavy chain designated "*On*-sIgM" and a variable region that is connected to 4 constant regions to form the L_H-V_H-C_μ1-C_μ2-C_μ3-C_μ4 pattern. Comparisons of the nucleotide and amino acid sequences of *On*-sIgM with IgM heavy chains of other organisms showed the highest similarity scores of 62.6 and 55.4%, respectively, to the orange-spotted grouper (*Epinephelus coioides*). Structural analysis of 126 cDNAs encoding variable domains of the IgM heavy chain revealed that at least 9 V_H families, 6 D_H segments and 4 J_H families were utilized using several mechanisms to generate the repertoire of antigen-binding domains. Variation analysis of the variable domains indicated that the amino acid sequences of the framework regions (FRs) were less variable than those of the complementarity determining regions (CDRs), among which the most variable was CDR3. Tissue expression profile analysis using quantitative real-time RT-PCR of healthy Nile tilapia showed that the IgM heavy chain gene was ubiquitously expressed in all 13 tested tissues, but the highest expression level was observed in the head kidney, followed by the spleen, intestine and peripheral blood leukocytes (PBLs). Furthermore, Southern blot analysis of the constant region of the IgM heavy chain gene of 3 different fishes indicated that Nile tilapia genomes may contain 2 copies of the IgM gene.

Key words: Nile tilapia, IgM heavy chain, variable region, diversity, secreted form, southern blot.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish that is cultured worldwide and is an important economic aquatic animal. The global production of tilapia was 3.6 million tons in 2011, and yearly increases are thought to continue. In 2010, tilapia production was focused in 2 leader countries (China and Egypt), with productions of 1,331,890 and 557,049 tons, respectively. In 2014, 174,872 tons were produced in Thailand as result of

increased aquaculture throughout the country, ranging from earthen ponds to intensive cage-culture systems, making Thailand the 5th largest world tilapia producer (FAO, 2014). This activity has also been promoted by related industries and supply chains, such as hatcheries, feed manufacturers and distributors, to support the huge domestic consumption. However, this intensive culture system has a high risk of disease outbreaks. The bulk of fish deaths occurs due to protozoan, fungal and bacterial infections, typically due to *Aeromonas hydrophila, Streptococcus agalactiae* and *Flavobacterium columnare*, and result in lost tilapia yields in culture systems (Mohamed and Refat, 2011; Pridgeon et al., 2011; Rodkhum et al., 2011). To circumvent these problems, fish immunity must improve to increase the efficiency of disease prophylactic and therapeutic methods. The immune systems of vertebrates govern homeostasis, prevention and surveillance and are generally divided into 2 parts: innate and adaptive immune systems. The adaptive or acquired immune system, which was discovered in cartilaginous fish, is distinguished from the innate immune system by antibody (immunoglobulin) production by plasma B cells and the functions of cytotoxic T cells associated with the degranulation process. Antibody production and degranulation are potent and effective methods used to specifically eliminate pathogenic infection. Immunoglobulins are important molecules in jawed vertebrates, ranging from gnathostomes to tetrapods, but are not found in invertebrates (Flajnik, 2002; Flajnik and Du Pasquier, 2004). The fundamental functions of immunoglobulins include toxic neutralization, the promotion of phagocytosis by opsonization and activation of the complement system (Walport, 2001; Holland and Lambris, 2002). An immunoglobulin molecule is composed of 2 heavy chains and 2 light chains that are joined by inter- and intra-disulfide bonds.

In bony fish, 3 major isotypes of immunoglobulins exist: IgM, IgD and IgT/IgZ (Hikima et al., 2011; Salinas et al., 2011). Immunoglobulin isotypes are determined by the constant region (C_H), which also dictates the effector function of the molecule in different types of immune responses. The variable region contains the antigen-binding site (Roitt et al., 2001) and is located at the N-terminus of the heavy and light chains. Heavy chains are composed of a variable segment (V_H), a diversity segment (D_H), which is not found in the light chain, and a joining region (J_H). The variable regions of the heavy and light chains consist of 4 framework regions (FR1-4) and 3 complementarity determining regions (CDR1-3), or hyper-variable regions. The CDRs are highly variable in Nucleotide sequence because of considerable contact with antigens (Pilstrom and Bengten, 1996).

The mechanisms used to generate the diverse immunoglobulins in higher vertebrates can be summarized into at least 7 events that consist of combinatorial diversity, junctional imprecision, junctional diversity, gene conversion, secondary $V_{H/L}$ gene recombination, somatic hypermutation and heavy/light chain pairing. During B cell development, the diversity of antigen-binding elements begins with rearrangement mediated by recombination-activating gene (*RAG*), which initiates the assembly of the antigenic binding domain of immunoglobulins, Artemis (DNA repair proteins) and terminal deoxynucleotidyl transferase (TdT), which are utilized for P and N nucleotide addition, respectively (Lieber, 1992; Kuo and Schlissel, 2009).

In Osteichthyes, secreted IgM (sIgM) is one of the major proteins in the serum and is generated during immune responses against pathogenic infection. IgM is classified as the primordial immunoglobulin of the adaptive immune response and is found in monomeric and tetrameric forms in circulating blood (Acton et al., 1971; Wilson and Warr, 1992). IgM can exist in 2 forms, sIgM and membrane-bound (mIgM), which are generated *via* alternative RNA splicing of the primary transcript of the µ gene (Ross et al., 1998). sIgM consists of the variable region and 4 constant domains in the heavy chain, whereas mIgM contains variable region, 3 constant domains and 2 additional transmembrane domains (T_M1 and T_M2) and acts as a B cell receptor for initial antigen binding (Dylke et al., 2007). To date, the cloning and characterization of the IgM gene has intensively been reported in holostean, cartilaginous and teleost fish (Rauta et al., 2012). However, information about the mechanisms important for generating diversity for antigen binding is reported in some teleost fish but still lacking in Nile tilapia.

The aim of this study was to increase the understanding of the teleost immune system, specifically IgM, which is the most vital humoral molecule for adaptive immune responses.

This study performed molecular characterization of the full-length cDNA of the IgM heavy chain gene of Nile tilapia and the diverse expression of its variable domain were intensively investigated. In addition, tissue distribution analysis was performed using quantitative real-time RT-PCR, and genomic structural analysis of the gene was performed using Southern blot analysis. Information from the current study may provide a better understanding of the adaptive immune system of Nile tilapia.

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Table 1. Oligonucleotide primers used for PCR analysis.

Gene name	Oligonucleotide primer	Sequence 5' → 3'	Amplicon size	Experiment
IgM heavy chain	- IgMuF1	AGGAGACAGGACTGGAATGCACAA	-	3' RACE-PCR
IgM heavy chain	- IgMuR1	TTGTGCATTCCAGTCCTGTCTCCT	-	5' RACE-PCR, Variable domain analysis
IgM heavy chain	- IgMuF2	GGATGATACCTATACTGCCTCCTG	174	Real-time PCR
IgM heavy chain	- IgMuR22	AATCTAGTCTGATCATTCAGGTCA	174	Real-time PCR
ß-actin	- ß-actinF2	ACAGGATGCAGAAGGAGATCACAG	155	Real-time PCR
ß-actin	- ß-actinR2	GTACTCCTGCTTGCTGATCCACAT	155	Real-time PCR
-	- UPM-long	CTAATACGACTCACTATAGGGCAAGCATGG TATCAACGCAGAGT	-	RACE-PCR
-	- UPM-short	AAGCAGTGGTATCAACGCAGAGT	-	RACE-PCR
Constant region of IgM heavy chain	- SB F	GGATGATACCTATACTGCCTCCTG	533	Southern blot
Constant region of IgM heavy chain	- SB R	GGTGAACAACACAGAAGCGTGT	533	Southern blot

MATERIALS AND METHODS

Experimental animals

Healthy adult Nile tilapia weighing 500 to 600 g were obtained from the Department of Aquaculture, Faculty of Fisheries, Kasetsart University. The fish were maintained in aerated water tanks and fed with commercial feed twice a day for a week.

Cloning of the full-length cDNA of the IgM heavy chain gene of Nile tilapia

Total RNA from the head kidney and spleen of an adult Nile tilapia was extracted using TRIzol reagent (Gibco BRL, USA) according to the manufacturer's instructions. The mRNAs were consequently prepared using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, USA). Five hundred micrograms of mRNA from each organ were pooled, 1 µg of mixed mRNA was used per reaction, and 5' and 3' first-strand cDNA were synthesized using the BD Smart RACE cDNA Amplification Kit (Clontech, USA). The cDNAs were then used as templates for 5' and 3' RACE PCRs, which were conducted using the specific primers IgMuF1 and IgMuR1, respectively (Table 1). These primers were designed from the EST clone HK0156 encoding the partially constant region of the Nile tilapia IgM (GenBank accession no. FF279636). The PCR conditions included pre-denaturation for 5 min at 95°C; 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s; and a final elongation step at 72°C for 5 min. The 5' and 3' RACE PCR products were purified using the HiYield™ Gel/PCR Fragments Extraction Kit (RBC Bioscience, Taiwan), ligated into the pGEM T-Easy cloning vector (Promega, USA) and transformed into *Escherichia coli* strain JM 109, which was grown on Luria Bertani (LB) agar containing ampicillin (0.01 g/mL), IPTG (100 mM) and X-gal (50 mg/mL). Each plate of transformants was incubated at 37°C for 18 h. Positive clones, that is, white colonies, were selected, and plasmids were extracted using the Plasmid DNA Extraction Manual Kit (Bio Excellence, Thailand). Nucleotide sequencing of the selected clones in the 5' and 3' directions was performed by Macrogen, Inc. (Korea) using the M13F and M13R primers with the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Characterization of the full-length cDNA of the IgM heavy chain gene of Nile tilapia

After sequencing, the nucleotide sequences were screened for vector contamination, and vector sequences were removed using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/ VecScreen.html). The nucleotide sequences from the 3' and 5' fragments were multiply aligned to find overlapping regions and compared with nucleotide and amino acid sequences of other vertebrate IgMs in the GenBank database using the BLASTN and X programs (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The coding sequences, conserved residues and signature motifs that were important for immunoglobulin functions and structure were determined using the IMGT (International ImMunoGeneTics Information System) database (http://www.ebi.ac.uk/imgt/) and other publications. The full-length cDNA of the IgM heavy chain in Nile tilapia was examined to predict its signal peptide sequence using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/ SignalP/). The similarity and identity of the nucleotides and amino acids of the IgM heavy chain of Nile tilapia and other vertebrates were calculated using the MatGat 2.02 program (http://bitincka.com/ledion/matgat).

Phylogenetic analysis

The deduced amino acid sequences of the constant domain of the IgM heavy chain, consisting of the Cµ2-Cµ4 domains, in Nile tilapia and other vertebrates (gnathostomes to mammals) were multiply aligned using CLUSTALW. Cµ1 was excluded because it is known as the high conserved region resulted from the evolutionary duplicated to generate other C regions. The IgD heavy chains of Mandarin fish, *Siniperca chuatsi* (ACO88906), and grouper fish, *Epinephelus coioides* (AEN71108), were used as outgroups for the phylogenetic tree. Then, the evolution of the IgM heavy chain gene was determined using the UPMGA method by performing 1,000 bootstrap resampling replicates with the MEGA program, version 5.05 (http://www.megasoftware.net).

Construction of a cDNA library of the variable domain of the IgM heavy chain gene

The cDNA library was constructed using 5' RACE PCR with the

specific primer IgMuR1 (Table 1). Briefly, the previously prepared, ready-to-use, first-strand cDNA template for 5' RACE PCR was amplified, cloned and sequenced using the same protocols described above.

Diversity analysis of the variable domain

After sequencing, the entire nucleotide sequences of randomly selected clones were analyzed for homology with other sequences available in the GenBank database using the BLASTN and BLASTX programs, as previously described. A representative sequence from each redundant group was arbitrarily chosen for further family classification. The resulting 126 cDNA sequences were analyzed to find the leader sequence, FR and CDR according to the IMGT standardization numbering. Each of the V_H families, D_H segments and J_H families was classified using the CAP3 program (http://bioweb.pasteur.fr/seqana/ interface/cap3. html). The V_H family was grouped based on the percentages of nucleotide sequence identity in the same V_H family greater than 80% (Brodeur and Riblet, 1984). Then, the similarity and identity of the V_H, D_H and J_H amino acids were calculated using MatGat 2.02, and multiple alignments were performed using the CLUSTALW program. To examine the degree of sequence variability in the variable region of Nile tilapia IgM, the deduced amino acid sequences were multiple-aligned and calculated as the position variability using the Kabat and Wu method (Kabat and Wu, 1971) and Shannon analysis (Stewart et al., 1997).

Tissue distribution of IgM heavy chain gene by quantitative real-time PCR

Total RNA from the brain, gills, gonad, heart, head kidney, intestine, liver, muscle, skin, spleen, stomach, peripheral blood leukocytes and trunk kidney of a healthy Nile tilapia was extracted using TRIzol reagent (Gibco BRL, USA). The contaminating genomic DNA was digested with RNAse-free DNAse I (Fermentas, USA), and first-strand cDNA synthesis was performed using 1 µg of total RNA from each tissue with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). First-strand cDNA from the 13 tissues was quantitatively examined using the IgMuF2 and IgMuR22 primers (Table 1). The expression levels were normalized to the expression level of beta-actin mRNA using the β-actinF2 and β-actinR2 primers (Table 1). The quantitative real-time RT PCR was conducted using an Mx Pro^{TM} 3005P QPCR (Stratagene, USA), and the mRNA expression of the IgM heavy chain and beta-actin genes was detected using Brilliant II SYBR Green qPCR Master Mix (Stratagene, USA). The cycling conditions consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. For each sample, 3 replicates were performed for the IgM heavy chain and β-actin mRNAs. Standard curves were constructed to examine the efficiency and specificity of both specific primer sets. PCR efficiencies for IgM heavy chain and β-actin genes were 2.015 and 2.023, respectively. The relative expression ratio of the IgM heavy chain gene in Nile tilapia was calculated according to the $2^{-\Delta\Delta C}{}_T$ formula (Livak and Schmittgen, 2001). Statistical analysis was performed using the SPSS program, version 13.0. Differences in the expression levels of the Nile tilapia IgM heavy chain gene in the 13 tissues were analyzed using one-way analysis of variance (ANOVA), and the means were compared using Duncan's new multiple range test. The significance level was established at $P<0.05$.

Southern blot analysis

Genomic DNA was isolated from the whole blood of 3 different Nile tilapias and was subjected to phenol-chloroform treatment, as described by Taggart et al. (1992). Ten micrograms of DNA from each fish were completely digested with the *Eco* RI and *Pst* I restriction enzymes, and electrophoresis in a 1% agarose gel was performed to separate the DNA fragments. The DNA fragments were then transferred to a nitrocellulose membrane using the capillary blotting method with 20X SSC, and the membrane was dried and baked at 80°C for 2 h in a hot-air oven. Probes specific for the Cµ2-Cµ3 constant regions were prepared by PCR using the designed primers SBF and SBR (Table 1). PCR probes were labeled with Digoxigenin-11-dUTP using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) according to the instruction manual. The membranes were incubated in hybridization solution (DIG Easy Hyb) with denatured, DIG-labeled DNA probe at 68°C overnight. After hybridization, the membranes were stringently washed twice in ample 2X SSC, 0.1% SDS at 25°C for 5 min and in 0.5X SSC, 0.1% SDS at 68°C for 15 min under constant agitation. Then, immunological detection of the membrane was carried out following the procedure recommended by the manufacturer. Finally, color detection with NBT/BCIP was performed to investigate the intensity of the bands by photography.

RESULTS

Cloning and characterization of a full-length cDNA encoding the IgM heavy chain gene in Nile tilapia

A complete full-length cDNA of the IgM heavy chain gene in Nile tilapia was successfully cloned using 3' and 5' RACE PCR. The full-length cDNA was 1,921 nucleotides in length and composed of a 45-nucleotide 5' untranslated region (UTR) that was followed by the open reading frame (ORF) beginning with ATG, the first translated codon. The length of the ORF was 1,740 bp and encoded 580 amino acids, and the leader peptide was predicted to consist of 26 amino acids. Translation terminated at nucleotide position 1,786, which encoded TAG, the stop codon. The length of the 3' UTR was 90 nucleotides and included the polyadenylation signal (AATAAA) and poly A tail (Figure 1). The deduced amino acid sequence of the Nile tilapia IgM heavy chain gene included a typical heavy chain sequence for secretory IgM, which was termed "*On*-sIgM". Its organization began with 1 variable region and 4 constant regions that formed a L_H-V_H-$C_\mu1$-$C_\mu2$-$C_\mu3$-$C_\mu4$ pattern, which is different from the teleost fish IgM membrane-bound form that is generally rearranged as L_H-V_H-$C_\mu1$-$C_\mu2$-$C_\mu3$-T_M1-T_M2 (Saha et al., 2005; Tian et al., 2009). The potential N-linked glycosylation sites were found as NSS in the Cµ2, NKT in the Cµ3 and 2 NTTs in the Cµ4 domain (Figure 1). Comparisons of *On*-sIgM (GenBank accession number KC677037) with known IgM heavy chain cDNAs of other higher vertebrates showed that the nucleotide identity scores were between 38.0 to 47.3% and the amino acid identity and similarity scores ranged from 24.2 to 28.8% and 45.7 to 51.5%, respectively (Table 2). On the other hand, comparisons of *On*-sIgM with known IgM heavy chain cDNAs of other cartilaginous and teleost fishes indicated that the nucleotide identity scores were

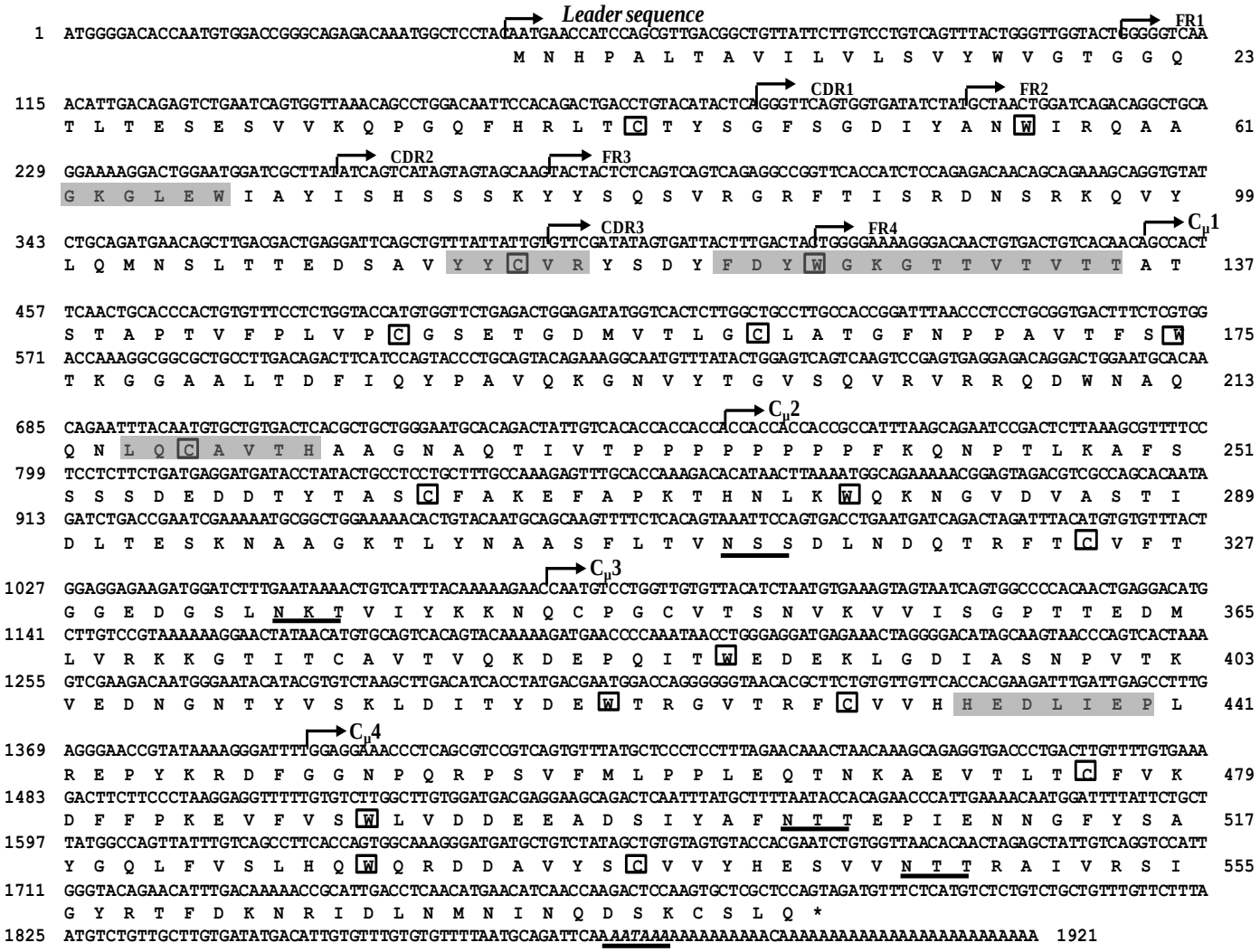

Figure 1. Nucleotide and deduced amino acid sequences of *On*-sIgM. The predicted amino acid sequence is marked under the nucleotide sequence. The conserved cysteine and tryptophan residues are boxed. The conserved blocks GKGLEW at FR2, YYCVR at FR3 and FDYWGKGTTVTVTT at FR4 and the immunoglobulin signature motif, LQCAVTH, are highlighted in gray. Four potential glycosylation sites are underlined. A typical polyadenylation signal, AATAAA, is italicized and underlined. The TAG stop codon is indicated with an asterisk.

between 38.0 to 67.0% and the amino acid identity and similarity scores ranged from 26.4 to 55.4% and 46.1 to 75.0%, respectively. Noticeably, the greatest amino acid similarity to *On*-sIgM (75.0%) was with the closely related orange-spotted grouper, *E. coioides* (Table 2).

Evolutionary relationship between the Nile tilapia IgM heavy chain gene and other vertebrates

The relationship between the Nile tilapia IgM heavy chain gene and other vertebrates was examined by phylogenetic analysis using the deduced amino acid of the IgM heavy chain constant region, Cμ2-Cμ4. In the evolutionary tree, all of the IgM heavy chain genes were clearly separated from the IgD heavy chain genes of the Mandarin and grouper fishes, which were used as the outgroups of the tree. The tree could be split into 2 major clusters that included superclasses Tetrapoda and Pisces. The first group (superclass Tetrapoda) was composed of human, dolphin, cow, mouse, rat, platypus, salamander, duck, chicken, turtle and newt. Interestingly, classes Chondrichthyes (cartilaginous fish) and Sarcopterygii (lobe-finned fish; lungfish) were also grouped into this branch. Only class Osteichthyes (bony fish) was grouped into the second group. *On*-sIgM was classified into the group of Osteicthyes in superclass Pisces and was closely related to the orange-spotted grouper (order Perciformes), which was also similar based on homology analysis (Figure 2).

Table 2. Comparisons of Nile tilapia IgM heavy chain sequences with those of other vertebrates.

Name	Accession number	Identity (%)		Similarity (%)
		Nucleotide	Amino acid	
Higher vertebrates				
Human	CAA47708	40.2	28.8	47.0
Dolphin	AAG40853	40.2	26.9	45.7
Cow	AAN60017	47.3	26.4	46.2
Mouse	CAC20701	41.2	25.8	46.2
Platypus	AA037747	38.0	28.2	46.9
Salamander	CAE02685	41.4	27.2	47.6
Duck	AAA68605	39.3	24.2	51.5
Chicken	CAA25762	38.8	25.9	47.9
Cartilaginous fish				
Antarctic skate	ACU11614	47.7	28.2	46.1
Nurse shark	AAT76789	38.1	28.9	49.0
Teleost fish				
African lungfish	AAO52809	38.0	26.4	47.2
Long nose gar	AAC59688	41.9	33.9	52.1
Bowfin	AAC59687	52.1	34.4	54.0
European eel	ABM87939	52.0	34.7	57.5
Zebrafish	AAT67447	43.5	34.2	55.1
Grass Carp	ABD76396	43.6	34.1	54.2
Haddock	CAH04753	46.1	34.8	55.5
Rainbow trout	AAB27359	50.6	41.2	64.9
Atlantic salmon	AAB24064	50.8	40.3	64.0
Japanese pufferfish	BAD26619	52.2	45.1	63.6
Snakehead	ACF49353	60.3	48.5	67.2
Atlantic halibut	AAF69488	56.0	49.2	65.4
Japanese flouder	BAB60868	56.6	49.2	66.1
Orange-spotted grouper	AAX78211	62.6	55.4	75.0
Mandarin fish	AAQ14845	60.2	52.2	67.4
Tristan klipfish	ACH87158	67.0	51.6	69.6
Black rockcod	AAL99934	58.2	49.1	65.9
Antarctic fish	ABW77756	56.5	48.6	68.2
Ploughfish	ABY54906	57.9	48.8	66.1
Antarctic fish	ABW77754	56.8	48.6	65.5
Blackfin icefish	AAL99930	64.4	49.9	67.2
Antarctic fish	ABW81218	57.6	48.7	66.5

Structural and diversity analyses of the variable domain of the IgM heavy chain gene in Nile tilapia

The putative V_H, D_H and J_H segments of the non-redundant 126 cDNA clones (GenBank accession number KC708098- KC708223) could be classified into 9 families, 6 segments and 4 families, respectively, based on the percent nucleotide identity. In the V_H domain classification, the range of nucleotide identity for each V_H family was between 51.5 to 66.5%. The nucleotide and amino acid identity between the clones within each family ranged from 80.1 to 99.7% and 80.2 to 99.1%, respectively. Families V_H II and V_H IV were more frequently employed than other families because they showed utilized frequencies of 30.2 and 26.9%, respectively. The V_H I, III, V, VI and VII families exhibited utilized frequencies of 10.3, 18.2, 7.9, 3.2 and 1.6%, respectively; however, the V_H VIII and V_H IX families possessed only

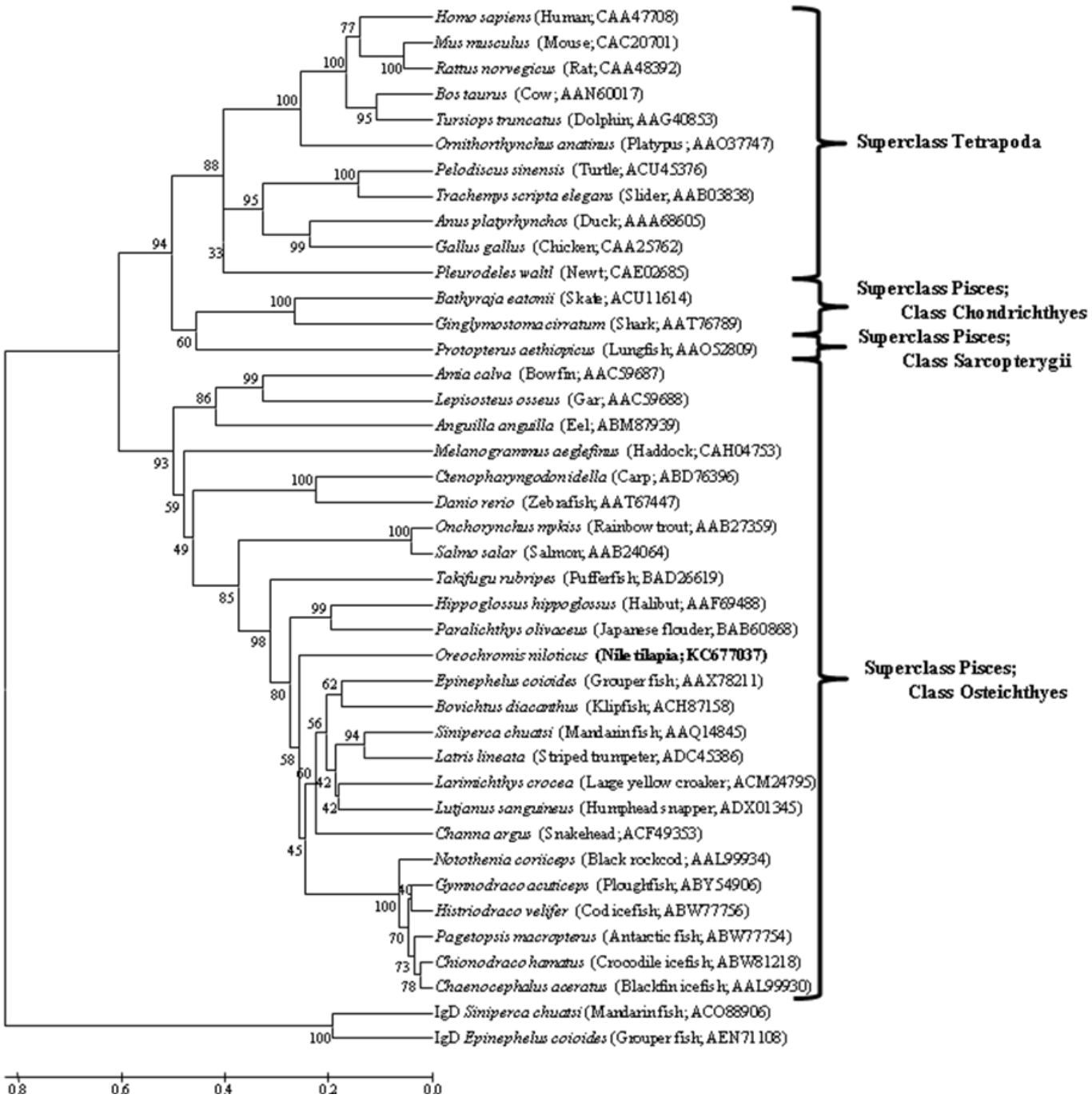

Figure 2. Phylogenetic tree showing the relationships between the Nile tilapia IgM heavy chain amino sequences and those of other known vertebrates (Cμ2-Cμ4). The numbers at the relevant branches refer to bootstrap values of 1,000. Values indicate the percentage along the branch. Common names and accession numbers for the sequences are indicated in parentheses behind their scientific names. The IgD heavy chain ($C\delta_x$-δ_n) of the Mandarin fish *Siniperca chuatsi* (Basilewsky, 1855) (ACO88906) and grouper fish *Epinephelus coioides* (Hamilton, 1822) (AEN71108) were used as outgroups.

1 clone (0.8%) that was used for V_H gene rearrangement of the IgM heavy chain (Figure 3). Arbitrary classification of the D_H segments placed them into 6 groups, with the core nucleotide sequences in each group as follows: GCGGCG, TGGGA, GGCTAC, GGTGCT, GACGAA and TACAA. Additionally, P and N nucleotide additions were investigated for the entire group. In particular, palindromic sequence additions were discovered in the following

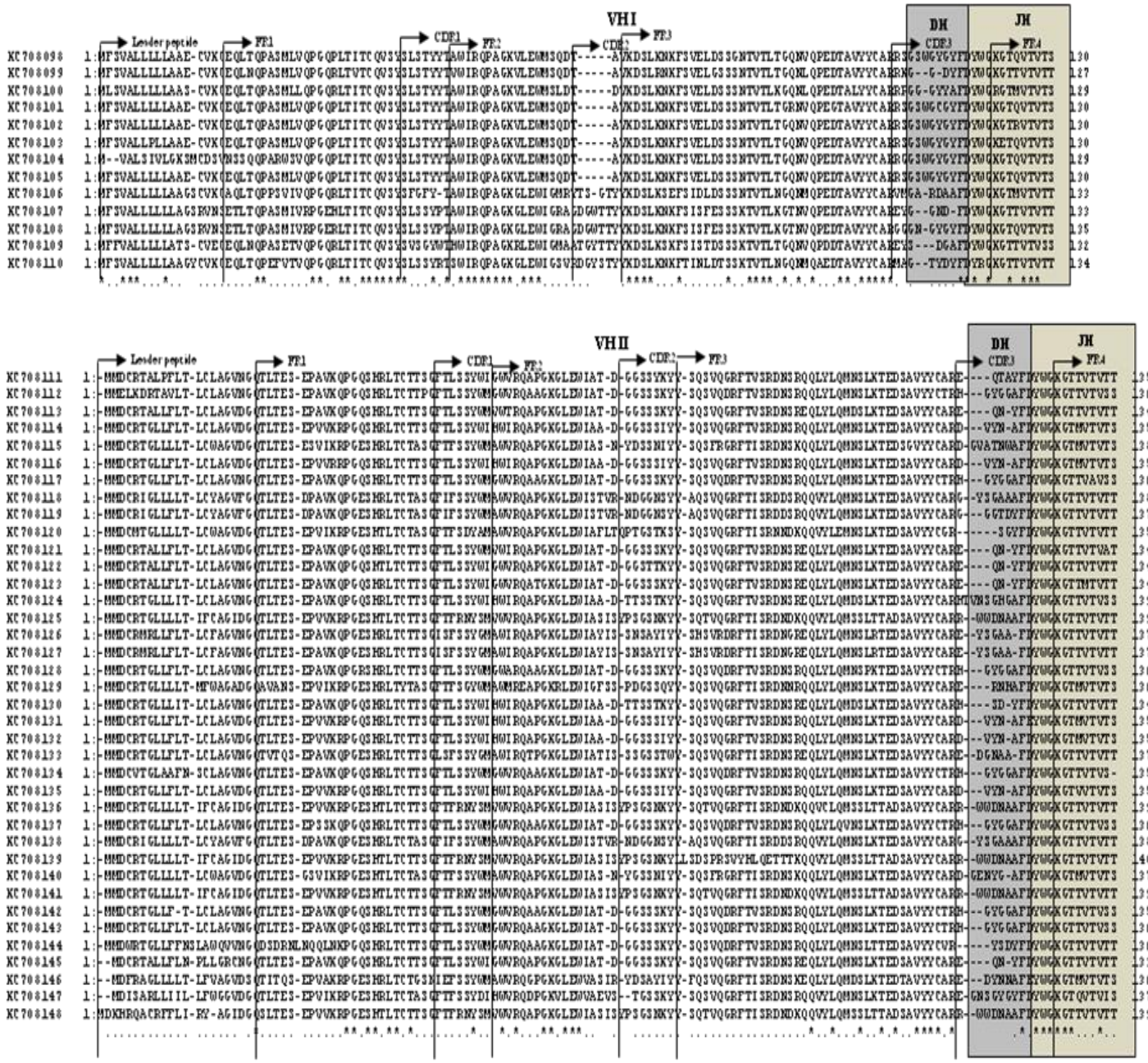

Figure 3. Classification of the variable region of the Nile tilapia IgM heavy chain. The FR and CDR domains were identified using the IMGT database. Dashes indicate gaps that were introduced for maximal alignment, and the amino acid identity is indicated by asterisks. Dots indicate residues conserved in most sequences. The accession number of each sequence is shown before the first point of the sequence.

forms: GCGC, CGCG, GCGGCGCCGC, GCTAGC, CGTACG, CCGG, CAGCTG, AGCT, ACGT, TTTAAA, GGATCC and AATT. The highest utilized frequency was observed for the D_H VI segment (28.6%), which coincided with the highest redundancy clones (21.4%), while the D_H IV segments showed the lowest utilized frequency of 3.2% (Figure 4).

The boundary of the J_H segment was determined as the first codons encoding the FDYWG motif; as a result, the J_H segment could be classified into 4 major groups. The J_HIII segment in the FDYWGKGTTVTVTT form was the most frequently (43.7%) used in rearrangements, while the least frequently used segment was J_HI (7.9%) (Figure 5). Moreover, the diversity and variance of the CDR3

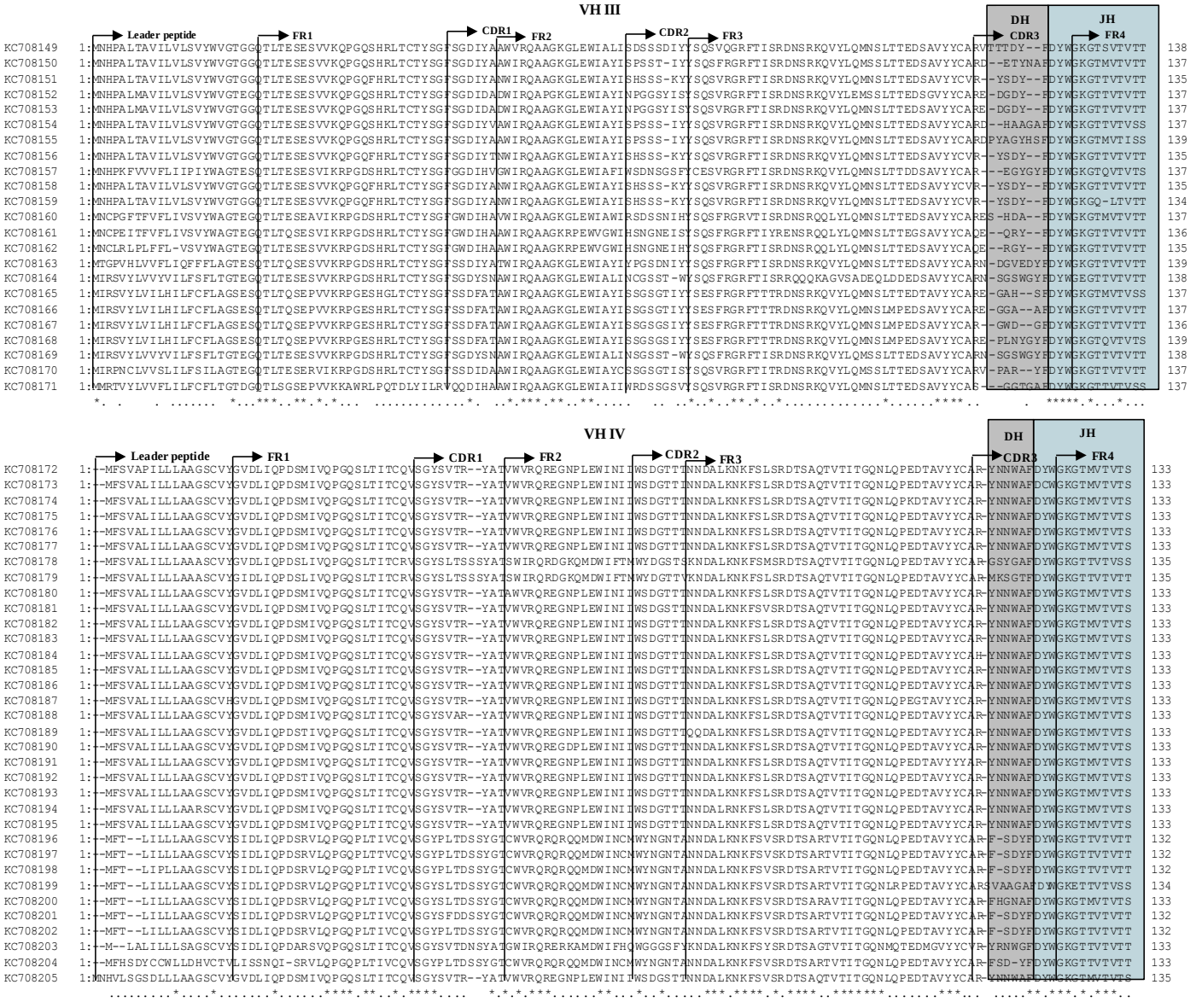

Figure 3. Contd.

region of 126 cDNA clones encoding the Nile tilapia IgM heavy chain gene were examined. The results showed that its nucleotide length ranged from 24 to 42 bp (8 to 14 amino acid residues), and 10 amino acid residues of CDR3 were the most frequently used to create the diverse repertoire of the variable domain. The average length and length variability of the CDR3 region were 10.97 and 1.99, respectively (Figure 6).

Additionally, diversity analysis of the variable domain of the Nile tilapia IgM heavy chain was relatively characterized using the Kabat and Wu method and Shannon analysis, which are general mathematical tools used to estimate variability. The result of these methods coincidently indicated that the amino acid sequences of the FRs were distinctively less variable than those of the CDRs; in particular, CDR3 showed the highest variability at position 106, followed by CDR2 and CDR1 (Figure 7).

Tissue distribution of the Nile tilapia IgM heavy chain gene

Quantitative real-time RT PCR analyses of the expression profile of the Nile tilapia IgM heavy chain gene indicated that the mRNA transcripts were expressed in 13 tissues. The highest expression level was observed in the head kidney (12.4-fold greater compared to the brain), which significantly differed from

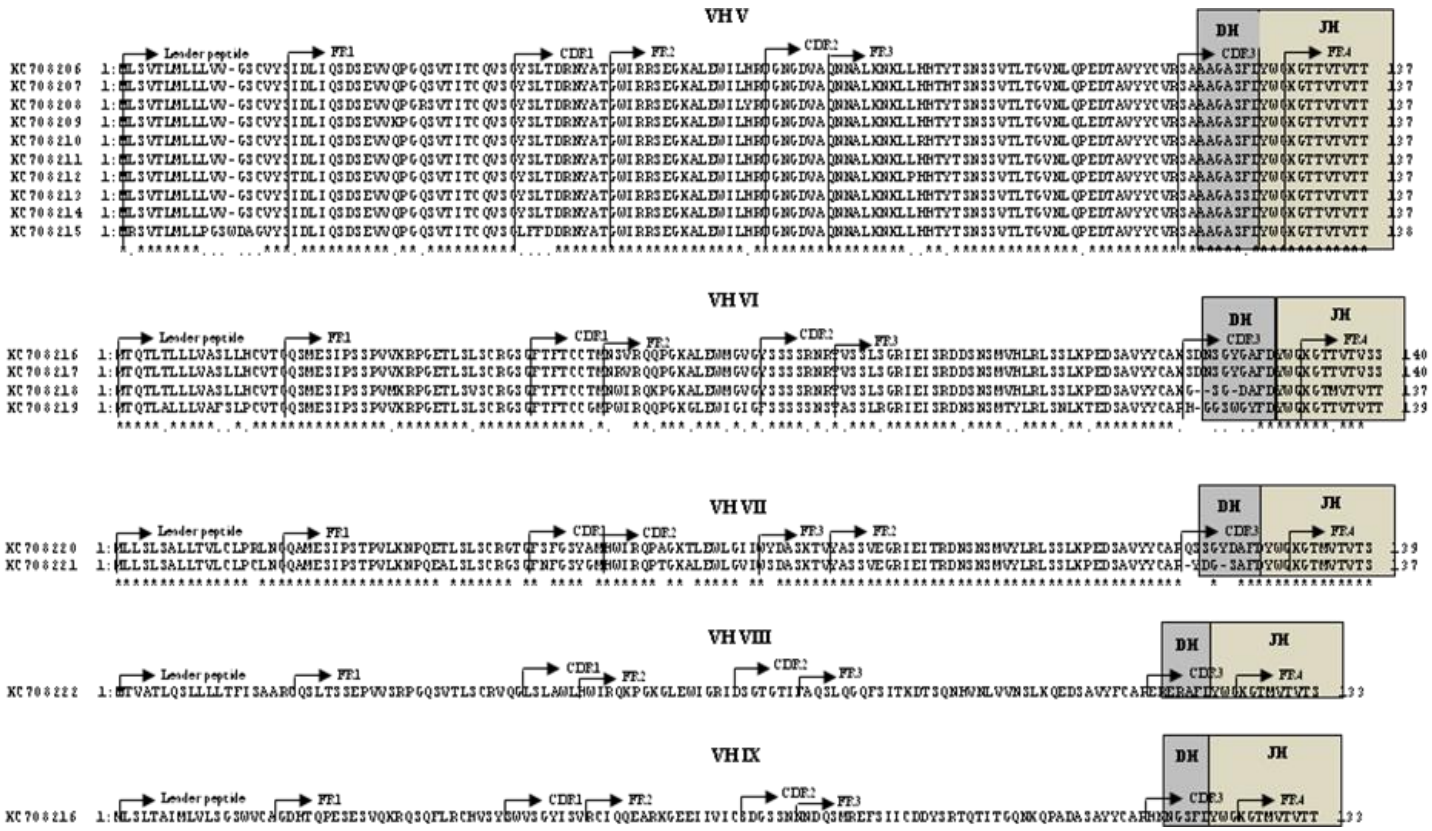

Figure 3. Contd.

that of other tissues, and was followed by the spleen, intestine and peripheral blood leukocytes (PBLs). Low expression was observed in the muscle and heart (Figure 8).

Southern blot analysis

Structural analysis of the constant region of the Nile tilapia IgM heavy chain gene was performed after digesting the genomes from 3 different fishes with the *Eco* RI and *Pst* I restriction enzymes. The hybridization of a specific probe ($C_{\mu}2$-$C_{\mu}3$ exon) illustrated that the bands appeared in the same pattern and with the same size and intensity in each fish genome. The sizes of these bands were approximately 20 and 7 kb for the *Eco* RI digestion and 22 and 10 kb for the *Pst* I digestion (Figure 9).

DISCUSSION

Molecular cloning and characterization of a cDNA encoding the IgM heavy chain gene in Nile tilapia revealed that 3' RACE PCR only amplified 1 distinctive band and this band was identical to only the secreted form of the IgM heavy chain. In this experiment, the specific primer that was used for the 3' RACE PCR was designed based on the Cμ1 region. Theoretically, the mIgM, IgD and IgZ/T transcripts, which normally contain the Cμ1 domain (Hikima et al., 2011), must be simultaneously amplified with sIgM, but we could not identify all of them in this experiment. It is possible that those mRNA levels were low in the spleen and head kidney, that most mRNAs were primary IgM-IgD transcripts or that the lengths of these mRNAs were too long to amplify. Additionally, these mRNAs may have short half-lives. Based on our these results, attempts to find other forms of IgM and other heavy chain isotypes failed with the currently used techniques, indicating that sIgM was the most abundant group of immunoglobulin heavy chain transcripts. Thus, further study is needed for a more complete understanding of the immunoglobulin heavy chains in this fish. The organization of *On*-sIgM found in this current study was rearranged to form a leader sequence and a variable region, which was followed by a constant region (Cμ1-Cμ4). This type of rearrangement is present in the secreted and soluble IgM forms found in the circulating blood. In contrast, the teleost IgM membrane-bound form, which possesses a transmembrane (TM) domain, contains a constant region that is rearranged as Cμ1-Cμ3-TM. To compare the

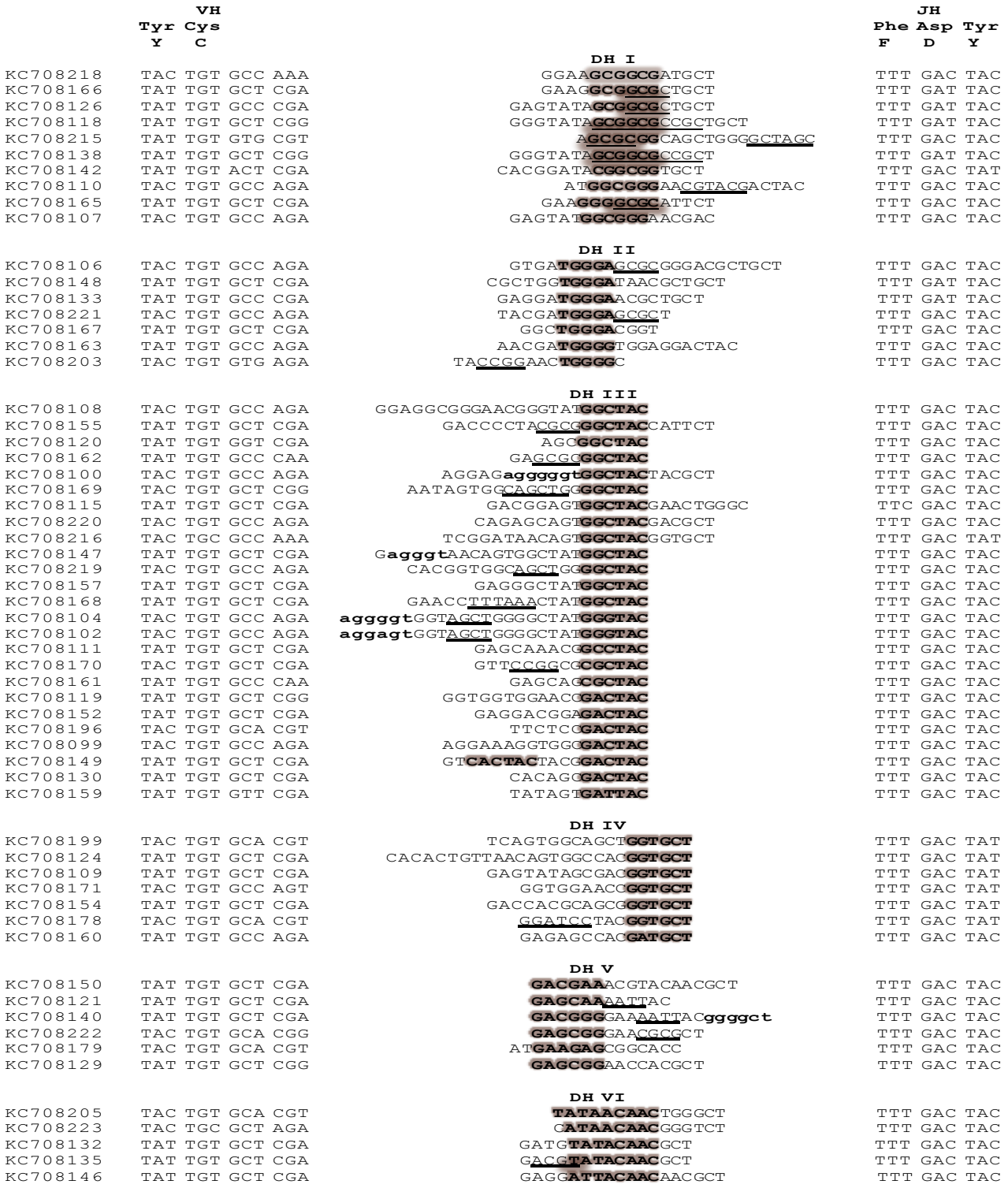

Figure 4. Classification of the VH/DH/JH junctions. Core DH nucleotides are shaded in gray. Palindromic sequences are underlined, and inverted (D-D joining) sequences are shown in small letters. The accession number of each sequence is shown before the first point of the sequence..

amino acid sequences of *On*-sIgM to those of other vertebrates, we selected the Cμ1-Cμ4 regions for multiple alignments, while the Cμ2-Cμ4 regions were used for phylogenetic analyses because the Cμ1 gene was

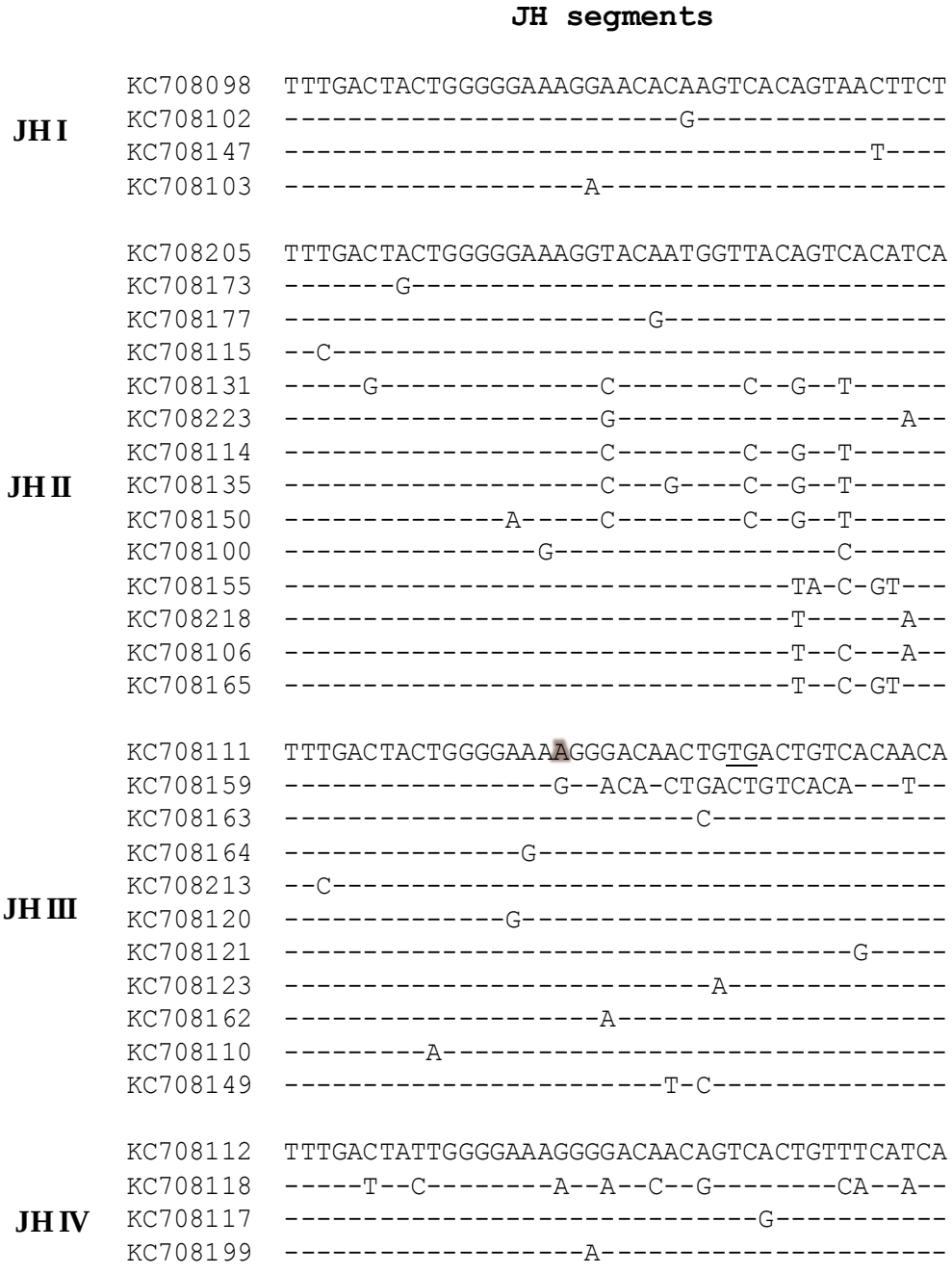

Figure 5. Nucleotide sequences of the four putative J_H segments. Nucleotide identities of the J_H III segment are indicated as dashes. Nucleotide deletion is indicated as highlight letter and nucleotide addition is underlined (for clone KC708159 compared with clone KC708111). The accession number of each sequence is shown before the first point of the sequence.

evolutionally duplicated to form other C genes (Bengten et al., 2002).

The results of the evolutionary relationship analysis showed that the IgM heavy chain of cartilaginous fish was grouped in the higher vertebrate cluster because these primitive fish do generate humoral immune responses (Coscia et al., 2012). Interestingly, the cartilaginous fish IgM heavy chain gene of the lung fish (subclass Dipnoi) was also clustered in the higher vertebrate branch because the lung fish is closely related to crossopterygians (coelacanths), which are ancestors of amphibians and higher vertebrates. Likewise, Ota et al.

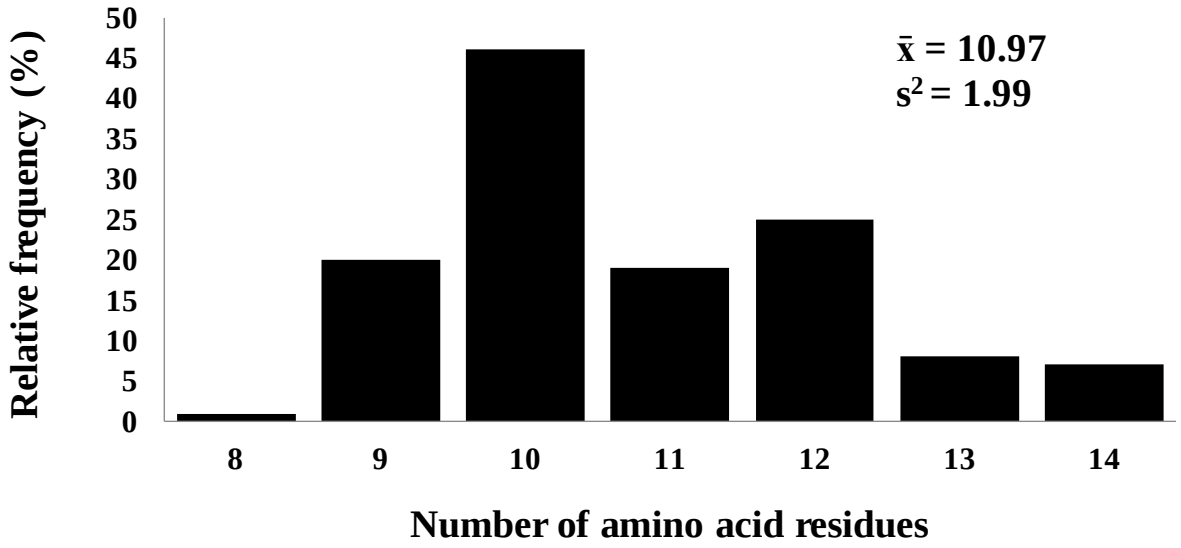

Figure 6. The length distributions of the CDR3 regions. The CDR3 regions were calculated from the 126 amino acid sequences of the variable domain of the IgM heavy chain. The average number and variance of the amino acid number are indicated by $\bar{x}$ and s^2, respectively.

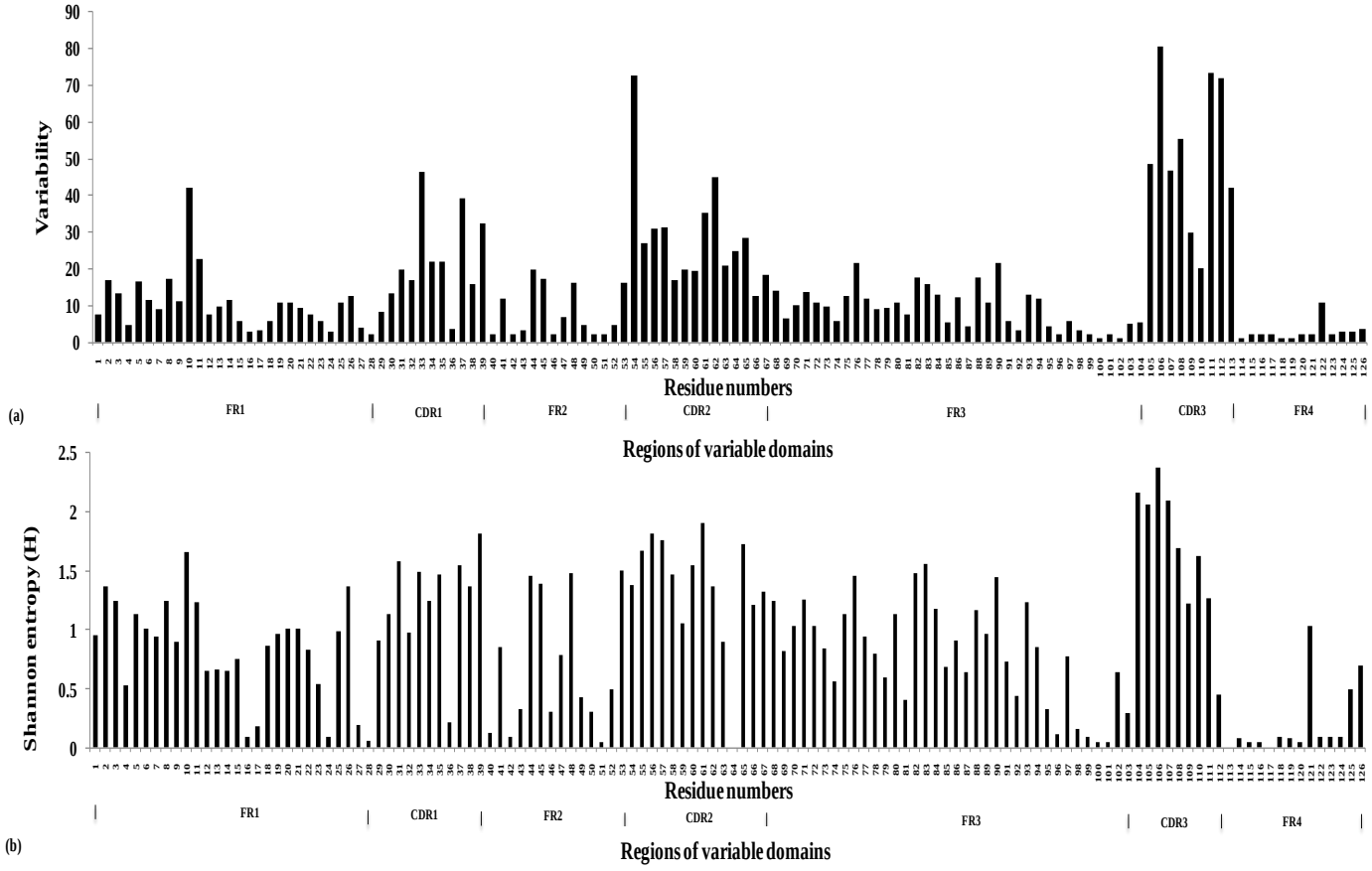

Figure 7. Variability plots of the 126-amino-acid sequences of the variable domains of the IgM heavy chain of Nile tilapia. The calculation was performed using the variability formulas of the (a) Kabat and Wu method (Kabat and Wu, 1971) and (b) Shannon analysis (Stewart *et al*. 1997).

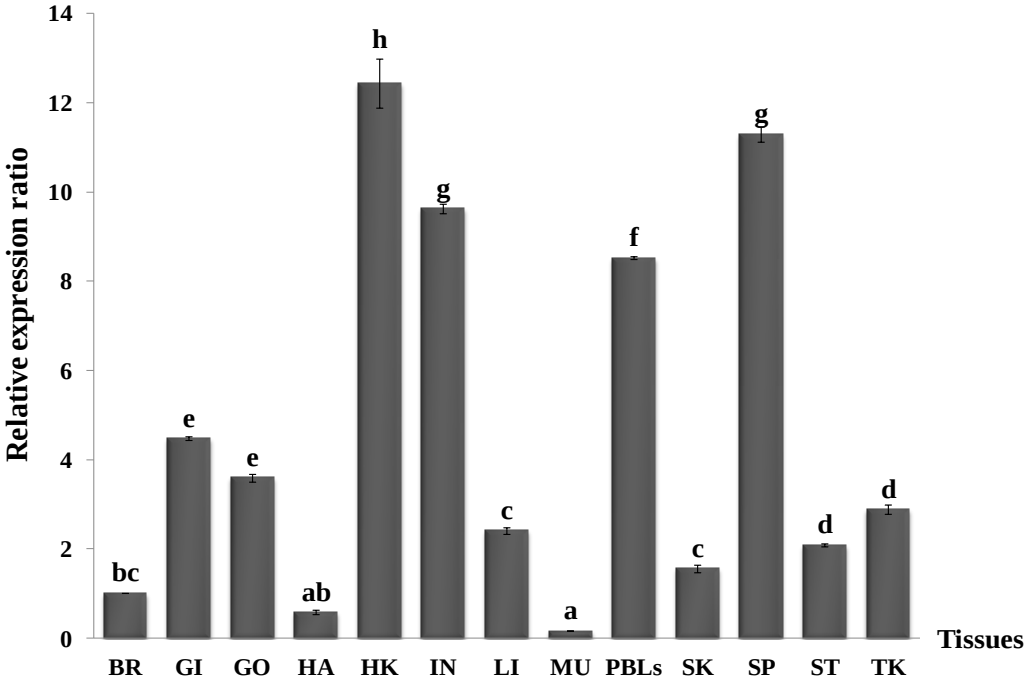

Figure 8. Quantitative real-time PCR analysis of the IgM heavy chain in 13 tissues of Nile tilapia. Significant differences are indicated with different letters on each bar (P < 0.05). BR; brain, GI; gills, GO; gonad, HA; heart, HK; head kidney, IN; intestine, LI; liver, MU; muscle, PBLs; peripheral blood leukocytes, SK; skin, SP; spleen, ST; stomach, TK; trunk kidney.

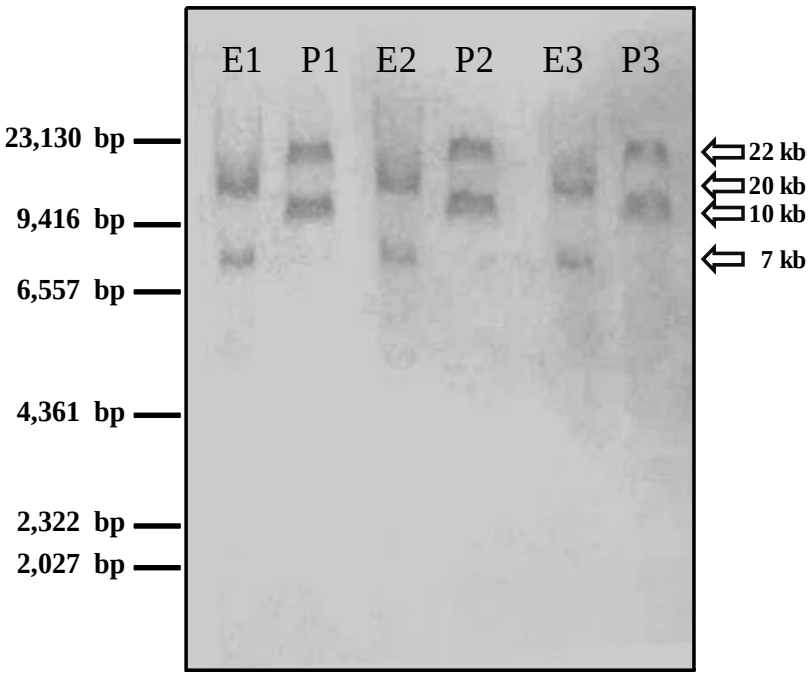

Figure 9. Southern blot hybridization of the Cμ2-Cμ3 constant region of the IgM heavy chain gene in Nile tilapia. Genomic DNA of 3 different fishes was isolated from whole blood and digested with the Eco RI (E) and Pst I (P) restriction enzymes. The band sizes were estimated by comparison with the lambda Hind III ladder shown on the left.

(2002) found that the IgM heavy chain genes of lung fishes (lobe-finned fishes) were closely related to those of tetrapods rather than neopterygians, which are primitive bony fish including bowfins and sturgeons.

When constructing the variable domain cDNA library of *On*-sIgM, the primer was designed in the Cμ2 region to eliminate contamination with the variable domains of IgD transcripts. The classification of putative V_H, D_H and J_H segments indicated that the V_H II family (30.2%) was an important group for the IgM heavy chain gene repertoire. The V_H II family demonstrated different initiation codons, AUGAUG, compared with other families, and it was suggested that these codons may encourage simpler and faster translation initiation, in accordance with the report of Kozak (1998) and Coscia and Oreste (2003). Recently, many studies on the diversity of the variable domain of the immunoglobulin heavy chain have analyzed the mRNA and genomic levels in higher and lower vertebrates. Interestingly, these compiled data have demonstrated that the numbers of V_H family members in higher vertebrates tend to be low, although some organisms, such as mouse and frog, demonstrate large numbers of V_H family members. However, the V_H family numbers in teleost fish were somewhat higher than those of higher vertebrates, possibly because the diversity generation mechanisms of the variable domain of the IgM heavy chain in higher vertebrates is more variable than that of teleost fish. Hence, large numbers of V_H family members were necessary to generate diversity of the variable domain in teleost fish, especially in Nile tilapia.

Recently, many studies on the diversity of the variable domain of the Ig heavy chain gene have demonstrated the mRNA and genomic levels in higher and lower vertebrates (Table 3). Most of the V_H families in teleost fish have been mainly studied at the transcriptional level. Nile tilapia demonstrated 9 V_H families, which was moderate amount compared to those of vertebrate V_H families (Table 3). These numbers were close to human, that is, 7 V_H families (Matsuda et al., 1998) and an Atlantic charr, that is, 8 V_H families (Andersson and Matsunaga, 1998). Atlantic salmon revealed the greatest number of V_H families, that is, 18 families (Yasuike et al., 2010) followed by zebrafish, channel catfish and rainbow trout, that is, 14, 13 and 13 families, respectively (Danilova et al., 2005; Yang et al., 2003; Brown et al., 2006). Furthermore, when 9 V_H families of Nile tilapia were analyzed a distinctive distribution of P and N nucleotide addition, inversion (D-D joining) and nucleotide deletion was observed in the generation of putative D_H segment diversity, similar to the report of Hsu et al. (1989) and Coscia and Oreste (2003). Additionally, the J_H segments were rarely diverse, with the nucleotide sequences differing by only 1 to 9 residues, and these differences may be the effect of allelic variants (Stenvik et al., 2000). Moreover, nucleotide addition and deletion of the J_H III segment by 2 essential enzymes, TdT and RAG, were also observed in clone number KC708159 (Figure 6). CDR3 region analysis demonstrated that the diversity of the CDR3 regions had a rather low exhibition level. Commonly, the length distribution and variance values of the CDR3 regions in higher vertebrates are higher than those of lower vertebrates, especially cold-blooded vertebrates. Short CDR3 regions in cold-blooded vertebrates may restrict diversity generation of the antigen-binding site of antibody molecules (Roman et al., 1995). Additionally, highly specific affinity binding with an antigen was discovered for CDR3 regions with high variability (Casali and Schettino, 1996; Kabat and Wu, 1991). Diversity analysis of the variable domain of *On*-sIgM indicated that the amino acid sequences of the FRs were less variable than those of the CDRs, with the greatest variability observed for CDR3.

Comparison of variable domain residues using Shannon's method and Kabat and Wu analysis showed that the calculated amino acid variability (according to the Kabat and Wu method) was more sharply shown in the variable pattern. Based on the current data, the diversity generation mechanisms of the variable domain repertoire of *On*-sIgM were likely obtained from combinatorial diversity, junctional imprecision and junctional diversity. At least 9 V_H families, 6 D_H segments and 4 J_H families of *On*-sIgM were used to generate diversity through random linkage resulting from RAG enzyme activity (Tonegawa, 1983). Moreover, Artemis and TdT may be used to promote junctional diversity through the deletion and addition of P and N nucleotides at the $V_H/D_H/J_H$ junction site (CDR3 region). However, it may be expected that other mechanisms, such as somatic hypermutation, secondary $V_{H/L}$ gene recombination and heavy/light chain pairing, occur to increase the antigen binding capability, antibody diversity and antigen recognition in Nile tilapia immune responses.

In this experiment, quantitative real-time RT-PCR was employed to study the expression profile of *On*-sIgM in various tissues of Nile tilapia. The highest expression level was found in the head kidney, followed by the spleen, intestine and PBLs, as these organs act as major lymphoid organs. Generally, the head kidney, spleen and intestine are acknowledged as hematopoietic tissues that play crucial roles in blood cell generation (Abbas et al., 2007). Moreover, blood-borne antigens are stored at the germinal center within the spleen, where multiple defense mechanisms emerge to recognize and neutralize antigens using specific antibodies (Grontvedt and Espelid, 2003; Saha et al., 2005). Therefore, these organs may provide larger population numbers of pro-B cells, pre-B cells, immature B cells and mature B cells than other organs. However, we found *On*-sIgM transcripts in other non-lymphoid organs, which suggested that large numbers of mature B cells may normally circulate and infiltrate into these organs (Mao et al., 2012).

Surprisingly, Southern blot analysis of the constant region of the IgM heavy chain gene in 3 different fishes indicated that the Nile tilapia diploid genome might

Table 3. The number of V_H gene families in teleost fish and other vertebrates.

Vertebrate species	V_H gene families	Study levels		References
		Transcriptional	Genomic	
Nile tilapia	9	/		This study
Pufferfish	2		/	Peixoto and Brenner, 2000
Zebrafish	14	/		Danilova et al., 2005
Emeral rockcod	2	/		Coscia and Oreste, 2003
Channel catfish	13	/	/	Yang et al., 2003
Atlantic cod	4	/		Stenvik et al., 2000
Atlantic salmon	18	/		Yasuike et al., 2010
Rainbow trout	13	/		Brown et al., 2006
Atlantic charr	8	/		Andersson and Matsunaga, 1998
Goldfish	3		/	Wilson et al., 1991
Sturgeon	3		/	Lundqvist et al., 1998
Nurse shark	5	/		Rumfelt et al., 2004
Frog	11	/		Haire et al., 1990
Chicken	1		/	Ota and Nei, 1995
Rabbit	1		/	Mage et al., 1984
Pig	1		/	Sun et al., 1994
Mouse	15		/	Mainville et al., 1996
Human	7		/	Matsuda et al., 1998
Nile tilapia	9	/		This study
Pufferfish	2		/	Peixoto and Brenner, 2000
Zebrafish	14	/		Danilova et al., 2005
Emeral rockcod	2	/		Coscia and Oreste, 2003
Channel catfish	13	/	/	Yang et al., 2003
Atlantic cod	4	/		Stenvik et al., 2000
Atlantic salmon	18	/		Yasuike et al., 2010
Rainbow trout	13	/		Brown et al., 2006
Atlantic charr	8	/		Andersson and Matsunaga, 1998
Goldfish	3		/	Wilson et al., 1991
Sturgeon	3		/	Lundqvist et al., 1998
Nurse shark	5	/		Rumfelt et al., 2004
Frog	11	/		Haire et al., 1990
Chicken	1		/	Ota and Nei, 1995
Rabbit	1		/	Mage et al., 1984
Pig	1		/	Sun et al., 1994
Mouse	15		/	Mainville et al., 1996
Human	7		/	Matsuda et al., 1998

contain 2 copies of this gene. This finding was confirmed by cloning, sequencing and a search for *Eco* RI or *Pst* I restriction sites within the intron linking the Cμ2 and Cμ3 exons, which were not found between these exons. Moreover, the nucleotide length between the Cμ2 and Cμ3 exons was determined to be approximately 104 bp (GenBank accession no. KJ558374). Generally, the length of the intron between the Cμ2 and Cμ3 exons of the IgM gene in teleost fish is not larger than 4 kb (Bengten et al., 2002; Srisapoome et al., 2004). Hence, these results indicate that Nile tilapia might possess a pseudo Cμ gene, similar to the channel catfish *Ictalurus punctatus*, or a true second Cμ gene cluster to increase the diversity of the immunoglobulin heavy chain gene.

Conclusion

The results of recent studies imply crucial functional roles for IgM, for which diversification at variable domains is generated through a number of variations that increase antigen recognition through the actions of the TdT and

RAG enzymes. Our work suggests that fish such as the Nile tilapia may possess additional Cμ loci in their genomes to create more diverse Ig heavy chains, which may be important for the generation of specific immune responses against pathogens.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

A valid measure to eliminate the influence of polysaccharides and polyphenols in recalcitrant longan (*Dimocarpus longan* L.) during DNA isolation

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Large amounts of polysaccharides, polyphenols, tannins, proteins, and other secondary metabolites in recalcitrant longan leaves make it difficult to obtain high quality genomic DNA during extraction. To obtain good quality of nucleic acids from local longan leaves and for its downstream applications, a new protocol was developed. It consists of rapid isolation of stable nuclei, which hinders covalent interactions with phenolics, followed by DNA extraction. The yield and quality of the resulting DNA were satisfactory and suitable for PCR analysis and digestion with a restriction enzyme. Here, a valid combination measure (β-mercaptoethanol, PVP40 and PVPP were used at different stages) was created to eliminate the influence of polysaccharides and polyphenols in recalcitrant longan during DNA extraction, which will facilitate the development of molecular quantitative genetics of longan.

Key words: Longan, extraction buffer, DNA isolation, PCR products.

INTRODUCTION

Longan (*Dimocarpus longan* L.) is an evergreen tree of the Sapindaceae family, which is widely cultivated in Southern China, India, and Southeast Asia (Jiang et al., 2002). Longan fruit is one of the most favored tropical fruits in China (Zheng et al., 2012). In traditional medicine, the flesh of the fruit is administered as a stomachic, febrifuge (antipyretic) or vermifuge (anthelmintic), and is regarded as an antidote for poison. A decoction of the dried flesh is taken as a tonic or as treatment for insomnia or neutrasthenic neurosis (Sudjaroen et al., 2012).

However, leaves of recalcitrant longan have high polysaccharides, polyphenols, tannins, proteins, and other secondary metabolites, which contribute to the difficulty in DNA extraction and present a major problem during the purification of longan DNA. When cells are lysed, nucleic acids come into contact with these polysaccrides (Sun et al., 2010). In the oxidised form,

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these polyphenols bind covalently and irreversibly to proteins and nucleic acids (Maréchal-Drouard and Guillemaut, 1995), resulting in a brown gelatinous material that reduces the yield and quality of the extracted DNA (Porebskim et al., 1997) and prevents it (DNA pellet) from redissolving completely. Furthermore, DNA that is able to dissolve in the presence of these polysaccharides is shown to inhibit PCR amplification and the activity of several restriction enzymes (Sahu et al., 2012).

The availability of high quality intact genomic DNA is a precondition for almost every molecular genetic analysis. Many plant tissues, especially in recalcitrant longan, are rich in polysaccharide contaminants, making isolation of good quality DNA for PCR, gene mapping, diversity assessments, and other molecular analyses a challenge. The isolation of DNA from tissues with high levels of polysaccharides and polyphenols had been reported using traditional methods (Dabo et al., 1993; Lodhi et al., 1994; Permingeat and Romagnoli, 1998; Leftort and Douglas, 1999; Chaudhry et al., 1999; Khanuja et al., 1999; Zhang and Stewart, 2000; Michiels et al., 2003; Puchooa, 2004; Zidani et al., 2005; Cota-Sanchez et al., 2006; Kotchoni and Gachomo, 2009; Azmat et al., 2012)，however, these methods did not give satisfactory results with longan, because they were not effective in eliminating the effects of polysaccharides and polyphenols in the process of DNA isolation.

PVPP was mainly applied in the beverage industry, where polyphenol adsorption leads to the stabilization of beer, wine, and fruit juices (Sarioglu 2007; Leiper et al., 2005). A research of comparison of polyvinylpoly-pyrrolidone (PVPP), silica xerogel and a polyvinyl-pyrrolidone (PVP)–silica co-product for their ability to remove polyphenols from beer was carried out (Mitchell et al., 2005), demonstrating that the PVPP had the greater binding capability compared to the PVP-silica co-product. Many researches on DNA isolation from environmental samples were conducted, however, high quality DNA could not be achieved using previous methods because these samples often contains enzyme inhibitors disruptive to downstream molecular applications; to eliminate these inhibitors from sediment samples or cells collected from freshwater ecosystems, PVPP was used to eliminate the influence of polysaccharides and polyphenols during DNA extraction and the yield and quality of the resulting DNA were satisfactory and suitable for PCR analysis, along with cloning and gene sequencing (Berthelet et al., 1996; Arbeli and Fuentes, 2007; Yilmaz and Phlips, 2009). These results indicate that PVPP was a valid reagent to eliminate the influence of polysaccharides and polyphenols tannins, proteins, and other secondary metabolites during DNA isolation. However, PVPP was not used to remove polysaccharides and polyphenols in previous methods of recalcitrant fruits DNA extraction.

Due to problems with DNA isolation, only a few studies at the molecular level in longan were carried out. Thus, an appropriate protocol for DNA isolation of longan is necessary to be developed. After following published protocols and failing to obtain high quality DNA free of polyphenolic compounds, we developed an improved genomic DNA extraction protocol for longan.

In the present study, we report a valid measure to eliminate the influence of polysaccharides and polyphenols in recalcitrant longan (*Dimocarpus longan* L.) during DNA isolation and successfully collected good quality DNA from longan validated for PCR applications. The protocol developed in the present study will facilitate the development of molecular quantitative genetics of longan.

MATERIALS AND METHODS

Reagents for extraction of genomic DNA

The protocol for extraction of genomic DNA used in our experiment was modified from Paterson Paterson et al. (1993). Extraction buffer (adjusted pH to 8.0 after sterilization) included 2.0% CTAB (w/v), 2.8% PVP-40 (polyvinylpyrrolidone, molar weight 40 000) (w/v), 1.5 M NaCl, 20 mM thylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl (pH 8.0), β-mercaptoethanol 5% (added just before use); chloroform-isoamyl alcohol 24:1 (v/v); ethanol (75%, 100%); CTAB/NaCl (including 0.14 M CTAB, 0.5 M NaCl); 3 M sodium acetate; RNase A 10 mg/mL; TE buffer [including 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]; PVPP (polyvingypyrrolidone) (solid powder).

Plant materials

Young leaves from local commercial cultivars of longan (Table 1) were collected from the National Field Genebank for Tropical Fruit (Zhanjiang), meanwhile, banana leaves contained a moderate amount of polysaccharides and polyphenols were used as control. Leaf samples were stored at −80°C until analysis.

Extraction protocol for genomic DNA

The following protocol was employed to extract good quality genomic DNA from young leaves of longan: (1) The extraction buffer was preheated at 65°C. (2) 2-3 g of leaf sample and 0.25 g of PVPP (polyvingypyrrolidone) (solid powder) were placed together in a pre-cooled pestle and mortar and ground to a fine powder using liquid nitrogen; (3) The powdered leaf sample was transfer to a 15 ml centrifuge tube and 5 ml of hot extraction buffer added to the tube before the frozen powder starts to thaw. (4) The tube was several times inverted to mix the ingredients thoroughly and incubated at 65°C for 60 min. (5) An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added and mix gently by inverting the tube to form an emulsion. (6) It was spined for 7 min at 10,000 rpm at room temperature. (7) The supernatant (top aqueous phase) was carefully transfered to a new centrifuge tube, and 1/10 volume of CTAB/NaCl added to equal volume of chloroform-isoamyl alcohol (24:1, v/v), and mix gently by inverting the tube to form an emulsion. (8) It was spun for 10 min at 10,000 rpm at room temperature. (9) The supernatant (top aqueous phase) was carefully transferred to a new centrifuge tube, 5 ml of chilled isopropanol added, and mix gently by inverting the tube. Tubes were placed at −20°C for 30 min

Table 1. DNA yield and purity from mature or young leaves of ten longan cultivars

S/N	Cultivar name	Concentration(ng/μl)	A260/A280
1	Youxuanshixia	158	1.80
2	Benzhan	162	1.84
3	Guixiang	173	1.92
4	Chailuo	168	1.83
5	Liqiuben	180	1.97
6	Nanhujiaohe	172	1.96
7	Xipuben	185	1.81
8	Cizaozaobai	175	1.95
9	Xuezhuangben	186	1.84
10	Caohuiben	190	1.86

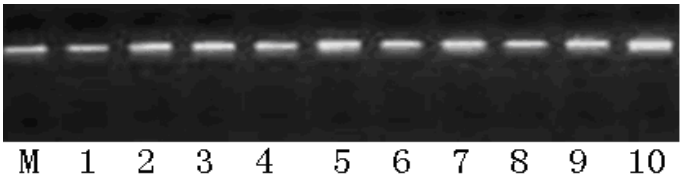

Figure 1. Agarose gel electrophoresis of DNA extracted from 10 cultivars of longan. M, λ DNA; concentration: 150 ng/ul; 1, Youxuanshixia; 2, Benzhan; 3, Guixiang; 4, Chailuo; 5, Liqiuben; 6, Nanhujiaohe; 7, Xipuben; 8, Cizaozaobai; 9, Xuezhuangben; 10, Caohuiben.

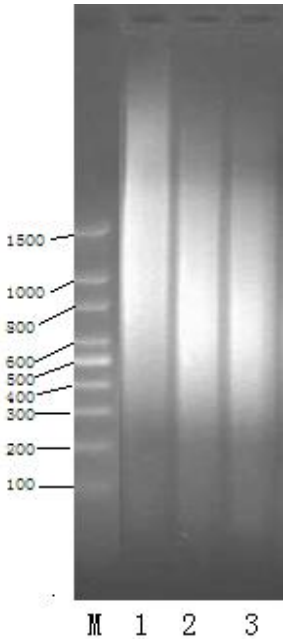

Figure 2. Genomic DNA of longan digested with EcoR I restriction enzyme (M, 100 bp DNA ladder marker).

(the precipitated DNA will be visible at this step). (10) It was spun at 10,000 rpm for 5 min and discarded the supernatant. (11) Wash the pellets 2-3 times with 75% ethanol. (12) The tube was inverted on a paper towel and the pellets air-dried. (13) The DNA in 500 μl TE buffer was resuspended, and treated with 25 μl RNase A, and incubated at 37°C for 30 min. (14) 500 μl of chloroform-isoamyl alcohol was added and mixed gently. (15) It was spun for 10 min at 10,000 rpm and the supernatant transferred into a new 1.5 ml eppendorf tube. (16) 1/10 volume 3 M sodium acetate was added, mix and precipitate the DNA with two volumes of chilled 100% ethanol precipitated. (17) It was spun for 5 min at 8000 rpm, supernatant discarded, and the pellet washed with 75% ethanol. (18) The pellet was air-dried and dissolved in 200 μl TE. (19) The DNA concentration was measured by taking absorbance at 260 nm or by running aliquots on a 1% agarose gel. (20) The DNA concentration and purity of these samples were determined by estimating the ratio of absorbance at 260 nm to that at 280 nm (A260/A280).

The extracted DNA was validated for PCR application as described by Mei et al. (2004). Preparation of polyacrylamide gel and electrophoresis of PCR products were carried out according to Benbouza et al. (2006). Gels were silver stained according to the steps described by Liang et al. (2014).

RESULTS AND DISCUSSION

The quality and concentration of longan DNA prepared with developed procedure was tested for yield and quality using UV spectrophotometer; the A260/A280 of the extracted DNA ranged from 1.8-2.0 (Table 1) and the result indicated that we achieved high quality longan genomic DNA. The use of adjusted β-mercaptoethanol, PVP40 and PVPP specifically helps the release of DNA from polysaccharides and polyphenols. The poly-saccharides settle in the bottom with NaCl while the precipitated DNA remains suspended in the upper isopropanol layer. The quality of the extracted DNA was observed by 1% agarose gel electrophoresis (Figure 1).

The purity, quality and intactness of the extracted DNA were also evaluated by digestion with restriction enzymes. Briefly, each digestion in a total volume of 20 μl (4 h at 37°C) with EcoR I (10 unit/μl) according to Barzegari et al. (2010), the result indicated that the quality of the extracted DNA was suitable for digestion with restriction enzymes (Figure 2).

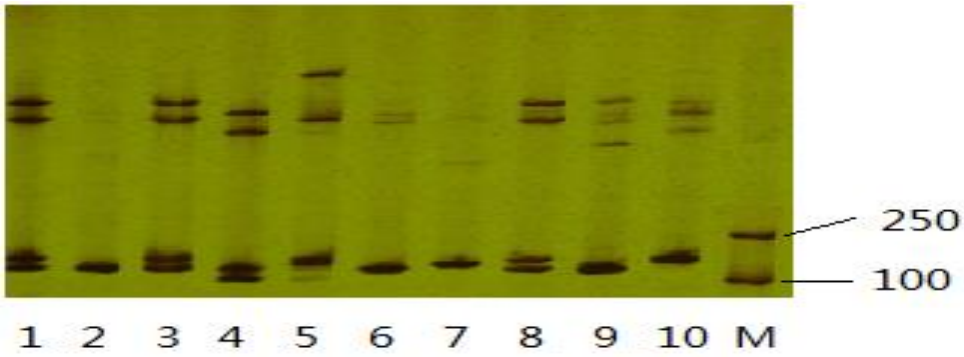

Figure 3. Revelation of the amplification products of SSR primer pair on polyacrylamide gel SSR primer sequences: Forward AAAAGGGGCCAAAATG. Reverse GGCAGAGTTCGGGATTTT. 1, Youxuanshixia; 2, Benzhan; 3, Guixiang; 4, Chailuo; 5, Liqiuben; 6, Nanhujiaohe; 7, Xipuben; 8. Cizaozaobai; 9. Xuezhuangben; 10. Caohuiben; M: DNA marker.

In order to further detect the quality of the extracted longan DNA, SSR primers (Figure 3) were used to amplify genomic DNA of longan; PCR conditions were as described by Benbouza et al. (2006). PCR products were silver stained according to the steps described by Liang et al. (2014). The results (Figures 2 and 3) show that extracted DNA was suitable for PCR analysis.

We presented an efficient and reliable method to extract high-quality genomic DNA from longan. The tissues of longan have large amounts of polysaccharides, polyphenols, tannins, proteins, and other secondary metabolites. Therefore, the most critical issue is to remove interfering substances that often coprecipitate with the extracted DNA, which presents a major contamination problem in the purification of longan genomic DNA. Previous procedures described by Dabo et al. (1993), Lodhi et al. (1994), Permingeat and Romagnoli (1998), Leftort and Douglas (1999), Chaudhry et al. (1999), Khanuja et al. (1999), Zhang and Stewart (2000), Michiels et al. (2003), Puchooa, (2004), Zidani et al. (2005), Cota-Sanchez et al. (2006), Kotchoni and Gachomo (2009) and Azmat et al. (2012) were used to isolate gemomic DNA of longan and banana (which contained a moderate amount of contaminants used as control). Unfortunately, these procedures were inefficient for extracting genomic DNA from mature longan leaves, making it useless for most of molecular manipulations, however, we obtained high quality banana genomic DNA (supplementary Figure 1). These results show that polyphenols and polysaccharide were the main reason for failure of longan DNA extraction, the presence of these metabolites can hamper the DNA isolation procedures and reactions such as DNA restriction, amplification and cloning. In order to eliminate the effects of polysaccharides and polyphenols during genomic DNA extraction, we adjusted the amount of β-mercaptoethanol and PVP40 in the DNA extraction buffer of longan (reagents for extraction of genomic DNA); β-mercapto-ethanol and PVP40 were effective and commonly used reagents for removing polyphenols and polysaccharide. However, the improvement effect of DNA extraction was not obvious. These results show that relying only on the β-mercaptoethanol and PVP40 could not eliminate effectively the influence of the polyphenols and poly-saccharide during longan genomic DNA extraction.

PVPP was first reported and used effectively to remove the brown algal phlorotannins by Toth and Pavia (2001); the results indicated that insoluble polyvinylpolypyrrolidone (PVPP) can be used to specifically remove tannins and phlorotannins from plant and algal phlorotannins. Nevertheless, PVPP was not used to remove polysaccharides and polyphenols in previous methods of DNA extraction. In order to detect the extraction effects of longan DNA using PVPP only, longan and banana leaf sample of 2-3 g respectively and 0.25 g of PVPP together in a pre-cooled pestle and mortar were ground to fine powder using liquid nitrogen; when no β-mercaptoethanol and PVP40 were added into traditional DNA extraction solution, the same result occurred (supplementary Figure 2). Interestingly, combination (β-mercaptoethanol and PVP40 were added into traditional DNA extraction solution, meanwhile, PVPP were used at grinding step stage) was adopted to eliminate the influence of polysaccharides and polyphenols, longan genonmic DNA degradation was avoided and DNA with good spectral qualities was obtained (Figure 1).

Leaves of recalcitrant longan have high polysaccharides, polyphenols, tannins, proteins, and other secondary metabolites, which present a major problem during the purification of longan DNA. Previous methods were tried to isolate genomic DNA of longan leaves but failed to obtain intact DNA due to irreversible binding of phenolic

compounds and coprecipitation of polysaccharides when cells were lysed nucleic acids come into contact with these polysaccrides. Therefore, the main problem of longan genomic DNA extraction was how to eliminate the influence of polysaccharides and polyphenols which degrade genomic DNA at different stages of extraction. In present experiment, a valid combination (β-mercaptoethanol, PVP40 and PVPP were used at different stages) was adopted to eliminate the influence of polysaccharides and polyphenols. From perspective of elimination of polysaccharides and polyphenols, the present method and measure was efficient and reliable.

Present research provided an opportunity to successfully collect good quality DNA for PCR applications in recalcitrant longan. This protocol has the potential to extract DNA from the young and mature leaves of other species high in polysaccharides and polyphenols as well (data not shown). The improved method has been used successfully to extract DNA from mango, litchi and so on in our laboratory, those plants with high in polysaccharides and polyphenols likewise. The protocol developed in the present study will facilitate the development of molecular quantitative genetics of recalcitrant longan.

Author contributions

QL LD, Conceived and designed the experiments. QL DW DG, Performed the experiments, QL DW DG, analyzed the data. DW DG, contributed reagents/materials and QL DW wrote the paper. All the authors discussed the results and commented on the manuscript.

Conflict of interests

The author(s) did not declare any conflict of interest.

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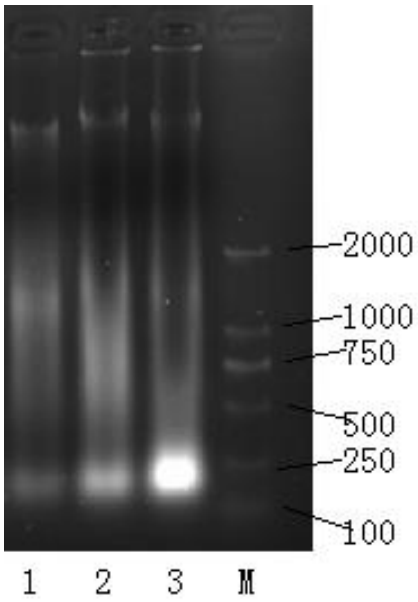
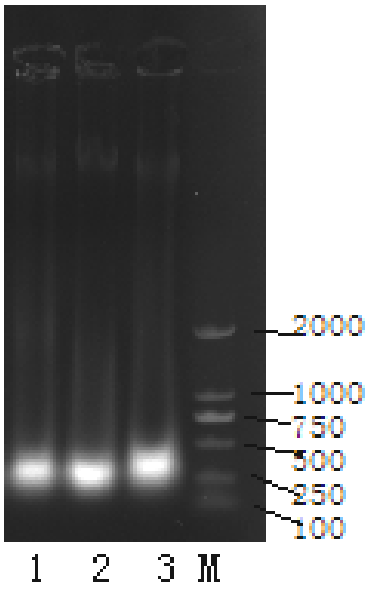


Supplementary Figure 1. DNA extraction from mature leaves of longan using traditional method.



Supplementary Figure 2. DNA extraction solution including PVPP only; no β-mercaptoethanol and PVP40 were added

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Full Length Research Paper

Hydroponic technology for lily flowers and bulbs production using rainwater and some common nutrient solutions

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This experiment was carried out to investigate the potential of nutrient film technique (NFT) hydroponic system for flowers and bulbs production of the Asiatic hybrid lily cv. "Blackout" using rainwater and some common nutrient solutions (Hoagland No. 2 Basal Salt Mixture, Murashige and Skoog Basal Salt Mixture and White's Basal Salt) with rock wool cubes as medium with or without removal of flower buds and mother bulb scales. The results show that the NFT hydroponic system was an excellent method to produce lily flowers in 55 days. The rainwater could be applied as nutrient solution in this system to produce lily flowers in a good quality making this system easier and cheaper way to get into cut lily production. The results of analytical chemistry indicated that the rainwater contained some amounts of macro and micro elements in forms that plants can absorb and had a good value of pH (6.20) with favorite high ratio of NO_3:NH_4 making this water to be more efficient for plant growth and development as nutrient solution. The analytical results of nutrient solutions at flowering time showed that these solutions had different amounts of nutrients, values of pH, electrical conductivity (EC) and the ratio of NO_3:NH_4. The present results indicate that the number and quality of flowers were influenced by different nutrient solutions even so the flowers of all treatments were in a good quality. The NFT hydroponic system was shown to be the most effective for bulblets and daughters production, but different solutions showed different results and the Hoag solution and MS solution gave the best results related to the production of these propagated storage organs.

Key word: Asiatic lily, nutrient film technique (NFT), hydroponic, rainwater, nutrient solutions, removal treatment, flower, bulblet, daughter.

INTRODUCTION

In recent years, hydroponic forcing techniques of cut flowers have significantly increased in flower industry around the world and a great progress have been done to these commercial forcing techniques which are widely

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Abbreviations: Hoag S, Hoagland No. 2 basal salt mixture solution; **MS S,** Murashige and Skoog basal salt mixture (MS) solution; **White S,** White's basal salt solution; **NFT system,** nutrient film technique system; **EC,** electrical conductivity.

used in Netherlands to produce cut flowers of some important ornamental crops such as tulip using the bulb fust hydroponic system (Grassotti and Gimelli, 2011; Miller, 2012; Gude, 2011; Rina and Hiroshi, 2012). It is well known that the nutrient film technique (NFT) system is highly productive, and can be used for cut production and for propagation; one of the best aspects of this system is that the plant roots were bathed constantly in thin film of an oxygen-rich nutrient solution which allow the roots to uptake adequate supplies of water, oxygen and nutrients (Libia et al., 2012). In hydroponic technology, there are many common nutrient solutions that were used however; they provide every nutrients necessary for plant growth within the common ranges of the macro and micro elements (mg/l) and for the most plants species, they are nitrogen (100 to 255), phosphorus (30 to 50), potassium (100 to 300), calcium (80 to 140), magnesium (30 to 70), sulfur (50 to 120), iron (1 to 3), copper (0.08 to 0.2), manganese (0.5 to 1), zinc (0.3 to 0.6), molybdenum (0.04 to 0.08), boron (0.2 to 0.5) furthermore; the plant growth can be affected by pH of solution , the pH range of 5.5 to 6.5 is optimal for the availability of nutrients in nutrient solutions for most plants (Libia et al., 2012; Toshiki, 2012).

Several studies focused on the chemical composition of rainwater indicated that it contains the elements plants need to grow; it contains a trace amounts of some macro and micro elements in forms that plants can absorb but the analysis of rainwater was different from region to region and the nitrogen compounds such as ammonium and nitrates, if they exist in the air, they can mix with water and come down with rainwater (Ellen et al., 2004; Wood et al., 1999). Lily can be propagated through many vegetative means; the bulblets is considered to be the key of lily propagation, it can be regenerated from bulb scale through scaling, from leaf through cutting propagation and from various organs through tissue culture technique *in vitro* (Duong et al., 2001). The technique of removal of some organs was used to stimulate the plants to produce more bulblets by remobilization of the carbohydrates between the sources and the sinks (Asker, 2013; Leclerc et al., 2005).

The objective of the present experiment on Asiatic hybrid lily cv. Blackout was to develop efficient and low cost system for lily flowers and bulbs production using nutrient Film technique with application of rainwater as nutrient solution and some common nutrient solutions with or without flower buds and bulb scales removal.

MATERIALS AND METHODS

This experiment was conducted at the computerized greenhouses with environmental control systems at School of Biomedical and Biological Sciences, University of Plymouth during the year 2014 in a range of temperature around 20 to 25°C. It is designed to study the utilization of the hydroponic technology to produce flower and bulb of Asiatic hybrid cv." Blackout" using rainwater as nutrient solution in addition to some common nutrient solutions: Hoagland No. 2 Basal Salt Mixture, Murashige and Skoog Basal Salt Mixture (MS) and White's Basal Salt which were prepared in concentration of 1/6 strength at pH 6. The experiment had eight treatments, which included four nutrient solutions with or without removal treatments; each treatment contains 9 plants, the flower buds were removed when they became 2 cm long and mother bulb scales were removed at the same time in treated plants. To investigate the chemical analysis of nutrient solutions, the highly modern equipments ICP- MS Instrument and ICP- OES Instrument were used to determine all nutrient elements except nitrogen compounds which were determined by Colorimetric Assay Kits using UV spectrophotometer. The pH and electrical conductivity EC (mS- cm^{-1}) values of solutions were determined using pH meter during the experiment.

NFT tanks with constant flow of nutrient solution were used with rock wool cubes as growing medium; the tang measures were 112 cm long × 49 cm wide × 21 cm high) and holds 20 L of solution and with a tray on top were used which was fed by a pump to create a constant flow of solution for plants, and these solutions were changed once during the experiment. The healthy bulbs were purchased from Hyde and Sons Nursery-UK, rock wall cubes from hydro grow com. nutrient solutions from Sigma-Aldrich Company Ltd, and the composition of these solutions was as follow: A) Hoagland No. 2 Basal Salt Mixture: ingredients include potassium nitrate, calcium nitrate, magnesium sulphate, ammonium phosphate monobasic, manganese chloride.$4H_2O$, boric acid, molybdenum trioxide, zinc sulphate.$7H_2O$, copper sulphate.$5H_2O$, ferric tartrate 606.60, 656.40, 240.76, 115.03, 1.81, 2.86, 0.016, 0.22, 0.08 and 5.00 mg/L respectively, and this was formulated to contain 1.63 g of powder per liter of medium, pH 4.2 to 5.2. B) Murashige and Skoog Basal Salt Mixture (MS) and the ingredients included ammonium nitrate, boric acid, calcium chloride (anhydrous), cobalt chloride hexahydrate, cupric sulfate pentahydrate, disodium EDTA dihydrate, ferrous sulfate heptahydrate, magnesium sulfate (anhydrous), manganese sulfate monohydrate, potassium iodide, potassium nitrate, potassium phosphate monobasic, sodium molybdate dihydrate, Zinc sulfate heptahydrate with 1,650.0, 6.20, 332.20, 0.0250, 0.0250, 37.260, 27.80, 180.70, 16.90, 0.830, 1900, 170.0, 0.250 and 8.60 mg/L respectively, and this was formulated by 4.33 g of powder per liter of medium, pH 3.5 to 4.5. C) White's Basal Salt Mixture and the ingredients: boric acid, calcium nitrate (vacuum-dried), Ferric sulfate, magnesium sulfate anhydrous, manganese sulfate monohydrate, potassium chloride, potassium iodide, potassium nitrate, sodium phosphate monobasic, sodium sulfate anhydrous, zinc sulfate heptahydrate 1.50, 200.0, 2.50, 360.0, 5.040, 65.0, 0.750, 80.0, 16.50, 200.0 and 2.670 mg/L respectively, and this was formulated by 0.934 g of powder per liter of medium, pH 4.2 to 5.2. Data of number of flowers per plant and number, weight (g) length (cm) surface area (cm^2) of petals per flower were collected at flowering time of untreated plants. Data of bulblets number and weight (g), daughter's number and weight (g) and weight of bulb roots, stem roots, bulblets roots per plant were collected after 16 weeks from planting of all experiment plants.

The statistical analysis system (SAS, 2012) was used to effect different factors in study parameters. Significant difference-LSD test was used in this study to significantly compare between means at the (0.05) level of significance.

RESULTS

Plate 1, shows the Asiatic lily plants cv. "Blackout" grown in NFT hydroponic system in different nutrient solutions in greenhouse. The flowers completely opened after 55 days from planting with good quality in all treatments. Plate 2, showing the Asiatic lily plants cv. "Blackout"

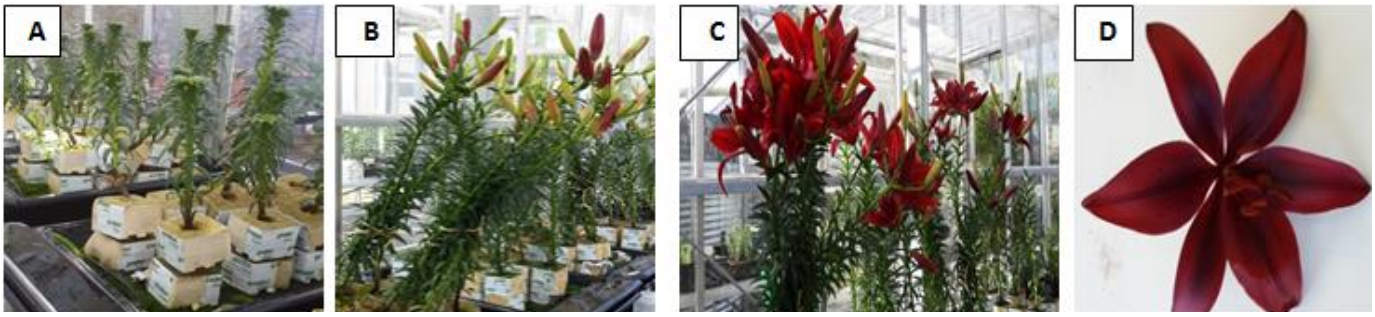

Plate 1. Showing that the Asiatic lily plants cv. "Blackout" grown in NFT hydroponic system in different nutrient solutions in greenhouse. The flowers completely opened after 55 days from planting. **A)** The growing system contained the NFT tanks, with constant flow of nutrient solutions circulation where the rock wool cubes were used as growing medium. **B)** The Asiatic hybrid lily plants grown in NFT hydroponic system in greenhouse at developmental stage of flower buds coloring. **C)** The plants with full open flowers in 55 days. **D)** The flowers had a good quality.

Plate 2. Showing that the Asiatic lily plants cv. "Blackout" grown in NFT hydroponic system in different nutrient solutions in greenhouse. The plants produced very large bulblets, daughters and three types of roots systems after 16 weeks from planting. **A)** The plants produced many bulblets on stem just above the mother bulbs. **B)** Large bulblets with high growth of bulblet root system were formation. **C)** The NFT hydroponic system produced a large daughter. **D)** Three types of roots were developed of the Asiatic plants, bulb roots, stem roots and bulblets roots. **E)** The bulblets of plants of Asiatic lily plants cv. "Blackout" produced many new sprouts without undergo to dormancy process.

grown in NFT hydroponic system in different nutrient solutions in greenhouse. The plants produced very large bulblets, daughters and three types of roots systems after 16 weeks from planting. Table 1 indicates the results of chemical analysis of nutrient solutions at flowering time and these solutions had different amounts of macro and micro elements, however; in all nutrient solutions at flowering time, there were still high proportion of the nutrients were not used by plants, the MS S. had highest concentrations of all elements except molybdenum compared to other nutrient solutions. The results of chemical analysis of rainwater of Plymouth city indicated that this water contained macro and micro elements in concentrations (mg/L): NO_3 (0.063), NH4 (0.01), other K (0.288575), P (0.094), Ca (1.0655), Mg (0.658075), S (0.7789), Mn (0.006491) Cu (0.000089), Zn (0.06349), Mo (0.000052), and B (0.0043618). At the same table, the nitrate amount was higher than ammonium in all nutrient solutions, the highest value of NO_3:NH_4 ratio was found in Hoag solution compared to others. Figure 1 shows the time course of electrical conductivity ($mS.cm^{-1}$) and pH of second nutrient solutions (the solutions were changed one time after 20 days from planting). The pH of second solutions were dropped through the period between 20 to 55 days of planting from 6.20 to 5.95, 6 to 5.45, 6 to 4.1, 6 to 5.8 and the EC ($mS.cm^{-1}$) were changed from 0.071 to 0.11, 0.502 to 0.306, 1.33 to 1.004, 0.406 to 0.296, in rainwater, Hoag, MS, and White solutions, respectively, however; the MS S. had the maximum value of EC and the minimum value of pH compared to other solutions.

Figure 2 shows that the number and quality of lily flowers as influenced by different nutrient solutions; the highest flower number per plant (6) was observed on plants grown in White S. while the lowest flower number (5) was found on plants of rainwater. The highest flower quality parameters were obtained in plants grown in Hoag S. compared to other plants which grow in other solutions, the flowers of plants in this solution had petals with weight of 5.91 g, length of 56.2 cm, width of 21.47 cm and surface area of 126.11 cm^2 per flower. Table 2 results show that the application of flower buds and bulb

Table 1. Indicates that the chemistry of analysis of rainwater and some common nutrient solutions in hydroponic system of Asiatic hybrid lily "Black out" at the start of experiment and after 55 days of planting.

Time after planting	Nutrient Solutions	Elements (mg/L)														
		NO3-N	NO2-N	NH4-N	K	P	Ca	Mg	S	Na	Fe	Mn	Cu	Zn	Mo	B
At the start	Rainwater	0.063	0.008	0.01	0.288575	0.094	1.0655	0.658075	0.7789	3.491	—	0.006491	0.000089	0.06349	0.000052	0.0043618
After 55 days	Rainwater	0.648	0.015	0.093	0.5668	0.189289	3.31975	1.368	0.995575	6.897	0.0344525	0.01253	0.0012018	0.042845	0.00006	0.008722
	Hoag S.	11.6	0.048	0.188	2.22375	—	21.845	7.08425	1.0617	8.55225	0.0526025	0.0018693	0.0036068	0.0061415	0.07689	0.0552475
	MS S.	82.4	0.027	11.92	65.43	—	37.765	11.52	2.71975	9.63	4.88625	0.897375	0.0111605	0.2316	0.008	0.130875
	White S.	0.554	0.018	0.074	0.1263	0.2355	10.5375	11.6	3.63225	11.97	0.0618225	0.001342	0.0033533	0.0163975	0.000005	0.033075

Table 2. Indicates that the effect of types of nutrient solutions and removal treatment on the development of bulblets , daughters and roots in Asiatic hybrid lily" Blackout" in hydroponic system

Factors		Parameters						
		Bulblets		Daughters		Bulb roots	Stem roots	Bulblet roots
		No.	WT (g)	No.	WT (g)	WT (g)	WT (g)	WT (g)
Treatments	Control	8.72	5.81	2.03	25.11	9.5	1.86	1.33
	Removal	8.91	10.34	1.97	23.74	9.92	4.04	2.58
LSD value	--	1.27	1.75 *	0.181	3.42	1.68	0.566 *	0.409 *
Nutrient	Rainwater	7.78	6.48	1.77	18.9	9.29	2.44	1.66
Solutions	Hoag S.	9.11	8.52	2.11	30.64	10.08	4.56	2.89
	MS S.	10.22	11.42	2.11	25.59	7.97	2.35	1.45
	White S.	8.16	5.88	2	22.57	11.51	2.47	1.83
LSD value	--	1.79*	2.48*	0.257*	4.84 *	2.38*	0.801*	0.578*
Solutions x	1 rainwater	7.22	3.76	1.77	19.27	8.17	1.29	0.75
Treatments	2 rainwater	8.33	9.2	1.77	18.53	10.4	3.59	2.57
	1 Hoag S.	9.22	6.02	2	29.87	10.32	1.88	2.11
	2 Hoag S.	9	11.01	2.22	31.4	9.83	7.23	3.66
	1 MS S.	9.66	8.85	2.11	27.36	8.32	2.15	1.06
	2 MS S.	10.77	13.99	2.11	23.82	7.62	2.54	1.84
	1 White S.	8.77	4.62	2.22	23.95	11.18	2.13	1.41
	2 White S.	7.55	7.14	1.77	21.18	11.83	2.81	2.24
LSD value	--	2.545 *	3.51 *	0.363*	6.85 *	3.37 *	1.13 *	0.818*

LSD test was used to significant compare between means at the 5% level of significance. *NS. Significant at P > 0.05 and not significant, respectively. Treatment 1 = Control. Treatment 2 = Flower buds and bulb scale removal.

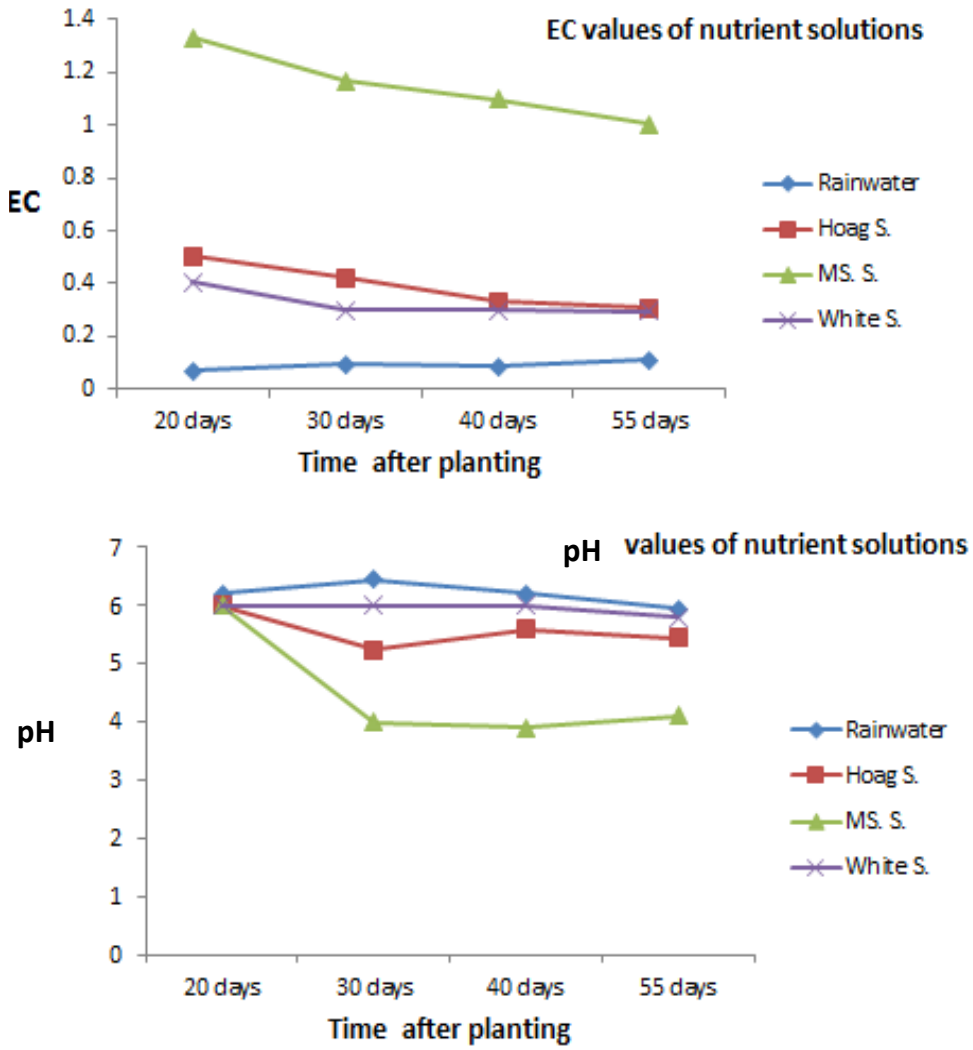

Figure 1. Indicating that the time course of pH and EC electrical conductivity mS.cm^{-1} of the rainwater and some common nutrient solutions (second solutions) during the experiment. The solutions were changed one time after 20 days from planting.

scales removal practice greatly increased the weight of bulblets by 77.97% and the weight of stem roots by 117.2%; the development of bulblets, daughters and roots were influenced by the type of nutrient solutions however; the plants grown in MS S. produced highest weight (11.42 g) and number (10.22) of bulblets with the minimum value of all types of roots while Hoag S plants gave the maximum weights of daughters (30.64 g), stem roots (4.56) and bulblets roots (2.89). The rainwater plants showed the minimum values of the daughters by number (1.77), weight (18.9 g) and the bulblets by number (7.78), while the largest bulb roots (11.51) were recorded in White S compared to others. The removal treatments showed the best results for bulblets production by number (10.77), weight (13.99 g) in MS S and for daughter formation by number (2.22) and weight (31.4 g) in Hoag S.

DISCUSSION

The present results show that the (NFT) hydroponic system achieved optimal performance to produce lily flowers in 55 days and that may be because the plant roots were bathed constantly in thin film of an oxygen-rich nutrient solution which allow the roots to uptake adequate supplies of water, oxygen and nutrients in this system. The results of analytical chemistry indicated that the rainwater contained some amounts of macro and micro elements in forms that plants can absorb and the pH was 6.22; the NO_3:NH_4 ratio was high. All these points clearly suggest that rainwater is good for plant growth and that may explain the results of this experiment which show that the rainwater could be applied in NFT hydroponic system as nutrient solution to produce lily flower in a good quality at low cost. The chemistry of nutrient

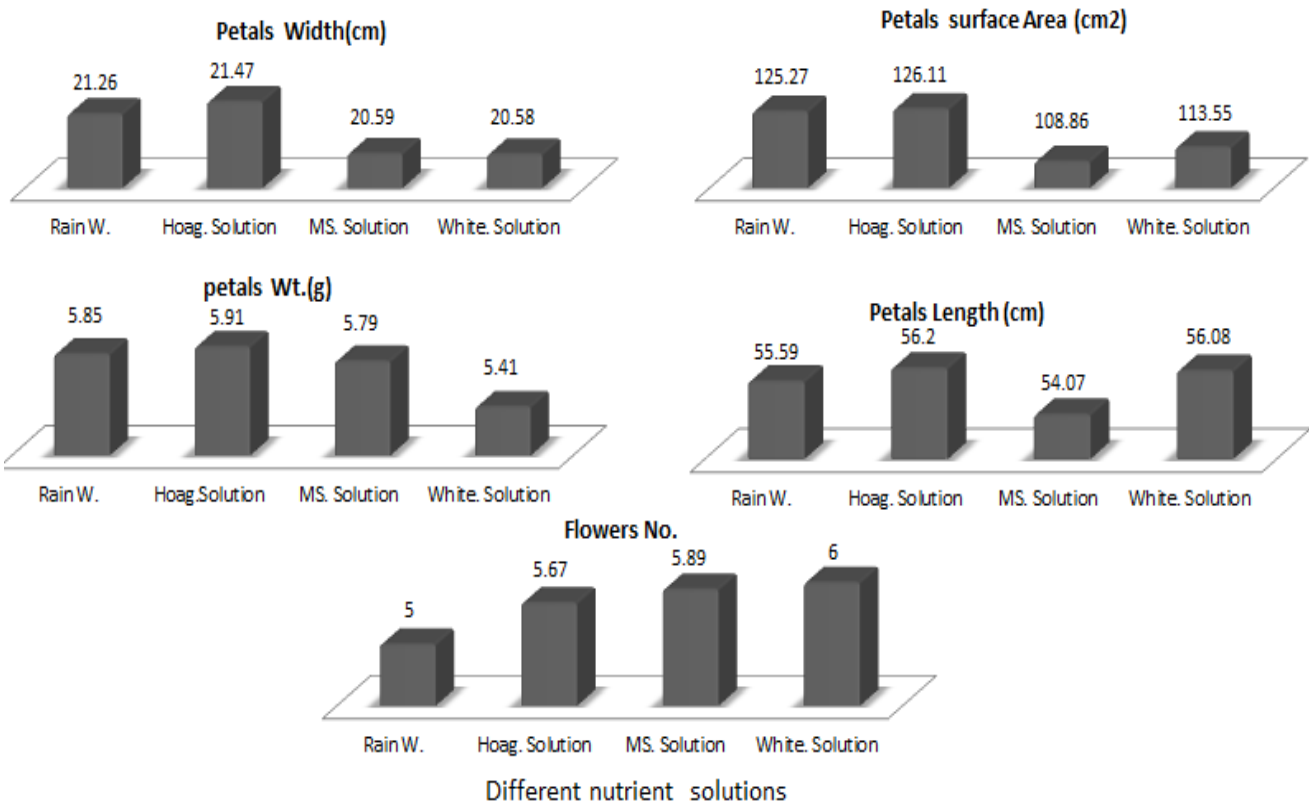

Figure 2. The effect of different nutrient solutions on number of flowers per plant and flower quality parameters such as weight (g), length (cm), width (cm), surface area (cm^2) of petals per flower of Asiatic hybrid lily cv ' blackout' plants grown in NFT hydroponic system. LSD at ($p < 0.05$) of flowers No. 0.943,* LSD at ($p < 0.05$) of petals wt. (g) 0.439*, length 1.740 *, width 0.723* and surface area 8.656 *,*NS. Significant at $p < 0.05$ and not significant, respectively.

solutions at flowering time indicated that there were still high proportion of the nutrients not used by plants and that may be because the NFT hydroponic system was efficient and precise system to feed the plant roots by nutrient, water and oxygen, hence, the lost amounts of nutrients were low during the experiment and the nutrient requirements of plants grown in this system were low and this conclusion agrees with several studies done on Anthurium, gerbera and geranium which mentioned that when nutrient solutions are applied continuously, plants can uptake ions at very low concentration (Dufour and Guerin, 2005; Zheng et al., 2005; Rouhpael and Colla, 2009).

The nutrient solutions had different results related to flowers and bulb development and that may be due to their differences in amounts of nutrients, values of pH, EC and the NO_3:NH_4 ratio of solutions. It is well known that these factors have great effect on plant development (Chad et al., 2009; Miller, 2012; Toshiki, 2012). In case of bulb production, the plants grown in rainwater had less and smaller daughters with less bulblets compared to others. That may be because the nutrient requirements of lily plants during bulbing stage were high and the low nutrients amount in rainwater were able to cover the nutrient demand of lily plants to produce stem, leafs and flower with assistant of mother bulbs but were not enough to cover the nutrient requirements of plants to produce bulbs and this conclusion agrees with the results of Wu et al. (2012) which reported that the mother bulb is mainly considered as a carbohydrate source for vegetative development in early developmental stage and flowering stage; the flowers utilize and consume the carbohydrate which is received from the current photosynthesis and bulb scales and the bulb in this stage is considered as a combination of sink and source and finally as a sink in bulbing stage. It seemed that removal treatment was effective tool to stimulate the lily plants to produce more bulblets which is the vital key for lily propagation and this results agrees with that reported by Asker (2013) and Leclerc et al. (2005).

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Chemical and quantitive study of hepatotoxins from fresh water cyanobacteria on vertebrate animals

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Toxicity of the freeze-dried micro-aquatic planktonic cyanobacterium *Oscillatoria agardhii*, dominating and isolated from Makkah –KSA was studied. Microcystins were detected from freeze-dried cells using high pressure liquid chromatography HPLC. The histopathological examination of mice liver injected on week basis with diluted 1/5 of the lethal dose, (105 mg/ Kg body weight), of toxin extract revealed sever changes in liver histology and displayed apparent signs of degenerative hepatic structure. Cytoplasmic vacuolation and parenchyma exhibit hepatocytes degeneration. Hepatic vasculature and biliary system were blocked and severely damaged. The effect of the extract on blood contents and liver function of white mice was investigated. Mice were divided into three groups and injected intraperitoneally (i.p) with weekly reciprocal doses of toxin extract as 21, 42 and 63 mg/ mice, respectively, for seven weeks. Mice injected with 63 mg were the mostly affected and showed signs of acute cellular and physiological damage involving oligocythemia, leucoytosis, marked increase in serum urea, cholesterol, triglycerides, creatinine, Aspartate Aminotransferase (AST), alanine aminotransferase (ALT), hematocrit (HCT) and mean cell hemoglobin concentration (MCHC), while blood platelets showed abnormal increase which suggest an inhibitory action on haemopiesis. In addition, the abnormal pathophysiology observed here reflects the sever toxic effect from the crude extract of *O. agardhii* on both liver and kidney.

Key words: Cyanobacterium, blood, histopathology, mice.

INTRODUCTION

Many species of bacteria secret varity of toxins (Osman et al., 2007; El-Menofy et al., 2014; Osman et al., 2015). Only cyanobacteria produces cyanotoxins source of natural product of toxins known as cyanotoxins, which might occur in fresh, brackish and marine water bodies. Poisoning cases are attributed to cyanobacterial toxins known since the late 19th century (Francis, 1878). Fatalities were high among animal livestock, pets and

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Abbreviations: AST, Aminotransferase; **ALT,** alanine aminotransferase; **HCT,** hematocrit; **MCHC,** mean cell hemoglobin concentration; **HPLC,** high pressure liquid chromatography; **i.p,** intraperitoneally; **FS,** field stimulation; **SPE,** solid phase extraction; **IST,** international sorbent technology; **RBC′s,** red blood cell; **HB,** hemoglobin; **WBC′s,** white blood cell count.

wildlife following ingestion of water contaminated with toxic cyanobacterial cells or toxins. Toxins-producing cyanobacteria pose a worldwide health threat to both human and animal due to their presence in both drinking and recreational water (Mutwally and Jamel Al-Layl, 1992; 1993; Ismail and Jamel Al-Layl, 1995; Jamel Al-Layl, 1996; Dittmann and Wiegand, 2005; Boaru et al., 2006). Recently, studies on the classification and characterization of cyanobacteria strains have been increasing because these strains possess commercially valuable toxins. To date, several researchers have identified and characterized bacterial strains using phenotypic characteristics, DNA-DNA relatedness data, and analysis of the 16SrRNA sequence (Assaeedi et al., 2011; Osman, 2012; EL-Ghareeb et al., 2012; Abulreesh et al., 2012; Assaeedi and Osman, 2012; Osman et al., 2013; Organji et al., 2015). Human illness resulting from exposure to blue-green algal toxins is less common than poisonings of wild and domestic animals (Yoo et al., 1995; Frazier et al., 1998). A large number of reports of water blooms having toxic cyanobacteria in lakes, dams and rivers have appeared in the last decade (Dittmann and Wiegand, 2005; Boaru et al., 2006). On the other hand, cyanobacterial produce microcystins toxic materials, which affect either the liver or the nervous system (Lawton and Codd, 1988; Mutwally and Jamel Al-Layl, 1992; 1993; Berry et al., 2008).

Microcystins released by cyanobacteria have sever toxic effects on liver, kidney, blood and other organs in humans and animals (Milutinovic et al., 2003). This could be true for some countries but in many other countries the most dominant species are *Microcystis aeruginosa* (Namikoshi et al., 1992). Cyanobacteria, blue-green algae, are a distinctive group of prokaryotic microorganisms. There are two main types of toxins in cyanobacteria. These are neurotoxic alkaloids (anatoxins), saxitoxin, derivates and hepatotoxic peptides, microcystine, cyanoginosin, cyanoviridin and cyanogenosin (Anon, 1988). Mutwally and Jamel Al-Layl (1992; 1993) found that the extract of blue-algae (*Ossoillatoria agardhii*) (10^{-5}, 5×10^{-5} and 10^{-4} M), inhibited the spontaneous activity (Spt.act), 100 mM K+-contractures and field stimulation-(FS) responses of *Locusta migratoria* foregut and hindgut muscles in dose-dependent manner. They concluded that the neurotoxin effect was stronger on the tonic responses than on the phasic one and the site action of this extract affected the neuronal plexus driving both muscles. Mutwally (1993) reported the toxicity and liver tumor promotion of cyanotoxins microcystins. Jamel Al-Layl and Jamal Al-lail (1993) reported the first record on toxic cyanobacteria including *Oscillatoria* spp., Microcystis sp. and yellow Microcysits sp. from Makkah area, the west province of Saudi Arabia. In this study we isolated and purified cyanotoxins (Microcystin) from *O. agardhii* by means of High Pressure Liquid Chromatography (HPLC). The toxicity of different animal tissues after dosing with lethal and sub-lethal doses was investigated. Finally, the long term effect of sub-lethal doses on liver function and blood contents was measured.

MATERIALS AND METHODS

Isolation of cyanobacterial strain

Cyanobacterial strain *O. agardhii* was the most abundant cyanobacterial strain found in farm land under investigation in Makkah, KSA. The strain was purified using serial streaking techniques on Agar plates rich with CT medium (Watanabe and Ichimura, 1977) and allowed to grow in 10 L batch culture. The yielded cells mass were spin down and kept at -20°C.

Animals

White female Balb/c mice with average weight of 25 ± 2 g provided and delivered from the animal unit, Biology Department, Faculty of Applied Sciences, Umm Al-Qura University.

Toxicity assay

Preparation of cyanobacterial cells extract

Cyanobacterial cells were grown till late log phase (3 to 4 weeks), and then centrifuged at large scale centrifuge at 8000 rpm. Pellet was washed with 50 ml distilled water prior freeze-drying in Edward freeze dryer (modylyo, MKII, Edwards, UK.). The freeze dried cells approximately 5 to 10 g was homogenized and extracted with 250 ml sterilized distilled water for 2 h followed by centrifugation at 10000 rpm for 20 min. The supernatants were pooled and collected and kept at -20°C.

Mice bioassay

Each female mouse Balb/c weighing 20 to 25 ± 2.0 g received 1 ml of the aqueous cellular extract. The animals were injected intraperitoneally (i.p) and kept under continuous observation for 48 h. Control animals received 1 ml of sterilized distilled water. LD_{50}, (mg dry weight /kg of body weight), was calculated according to Keleti and Laderer (1974). The low value in LD_{50} is regarded as the higher toxin content within the cell extract.

Toxin extraction

The method of toxin extraction was done by using 1 to 5 g of dry homogenized extract, and then stirred in 75 ml of 100% methanol for overnight followed by centrifugation at 10000 rpm for 20 min. The supernatants were collected and kept at 4°C. The supernatants were dried under stream of air to dryness. The extract was re-suspended in distilled water (20 ml). Solid phase extraction (SPE) was achieved by passing the water suspension through methanol pre-activated C18 column (IST, International Sorbent Technology, Mid Glamorgan, U.K). The MCYSTs were eluted with 80% methanol in water (v/v). The methanol elution (10 ml) was then dried to complete dryness and re-suspended in 1 ml distilled water and kept at -20°C for further analysis.

High pressure liquid chromatography (HPLC) analysis

Solid phase extraction (SPE) fractions were further analyzed using HPLC. The elute fractions were dissolved in 15% methanol /ddH_2O

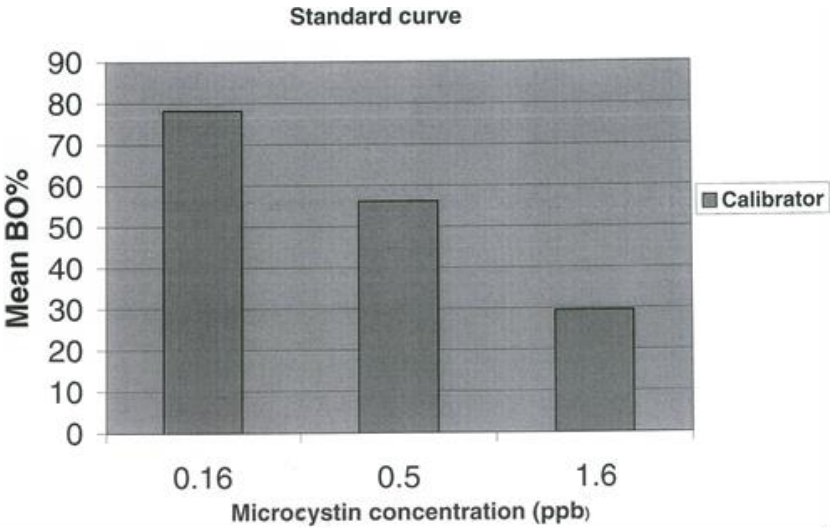

Figure 1. .Standard curve for cyanobacterial toxins determination in partial cells extract.

(v/v). 20 µl of the SPE fraction was injected onto a monochrome (5 µm 50 × 2.1 mm) C18 column (Metachema Technologies, Inc, Torrance, CA). The flow rate was adjusted to 0.25 ml/min. Separation was achieved by gradient elution. The mobile phase (A: ddH_2O/0.1% formic acid and B: acetonitrile / 0.1% formic acid) regimes were as followed: 0.00 to 5.0 min 70 to 30% A - 5.00 to 8.0 min 30 to 7% A.

ELISA technique

Enzyme linked Immunosorbent assay (ELISA) was carried out according to the method of Carmichael et al., (1999). The suspension extract from SPE step was tested for containing MCYSTs by using commercially available ELISA kit. Direct inhibition assays was done using polyclonal antibodies (Envirologix Inc., Portland, ME, and USA). Microtiter plate precoated with MCYST-LR was used for the quantitative amount of MCYST present in the cells extract. 125 µl of microcystin assay diluent were added to each well. 20 µl of NC (Blank without MCYST), MCYST-LR different calibrators concentrations (0.16, 0.5 and 1.6 ng/ ml^{-1}) and the unknown samples, were added to their designated wells. The plate was then covered with parafilm and allowed to be incubated for 30 min at room temperature on orbital shaker at 200 rpm. 100 µl of microcystin enzyme conjugate were added to each well, mixed thoroughly and incubated for 30 min. The incubated plate was then washed using washing solution (phosphate-buffer saline-Tween 20, pH 7.4). 100 µl of substrate were added to each well and incubated for 30 min at ambient temperature. Finally, 100 µl of (1.0 N HCl) were added to stop the reaction. A color of the wells contents will turn to yellow. The plate was read on semi-log scale at 450 nm. Optical density (OD) of each well contents were measured and standard curve graph of the microcystin concentration vs. BO% (maximum amount of microcystin-enzyme conjugate that was bounded by the antibodies in the absence of any microcystin in the sample).

Liver histopathology examination

Liver samples from injected animals were excised at the end of the experiment and fixed in Boiun solution for 24 h, followed by passage through dehydration series (v/v) of 70% ethanol for 24 h, 80% ethanol for 1 h, 90% ethanol for 2 h and finally in 100% ethanol for 2 h. The liver tissues were then replaced in histoclear agents (xylene) for 15 min. The samples were impregnated in embedding soft wax (melting at 54°C) for 1 h and then embedded in hard wax (melting at 60°C). Sectioning of the fixed liver tissues was achieved by cutting 5 µm slices using type 1512 microtome (litz, Wetzlar, GMBH, West Germany). The sections were stained with eosin and hematoxlyin and viewed under light microscope.

RESULTS

Toxins from cyanobacteria were determined at the beginning using mouse- bioassay. The strain under this investigation namely *O. agardhii* has an estimated LD_{50} of 105 mg dry weight of cyanobacterial cells / kg body weight. ElISA has successfully be employed for quantitation of MCYTS in drinking water (Ueno et al., 1996). The ELISA results can be related to toxicity (Harada et al., 1996). However, this method is currently considered to be the most quick and reliable used method for toxicity level evaluation and early predication. It had showed that cell free extract of *O. agardhii* cross-react with microcystin antibodies in the microtiter plate. Standard curve was drowned and the total BO% was calculated (Figure 1). 3.8 ng/mg^{-1} of MCYTS was detected from cells extract of *O. agardhii*. Figure 2 shows the HPLC profile of *O. agardhii* cells extract. Major product ions of MCYST-LR standard are also observed and compared with that of *O. agardhii*. It was concluded that *O. agardhii* cells extract contain MCYST-LR; chromatographic properties of toxin were compared with that of standard MCYST-LR toxin. These results were confirmed by collecting the separated peaks produced by HPLC analysis method, evaporated to dryness and

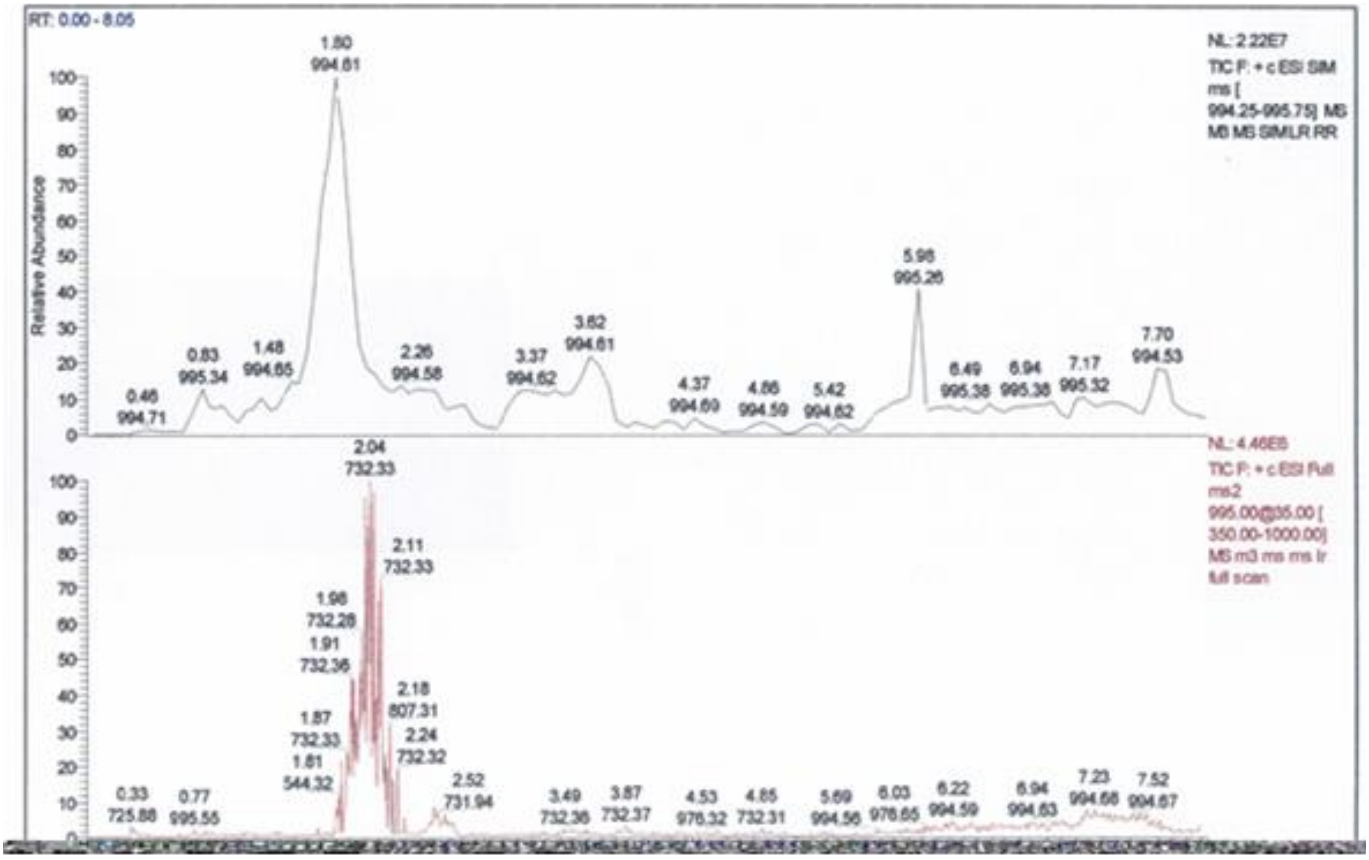

Figure 2. HPLC typical profile of microcystin –LR eluted from cyanobacterium *Oscillatoriaa agardhii* cell free extract. Peak at 2.0 min; retention time with MW at 994.

resuspended in water. Similar properties of the eluted toxins were compared with that of standard MCYST-LR toxin. Results from blood samples were collected by means of cardiac puncture weekly according to Waynforth (1980). Treated mice exposed to different doses of cells extract of *O. agardhii* were studied. The haematological parameters investigated were red blood cell (RBC's) count, hemoglobin content (HB), haematocrite value (HCT), mean cell hemoglobin concentration (MCHC), white blood cell count (WBC's), platelets, serum glucose, albumin, cholesterol, triglycerides, urea, creatinine, lymphocyte; AST, and ALT were measured to evaluate the pathophysiological changes induced by MCYST-LR. Histopathological results of mice liver exposed for prolonged duration of exposure of sub-lethal doses are presented in Figures 3 to 7. The data in these plates show sever liver damage and abnormalities in the structures followed by disappearance of normal organization of liver tissue.

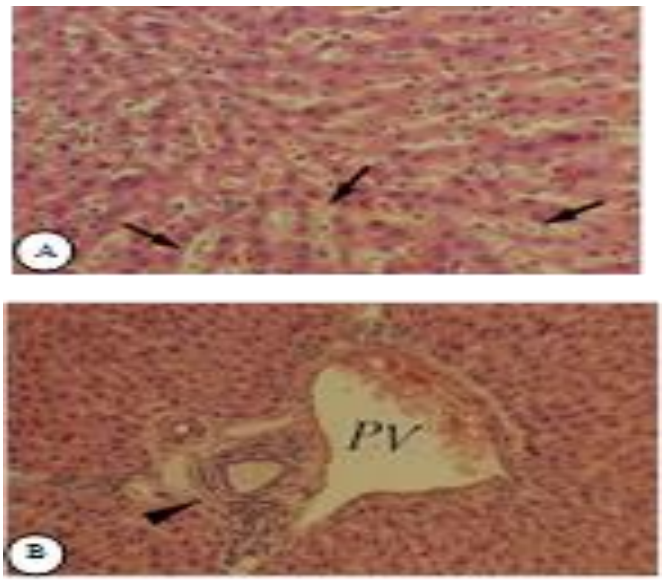

Figure 3. A. A photomicrograph revaeling marked dilatation in blood sinusoids arrows with conomitant hepatic cords atrophy (X100). **B.** A photomicrograph showing mild eosinophilic infiltration and collagenous fibers deposition head of arrows with conomitant hepatic cords atrophy (X40).

DISCUSSION

In the present study, we successfully isolated a dominate

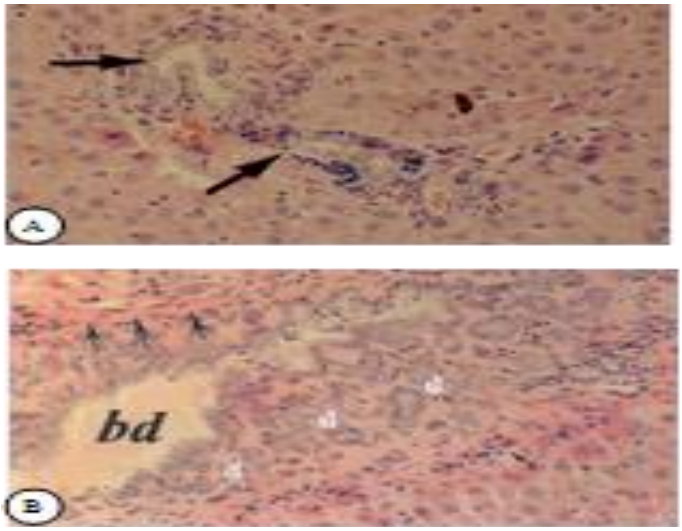

Figure 4. A. A photomicrograph showing deformity of biliary architecture with mild eosinophilic infiltration arrows (X100). **B.** A photomicrograph showing sever construction in bile duct (bd) interior wall with a well marked disarrangement of lining cell. Arrows are pointed to thick fibrotic layer surround with degenerating of hepatic tissue (X164).

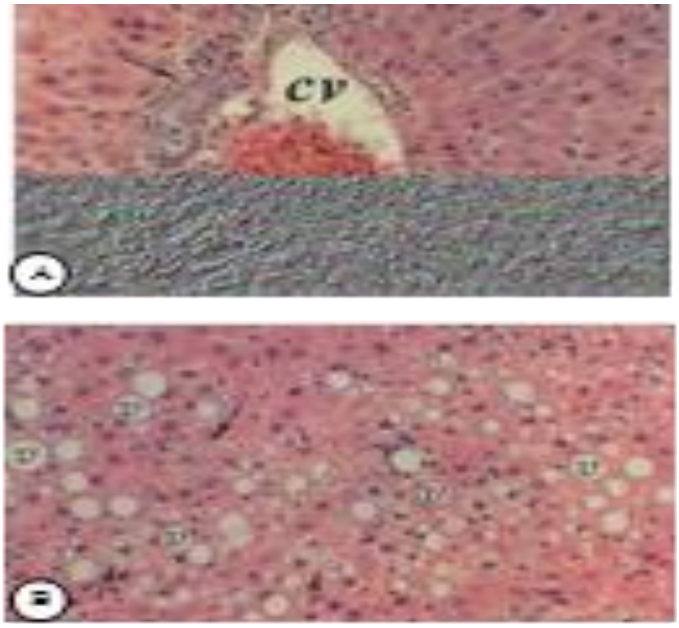

Figure 5. A. A photomicrograph showing intial fibrosis in the area surounded the central vein (CV). Head of arrowspointed to thick fibers with mild eosinophilic infilteration (X164). **B.** A photomicrograph showing several vacoulation (V) distributed in parenchyma. Arrows are pointed to foci of fat cells, with substituted the hepatocytes (X164).

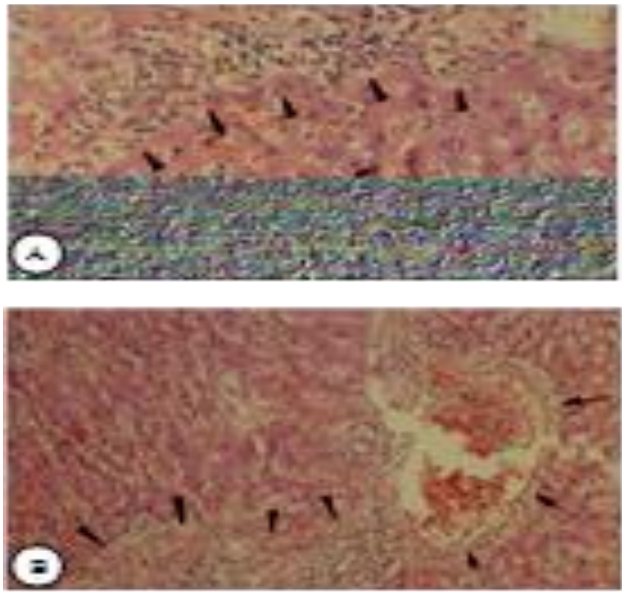

Figure 6. A/ A photomicrograph reveling marked dilatation in a portal vein (head of arrows) by mean of liver congestion. Note also the sinusoiddal dilation (arrows). X100. **B.** A micrograph showing area of premalignant changes (heads of arrows note the disappearnce of hepatic architecture. Note the portal vein is congested and the bile ductible are surrounded with heavy infilteration of inflammatory cells (arrows) 40X.

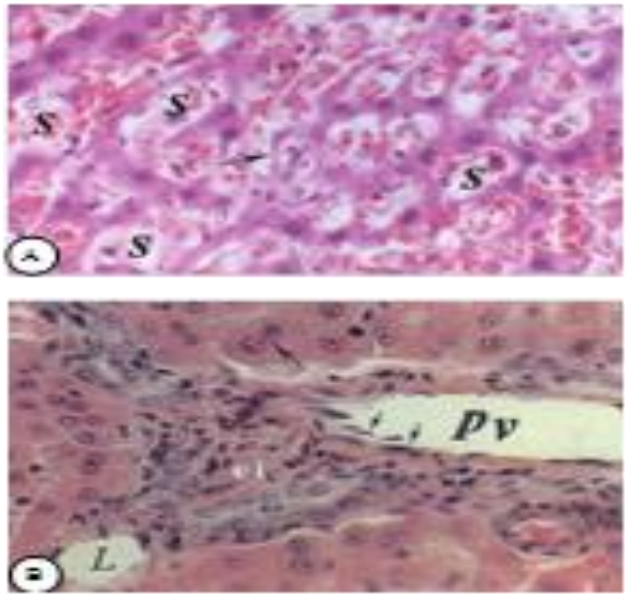

Figure 7. A. A photomicrograph showing well marked dilatation of blood sinusoids (S) accompanied by atrophy of hepatic cords (X330). **B.** A photomicrograph showing damage of endothelial lining of portal vein (Pv). The adjacent area shows eosinophilic infiltration with sever damage to bile ductile (BD) (X330).

species of *Oscillatoria* sp. from Makkah Al-Mukarmah area. The identification was confirmed by morphological observation. Microcystins (MCYTS) was detected from the crude cells extract by ElISA and HPLC. HPLC

technique was applied to isolate and characterize different forms of Microcystin. HPLC profile was typical of MYCTS-LR produced by different strains of MCYTS producing cyanobacteria. The major product ions peak were found in MCYSt-LR at 2.0 min retention time. This confirmed the presence of MCYST-LR in *O. agardhii* cells extract. Efficacy of water treatment procedures of recent evidences suggest that parts per billion levels of certain cyanobacterial toxins (microcystins and nodularins) may be associated with non-lethal acute chronic health effects and should be monitored and controlled in domestic water supplies. Several nations, including Canada, Australia and the United Kingdom are currently moving towards the development of health guidance levels for microcystins in drinking water. When evaluating water treatment procedures for the removal of cyanobacterial toxins, one is faced with problems regarding soluble and suspended substances. Extensive growth of cyanobacteria as indicators of polluted waters and their presence in the food chain have been recognized. The reason could be lesser awareness of problem and need for more surveys in the third world countries. This study gives an account of the toxic potential of these cyanobacteria, which cause hepatotoxicity.

Cyanobacterial toxins involve in food supplements may lead to greater health hazards. It is of great demand to test all potential sources of cyanobacterial contamination such as water storage reservoirs, drinking water and food supplements. The problems of toxic cyanobacteria in environment also have other serious implications. For instance, Non-toxic cyanobacteria that regarded as safe and promising sources of food dietary. Cyanobacteria are currently regarded as bio-fertilizer and the practice of Anabaena– Azolla symbiotic system has been followed in wetland rice farming for ages. For their success, it is essential to remove the undesirable effects such as toxins, odour and taste, filter clogging and water flow blocking. Cyanobacteria have been preferential proli-feration in wetlands waters. This is even more serious with water bodies subjected to thermal pollution through heated effluents, such as ones from cooling towers of thermal power plants. Care has to be taken in avoiding toxic cyanobacteria blooms in such cases. It is suggested that cyanobacteria species and their toxicity, should also be among regulatory parameters for environmental monitoring, impact assessment and eco-epidemiological surveys along with drinking water related activities and over all water resources management.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Biochemical and kinetic characterization of geranylgeraniol 18-hydroxylase (CYP97C27) from *Croton stellatopilosus* Ohba

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Geranylgeraniol 18-hydroxylase (EC 1.14.13.110) that exists solely in *Croton stellatopilosus* Ohba catalyses the last committed step of plaunotol biosynthetic pathways by conversion of geranylgeraniol (GGOH) to plaunotol. This enzyme and its gene are an attractive target for development of plaunotol production and its detailed biochemical properties need to be understood. Recently, even though the gene (*CYP97C27*) coding for GGOH 18-hydroxylase has been identified, cloned, and expressed in *Escherichia coli* system, the enzyme activity has been detected mainly in the insoluble fraction (20,000 *g*). This means that biochemical and kinetic studies could not be undertaken. However, our previous study indicated that this enzyme activity was easily and specifically detected in the microsomal fraction (100,000 *g*) of a crude enzyme extract. Therefore, in this report we describe a comprehensive biochemical characterization of GGOH 18-hydroxylase activity in the microsomal fraction from *C. stellatopilosus* Ohba. The oxygen-dependent enzyme activity of GGOH 18-hydroxylase was inhibited by carbon monoxide and the inhibition was partially reversible upon illumination with white light. Kinetic studies of the GGOH 18-hydroxylase showed high affinity to GGOH and NADPH with apparent K_m values of 0.8 and 53 µM, respectively. Furthermore, the enzyme activity was inhibited by P450 inhibitors, including ancymidol, metyrapone, miconazole, potassium cyanide and cytochrome c, with the IC_{50} values of 428, 65, 75, 66 and 8 µM, respectively. Based on the biochemical and kinetic characteristics, the GGOH 18-hydroxylase in the microsomal fraction is likely a P450 encoded by *CYP97C27* gene as previously described.

Key words: Plaunotol, cytochrome P450, enzyme activity, enzyme inhibitor, microsome.

INTRODUCTION

Plaunotol, an acyclic diterpenoid compound, has important chemotherapeutic activities; it exhibits an anti-inflammatory action in association with peptic ulcers and antimicrobial activities against *Helicobacter pylori* and *Staphylococcus aureus* (Koga et al., 2002; Inoue et al., 2004; Premprasert et al., 2013). It was originally found in

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Abbreviations: GGPP, Geranylgeranyl diphosphate; **GGOH,** geranylgeraniol; **PMSF,** phenylmethylsulfonyl fluoride; **KCN,** potasium cyanide; **CO,** carbon monoxide.

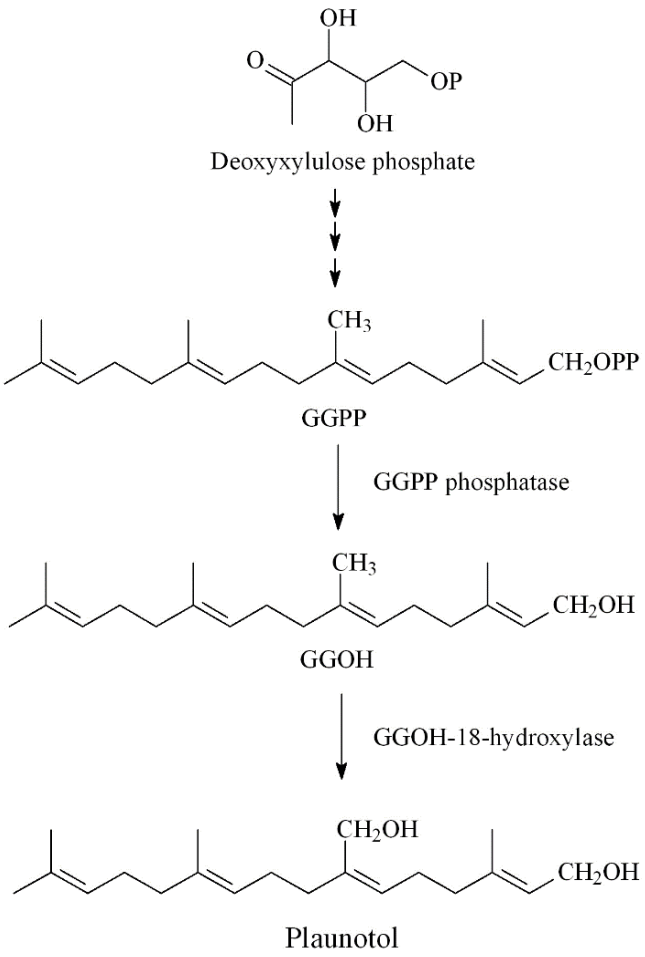

Figure 1. The biosynthetic pathway of plaunotol in *C. stellatopilosus* via deoxyxylulose phosphate pathway (modified from Nualkaew et al., 2006).

leaves and stems of *Croton stellatopilosus* Ohba (Euphorbiaceae), a medicinal plant growing in tropical Southeast Asian countries, especially Thailand. It appears to be accumulated mainly in the chloroplasts (Wungsintaweekul and De-Eknamkul, 2005; Sitthithaworn et al., 2006). Previous study on plaunotol biosynthesis in *C. stellatopilosus* has revealed that biosynthesis of plaunotol was carried out via the deoxyxylulose phosphate pathway in which geranylgeranyl diphosphate (GGPP) and geranylgeraniol (GGOH) were the intermediate precursors (Nualkaew et al., 2005; Wungsintaweekul and De-Eknamkul, 2005). As shown in Figure 1, GGPP is dephosphorylated by phosphatase to form GGOH, then the GGOH is subsequently hydroxylated at the C-18 position by GGOH 18-hydroxylase (EC 1.14.13.110) to form plaunotol, where the hydroxylation of GGOH exhibits the requirement of NADPH as a reducing equivalent of the reaction (Tansakul and De-Eknamkul, 1998; Nualkaew et al., 2005; Nualkaew et al., 2006). This suggested that the GGOH 18-hydroxylase is a member of cytochrome P450 monooxygenases (CYP), similar to other P450 hydroxylases involved in plant secondary metabolisms (Collu et al., 2001; Jennewein et al., 2003). As yet, there are no more details with regard to the biochemical and kinetic properties of GGOH 18-hydroxylase although the corresponding gene (*CYP97C27*) has been identified, cloned and expressed in an *Escherichia coli* system (Sintupachee et al., 2014). The expressed GGOH 18-hydroxylase protein was highly detectable in the insoluble fraction of the crude enzyme, however it was difficult to obtain in highly purified solubilized form and thus it was not amenable to detailed characterization. However, all P450 enzymes in plants have been reported to be localized to the microsomal fraction (Schuler, 1996) and the activity of our enzyme of interest (GGOH 18-hydroxylase) has been shown to exist in the 100,000 *g* microsomal pellet fraction of crude enzyme extract. Therefore, in this work, we investigated the biochemical and kinetic properties of GGOH 18-hydroxylase in the 100,000 *g* microsomal fraction.

MATERIALS AND METHODS

Plant materials and chemicals

Fresh mature leaves of *C. stellatopilosus* Ohba were kindly provided by the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand, and maintained at -20°C until used. All chemicals were of the highest purity available. Geranylgeraniol (GGOH), β-nicotinamide adenine dinucleotide phosphase (reduced form) tetrasodium salt (NADPH), β-mercaptoethanol, ancymidol, metyrapone, miconazole and cytochrome c were purchased from Sigma Chemical Co., (USA). Tricine and dithiothreitol (DTT) from USB (USA). Phenylmethylsulfonyl fluoride (PMSF) and potasium cyanide (KCN) from Fluka (Switzerland). Ethyl acetate and absolute ethanol were of HPLC grade and purchased from Lab-Scan Asia (Thailand). Plaunotol was obtained from Kelnac® soft gelatin capsules (Sankyo, Co., Japan).

Preparation of microsomal fraction containing GGOH 18-hydroxylase activity

All enzyme preparation steps were performed at 0 to 4°C. The GGOH 18-hydroxylase was prepared and purified according to the method as previously described (Chanama et al., 2009). Briefly, 30 g of frozen mature leaves were ground rapidly to a powder in liquid nitrogen using a prechilled mortar and pestle. Then, the fine powder was extracted in 60 ml of extraction buffer containing 83 mM Tricine-NaOH (pH 7.8), 0.4 M sucrose, 10 mM EDTA, 10 mM $MgCl_2$, 10 mg/ml BSA, 1 mM DTT, 0.8 mM PMSF and 5 mM β-mercaptoethanol, and the mixture was stirred for 10 min. The homogenate was filtered through several layers of cheesecloth, centrifuged at 3,000 *g* for 10 min and the supernatant collected prior to spinning for 20 min at 20,000 *g*. Resulting supernatant was further ultracentrifuged at 100,000 *g* for 60 min and the microsomal precipitate was suspended in 3 to 5 ml of 0.1 mM Tricine-NaOH (pH 7.8) containing 0.2 M sucrose, 1 mM EDTA, 1 mM DTT, 15% glycerol and 5 mM β-mercaptoethanol. The final enzyme preparation containing the GGOH 18-hydroxylase was stored at -80°C, and protein concentration of the enzyme was measured using the Bio-Rad protein assay (Bradford, 1976).

GGOH 18-hydroxylase activity assays

Enzyme activity of GGOH 18-hydroxylase was assayed as previously described (Chanama et al., 2009). The reaction mixture consisted of 83 mM Tricine-NaOH (pH 7.8), 0.8 mM NADPH and 57 µM GGOH (substrate). The reaction was initiated by the addition of 100 µl of enzyme preparation (~ 250 µg of protein) to the mixture. The reaction mixture was incubated at 30°C for 30 min and then stopped by extraction twice with an equal volume of ethyl acetate. The extracts were pooled and dried, and the residue was redissolved in ethyl acetate for analysis of plaunotol. Plaunotol content in the extract was determined using a TLC-densitometric technique as previously described. Briefly, the extract of enzyme product was applied onto silica gel_{60} F_{254} plates (CAMAG) using Linomat IV (CAMAG). The plate was developed by ethyl acetate and scanned at wavelength of 210 nm to obtain chromatogram of the sample. The plaunotol content was then estimated on the basis of the standard calibration curve of pure plaunotol compound. The assays were performed in triplicate. The enzyme activity is expressed as katal unit (kat). One katal is the amount of enzyme required to convert GGOH substrate to one mole of plaunotol product per second. For kinetic studies of the GGOH 18-hydroxylase, the maximum velocity (V_{max}) and the Michaelis constant (K_m) values were determined under the standard assay conditions with substrate concentrations ranging from 0.2 to 10 µM for GGOH and from 0.02 to 1.0 mM for NADPH.

Inhibition experiments

The inhibition by gaseous carbon monoxide (CO) and reversal of CO inhibition by white light were performed in 2 ml septum-capped glass vials containing all the reaction components except GGOH and NADPH. The vials were placed on ice, and each gas (CO, N_2 or air) was bubbled through the reaction mixtures. The reactions were initiated by the addition of GGOH and NADPH, and then incubated at 30°C for 30 min in the dark or under white light (for light reversal of CO inhibition). After incubation, the reaction products were extracted and analysed as described above. The control vials without the addition of inhibitors were carried out in the dark. The cytochrome P450 inhibitors: ancymidol, metyrapone, miconazole, KCN and cytochrome c were tested. The concentrations of ancymidol, metyrapone, miconazole and KCN were adjusted from 0.01 to 6.0 mM and cytochrome c from 0.003 to 0.3 mM. The enzyme inhibition tests were carried out by addition of the inhibitors to the standard assay. After completion of the reactions, the enzyme products (plaunotol) were extracted and analysed by TLC. The inhibition is expressed as IC_{50} value, that is, the concentration of an inhibitor where the enzyme activity is reduced by 50%.

RESULTS AND DISCUSSION

The enzyme GGOH 18-hydroxylase found in *C. stellatopilosus* catalyses the conversion of GGOH to plaunotol by addition of hydroxyl group (-OH) to acyclic diterpenoid GGOH at position of C-18 and the hydroxylation reaction is highly specific to this acyclic diterpene substrate and not for other terpenoid compounds (C-10: geraniol, C-15: farnesol) (Tansakul and De-Eknamkul, 1998). In addition, the activity was determined 1.5-fold higher in 100,000 *g* microsomal fraction than in the 20,000 *g* insoluble fraction under the presence of NADPH and aeration (Chanama et al., 2009) and also observed in other plant sources (Bolwell et al., 1994, Pierrel et al., 1994). In this work, we studied the kinetic properties of the GGOH 18-hydroxylase enzyme in the 100,000 *g* microsomal fraction with its substrate and cofactor. The results showed that the GGOH 18-hydroxylase was saturated at approximately 7 µM GGOH and 500 µM NADPH, and apparent K_m values for the hydroxylation of GGOH and for NADPH were 0.8 and 53 µM, respectively (Figure 2). The very low K_m value of the hydroxylase for GGOH implies the high affinity of the GGOH 18-hydroxylase for the GGOH substrate, and this result is in good agreement with the K_m values obtained for the hydroxylation of laurate by lauric acid (P450) monooxygenase from Jerusalem-Artichoke (*Helianthus tuberosus*) (0.97 µM) (Salaun et al., 1978) and from wheat (*Triticum aestivum*) (8 µM) (Zimmerlin et al., 1992). Interestingly, the apparent K_m value of this enzyme for diterpenoid GGOH was 20 to 60 fold lower than those for the hydroxylations of other fatty acid (16-hydroxypalmitic acid, 50 µM), acyclic monoterpene (geraniol, 15 µM), cyclic monoterpene (limonene, 18 to 21 µM), cyclic diterpene (taxoid, 50 µM) and phenolic compound (cinnamic acid, 35 µM) from other plant species (Soliday and Kolattukudy, 1978; Karp et al., 1990; Hallahan et al., 1992; Petersen, 1997; Jennewein et al., 2003). In addition, consumption of NADPH in the hydroxylation is essential for the enzyme activity and in a good agreement with the result reported by Tansakul and De-Eknamkul (1998). The K_m value observed in this study certainly supports the view that GGOH and NADPH are actual substrate and cofactor in the enzyme system.

To investigate whether the GGOH 18-hydroxylase present in the microsomal fraction belongs to a member of cytochrome P450 monooxygenase, several of the following criteria, that is, a requirement for molecular oxygen (O_2), inhibition by CO and reversal of the CO inhibition by light, and inhibition by specific cytochrome P450 inhibitors were examined (Krochko et al., 1998; Kim et al., 2004). To address whether molecular oxygen (O_2) was essential for GGOH 18-hydroxylase activity, replacement of dissolved oxygen in the reaction mixture by nitrogen gas prior to initiating reaction was performed. The reaction exhibited a dramatic decrease in enzyme activity (85% inhibition) (Table 1). Moreover, carbon monoxide, which is known to bind effectively to Fe(II)-heme region of the cytochrome P450 (Krochko et al., 1998), also exhibited the strongest inhibition when it was introduced into the enzyme system in the dark. The relative activity of GGOH 18-hydroxylase inhibited by CO was reduced to as low as 5.87% of control (94% inhibition). This inhibition was partially reversible upon illumination with visible light (relative activity of 35%) as shown in Table 1. This partial reversion of CO-inhibition by white light (particularly at wavelength of 450 nm) could be due to the photolysis of Fe (II)-CO complex which exists in the GGOH 18-hydroxylase. The effects of specific cytochrome P450 inhibitors (ancymidol,

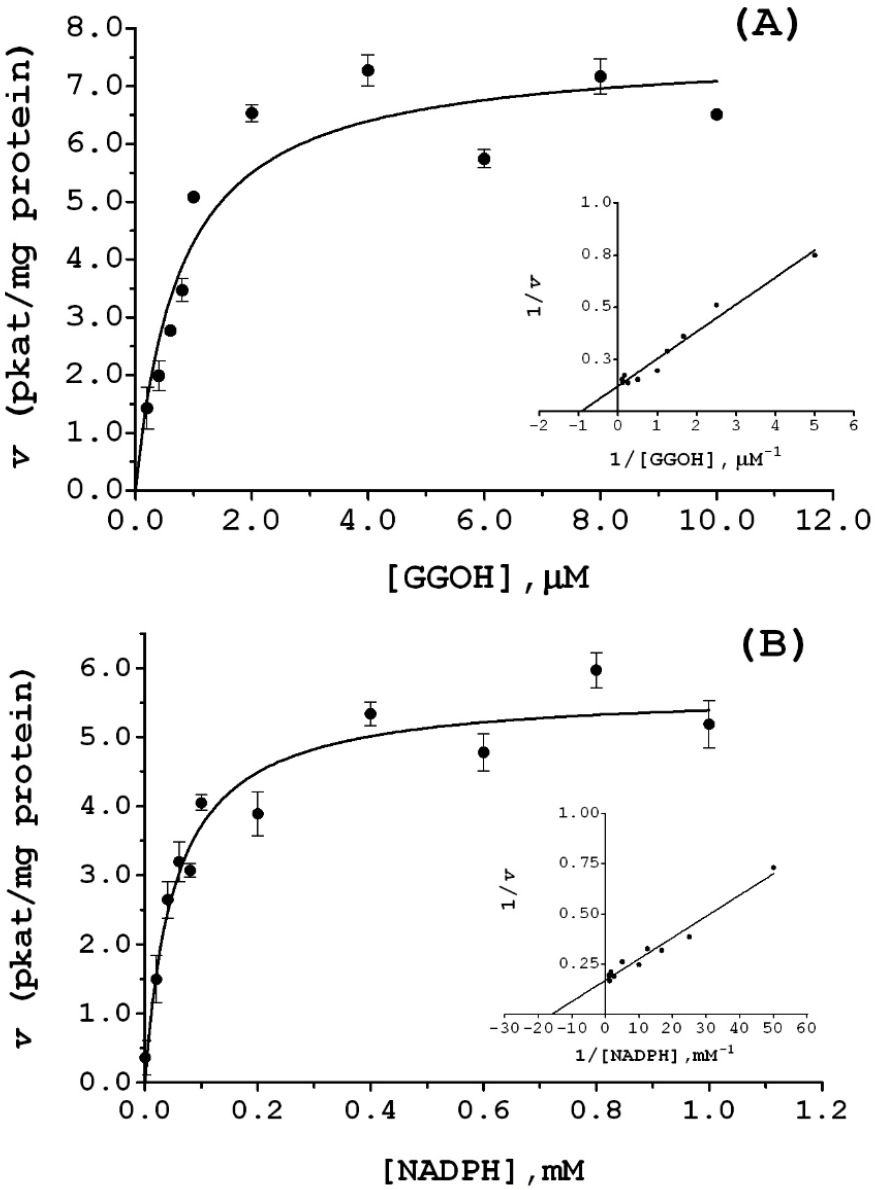

Figure 2. Michaelis-Menten plots showing the variation of initial velocity of GGOH 18-hydroxylase reaction as a function of GGOH (A) and NADPH (B) concentrations. The double reciprocal plots 1/V as a function of 1/[GGOH] and of 1/[NADPH] are shown in the insets. The values are means (±SEM) of three separate experiments.

Table 1. Effect of oxygen (air), nitrogen (N_2) and carbon monoxide (CO), and white light on the activity of GGOH 18-hydroxylase from *C. stellatopilosus*.

Treatment	Relative activity [a] (% of control)	Inhibition (%)
Control (air, dark)	100.0	00.00
N_2	14.47	85.53
CO (dark)	05.87	94.13
CO (white light)	35.56	64.44

[a]The enzyme activity of the control was 1.77 pkat/ mg protein.

metyrapone, miconazole, cytochrome c and KCN) were used in this study. All cytochrome P450 inhibitors inhibited the enzyme activity of GGOH 18-hydroxylase completely or to a lesser extent and with variable concentrations for half-maximal activity (IC_{50} values) (Figure 3). Ancymidol, metyrapone, miconazole and KCN exhibited 100% inhibitory effects on the hydroxylation reaction at concentrations ranging from 0.01 to 6 mM with IC_{50} values of 428, 65, 75 and 66 µM, respectively.

On the other hand, cytochrome c which is known to remove electrons competitively from NADPH-cytochrome

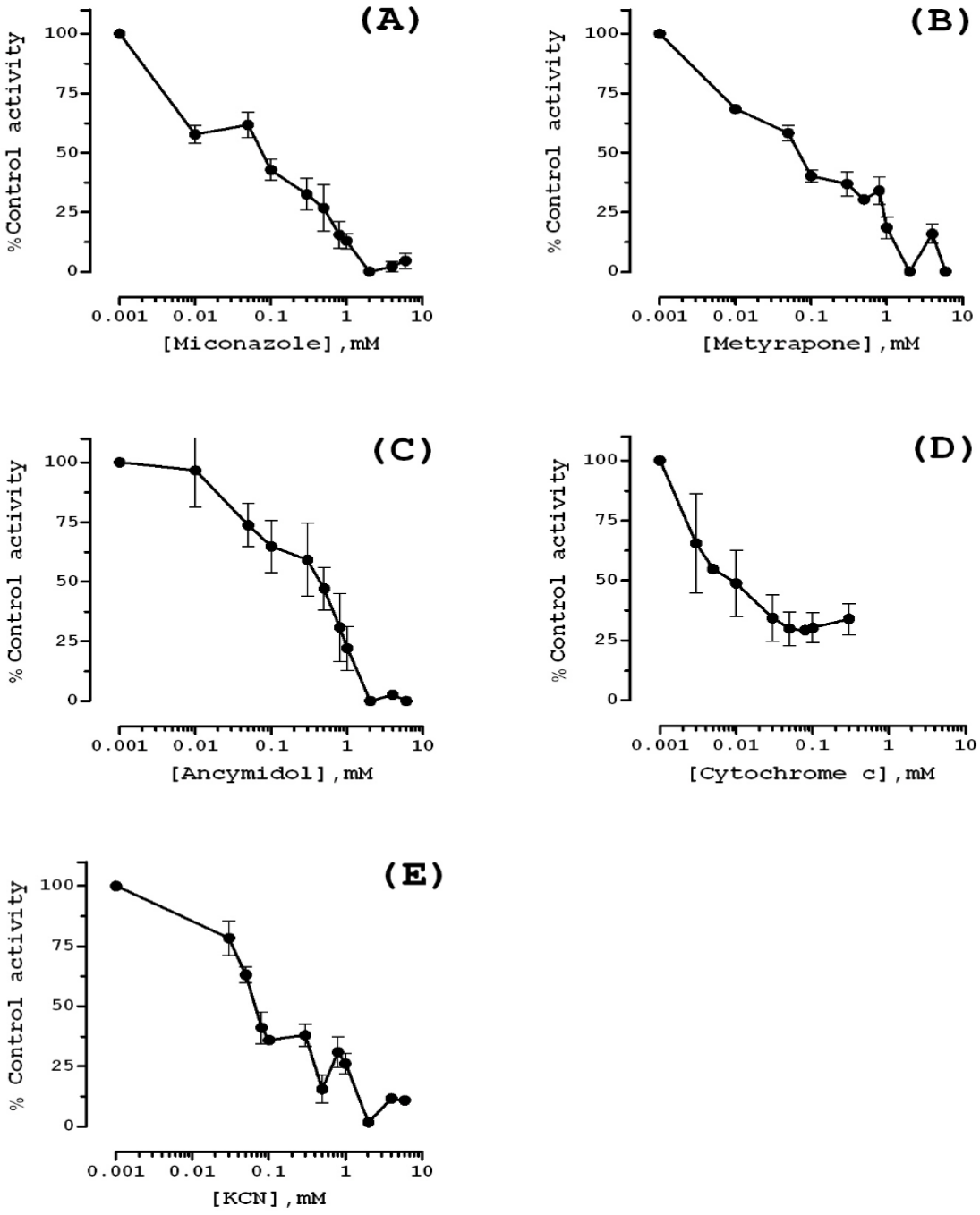

Figure 3. Inhibitions of GGOH 18-hydroxylase activity by cytochrome P450 inhibitors. The values are means (±SEM) of three separate experiments.

P450 reductase complex (Petersen, 1997), showed about 75% inhibition with the lowest IC_{50} of 8 µM. Among these inhibitors tested, cytochrome c seemed to be the most potent inhibitor against GGOH 18-hydroxylase (IC_{50} of 8 µM). These results strongly support a typical feature of plant cytochrome P450s of the enzyme (Friederich et al., 1999; Yamamoto et al., 2000; Katano et al., 2001). According to the biochemical and kinetic properties of the GGOH 18-hydroxylase, that is, high affinity to GGOH substrate, requirement for molecular oxygen and NADPH, inhibition by P450 inhibitors, inhibition by CO, and reversal of the CO inhibition by light, it is likely that the GGOH 18-hydroxylase in the 100,000 *g* microsomal fraction of *C. stellatopilosus* is the CYP97C27 previously identified by RT-PCR methodology from leaves of *C. stellatopilosus* (Sintupachee et al., 2014).

Conflict of interests

The authors did not declare any conflict of interest.

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Investigation of parietal polysaccharides from *Retama raetam* roots

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This study characterizes the cell wall hemicellulose and pectins polymers of *Retama raetam*. This species develops a particularly important root system and is adapted to arid areas. The cellulose, hemicelluloses and pectins were extracted. The cellulose remains the major component of the wall (27% for young roots and 80% for adult roots), hemicelluloses (14.3% for young roots and 3.6% for adult roots) and pectins (17.3% for young roots and 4.1% for adult roots). The monosaccharidic composition of water soluble extracts determined by gas liquid chromatography (GLC) and completed by infrared (FTIR) spectroscopy of hemicellulosic shows the presence of xylose as a major monosaccharide in the non-cellulose polysaccharides (47.8% for young roots and 59.5% for adult roots). These results indicate the presence of the homogalacturonans and rhamnogalacturonans in pectin. This study constitutes the preliminary data obtained in the biochemical analysis of the parietal compounds of the roots of a species which grows in an arid area in comparison with those of its aerial parts.

Key words: *Retama raetam*, roots, cell wall, investigation, polysaccharides, monosaccharidic.

INTRODUCTION

The cell wall compartment is the subject of many studies because of the important roles that it plays in the vegetal cell (growth, protection, defence against the phyto pathogenes). The components of the cell wall are subjected to applied research since they constitute 80% of the vegetal biomass (Robert and Roland, 1989). The structural diversity of plant cell wall polysaccharides has brought lots of application in many diverse domains. For

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example, polysaccharides can be used in the metal biosorption (Hachem et al*.,* 2012) or in the cholesterol biosorption (Copikova et al*.,* 2013). Some hemicelluloses present immune-stimulating properties (Caffall and Mohnen, 2009) or inhibiting properties of cellular proliferation (Barbat et al., 2010).

Few studies have been conducted in relation to the walls of roots (Leucci et al., 2008). This is the reason a biochemical study of the walls of the roots of *R. raetam* was undertaken. This species belonging to the Fabaceae family has a very productive vertical and horizontal root system which can reach 20 m. This in turn increases substantially the stabilization of the soil. Moreover, the *Retama* species contributes to the bio fertilisation of poor grounds because of their aptitude to associate with fixing nitrogen bacteria *Rhizobia* (Boulila et al., 2009; Selami et al*.,* 2014). Therefore, the genus of the *Retama* is included in a re-vegetation program for degraded areas in semi-arid Mediterranean environments (Caravaca et al*.,* 2003). This preliminary study on the biochemical analysis of the parietal compartment of *R. raetam* roots could help in developing future valorization strategies of cell wall polysaccharides (bioactives molecules, cellular signalisation).

MATERIALS AND METHODS

Biological materials

Seeds, stems and leaves of *R. raetam,* were collected in Oran, Algeria, in 2012, and identified by Pr. M. Kaid Harche, University of Sciences and Technology, Mohamed Boudiaf. A voucher specimen (F 2652) was deposited at the Herbarium of the Department of Biotechnology, University of Sciences and Technology, Mohamed Boudiaf, Oran, Algeria.

Plant samples consist of *R. raetam* young and adult roots. In the case of the young roots, plantlets were obtained from 10 days old germinations. Two centimeter long fragments were excised from the above apical zone of roots. For adult roots, 2 cm long-fragments were excised from the differentiated zone of 5 year old plants growing in natural conditions (USTO university campus). The samples were collected in March 2012.

Extraction of cell wall polysaccharides

The sequential and selective extraction of parietal polysaccharides of the roots of *R. raetam* was carried out according to Bailey (1967) and Carpita (1984). The extractions were carried out under magnetic agitation and the residues were separated from the supernatants by filtration on sintered glass (porosity 3). Dialysis was carried out in a Spectrapor membrane whose cutting threshold lies between 6000 and 8000 Da. The steps are presented in Figure 1.

Colorimetric assay of total sugars

The uronic acid contents of the polysaccharidic fractions were determined following the meta-hydroxydiphenyl method (Blumenkrantz and Asboe Hansen, 1973). Glucuronic acid was used as standard. The determination of the content of neutral sugars of polysaccharidic fractions was carried out by the method with phenol/H_2SO_4 (Dubois et al., 1956) with glucose and xylose as standards. A correction by calculation was made in order to take into account the interferences due to the presence of the uronic acids as established by (Montreuil et al., 1963):

$[ON] = (DO_{Phenol} - b^* [AU]) / a$
$[AU] = (DO_{MHDP} -(a' / a) * DO_{Phenol}) / ((ab'-a'b) / a)$
[ON]: Concentration in neutral sugars
[AU] = uronic acids concentration
a = Slope (Glc) of the assay of the neutral sugars
b = slope (GalA) for the assay of the neutral sugars
$DO_{Phenol} = a [ON] + b [AU]$
a' = Slope (Glc) for the assay of the uronic acids
b' = slope (GalA) for the assay of uronic acids
$DO_{MHDP} = a' [ON] + b' [AU]$.

Qualitative analysis by gas liquid chromatography (GLC)

Prior to analysis, polysaccharidic fractions were submitted to methanolysis. The methylglucosides released obtained were then derivatized by trimethylsilylation.

Preparation of themethyl glycosides trimethylated derivatives

The analysis was carried out on 200 to 500 µg of freeze-dried polysaccharide powder, added to an internal standard, mesoinositol (MI), at a rate of 10% of the quantity of polysaccharide. This method was used for the direct analysis of crude plant powders (Marga et al*.,* 1995). It can also be used for the study of the monosaccharidic composition of the residues of extraction.

Methanolysis

Monosaccharides were released as methylglycosides by adding 1 mL of 1 M hydrochloric methanol to the anhydrous polysaccharide sample. After 24 h, at 80°C in a sealed tube, the methanolisis was stopped by evaporation of the hydrolyzate under a nitrogen flux. The residue was dissolved in 1 mL methanol and then delipidated by three successive extraction with heptane (3 x 1 mL). Finally, it was evaporated again under nitrogen flux.

Trimethylating

The methylglycosides were then trimethylated with 100 µL of N,O-bis-trim ethylsilyl- trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) in pyridine (100 µL), in the darkness during 2 h at 27°C. These samples were maintained at -20°C during 24 h and then directly injected in GLC.

Analysis of the trimethylsilylation derivatives by gas liquid chromatography

Methylglycosidestrimethylsylilated have been identified by GLC by comparison with authentic samples using a Perichrom PR 2100 chromatograph equipped with a capillary tube (0.32 mm by 60 m) OPTIMA® 1-Accent 0.25 µM (Macherey-Nagel) and with a flame ionization detector (FID). The carrier gas is nitrogen under a pressure of 75 kPa. The temperature of the injector was fixed at 260°C. The rise in the temperature of the furnace has been programmed from 130 to 210°C at a rate of $2°C.min^{-1}$, with a 5 min mitigation at 190°C, then from 210 to 260°C at a rate of $5°C.min^{-1}$.

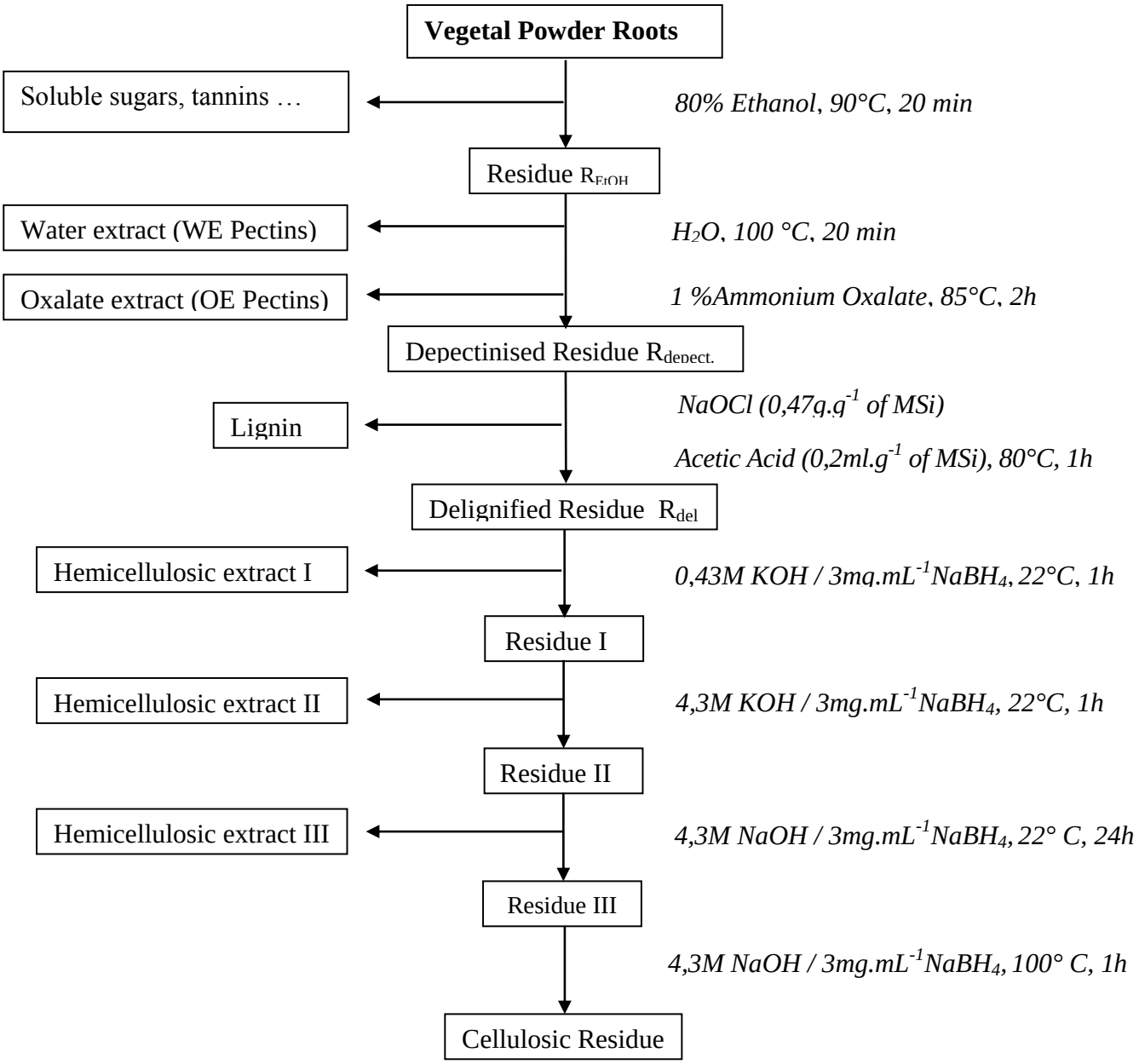

Figure 1. Selective and sequential extraction protocol of cell wall polysaccharides adapted from Bailey (1967) and Carpita (1984).

The chromatograph has been controlled by the software Winilab III (Perichrom). The results were expressed as a molar percentage after correction of the areas of the chromatographic peaks by the response factors.

Evaluation of the degree of methylesterification of pectins

A biochemical assay of methanol was carried out after saponification on extracted pectic fractions (WE and OE) in order to determine the degree of methylesterification of pectins. The methanol compound was determined by visible UV spectrometry: the coupling of Purpald with methanol produced by the enzymatic oxidation of methanol led to the formation of a purple compound dosed at 550 nm as described by (Anthon and Barrett, 2008).

The methanol concentration was estimated by reference to a methanol standard (0 and 40 µg.L $^{-1}$). 10 mg of WE and OE were mixed with 1 ml of 0.1 M NaOH in order to saponify the ester functions. The suspension was maintained to 4°C during 15 h. After neutralization of excess NaOH by adding hydrochloric acid, the suspended particles were eliminated by centrifugation (12000 *g*, 5 min, and 4°C). The supernantant constitute the sample for methanol assay. A volume of 100 µL (or diluted sample) or standard was added to 90 µL of 200 mM potassium phosphate buffer, pH 7, then 10 µL of enzymatic

Table 1. Extraction yields of extracted fractions from young roots (YR) and adult roots (AR). All fractions were dialysed before lyophilisation and weighting.

Parameter		Extracted Quantity in g	Extraction yields expressed in percent of dry mass	Uronic acid content (%)
Ethanolic extract	YR	0.3	14.6	Nd
	AR	1	6.4	Nd
WE	YR	0.2	9.7	16.2
	AR	0.4	2.6	15.6
OE	YR	0.1	7.6	32.5
	AR	0.2	1.5	20.8
Hemicellulosic Fractions	YR	0.2	14.3	11.1
(I , II and III)	AR	0.6	3.6	12.4
Cellulosic fractions	YR	0.5	27	Nd
	AR	12.8	80	Nd

WE, water extract; OE, oxalate extract; YR, young roots; AR, adult roots; Nd, not detected.

alcohol oxydase solution (Alcohol oxidase from *Pichia pastoris*, Sigma) freshly prepared in the same phosphate buffer. The samples was homogenized, then incubated for 10 min at 30°C. Then 200 µL of Purpald at 5 $mg.mL^{-1}$freshly were prepared in 0.5 M NaOH were added. The samples were homogenized then incubated for 40 min at 30°C. Finally, 600 µL of water were added. After homogenisation, the absorbance was measured with a UV-Visible spectrophotometer (Shimadzu, PharmaSpec UV-1700). The results were expressed in percentage of the molar quantity of methanol in relation to the molar quantity of galacturonic acid of the extract determined by GLC.

Characterization by infrared (IR) spectroscopy

Polysaccharidic fractions were characterized by infrared (IR) spectroscopy with a 1000 FT-IR Perkin Elmer Spectrum spectrometer in the 400 to 4000 cm^{-1} frequency range.

RESULTS AND DISCUSSION

Yield of extraction of polysaccharidic components

The quantities of *R. raetam* root parietal components are represented in Table 1. The compounds extracted by ethanol represent 14.6% of the mass of the young roots and 6.4% of the adult roots. This difference in percentage was probably due to a more efficient elimination in adult roots of pigments, tanins or suberin. The WE fractions were relatively important compared to OE, and contained from 15.6 to 32.5% of uronic acids. The OE fractions was represented as 7.6% against 9.7% for young roots and 1.5% against 2.6% for adult roots expressed in percentage dry mass. The percentage of pectic substances in adult roots seemed lower than that of young roots. This could be explained by the inaccessibility of these polymers since strong linkages (covalent) between lignins, hemicelluloses and these pectins may exist; this impair the attack by chemical treatment.

Young roots showed a high hemicellulose content compared to adult roots (14.3% against 3.6%), and were mainly composed of contained neutral oses (89% in average). Conversely, the percentage of cellulose in adult roots (80%) was definitely higher than that in young roots (27%). However, the total extraction yields were higher for adult roots (94% of the DW) than for young roots (73% of the DW). It can be noticed that cellulose represented the majority (80%) of the cell wall, the hemicellulosic fraction represented 14.3% for young roots and 3.6% for adult roots. Hemicelluloses were less accessible in the secondary walls lignified tissues (Harche et al.,1989).

The percentages of the pectin fractions WE and OE were relatively low for young and adult roots (9.7 and 2. 6%) respectively, for WE and 7. 6% and 1.5% for OE. This could be explained by the presence of covalent links between pectins and other parietal components**,** but also linkages between pectins exist (rhamnogalacturonans II). According to Thibault and Saulnier (1991), the extraction of pectins (which form an eggbox structure) could be improved by a chelating agent of metals like ammonium oxalate (Golovchenko et al., 2012).

Xu et al*.* (2007) have recorded losses in weight of about 24% of the initial mass, following the treatment of depectinisation with hot water of the leaves of some Poaceae.

Determination of the monosaccharidic composition by GLC

The filtrates and the residues resulting from the various extractions have been characterized by GLC (Table 2).The analysis of the WE and OE of young and adult roots, confirmed their pectic nature by the high rate of galacturonic acid (13.4 and 14.6% for the WE of the young and adult roots respectively, 28.6% and 19% at the OE respectively). The rhamnose rates varied from 5

Table 2. Monosaccharidic composition of the extracts and the residues of extraction, from *R.raetam* young roots (YR) and adult roots (AR)

Fractions	Monosaccharides (% molar)							
	Ara	Rha	Xyl	Man	Gal	Glu	Gal A	Glc A
Young roots (crude extract)	23.9	6.6	9.1	2.2	10.7	30.2	15.4	1.0
Adult Roots (crude extract)	21.3	6.2	15.7	0.2	2.5	44.7	6.9	1.7
WE YR	19.4	7.9	8.0	5.0	17.5	25.2	13.4	3.4
WE AR	9.9	5.8	2.6	0.8	4.7	62.2	14.6	1.2
OE YR	19.0	10.2	12.0	0.2	11.9	17.3	28.6	0.2
OE AR	14.0	9.8	7.1	0.5	7.8	40.9	19.0	1.0
Hemicelluloses YR	30.6	11.5	47.8	0.4	17.9	10.0	10.3	1.4
Hemicelluloses AR	8.0	6.4	59.5	0.7	6.1	4.9	11.7	1.0
CelluloseYR	Nd	Nd	3.2	3.5	Nd	90.1	3.2	Nd
Cellulose AR	Nd	Nd	4.9	Nd	5.8	86.6	2.7	Nd

WE, water extract; OE, oxalate extract; YR, young roots; AR, adult roots; Nd, not detected.

to 10%, this led us to suppose that these fractions also contain rhamno-galacturonans which could be substituted by side chains of arabinans, of galactans and/or arabino-galactans. Other identified monosaccharides (mannose, glucuronic acid) were detected at low rates (less than 5%). Consequently, the ratio Gal A/Rha was decreased (2.8% to 1.9% for OE of young and adult roots respectively). This is typical of more ramified structural pectic features.

The hemicellulosic fractions extracted with KOH were rich in xylose (from 47.8 to 59.5 %) which indicated the presence of xylans. The presence of arabinose (8 to 30.6%) indicated the presence of arabinoxylans. Galactose and galacturonic acid were also present (from 6 to 17.9% and 10.3 to 11.7%, respectively); this could be explained by a co-extraction of pectic polymers.

The final residue of the extraction was largely composed of glucose (86 to 90%), monosaccharide characteristic parietal glucans such as cellulose. The presence of low levels of remaining xylose (3 to 5%) suggests that hemicellulosic polymers were co-extracted almost quantitatively during the extraction. According to Chaa et al*.,* (2008), the presence in these features of xylose and arabinose, after the various treatments of depectinisation seems to indicate that hemicellulosic polymers were co-extracted during this stage of depectinisation. Xu et al*.* (2007), noted that the treatment of depectinisation by hot water solubilizes, in addition to pectins, low molecular weight polysaccharides such as galacto-arabinoxylans and also recorded weight losses of about 24% of the initial mass after hot water treatment; the hemicelluloses fractions represented 35% of initial mass and the lignins 10.4%.

The presence of glucose in the WE of young roots and OE of adult roots with contents of 25.2, 62.2, 17.3 and 40.9% respectively suggested that this detected monosaccharides comes from the hydrolysis of the callose, a polysaccharide which is present in the walls of young and mature sieved tube (Currier, 1957). In fact, an amylase treatment has shown a persistence of glucose in the WE of adult roots (85% before treatment and 50% after) and in the OE of the adult roots (88% before treatment and 33.1% afterwards). These results make it possible to argue that in *R. raetam* roots, pectins were strongly related to the callose as observed in the pollen of Nicotiana (Kroh and Knuiman, 1982). We can also suggest that the strong proportion of glucose in the two fractions probably comes from the residual hydrolysis of intra-parietal saccharose. In fact, the conveying of saccharose in fabaceae was primarily performed by means of the apoplasmic pathway (Dinant and Lemoine, 2010). The hemicellulosic fractions extracted with KOH were rich in xylose (from 47.8 to 59.5%) which indicated the presence of xylans. The presence of arabinose (8 to 30.6%) indicated the presence of arabinoxylans (Chaa et al., 2008). Galactose was also present (from 6 to 17.9%); this could be explained by a co-extraction of pectic polymers. They also contained galacturonic acid at 10.3 to 11.7%. Nevertheless, the presence of xyloglucan features in the hemicellulosic fractions was not to be excluded because the Xyl/Glc ratio was from 5 to 12 for young and adult roots respectively.

The composition of the cellulose to glucose residue (Brannavall, 2007) confirms the selectivity of the extraction of other cell wall polysaccharides and thus the good efficiency of the extraction protocol implemented.

Degree of methylesterification (DM) of the pectins extracted from young and adult roots

The DM was established by the relation between the quantity of released methanol during saponification (UV-visible spectrophotometry assay after derivation with Purpald) and the galacturonic quantity of acid previously was determined by GLC. The values obtained were in

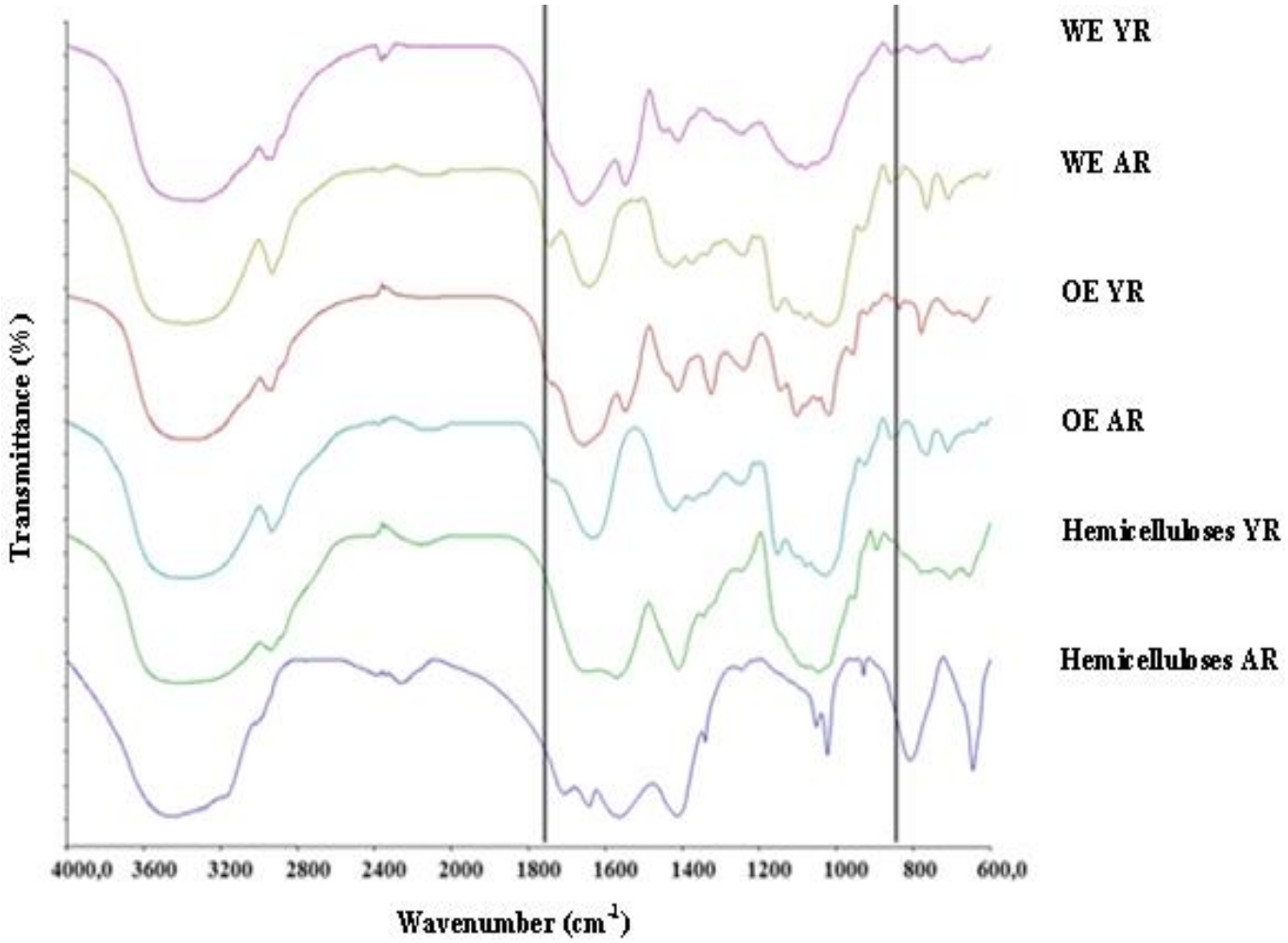

Figure 2. Infrared spectrum of polysaccharides obtained after different extraction steps.

conformity with the extraction strategy: in the pectic fractions (WE), the DMs were often high between (63 and 79%). A strong DM was often correlated with a small proportion of structure of the "egg box" type within pectins. Consequently, these were more easily extracted by hot water. In the case of the pectic fractions with low DM ranging between 50 to 62%, structural features rich in homogalacturonans might be present.

Characterization by infrared spectroscopy

The various parietal polysaccharide fractions were analysed by infrared (IR) spectroscopy (Figure 2). Some parietal polysaccharides have in their structure characteristic chemical groups which can be highlighted (Taboada et al., 2010). They are listed in Table 3.

The comparison of the different spectra (Figure 2) showed, in the case of the pectic fractions WE and OE from YR and AR, the presence of an intense band around 1742 cm^{-1} which corresponded to the elongation band for the C=0 of esters and acids. This band may be attributed to acidic oses esterified or not that are mainly found in pectins and in a lesser proportion in hemicelluloses (Habibi, 2004). Nevertheless,the disappearance of this band was observed in hemicelluloses spectra (YR and AR) after extraction with NaOH. The hemicelluloses spectra also showed typical arabinose absorption bands with low intensity in the vicinity of 1077 to 1154 cm^{-1} because of vibrations C-O-C (Peng and Wu, 2010; Chaa et al., 2008). This might be due to the presence of pectic substances with arabinans inside chains.

The spectra of hemicellulosic fractions of YP and AR showed absorption bands between 1017 and 1101 cm^{-1} with a maximum in the vicinity of 1051 cm^{-1} corresponding to elongation vibrations of linkage inside the cycle and C-OH. These signals are characteristic of xylans. This suggests that the xylose is the main constituent under the form of pyrans (Peng and Wu, 2010). The pectins and hemicelluloses spectra presented other bands around 1545-1570 cm^{-1}. This could be due to the possible presence of residual lignin (Xu et al., 2007).

Table 3. Attribution of the main bands observed on the spectra IR of the various polysaccharidic fractions obtained

Vibration	Attribution	Frequency cm^{-1}
$\sqrt{}_{(OH)}$	Polysaccharides and hydratation water	3313-3383
$\sqrt{}_{(CH)}$	Polysaccharides	2930-2935
$\sqrt{}_{(C=O)}$	Esters and acids	1742
Liaisons absorbed hydrogenes and water		1629-1656
Residual Lignine		1545-1570
$\delta_{(CH)}$ et $\delta_{(CH2)}$ et $\delta_{(O-H)}$	Polysaccharides	1240-1420
$\sqrt{}_{(COC)}$	Skeletal Pyranose	1077-1154
$\sqrt{}_{(C-C)}$	Polysaccharides	1017-1101
$\sqrt{}_{(C1-H)}$	β-D-Xylose	834-859

Conclusion

This preliminary biochemical study of the parietal compounds in the roots gives some information about the polysaccharidic composition of the cell walls of the young and adult roots of *R. raetam*. The ponderal dosage indicates that the cellulose remains a major component of the polysaccharides of the wall (27% for young roots and 80% for adult roots) compared to hemicelluloses (14.3% for young roots and 3.6% for adult roots) and to pectins (17.3% for young roots and 4.1% for adult roots).

The analysis of gas chromomatography and infrared spectroscopy reveal the presence of altering homo-galacturonan and rhamnogalacturonan I blocks which can be substituted by galactan and arabinan side chains in the pectin fraction and the presence of arabinoxylans in the hemicellulosic fraction.

Conflict of interests

The authors did not declare any conflict of interest.

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