African Journal of Biotechnology

Volume 14 Number 10, 11 March, 2015
ISSN 1684-5315

Academic Journals

ABOUT AJB

The **African Journal of Biotechnology (AJB)** (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajb@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajb@academicjournals.org.

Editorial Board

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

African Journal of Biotechnology

Table of Contents: Volume 14 Number 10, 11 March, 2015

ARTICLES

academicJournals

Vol. 14(10), pp. 843-850, 11 March, 2015
DOI: 10.5897/AJB2015.14488
Article Number: 2AFFF3F51256
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Analysis of mtDNA hypervariable region II for increasing the discrimination power from Middle and South of Iraq

Ameera Omran Hussein[1], Muhanned Abdulhasan Kareem[2] and Imad Hadi Hameed[1]*

[1]Department of Molecular Biology, Babylon University, Hilla City, Iraq.
[2]Babylon University, Centre of Environmental Research, Iraq.

Received 8 February, 2015; Accepted 2 March, 2015

Mitochondrial DNA is a useful genetic marker for answering evolutionary questions due to its high copy number, maternal mode of inheritance, and its high rate of evolution. The aims of this research were to study the mitochondria noncoding region by using the sanger sequencing technique and establish the degree of variation characteristic of a fragment FTA® Technology (FTA™ paper DNA extraction) utilized to extract DNA. A portion of a non-coding region encompassing positions 37 to 340 for HVII was amplified in accordance with the Anderson reference sequence. PCR products were purified by EZ-10 spin column then sequenced and detected by using the ABI 3730*xL* DNA analyzer. New polymorphic positions G92C, C113G, C150G, T156A, C194G, C198G, G207C, G225C and G228C are described and may in future be suitable sources for identification purpose. The data obtained can be used to identify variable nucleotide positions characterized by frequent occurrence most promising for identification variants.

Key words: D-loop, HVII, Middle and South of Iraq, mitochondrial DNA.

INTRODUCTION

The mitochondrial DNA (mtDNA) is a small circular genome located within the mitochondria in the cytoplasm of the cell. The mitochondrial genome can be divided into two sections: a large coding region, which is responsible for the production of various biological molecules involved in the process of energy production in the cell, and a smaller 1.2 kb pair fragment, called the control region. It is found to be highly polymorphic and harbors three hypervariable regions (HV), HV1, HV2 and HV3 (Kraytsberg et al., 2004). Mitochondrial DNA comprising of 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs are a small circle of DNA (Helgason et al., 2004). Mitochondrial DNA does not recombine and thus there is no change between parent and child, unlike nuclear DNA. MtDNA is only passed on from mother to child and this is an important fact (Brown et al., 1993; Giulietta et al., 2000). There is more sequence divergence in mitochondrial than in nuclear DNA (Giulietta et al., 2000; Stoneking et al., 2002).

Genetic analyses in population studies of the mitochon-

*Corresponding author. E-mail: imad_dna@yahoo.com.

drial genome can be done either by sequencing the mtDNA or through the use of restriction fragment length polymorphisms (RFLPs) (Young, 2009). RFLPs utilize restriction enzymes that can recognize the presence or absence of specific polymorphic DNA regions, and cut sites in the coding region of the mtDNA. Mitochondrial DNA is a useful genetic marker for answering evolutionary questions due to its high copy number, maternal mode of inheritance, and its high rate of evolution. In modern population genetics research, studies based on mitochondrial DNA (mtDNA) and Y-chromosome DNA are an excellent way of illustrating population structure while tracing uni-parental inheritance and ancestry—mtDNA is maternally inherited while the Y-chromosome is paternally inherited.

The aim of this study was to sequence the portion of the noncoding region of mtDNA in order to ascertain the degree of variation present in this fragment and to find those particular polymorphic positions that fulfill the conditions necessary for their future application in the identification process.

MATERIALS AND METHODS

Sample collection, mitochonderial DNA extraction and amplification

Population sample was collected from 380 healthy unrelated volunteer donors, recruited from Middle and South of Iraq. DNA was extracted from all dried blood samples on FTA cards following the manufacturer's procedure as described in Whatman FTA Protocol BD01 except that the Whatman FTA purification reagent was modified to half the volume (Dobbs et al., 2002).

A 1.2 mm diameter disc was punched from each FTA card with a puncher. The discs were transferred to new Eppendorf tubes and washed three times in 100 µl Whatman FTA purification reagent. Each wash was incubated for 5 min at room temperature with moderate manual mixing and the reagent was discarded between washing steps. The discs were then washed twice in 200 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), the buffer was discarded and the discs were left to dry at room temperature for 1 h. Amplification of HVII region was carried out using five sets of primers

A portion of a noncoding region for HVII was amplified in accordance with the Anderson reference sequence (Table 1). 20 µL of Master Mix was added into a PCR tube and 20 µL of Primer Mix also added. To the same PCR tube, 10 µL of extracting DNA was added after changing the pipette tip again. All the liquid were allowed to settle at the bottom of the tube, and not elsewhere. Check the volume in the PCR tube using the PCR tube with 50 µL in it. 95°C hold for 10 min, 30 cycles of: 94°C for 30 s, 52.5°C for 30 s, 65°C for 1 min. 72°C hold for 10 min. 4°C hold, ∞ infinity is the cycling protocol for amplification of mtDNA PCR.

Purification, cycle sequencing and sequence analysis of mitochonderial DNA

Purification of mitochonderial DNA by EZ10-spin column DNA cleanup kit 100 prep. The DNA Sequencing of the PCR products was done using the BigDye TM Terminator. Utilizing POP-7 polymer (Applied Biosystems) polymer lot number 1206453. The separation of the cycle sequencing products was carried out. Detection was by using the ABI 3730xL DNA analyzer, cap array size 96, cap array length 50. The reference sequence described by Anderson et al. (1981) was compared to the data observed. Within the coding region Mitochondrial DNA, sequencing results are studied from a consensus sequence derived from multiple sequence results. Data were analysed by Sequencher™ (SEQUENCHER™ 4.7 User Manual for Windows © 1991-2007) and aligned with the Anderson sequence (Anderson et al., 1981) using sequence Navigator software.

Statistical analysis

Genetic diversity for the analyzed DNA fragment was calculated according to the formula:

$$h=(1-\sum x_i^2)n/(n-1)$$

Where; n is sample size and xi is the frequency of i-th mtDNA type (Gu, 2001).

The probability of two randomly selected individuals from a population having identical mtDNA types was calculated.

$$(P=\sum x_i^2)$$

Where, p = frequencies of the observed haplotypes (Jones, 1972).

RESULTS AND DISCUSSION

The basic aim of this work was to assess the degree of variation characterizing a selected segment of the noncoding region of mtDNA of human populations from Iraq. The study enabled identification of 107 different haplotypes and 38 polymorphic nucleotide positions (Table 2).

The most frequent variant (H1) was consistent with the Anderson sequence. Substitutions determined during the study are transitions and transversion. This fact is consistent with abundant literature data revealing significant domination of transitions over transversions (Brown et al., 1982; David et al., 2013; Imad et al., 2015a; Mohammed and Imad, 2015). Sixteen (16) polymorphic positions G92C, A95T, C113G, C150G, T156A, T173A, G185C, C186G, A188G, C194G, T195A, C198G, G203C, G207C, G225C and G228C have transverse substitution (Table 3). All the other substitutions determined during the analysis are transitions. The number of analyzed markers has been increased to compensate for the increasing number of profiles in the databases in order to minimize accidental matches between unrelated individuals. Progression of new technology is therefore very slow and the use of SNPs has sometimes met a reluctant reception (Imad et al., 2015b and Mohammed et al., 2015.

Genetic diversity for the analysed DNA fragment was calculated according to the formula: D= 1-$\sum p^2$ and recorded 0.950%. The calculated value of the genetic diversity should be understood as high in the context of

Table 1. Primer sequence, region amplified, fragment size (bp) and PCR product length for HVII.

Primer name	Primer sequence (5' - 3') (forward; F, reverse; R)	Region amplified	Fragment size (bp)	PCR Product length
HVII-1	F: 5'- CACGCGATAGCATTGCGAGA -3'	76-95	20	189
	R: 5'- GTGGCTGTGCAGACATTCAA -3'	264-245	20	
HVII-2	F: 5'- AGCACCCTATGTCGCAGTATC-3'	108-128	21	158
	R: 5'-AGTGGCTGTGCAGACATTCAA-3'	265-245	21	
HVII-3	F: 5'- GAGCACCCTATGTCGCAGTAT-3'	107-127	21	160
	R: 5'-AAGTGGCTGTGCAGACATTCA-3'	266-246	21	
HVII-4	F: 5'- GCGATAGCATTGCGAGACG -3'	79-97	19	200
	R: 5'-TGTCTGTGTGGAAAGTGGCTG-3'	278-258	21	
HVII-5	F: 5'- CGCGATAGCATTGCGAGAC -3'	78-96	19	200
	R: 5'-GTCTGTGTGGAAAGTGGCTGT-3'	277-257	21	

Table 2. Variable positions and Haplotypes for HVII.

Anderson	85	89	92	93	95	113	114	131	143	146	150	151	156	173	178	182	183	185	186	188	189	194	195	198	200	203	207	210	217	225	228	235	239	242	247	248	250	257	No. of individuals
	G	T	G	A	A	C	C	T	G	T	C	C	T	T	A	C	A	G	C	A	A	C	T	C	A	G	G	A	T	G	G	A	T	C	G	A	T	A	
H1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	220
H2	-	-	A	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1
H3	-	-	-	G	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	3
H4	-	-	-	-	-	T	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	-	-	-	G	1
H5	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H6	-	-	-	-	-	T	-	-	-	C	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	1
H7	-	-	-	-	G	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	-	-	1
H8	-	-	-	-	-	-	T	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	2
H9	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	-	-	-	-	-	1
H10	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H11	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	3
H12	-	-	-	-	-	-	-	-	A	-	-	T	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H13	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	2
H14	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	2
H15	-	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H16	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	4
H17	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

Table 2. Contd.

H18	-	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	2
H19	-	-	A	-	-	-	-	-	-	-	-	-	-	C	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H20	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	3
H21	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H22	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	C	-	2
H23	-	-	-	-	-	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H24	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H25	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H26	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	G	-	-	3
H27	-	-	-	-	-	-	-	-	A	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	3
H28	-	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H29	-	-	-	-	-	T	-	-	-	C	-	-	-	-	-	-	-	-	-	G	-	T	-	-	-	-	A	-	-	-	-	-	-	-	A	-	-	-	1
H30	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	1
H31	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	A	-	-	-	3
H32	-	-	-	-	-	-	-	-	-	C	-	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H33	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	C	-	-	-	C	-	1
H34	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	1
H35	-	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	1
H36	-	-	-	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	2
H37	-	-	-	-	-	-	-	-	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	2
H38	-	-	-	-	G	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H39	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H40	-	-	-	-	-	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H41	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H42	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	1
H43	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1
H44	-	-	-	G	-	-	-	-	-	-	-	-	C	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	1
H45	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H46	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H47	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H48	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	G	1
H49	-	-	-	-	-	-	-	C	-	-	-	-	C	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H50	-	-	A	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H51	-	-	-	-	-	-	-	-	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H52	A	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	C	-	3
H53	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	3
H54	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	2
H55	-	-	-	-	-	T	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	2
H56	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H57	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H58	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

Table 2. Contd.

H59	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	1
H60	-	-	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	T	-	-	-	A	-	-	-	-	-	-	-	-	-	G	-	-	1
H61	A	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H62	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	T	-	-	-	-	3
H63	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	1
H64	-	-	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H65	-	-	-	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	1
H66	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	1
H67	-	-	A	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	2
H68	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H69	-	-	-	G	G	-	-	-	-	-	-	-	-	-	G	T	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H70	-	-	-	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	3
H71	-	C	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	2
H72	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H73	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H74	-	-	-	-	-	-	T	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	1
H75	A	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	A	-	-	-	4
H76	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	1
H77	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	2
H78	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H79	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	3
H80	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	1
H81	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	2
H82	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1
H83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	2
H84	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	1
H85	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	3
H86	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	3
H87	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H88	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	G	1
H89	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	1
H90	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	3
H91	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	1
H92	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	1
H93	A	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	G	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H94	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	1
H95	-	-	-	-	-	-	-	-	-	-	-	-	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	1
H96	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H97	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H98	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	C	-	-	-	-	-	-	-	C	-	1
H99	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	1

Table 2. Contd.

H100	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H101	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	1
H102	A	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H103	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H104	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	G	-	-	2
H105	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	2
H106	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	1
H107	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	1
Total	-	-	A	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	380

Table 3. Types of mutations in variable positions for HVII.

Positions	Mutation	Type of mutation	Presence in Mitomap	Frequency	Frequency (%)
85	Transition	G-A	Presence	0.043	4.3
89	Transition	T-C	Presence	0.0375	3.75
92	Transition	G-A	Presence	0.05	5
92	Transversion	G-C	New*		
93	Transition	A-G	Presence	0.0375	3.75
95	Transversion	A-T	Presence	0.0405	4.05
113	Transition	C-T	Presence	0.045	4.5
113	Transversion	C-G	New		
114	Transition	C-T	Presence	0.0475	4.75
131	Transition	T-C	Presence	0.0425	4.25
143	Transition	G-A	Presence	0.05	5
146	Transition	T-C	Presence	0.0325	3.25
150	Transition	C-T	Presence	0.035	3.5
150	Transversion	C-G	New		
151	Transition	C-T	Presence	0.045	4.5
156	Transition	T-C	Presence	0.0375	3.75
156	Transversion	T-A	New		
173	Transition	T-C	Presence	0.0325	3.25
173	Transversion	T-A	Presence		
178	Transition	A-G	Presence	0.0225	2.25
182	Transition	C-T	Presence	0.015	1.5
183	Transition	A-G	Presence	0.0475	4.75
185	Transition	G-A	Presence	0.015	1.5
185	Transversion	G-C	Presence		

Table 3. Contd.

186	Transition	C-T	Presence	0.0175	1.75
186	Transversion	C-G	Presence		
188	Transition	A-G	Presence	0.03	3
188	Transversion	A-G	Presence		
189	Transition	A-G	Presence	0.01	1
194	Transition	C-T	Presence	0.015	1.5
194	Transversion	C-G	New		
195	Transition	T-C	Presence	0.025	2.5
195	Transversion	T-A	Presence		
198	Transition	C-T	Presence	0.0125	1.25
198	Transversion	C-G	New		
200	Transition	A-G	Presence	0.0175	1.75
203	Transition	G-A	Presence	0.03	3
203	Transversion	G-C	Presence		
207	Transition	G-A	Presence	0.025	2.5
207	Transversion	G-C	New		
210	Transition	A-G	Presence	0.015	1.5
217	Transition	T-C	Presence	0.015	1.5
225	Transition	G-A	Presence	0.015	1.5
225	Transversion	G-C	New		
228	Transition	G-A	Presence	0.025	2.5
228	Transversion	G-C	New		
235	Transition	A-G	Presence	0.0405	4.05
239	Transition	T-C	Presence	0.0275	2.75
242	Transition	C-T	Presence	0.0225	2.25
247	Transition	G-A	Presence	0.035	3.5
248	Transition	A-G	Presence	0.045	4.5
250	Transition	T-C	Presence	0.02	2
257	Transition	A-G	Presence	0.0225	2.25
Genetic diversity*		D= 1-$\sum p^2$ = 0.95	= 95 %		

New*: new polymorphic positions; Genetic diversity* Genetic diversity for the analyzed DNA fragment was calculated according to the formula: D = 1-$\sum p^2$.

noncoding function of the analysed DNA fragment. The relatively high gene diversity and a relatively low random match probability were observed in this study. Holland et al. (2011) showed that the polymorphism of mtDNA coding area is less than that of mtDNA control region. Therefore, more efficient poly-morphic sites should be used to provide an improved discrimination power for forensic mtDNA testing (Nadia et al., 2011; Imad et al., 2015c; Muhanned et al., 2015).

As forensic markers, they should be phenotypic neutral to avoid landing investigators into serious situations of medical genetic privacy and ethnics, especially for mtDNA coding area whose mutation often correlated with an increased risk of some disease. With the whole mtGenome sequences being researched, we are optimistic that the polymorphism sites within mtDNA coding area will be useful in combination with control region SNPs so as to increase the discrimination power of mtDNA.

Conclusion

Sequence analysis of the noncoding region of mtDNA (HVII) conducted on a population of 380 unrelated individuals enabled identification 107 different haplo-types. New polymorphic positions G92C, C113G, C150G, T156A, C194G, C198G, G207C, G225C and G228C are described may be in future be suitable sources for identification purpose.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Dr. Khalifa from the Institution of medico-legal for all time put in to discuss the project and

helping us to put the project together. We would also like to thank Zainab Al-Habubi from the Department Biology for her guidance and help in the laboratory work.

REFERENCES

Anderson S, Bankier AT, Barrell BG (1981). Sequence and organization of the human mitochondrial genome. Nature. 290:457-465.

Brown JR, Bechenbach AT, Smith MJ (1993). Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (Acipenser transmontanus). Mol. Biol. Evol. 10:326-341.

Brown WM, Prager EM, Wang A (1982). Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18:225-239.

Dobbs LJ, Madigan MN, Carter AB (2002). Use of FTA gene guard filter paper for the storage and transportation of tumor cells for molecular testing. Arch. Pathol. Lab. Med. 126(1):56-63.

Giulietta DB, Ivane SN, Michele S (2000). Mitochondrial DNA sequences in prehistoric human remains from the Alps. Eur. J. Hum. Genet. 8:669-677.

Gu MB (2001). Polymorphism study of the mitochondrial DNA control region in Chinese Han population group. Chinese J. Forensic Med. 16(1):6-9.

Helgason A, Hrafnkelsson B, Gulcher JR (2003). A populationwide coalescent analysis of Icelandic matrilineal and patrilineal genealogies: evidence for a faster evolutionary rate of mtDNA lineages than Y chromosomes. Am. J. Hum. Genet. 72:1370-1388.

Holland MM, McQuillan MR, Hanlon KA (2011). Second generation sequencing allows for mtDNA mixture deconvolution and high resolution detection of heteroplasmy. Croat Med. J. 52(3):299-313.

Imad HH, Mohammed AJ, Muhanned AK (2015a). Forensic analysis of mitochondrial DNA hypervariable region HVII (encompassing nucleotide positions 37 to 340) and HVIII (encompassing nucleotide positions 438-574) and evaluate the importance of these variable positions for forensic genetic purposes. Afr. J. Biotechnol. 14(5):365-375.

Imad HH, Muhanned AK, Rafid HH (2015b). X-chromosome short tandem repeat, advantages and typing technology review. Afr. J. Biotechnol. 14(7):535-541.

Imad HH, Aamera JO, Abeer FM, Ghaidaa JM (2015c). Allele frequency data of 21 autosomal Short Tandem Repeat loci in Mesan and Basra provinces in south of Iraq. Egypt J. Forensic Sci. http://dx.doi.org/10.1016/j.ejfs.2014.10.003

Jones DA (1972). Blood samples: probability of discrimination Journal of Forensic Science Society. 12:355-358.

Kraytsberg Y, Schwartz M, Brown TA (2004). Recombination of Human Mitochondrial DNA. Science. 304(56731):28-981.

Mohammed AJ, Imad HH, Muhanned AK (2015). Detection of New Variant “Off-ladder” at the (D12S391, D19S433 and D1S1656 loci) and Tri-allelic Pattern at the D16S539 Locus in a 21 Locus Autosomal Short Tandem Repeat Database of 400 Iraqi Individuals. Afri. J. Biotechnol. 14(5):375-399.

Muhanned AK, Ameer IA, Imad HH, Mohammed AJ. (2015) A New Polymorphic Positions Discovered in Mitochondrial DNA Hypervariable Region HVIII From Central and North-Central of Iraq. Mitochondrial DNA. 1-5.

Nadia A, Maria P, Vincenza B (2011). In search of the genetic footprints of Sumerians: a survey of Y-chromosome and mtDNA variation in the Marsh Arabs of Iraq,” BMC Evol. Biol. 11: 288.

Stoneking M, Soodyall H (1996). Human Evolution and the Mitochondrial Genome. Curr. Opin. Genet. Dev. 731-736.

Young KL (2009). The Basques in the Genetic Landscape of Europe. PhD Dissertation. University of Kansas.

academicJournals

Vol. 14(10), pp. 851-858, 11 March, 2015
DOI: 10.5897/AJB2015.14423
Article Number: C03E1E551257
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Allele frequency present within the DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b of unrelated individuals in Iraq

Muhanned Abdulhasan Kareem[1], Mohammed Abdullah Jebor[2] and Imad Hadi Hameed[2]*

[1]Babylon University, Centre of Environmental Research, Hilla City, Iraq.
[2]Department of Molecular Biology, Babylon University, Hilla City, Iraq.

Received 13 January, 2015; Accepted 23 February, 2015

The Y chromosome is becoming a useful tool for tracing human evolution through male lineages marker. The aim of this study was to determine the genetic structure in Babylon province in Iraq and evaluate the importance of these STRs loci for forensic genetic applications. FTA® Technology was utilized to extract DNA from blood collected on FTA™ paper. We have analyzed 17 Y chromosomal STR loci (DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b) for evaluating allele frequencies and genetic diversity. A total of 94 unique haplotypes was identified among the one hundred individuals studied. The DYS456 had the highest diversity (GD = 0.752), while DYS392 locus had the lowest one (GD = 0.185). The light has been focused and directed in this study to establish the basic forensic genetic information, knowledge, data and statistics which might be so ultimately helpful practically in forensic science and criminology and to let evaluate and present the DNA weight evidences in Iraq medico-legal institute and courts of law.

Key words: Allele frequency, FTA™ paper, Iraq, STR DNA typing, Y filer™.

INTRODUCTION

Microsatellites are a group of molecular markers chosen for a number of purposes which include forensics individual identification and relatedness testing polymorphic (Yamamoto et al., 1999; Nakamura, 2009). Low quantities of template DNA is required (10 to 100 ng) (Markoulatos et al., 2002), when using microsatellites. There is a high genomic abundance of random distribution throughout the genome. There is also an abundance of polymorphism. A nuclear DNA present in one copy per cell and only in males is called the Y chromosome. It includes the sex determining region and known as a paternal lineage marker (Butler et al., 2002;

*Corresponding author. E-mail: imad_dna@yahoo.com. Tel: 009647716150716.

Carolina et al., 2010; Kuppareddi et al., 2010). The genetic information is inherited from the father to the son, and this information does not change except for mutational events (Hanson and Ballantyne, 2007). The individual STRs are inherited as a single unit because of the lack of recombination and this is called a haplotype and behaves as single allele per individual (Parson et al., 2003; Kwak et al., 2005).

The Y - chromosome is specific to the male portion of a male-female DNA mixed such as is common in sexual assault cases (Park et al., 2007). These STRs can also be useful in missing persons investigations, historical investigations, some paternity testing scenarios, and genetic genealogy (Park et al., 2007; Andrea et al., 2008). Although, they are often used to suggest which haplogroup an individual matches, STR analysis typically provides a person haplotype. Most tests on the Y chromosome examine between 12 and 67 STR markers (Jobling et al., 1997; Kayser et al., 1997). The Y chromosome is less variable than the other chromosomes. Many markers are thus needed to obtain a high degree of discrimination between unrelated males marker. The Y chromosome is becoming a useful tool for tracing human evolution through male lineages marker[13] as well as application in a variety of forensic situations[12] including those involving evidence from sexual assault cases containing a mixture of male and female DNA (Prinz et al., 1997; Prinz et al., 2001). Using Y-chromosome, specific methods can improve the chances of detecting low levels of male DNA in a high background of female DNA.

MATERIALS AND METHODS

Preparation of blood stain samples

Blood samples were randomly collected from healthy unrelated males living in middle and south of Iraq and sent to the genetic laboratories.

DNA extraction

DNA was extracted from all dried blood samples on FTA cards following the manufacture's procedure as described in Whatman FTA Protocol BD01 except that the Whatman FTA purification reagent was modified to half the volume. A 1.2 mm diameter disc was punched from each FTA card with a puncher. The discs were transferred to new Eppendorf tubes and washed 3 times in 100 µl Whatman FTA purification reagent. Each wash was incubated for 5 min at room temperature with moderate manual mixing and the reagent was discarded between washing steps. The discs were then washed twice in 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), the buffer was discarded and the discs were left to dry at room temperature for 1 h.

DNA amplification for Y- Chromosomal STR

PCR is the process used to amplify a specific region of DNA. It is possible to create multiple copies from small amount of template DNA. A commercial kit Y filer™ PCR amplification kit (Applied Biosystems, Foster City, CA) that amplifies 17 Y-STR loci: DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b and a segment of the amelogenin gene was used, according to manufacturer's instructions but in a total reaction volume of 25 µl. The master mix was homogenized by vortex for 3 s, then centrifuged briefly, then 25 µl of PCR amplification mix was pipetted into each reaction well. 1.2 mm punch from a card containing whole blood was loaded into the appropriate wells of the reaction plate. The positive amplification control, 1 µl of 2800 M Control DNA (10 ng/µl) was added to a reaction well containing 25 µl of PCR amplification mix. The preferred protocol used with the GeneAmp® PCR System 9700 thermal cycler is provided below. The estimated total cycle time was 1.5 h. PCR program is as follows: 96°C for 1 min, then 94°C for 10 s , 59°C for 1 min, 72°C for 30 s, for 25 cycles, then: 60°C for 20 min 4°C soak. After completion of the thermal cycling protocol, the amplified samples were kept or stored at –20°C in a light-protected box.

PCR amplicon analysis (capillary electrophoresis)

The major application of CE in forensic biology is in the detection and analysis of short tandem repeats (STRs). STR markers are preferred because of the powerful statistical analysis that is possible with these markers and the large databases that exist for convicted offenders' profiles. The ABI Prism1 3130xl Genetic Analyzer 16-capillary array system (Applied Biosystems, Foster City, CA, USA) was used following the manufacturer's protocols, with POP-7™ Polymer and Data Collection Software, Genemapper version 3.5 software (Applied Biosystems). The allele designations were determined by comparison of the PCR products with those of allelic ladders provided with the kit. Nomenclature of loci and alleles is according to the International Society of Forensic Genetics (ISFG) guidelines reported in Gill et al. (2001). By comparison of the size of a sample's alleles to size of alleles in allelic ladders for the same loci being tested in the sample, the STR genotyping was conducted.

Quality control

Allelic ladders, male DNA (positive internal control), female DNA (negative control) and the amelogenin (internal control), provided by Reliagene (Reliagene Tech.), were used in each reaction with the Y filer™ kit.

Statistical analysis for Y- Chromosomal STR

Analysis of data

Allele frequencies were calculated by direct counting.

Allele diversity (genetic diversity)

Allele diversity was calculated as described by Nei (1987).

$$D = \frac{n}{n-1}\left(1 - \sum_{i=1}^{n} p_i^2\right)$$

Where, *n* is the sample size and *pi* is the frequency of the *i*th allele.

Table 1. Allele frequencies and genetic diversity of (DYS635, DYS437, DYS448, DYS456, DYS458 and YGATA H4) Y-STR loci.

Allele	DYS635		DYS437		DYS448		DYS456		DYS458		YGATA H4	
	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	0.050	0.020
11	-	-	-	-	-	-	-	-	-	-	0.260	0.043
12	-	-	-	-	-	-	-	-	-	-	0.550	0.049
13	-	-	-	-	-	-	0.010	0.009	-	-	0.110	0.031
14	-	-	0.540	0.049	-	-	0.070	0.025	0.020	0.014	0.020	0.014
15	-	-	0.300	0.047	-	-	0.630	0.051	0.430	0.049	-	-
16	-	-	0.150	0.036	-	-	0.230	0.041	0.190	0.038	-	-
17	-	-	-	-	-	-	0.050	0.020	0.100	0.030	-	-
18	-	-	-	-	0.160	0.037	-	-	0.070	0.025	-	-
19	-	-	-	-	0.550	0.049	-	-	0.010	0.030	-	-
20	0.010	0.030	-	-	0.230	0.042	-	-	0.050	0.020	-	-
21	0.080	0.027	-	-	0.040	0.019	-	-	-	-	-	-
22	0.070	0.025	-	-	0.010	0.009	-	-	-	-	-	-
23	0.360	0.048	-	-	-		-	-	-	-	-	-
24	0.330	0.047	-	-	-		-	-	-	-	-	-
25	0.020	0.014	-	-	-		-	-	-	-	-	-
GD*	0.729		0.595		0.617		0.752		0.751		0.615	

Freq, Frequency; SE, standard error; GD, genetic diversity.

Standard error (SE)

The standard error (SE) of allele frequencies was calculated as:

$$SE\,(p_i) = \sqrt{[(1 - p_i)\,p_i]/N},$$

Where, pi denotes the frequency of the *ith* allele at any given locus and N equals the total number of individuals screened at this locus.

RESULTS AND DISCUSSION

Y-STR-Allele frequency and genetic diversity

Allelic genotyping of STRs does not require the use of complex molecular techniques, since amplifications and visualization of PCR products make it easy. Y-chromosome specific STRs (Y-STRs) are chosen as more informative in paternity testing, forensic applications and the study of population histories due to the haploid state of Y chromosome which ensures both the transmittance by the paternal lineages and the lack of recombination in NRY, excluding pseudoautosomal regions (PARs) (Betz et al., 2001; Corach et al., 2001; Dekairelle and Hoste, 2001; Honda et al., 2001; Gill et al., 2004). Allelic and haplotypic distributions of Y-STRs have shown significant differences in different geographical regions, ethnical groups and communities (Alaves et al., 2003; Gusmao et al., 2003; Rustamov et al., 2004; Yan et al., 2007). Therefore, allelic and haplotypic frequencies of Y-STRs should be determined in a male population prior to any interpretations of forensic analysis and paternity testing (Budowle et al., 2003). In this study, allelic and haplotypic frequencies involving 17 Y-STR loci have been determined with such a necessity in a representative group of Iraq population in order to make comparisons with other populations. Seventeen Y-STRs have been analyzed for diversity in 100 healthy and unrelated male individuals. Observed allele or genotype frequencies of the 17 Y-STR loci have been given in Tables 1, 2 and 3.

Gene diversity values for each Y-STR loci have been given (Table 1, 2 and 3). The lowest gene diversity (0.185) has been found in DYS392 locus, wherein the most frequent allele has been allele 11 with a frequency of 0.90. The highest gene diversity (0.752) has been found in DYS385 locus, wherein the most frequent allele has been allele 15 with a frequency of 0.630. Data comparison between our samples and a previously published sample from the Iraq population was performed for markers which are common to both studies using the exact test for population differentiation implemented in GENEPOP (Raymond, 1995). In another study on 17 Y-STR Y-chromosomal short tandem repeat loci from the Cukurova region of Turkey, the DYS391 recorded lowest gene diversity in this region was 0.51 and the highest was 0.95 for DYS385a/b and no significant differences were found when this data was compared with haplotype data of other Turkish populations (Ayse et al., 2011). In Northern Greece the haplotype diversity was 0.9992 in 17 Y STR loci typed in a population sample of unrelated male individuals. Haplotypes are presented for the

Table 2. Allele frequencies and genetic diversity of (DYS19, DYS385a, DYS385b, DYS389I, DYS389II and DYS390) Y-STR loci.

Allele	DYS19		DYS385a		DYS385b		DYS389I		DYS389II		DYS390	
	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE
11	-	-	0.100	0.030	-	-	-	-	-	-	-	-
12	-	-	0.040	0.019	-	-	0.180	0.038	-	-	-	-
13	0.390	0.046	0.540	0.049	0.030	0.017	0.500	0.020	-	-	-	-
14	0.400	0.094	0.120	0.031	0.270	0.043	0.320	0.038	-	-	-	-
15	0.140	0.036	0.010	0.009	0.150	0.036	-	-	-	-	-	-
16	0.020	0.014	0.060	0.024	0.150	0.036	-	-	-	-	-	-
17	0.010	0.009	0.070	0.025	0.020	0.014	-	-	-	-	-	-
18	-	-	-	-	0.190	0.039	-	-	-	-	-	-
19	-	-	0.020	0.014	0.150	0.036	-	-	-	-	-	-
20	-	-	-	-	0.030	0.017	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	0.030	0.017
22	-	-	-	-	-	-	-	-	-	-	0.040	0.019
23	-	-	-	-	-	-	-	-	-	-	0.450	0.049
24	-	-	-	-	-	-	-	-	-	-	0.280	0.043
25	-	-	-	-	-	-	-	-	-	-	0.150	0.036
28	-	-	-	-	-	-	-	-	0.090	0.028	-	-
29	-	-	-	-	-	-	-	-	0.200	0.040	-	-
30	-	-	-	-	-	-	-	-	0.510	0.020	-	-
31	-	-	-	-	-	-	-	-	0.100	0.030	-	-
32	-	-	-	-	-	-	-	-	0.070	0.025	-	-
GD*	0.668		0.670		0.819		0.608		0.727		0.693	

Table 3. Allele frequencies and genetic diversity of (DYS391, DYS392, DYS393, DYS438, and DYS439) Y-STR loci.

Allele	DYS391		DYS392		DYS393		DYS438		DYS439	
	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE
8	0.040	0.019	-	-	-	-	-	-	-	-
9	0.170	0.038	-	-	-	-	0.130	0.034	-	-
10	0.570	0.045	-	-	-	-	0.650	0.048	0.420	0.049
11	0.110	0.030	0.900	0.030	-	-	0.130	0.034	0.320	0.038
12	0.060	0.024	0.010	0.009	0.240	0.043	0.070	0.025	0.130	0.034
13	-	-	0.060	0.024	0.650	0.048	-	-	0.100	0.030
14	-	-	0.030	0.017	0.090	0.028	-	-	-	-
GD*	0.624		0.185		0.504		0.531		0.694	

Freq, Frequency; SE, standard error; GD, genetic diversity.

following loci: DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y GATA H4, DYS437, DYS438 and DYS448. This database study provides additional information for the application of Y chromosomal STRs to forensic identification efforts in Greece (Leda et al., 2008).

Y-STR- Haplotypes and Haplotype frequency

The observed numbers of haplotypes and their frequencies have been shown (Tables 4, 5 and 6). We identified 96 different haplotypes in our study sample. 89 of which (93%) were unique, one was found twice and one was found in three individuals. The most frequent haplotype was haplotype number 77. Haplotype 77 seems to be specific to Iraq. Haplotypes detected in this study group have been compared with seven other populations: German (n = 88), Indian (n = 25), Chinese (n = 36), Italians(n = 100) (Manfred et al., 2001), Tunis (n = 105) (Imen et al., 2005) and India (n = 154) (Kuppareddi et al., 2010) (Table 7). Haplotypic comparisons have high-lighted that significant differences from Iraq population

Table 4. Haplotypes and haplotypes frequency for the 17 Y-STR loci (haplotype 1- haplotype 32).

Haplotype	DYS635	DYS437	DYS448	DYS456	DYS458	YGATA H4	DYS19	DYS385a	DYS385b	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS438	DYS439	N	F
H1	24	15	20	15	14	12	13	11	16	12	30	23	10	11	13	10	13	1	0.0025
H2	24	15	18	15	14	13	13	11	16	12	30	23	8	11	13	9	13	1	0.0025
H3	24	15	20	15	15	12	13	11	16	12	31	23	8	11	12	9	10	1	0.0025
H4	24	15	19	15	15	11	13	11	14	12	31	23	8	11	13	11	10	1	0.0025
H5	24	15	19	16	15	10	13	13	14	12	31	23	10	11	13	10	10	1	0.0025
H6	24	15	19	16	17	12	13	13	14	12	28	23	10	11	13	10	13	1	0.0025
H7	24	15	19	14	17	12	13	13	14	12	28	23	9	11	14	12	13	1	0.0025
H8	24	15	19	16	17	11	13	13	14	12	28	23	10	11	13	12	10	1	0.0025
H9	24	15	20	16	17	14	13	13	14	12	29	23	10	11	13	9	10	1	0.0025
H10	24	15	19	16	15	14	13	13	14	12	29	23	9	11	14	12	11	1	0.0025
H11	24	15	19	13	15	11	13	13	14	12	29	23	9	11	14	10	11	1	0.0025
H12	24	15	21	15	15	10	13	13	14	14	29	23	10	11	12	11	11	1	0.0025
H13	25	15	21	15	15	13	13	13	14	14	29	23	10	11	13	10	11	1	0.0025
H14	21	15	22	15	15	12	13	13	14	13	29	23	10	11	14	10	10	1	0.0025
H15	21	15	19	15	20	12	13	13	18	13	29	23	10	11	12	9	10	1	0.0025
H16	21	15	19	15	16	12	13	13	18	13	28	23	9	11	13	9	13	1	0.0025
H17	21	15	19	15	18	12	13	13	18	13	28	23	8	11	13	12	10	1	0.0025
H18	21	15	19	14	18	13	13	13	16	13	28	23	10	11	13	12	10	1	0.0025
H19	21	15	19	16	18	12	13	14	16	13	31	23	10	11	13	10	10	1	0.0025
H20	24	15	21	16	15	12	13	14	16	12	31	23	10	11	13	10	10	1	0.0025
H21	24	14	21	16	16	12	13	14	16	12	31	23	10	11	12	10	10	1	0.0025
H22	24	14	19	15	15	12	13	13	13	12	28	23	10	11	12	10	10	1	0.0025
H23	24	14	19	15	20	12	16	13	13	13	28	23	10	11	14	9	10	1	0.0025
H24	24	14	19	15	15	12	16	13	13	13	30	23	10	11	14	10	10	1	0.0025
H25	24	14	20	15	15	12	15	13	18	13	30	23	10	11	13	10	10	1	0.0025
H26	24	16	18	15	19	12	15	13	14	13	30	25	10	11	13	10	10	1	0.0025
H27	24	16	18	16	15	12	15	13	14	13	32	23	10	11	13	10	10	1	0.0025
H28	21	14	19	15	15	12	15	13	18	13	33	23	11	11	13	10	10	1	0.0025
H29	24	14	19	15	20	12	15	13	18	14	30	21	10	11	13	10	10	1	0.0025
H30	24	14	20	15	19	12	15	13	18	14	29	24	10	11	13	10	10	1	0.0025
H31	21	14	20	16	15	12	15	13	18	13	29	24	11	11	12	10	11	1	0.0025
H32	24	14	20	15	15	11	15	13	18	14	29	25	10	11	13	10	11	1	0.0025

in this study ($p < 0.05$). Our data have also provided additional information to the framework of variation involving seventeen Y-STR loci as well as a further contribution to the Y-STR database for Iraq population. This supports the observations, by others (Jorde et al., 2000), that, especially among European populations, Y STRs are very powerful in the detection of genetic differences between populations, compared with autosomal STRs. This can be attributed to the greater sensitivity of nonrecombining Y-chromosomal markers to founder effects and genetic drift. A similar conclusion was reached recently by Forster et al. (2000), on the basis of a phylogenetic approach only. The use of Y STRs allows the simple construction of highly variable haplotypes. With these haplotypes, it is possible to analyze differences in population structure by a comparison of haplotype diversity and of the number of population-specific haplotypes.

Conclusion

We identified 96 different haplotypes in our study sample. 94 of which (97.9%) were unique, one was found twice and one was found in three individuals. The DYS385b and DYS458 had the highest diversity (GD = 0.8392 and 0.806, respectively), while loci DYS392 and DYS439 had the lowest (D = 0.2695 and 0.2991, respectively).

Table 5. Haplotypes and haplotypes frequency for the 17 Y-STR loci (haplotype 33- haplotype 64).

Haplotype	DYS635	DYS437	DYS448	DYS456	DYS458	YGATA H4	DYS19	DYS385a	DYS385b	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS438	DYS439	N	F
H33	22	16	20	15	15	11	16	13	18	14	29	21	10	11	13	10	11	1	0.0025
H34	24	15	20	15	16	12	14	13	18	14	29	23	10	11	13	10	11	1	0.0025
H35	24	15	18	15	16	10	14	13	18	13	29	23	10	11	13	10	11	1	0.0025
H36	22	15	18	14	16	13	14	13	18	12	29	23	10	12	12	10	11	1	0.0025
H37	22	15	19	15	16	11	14	13	18	14	29	24	10	11	13	10	11	1	0.0025
H38	22	14	20	15	15	12	14	13	16	13	30	23	10	11	13	10	11	1	0.0025
H39	24	14	19	15	15	10	14	13	19	13	28	23	10	11	13	10	11	1	0.0025
H40	24	14	19	14	15	12	14	13	19	13	30	24	10	11	13	10	11	1	0.0025
H41	24	14	19	15	15	12	14	13	19	13	32	21	9	11	13	10	11	1	0.0025
H42	24	14	19	16	15	12	14	13	19	13	32	25	9	11	12	10	11	1	0.0025
H43	22	14	19	15	16	12	14	13	15	13	31	25	10	11	12	10	11	1	0.0025
H44	24	14	19	15	20	12	14	13	15	13	29	25	10	11	12	10	11	1	0.0025
H45	24	14	19	15	19	12	14	12	15	13	32	25	10	11	13	10	12	1	0.0025
H46	24	14	18	15	16	12	13	12	15	13	30	25	10	11	13	10	12	1	0.0025
H47	24	14	18	15	16	11	13	12	15	13	31	23	10	11	12	10	12	1	0.0025
H48	23	14	19	15	16	13	13	12	14	13	31	23	11	11	14	10	12	1	0.0025
H49	23	14	19	15	15	12	13	13	14	12	31	24	10	11	13	10	12	1	0.0025
H50	23	14	19	16	15	12	13	13	14	13	30	24	10	11	12	10	10	1	0.0025
H51	23	14	18	16	15	11	13	11	14	14	30	24	10	11	12	9	10	1	0.0025
H52	23	14	20	15	15	11	15	11	14	14	30	25	11	11	12	10	13	2	0.0050
H53	22	14	20	15	16	11	15	11	14	13	30	23	10	14	12	10	14	1	0.0025
H54	22	14	18	15	20	11	14	11	14	12	30	23	10	14	13	10	10	1	0.0025
H55	23	14	20	15	19	11	13	13	19	14	30	24	11	14	13	10	13	1	0.0025
H56	23	14	20	15	18	12	13	13	19	13	30	24	11	11	13	10	12	1	0.0025
H57	23	14	20	14	18	11	14	15	16	12	30	24	11	11	14	11	10	1	0.0025
H58	23	14	20	15	17	12	13	17	16	14	30	24	11	11	13	9	10	1	0.0025
H59	23	14	19	17	17	11	13	17	15	13	32	22	12	11	14	10	10	2	0.0050
H60	23	16	19	15	17	11	14	17	15	13	32	24	10	11	13	10	10	1	0.0025
H61	23	14	19	16	17	13	13	17	15	13	30	25	10	11	13	9	10	1	0.0025
H62	23	14	18	16	17	11	13	17	15	13	30	25	10	11	13	11	10	1	0.0025
H63	23	15	19	15	17	11	13	16	15	14	30	25	9	11	12	11	12	1	0.0025
H64	23	15	19	14	15	12	17	16	15	14	32	25	10	11	12	11	10	1	0.0025

Table 6. Haplotypes and haplotypes frequency for the 17 Y-STR loci (haplotype 65- haplotype 96).

Haplotype	DYS635	DYS437	DYS448	DYS456	DYS458	YGATA H4	DYS19	DYS385a	DYS385b	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS438	DYS439	N	F
H65	23	15	19	17	16	12	14	16	15	14	29	25	10	13	13	10	9	1	0.0025
H66	23	14	20	17	19	12	14	13	15	14	30	23	12	13	13	10	9	1	0.0025
H67	23	14	20	15	19	11	13	13	15	14	30	23	10	13	12	10	14	1	0.0025
H68	23	14	19	16	19	13	13	13	15	14	30	24	9	11	13	10	13	1	0.0025
H69	23	14	19	16	15	12	13	11	18	13	30	24	9	11	13	9	10	1	0.0025
H70	23	14	19	16	18	12	13	13	18	14	29	24	9	11	13	12	10	1	0.0025
H71	20	14	19	15	15	12	13	13	18	13	29	24	9	11	13	11	10	1	0.0025

Table 6. Contd.

H72	20	14	20	16	16	12	14	13	18	13	29	23	12	11	13	11	10	1	0.0025
H73	20	14	19	16	15	10	14	13	19	13	30	23	10	11	13	11	10	1	0.0025
H74	23	14	19	16	15	11	14	13	19	13	30	22	10	11	13	11	11	1	0.0025
H75	23	14	19	14	15	11	14	13	19	13	30	23	9	13	13	11	11	1	0.0025
H76	23	14	19	15	15	12	14	13	14	13	30	23	9	11	13	10	11	1	0.0025
H77	23	14	18	15	15	13	14	13	14	13	30	24	11	11	13	10	11	3	0.0075
H78	23	16	18	15	15	12	14	13	14	13	30	24	10	13	13	9	11	1	0.0025
H79	23	15	18	15	16	12	14	19	14	13	30	24	10	13	13	10	11	1	0.0025
H80	23	16	18	15	16	11	14	19	16	14	30	24	9	11	13	10	14	1	0.0025
H81	23	16	19	15	19	11	14	13	16	14	30	23	11	11	13	10	14	1	0.0025
H82	23	16	19	15	19	12	15	16	19	14	30	25	10	11	13	10	10	1	0.0025
H83	23	16	19	15	16	12	14	16	19	14	30	25	10	11	13	9	10	1	0.0025
H84	23	14	19	15	15	12	14	16	19	14	30	25	10	11	13	10	10	1	0.0025
H85	23	16	20	15	15	11	14	14	19	14	30	24	9	11	13	10	10	1	0.0025
H86	23	16	20	16	16	13	14	14	17	14	30	24	11	11	13	10	14	1	0.0025
H87	23	14	19	15	16	11	14	14	17	14	30	24	12	11	13	10	14	1	0.0025
H88	23	15	19	15	19	12	14	14	20	14	30	24	11	11	12	11	12	1	0.0025
H89	23	14	19	15	15	12	14	14	20	14	30	25	9	11	12	10	11	1	0.0025
H90	20	16	20	15	18	12	15	14	20	14	30	24	10	11	12	9	11	1	0.0025
H91	20	16	19	15	16	12	15	14	18	14	30	24	10	11	13	10	11	1	0.0025
H92	20	16	19	15	15	11	14	14	19	13	30	24	12	11	13	12	10	1	0.0025
H93	20	16	19	16	16	12	14	14	19	13	30	24	10	11	12	10	11	1	0.0025
H94	20	15	19	15	15	12	14	14	16	13	30	23	10	11	12	11	12	1	0.0025
H95	20	14	19	15	16	12	14	17	16	13	30	23	9	11	13	10	12	1	0.0025
H96	20	14	19	17	15	12	15	17	16	13	30	22	12	11	12	10	12	1	0.0025

Table 7. Comparison of the haplotypes and haplotype diversity in different human population groups.

Parameter	Iraq[1]	Tunis[2]	German[3]	Italy[4]	China[5]	India[6]	India[7]
Individuals number	100	105	88	100	36	25	154
Haplotypes number	96	81	77	82	34	16	152
Unique haplotypes	89	67	39	53	28	13	150
Proportion of unique haplotypes	0.93	0.83	0.51	0.65	0.82	0.81	0.98
Non-unique haplotypes	7	14	38	29	6	3	2
Proportion of non-unique haplotypes	0.07	0.17	0.49	0.35	0.18	0.19	0.01
Ratio (unique : non-unique)	12.71	4.88	1.03	1.83	4.67	4.33	98
Haplotypes diversity	0.892	0.9932	0.9963	0.9941	0.9968	0.950	0.9935

[1]This study.[2]Reference: Imen et al. (2005); [3]Reference: Manfred et al. (2001); [4]Reference: Manfred et al. (2001); [5]Reference: Manfred et al. (2001); [6]Reference: Manfred et al. (2001); [7]Reference: Kuppareddi et al. (2010).

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We sincerely wish to thank Dr. Ali for providing us the opportunity to work on this project. We would also like to thank Ghaidaa J. Mohammed from the Department of Biology for her guidance and help in the laboratory work.

REFERENCES

Alaves C, Gusmao L, Barbosa J, Amorim A (2003). Evaluating the informative power of Y-STRs: A comparative study using European and new African haplotype data. Forensic Sci. Int. 134: 126- 133.

Andrea V, Nicoletta C, Fausta G, Anna P (2008). Population Data for 15 Autosomal STRs Loci and 12 Y Chromosome STRs Loci in a

population sample from the Sardinia Island (Italy). Leg Med. 11(1):37-40.
Ayse S, Husniye C, Yasar S (2011). Haplotype frequencies of 17 Y-chromosomal short tandem repeat loci from the Cukurova region of Turkey. Croat Med. J. 52(6):703-708.
Betz A, Bäbler G, Dietl G, Syeil X (2001). DYS STR analysis with epithelial cells in a rape case. Forensic Sci. Int. 118:126-130.
Budowle SSK, Lee HS, Chakraborty R (2003). Utility of Y chromosome STR haplotypes in forensic applications, Forensic Sci. Rev. 15:153-164.
Butler JM, Schoske R, Vallone PM, Kline MC (2002). A Novel Multiplex for Simultaneous Amplification of 20 Y-Chromosome STR Markers. Forensic Sci. Int. 129(1):10-24.
Carolina N, Miriam B, Cecilia S, Yolanda C (2010). Reconstructing the population history of Nicaragua by means of mtDNA, Y-chromosome STRs, and autosomal STR markers. Am. J. Phy. Anthropol. 143(4):591-600.
Corach D, Figueira RL, Marino M, Penacino G (2001). Routine Y-STR typing in forensic casework. Forensic Sci. Int. 118: 131-135.
Dekairelle AF, Hoste B (2001). Application of a Y-STR-pentaplex PCR (DYS19, DYS389I and II, DYS390 and DYS393) to sexual assault cases. Forensic Sci. Int. 118:122-125.
Forster P, Roh A, Lu¨nnemann P, Brinkmann C, Zerjal T, Tyler- Smith C,Brinkmann B (2000). A short tandem repeat-based phylogeny for the human Y chromosome. Am. J. Hum. Genet. 67:182-196.
Gill P, Brenner C, Brinkmann B, Budowle B (2001). DNA Commission of the International Society of Forensic Genetics: recommendations for forensic analysis using Y-chromosome STRs. Forensic Sci. Int. 124:5-10.
Gill P, Werrett DJ, Budowle B, Guerrieri R (2004). An assessment of whether SNPs will replace STRs in national DNA databases—joint considerations of the DNA working group of the European Network of Forensic Science Institutes (ENFSI) and the Scientific Working Group on DNA Analysis Methods (SWGDAM). Science Justice 44:51-3.
Gusmao L, Sanchez-Diz P, Alves C, Beleza S (2003). Grouping of Y-STR haplotypes discloses European geographic clines. Forensic Sci. Int. 134:172-179.
Hanson EK, Ballantyne J (2007). An ultra-high discrimination Y chromosome short tandem repeat multiplex DNA typing system. PLoS ONE 2(8): e688.
Honda K, Tun Z, Matoba R (2001). DNA testing of Klinefelter's syndrome in a criminal case using XY-chromosomal STR multiplex-PCR. J. Forensic Sci. 46(5): 1235-8.
Imen A, Leila A, Ahmed R (2005). Haplotypes of 13 Y-chromosomal STR loci in south Tunisia population (Sfax region). Legal Medicine 4627: 1-5.
Jobling MA, Pandya A, Tyler-Smith C (1997). The Y chromosome in forensic analysis and paternity testing. Int. J. Leg. Med. 110:118-124.
Jorde LB, Watkins WS, Bamshad MJ, Dixon ME, Ricker CE, Seielstad MT, Batzer MA (2000). The distribution of human genetic diversity: a comparison of mitochondrial, autosomal, and Y-chromosome data. Am. J. Hum Genet. 66:979-988.
Kayser M, Caglia A, Corach D, Fretwell N (1997). Evaluation of Y-chromosomal STRs: a multicenter study. Int. J. Legal Med. 110:125-133.
Kuppareddi BG, Suhasini M, Vijaya S (2010). Kanthimathi. Chromosome STR Allelic and Haplotype Diversity in Five Ethic Tamil Populations from Tamil Nadu, India. Leg. Med. 12:265-269.
Kwak KD, Jin HJ, Shin DJ, Kim JM, Roewer L, Krawczak M (2005). Y-Chromosomal STR Haplotypes and Their Applications in Forensic and Population Studies in East Asia. Int. J. Leg. Med. 119(4):195-201.
Leda K, Jessica L, Jodi A (2008). Population genetics of Y-chromosome STRs in a population of Northern Greeks. Forensic Sci. Int. 175 (2-3): 250-5.
Manfred K, Michael K, Laurent E, Patrick D (2001). An extensive analysis of Y-Chromosomal microsatellite haplotypes in globally dispersed human populations. Am. J. Hum. Genet. 68:990-1018.
Markoulatos P, Siafakas N, Moncany M (2002). Multiplex polymerase chain reaction: a practical approach. J. Clin. Lab Anal. 16:47-51.
Nakamura Y (2009). DNA variations in human and medical genetics: 25years of my experience. J. Hum. Genet. 54 (1):1-8.
Nei M (1987). Molecular Evolutionary Genetics, Columbia University Press, New York.
Park MJ, HY Lee, Chang U, Kang SC (2007). Y-STR analysis of degraded DNA Using reduced-size amplicons. Int. J. Leg Med. 121(2):152-157.
Parson WH, Niederstätter A, Berger B (2003). Improved specificity of Y-STR typing in DNA mixture samples. Int. J. Leg Med. 117 (2):109-114.
Prinz A, Ishii A, Coleman HJ (2001). Validation and casework application of a Y chromosome specific STR multiplex. Forensic Sci. Int. 120: 177-188.
Prinz M, Boll K, Baum H, Shaler B (1997). Multiplexing of Y chromosome specific STRs and performance for mixed samples. Forensic Sci. Int. 85:209-218.
Raymond FR (1995). GENEPOP (version 1.2). Population genetics software for exact tests and ecumenicism. J. Heredity. 86:248-249.
Rustamov A, Gümüş G, Karabulut HG, Elhan AH (2004). Y-STR polymorphism in Central Anatolian region of Turkey. Forensic Sci. Int. 139:227-230.
Yamamoto T, Uchihi R, Nozawa H, Huang XL, Leong YK, Tanaka M (1999). Allele distribution at nine STR loci-D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818,D13S317 and D7S820-in the Japanese population by multiplex PCR and capillary electrophoresis. J. Forensic Sci. 44 (1):167-170.
Yan J, Tang H, Liu Y, Jing Y (2007). Genetic polymorphisms of 17 Y-STRs haplotypes in Chinese Han population residing in Shandong province of China. Legal Med. 9:196-202.

academicJournals

Vol. 14(10), pp. 859-866, 11 March, 2015
DOI: 10.5897/AJB2014.14357
Article Number: 018346651259
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Preliminary assessment of insecticidal activity of Moroccan actinobacteria isolates against mediterranean fruit fly (*Ceratitis capitata*)

Salah Eddine Samri[1,2], Mohamed Baz[1], Abdelmounaim Jamjari[1], Houda Aboussaid[3], Said El Messoussi[3], Abdellatif El Meziane[2] and Mustapha Barakate[1]*

[1]Laboratory of Biology and Biotechnology of Microorganisms (LBBM), Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box. 2390 Marrakech, 40000 Morocco.
[2]Laboratory of Biotechnology Valorisation and Protection of Agro-Resources, Faculty of Science and Technology Gueliz, Cadi Ayyad University, P.O. Box 549, Marrakech 40 000, Morocco.
[3]Laboratory of molecular and Ecophysiological Modelisation Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box. 2390 Marrakech, 40000 Morocco.

Received 6 December, 2014; Accepted 2 March, 2015

Microbial insecticides are considered as the most sustainable and ecologically acceptable means of crop protection. Here we report the ability of some Moroccan actinobacteria isolates to produce larvicidal compounds against the Medfly (*Ceratitis capitata* Wied.). Thus, actinobacteria isolates were tested for their insecticidal activity through biological and chemical screening. The primary biological screening using the brine shrimp bioassay showed that 12 isolates out of 210 (5.71%) have been found to be highly toxic at the concentration of 100 µg mL^{-1}. Among these, isolates OS46, 37 and B62 were the most toxic with an LC_{50} of 0.26, 0.34 and 0.84 µg mL^{-1}, respectively. The freeze-dried fermentation of the selected isolates showed moderate to high insecticidal activity against the first instar larvae of *C. capitata*, and the most important pupation reduction was obtained for isolates 37 and B89 (0 and 6% of pupation, respectively). The chemical screening on thin layer chromatography of the crude extract of the most important isolates using specific insecticide family's reagents showed a variety of compounds depending on isolate with at least one active spot for each tested specific reagent. Finally, the inhibition of acetylcholinesterase activity test was carried out in order to assess the possible nature of insecticidal activity of selected isolates. The obtained results show that, except the isolate 37 which probably acts by a different mode of action, all other isolates were active. This finding could have an applicative value for the potentiality of utilizing Moroccan actinobacteria isolates as an alternative to chemical insecticides in pest management mainly against *C. capitata*.

Key words: Moroccan actinobacteria, insecticidal activity, biological screening, chemical screening, *Ceratitis capitata*.

INTRODUCTION

With an ever-increasing of human population, the demand placed upon the agriculture sector to supply more food is one of the greatest challenges for the agrarian community. An increased yield potential of

crops, however, is often constrained by different pest attacks (Oerke and Dehne, 2004). In fact, an average of 35% of global crop production is lost due to insect pest (Oerke, 2005). A case in hand concerns one of the most economically costly pest species worldwide; the Mediterranean fruit fly, *Ceratitis capitata* (Weidemann) (Diptera: Tephritidae) (Malacrida et al., 2007; De Meyer et al., 2008). This species shows a wide larval hosts range comprising more than 200 different species of fruits and vegetables (Lance et al., 2014). In Morocco, the Medfly survives in large forests (800,000 hectares) of endemic Argan trees and invades continuously bordering agricultural areas (Alaoui et al., 2010). The control of the Medfly has been mostly done with chemical insecticides (especially malathion) (Bolognesi, 2003, Dominiak and Ekman, 2013). However, their intensive use and abuse have led to several problems such as the pollution of the environment (Kumari et al. 2008; Ferencz and Balog, 2010), an increase in human diseases such as cancer and several immune system disorders (Osman, 2011), the selection of insect resistant populations (Rivero et al., 2010), and important outbreaks of secondary pests (Croft, 1990; Ruberson et al., 1998; Preetha et al., 2010).

Microbial insecticides have been recognized as an important alternative to the use for pest control (Ravensberg, 2015). Unlike most chemical insecticides, bioinsecticides are often very specific for a particular pest. They have less impact on the environment and water quality, and they offer more environmentally friendly alternative to chemical insecticides. They could also be used where pests have developed resistance to conventional pesticides (Popp et al., 2013). For these reasons, there is an increasing concern in society for lowering the use of chemical insecticides and moving to safer practices in crop protection (Jemâa et al., 2010 ; Chueca et al., 2013), such as the development of new environmental friendly microbial insecticides (Aboussaid et al., 2011). Among bacteria, actinobacteria are the most prolific source for all types of bioactive metabolites, including bioinsecticides (Berdy, 2005; Liu et al., 2008; Herbert, 2010; Karthik et al., 2011; Omura, 2011; Deepika et al., 2012; Saurav et al., 2013). Currently, the control of *C. capitata* by actinobacterial insecticide is based on spinosad (Barry et al., 2003; Vargas et al., 2003; Stark et al., 2004). However, in laboratory experiments, some pest insects have developed resistance to this insecticide (Hsu and Feng, 2006; Su and Cheng, 2013 ; Abbas et al., 2014). Thus, the use of others actinobacteria as a biocontrol agent against the Medfly could ensure environmental protection and commercial sustainability.

The present study has been undertaken to select Moroccan actinobacteria isolates with insecticidal activity using a primary biological screening against *Artemia salina,* successfully used as a model for biological screening of insecticidal activity (Xiong et al., 2004 ; Shiomi et al., 2005; Deng et al., 2008), and a secondary biological screening against the first instar larvae of *C. capitata* (Wiedemann). Finally, the chemical screening through specific insecticide reagent was done in order to assess the possible nature of insecticidal activity of selected isolates.

MATERIALS AND METHODS

Microorganisms

The 210 actinobacteria isolates used in this study were from the collection of Laboratory of Biology and Biotechnology of Microorganisms, Cadi Ayyad University, Marrakesh, Morocco. They were isolated from various Moroccan habitats including rhizospheric soils and endophytic of endemic aromatic and medicinal plants (Barakate et al., 2002). All strains were maintained in glycerol (20%) at -20°C.

Fermentation

Selected actinobacteria isolates were inoculated into a 500 mL baffled Erlenmeyer flasks containing 100 mL of the producing Bennett's liquid medium (Beef extract, 1 g L^{-1} ; glucose 10 g L^{-1} ; peptone, 2 g L^{-1} ; yeast extract, 1 g L^{-1} ; distilled water, 1000 mL ; pH 7.2). Flasks were incubated on a rotary shaker (250 rev min^{-1}) at 30°C for 48 h. 2 L of this culture was used as inoculum for a 20 L jar fermentor containing 18 L of the Bennett's liquid medium. Starting pH is at about 7.2 and the aeration was of 5 L min^{-1} with agitation of 120 rev min^{-1}. The fermentation was carried out at 30°C for at least seven days. The bacterial culture received from the fermentation was first filtered over the Celite and the water phase was in part freeze-dried and used for larvicidal bioassay of *C. capitata*. The remaining liquid fermentation was extracted three times with acetic ester with a ratio of 1:1 (v/v). The organic phases were collected together and evaporated in vacuum at 40°C until they became dry. The obtained crude extract was suspended in methanol for further uses in chemical screening and acetylcholine-esterase test inhibition.

Primary screening: Brine shrimp bioassay

The brine shrimp lethality assay is considered as a useful and effective tool for preliminary screening of insecticidal substances of microbial origin (Liou, 2000 ; Zhiyu et al., 2000). Our primary biological screening's brine shrimp bioassay was conducted using agar culture diffusion solution according to the method of (Xiong et al., 2004) with some modifications. The agar culture diffusion solution was prepared as follows: actinobacteria isolates were grown on Bennett's agar medium (Beef extract, 1 g L^{-1} ; glucose, 10 g L^{-1} ; peptone, 2 g L^{-1} ; yeast extract, 1 g L^{-1} ;agar, 15 g L^{-1} ; distilled water, 1000 mL ; pH 7.2) and incubated for at least 7 days at 30°C. The agar culture diffusion solution was prepared by picking out under sterile conditions 1 cm^2 of agar culture of the isolates and

*Corresponding author. E-mail: mbarakate@uca.ma. Tel: +212 524 43 46 49 ext. 433/436/517. Fax: +212 524 43 74 12.

put into Eppendorf tubes containing 1 mL of sterile distilled water. The tubes were kept at 4°C for 12 h. The mixtures were centrifuged at 1000 g for 15 min and the supernatants were pipetted out. Brine shrimp eggs (0.5 g) were hatched in 500 mL sterile seawater. The suspension was aerated by bubbling air and kept for 24 to 48 h at room temperature. A volume of 0.5 mL of hatched nauplii suspension (30 to 40 larvae) was transferred into separate wells of a plastic 24-well tissue culture plate and filled with 0.5 mL of each actinobacteria supernatant. The supernatant obtained from a free isolates agar culture was used as negative control. All experiments were conducted in six replicates. The mortality was adjusted using the Abbott's control-adjusted mortality (Abbott, 1925) expressed as follow :

$$M = \frac{(A-B-N)}{(G-N)} \times 100$$

Where, M = percent of the dead larvae after 24 h; A = number of the dead larvae after 24 h; B = average number of the dead larvae in the blind samples after 24 h; N = Number of the dead larvae before starting the test and G = Total number of larvae.

For the isolates showing a strong anti-brine shrimp activity, the LC_{50} and TL_{50} were calculated.

Secondary screening: Laboratory bioassay of *C. capitata*

Insect rearing

C. capitata used in these tests was obtained from a mass-reared stock maintained at the laboratory of Molecular modeling and Ecophysiology, University Cadi Ayyad, Faculty of Sciences Semlalia, Marrakesh, Morocco (Aboussaid et al., 2011). The flies were maintained and all the experiments were carried out at 27 ± 2°C and 75 to 85% relative humidity under 16:8 light and dark cycles. Insects were reared in clear plastic containers (20 × 14 × 7 cm) on a diet composed of wheat bran, sucrose, brewer's yeast, Nipagin, Nipasol, benzoic acide, and water at a volumetric ratio of (25:7:3:1:1:1:62). A mixture of sucrose and Brewer's yeast (4:1) was used as adult food.

Insecticidal bioassay

A preliminary screening of *C. capitata* was performed using a single-dose test according to Molina et al. (2010) with some modifications. Thus, the first 10 instar larvae were transferred to plastic recipients (30 × 70 mm) containing 5 g of diet (see insect rearing) mixed with 0.5 g of freeze-dried fermentation of selected actinobacterial isolates. The control receives 0.5 g of freeze-dried Bennett's liquid medium. Five repetitions were conducted for each experiment and the pupation was recorded after 14 days of incubation.

Chemical characterization

Chemical reagents

Fast Blue B salt, physostigmine, and 1-naphthyl acetate were purchased from Sigma (Germany). The palladium chloride (was obtained from Riedel-de Haën, the methyl yellow from Acros organics and the molybdatophosphoric acid from Fluka. Silica gel G/UV254 plates were purchased from Macherey-Nagel & Co., with the thickness between 0.2 and 0.25 mm. Other reagents were of analytical grade.

Detection of chemical insecticide families by TLC

The detection of chemical insecticide families was conducted for the most promising isolates from the biological screening on thin layer chromatography (TLC) using specific reagents of each chemical insecticide family: (i) for the organophosphates (OP), the crude extract was applied onto the TLC plate and chromatographed in a mobile phase consisting of n-hexane-acetone (8:2 v/v). The plates were first placed under UV radiation at the wavelength of 254 nm and sprayed with a 0.5% solution of palladium (II) chloride in 10% HCl then heated (Koujiro et al., 1997), an active yellow-brown to black colored spots indicating a possible production of an insecticide of organophosphate family. (ii) for the carbamates (CA), developing solvent was benzene-ethyl methyl acetone (19:1 v/v). The orange or purple specific spots were visualized after migration by treating the chromatogram with an ethanolic solution of Fast Blue B followed by 20% of aqueous solution of NaOH and viewed under short UV light (254 nm) (Tewari and Singh, 1979); (iii) for the pyrethroids (PY) family, the mobile phase consisted of n-hexane-benzene-acetone (9:1:1 v/v) then after, plates were sprayed with molybdatophosphoric acid and heated to 120°C to visualize gray spots in a yellow background (Ogierman and Silowiecki, 1981); (iv) for the detection of organochlorines (OC), the hexane-chloroforme-acetone (9:3:1 v/v) was used as developing solvent. The TLC plates were sprayed with the N,N-Dimethyl-p-phenylazoaniline (methyl yellow), and red spots were viewed under UV radiation at the wavelength of 254 nm (Krzeminski and Landmann, 1963). For all tests, the colored spot positions were determined using the retardation factor (Rf).

Screening for acetylcholinesterase inhibitors

In order to determine whether selected isolates have an insecticidal activity on insect's nervous system a screening assay was conducted for acetylcholinesterase (AChE) inhibition according to the method of Zhongduo et al. (2009). Thus, 500 U of acetylcholinesterase (EC3.1.1.7, Sigma product No. C2888) was first dissolved in 500 mL Tris-hydrochloric acid buffer (0.05 mol L^{-1}, pH = 7.8) containing 500 mg BSA (Sigma, Germany) and 100 µg of crude extract of actinobacteria isolate was dissolved in 1 mL methanol and applied to silica gel TLC plate. The plate was dried and sprayed with enzyme and 1-naphthyl acetate solution (150 mg of 1-naphthyl acetate dissolved in 40 mL of ethanol and diluted with 60 mL of distilled water). Then, it was put in a closed humid environment and kept at 37°C for 20 min. Subsequently, the Fast Blue B salt solution (50 mg dissolved in 100 mL of distilled water) was sprayed onto TLC plate and the inhibition of the enzyme-substrate reaction by the produced insecticide caused the formation of an azo dye resulting in bright zones on a purple colored background (Sherma and Fried, 2003). Physostigmine, known as acetylcholinesterase inhibitor, was used as positive control and the methanol as negative control.

Statistical analyses

All experiments were conducted in a randomized design and the data obtained were compared by one way ANOVA, and means were compared by Tukey's multiple range test, with the level of significance set at $p<0.05$. The lethal concentration (LC_{50}) values were calculated using the EPA computer probit analysis program (Version 1.5). The lethal time (TL_{50}) were calculated according to the SAS LIFEREG procedure fitting a Weibull model to the time interval data with single censored-time observation using JMP version 8.0.

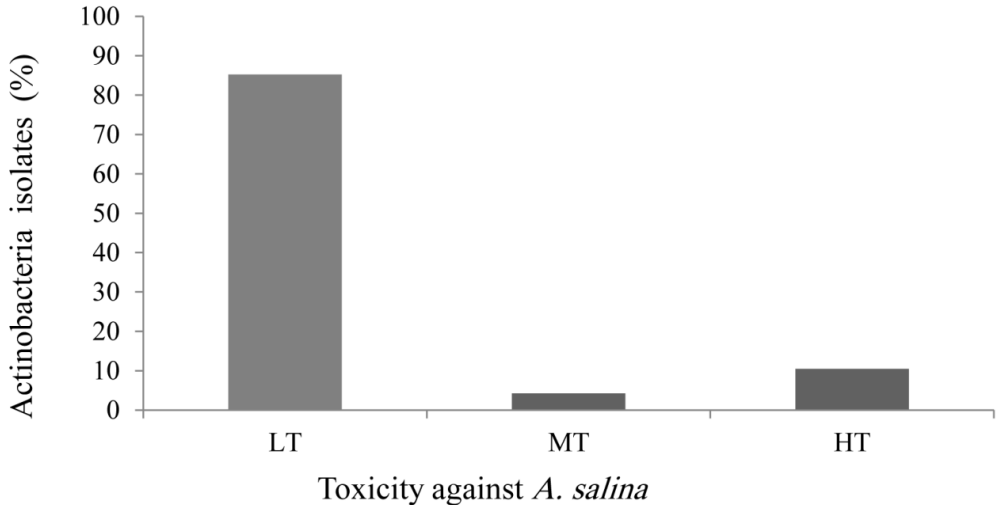

Figure 1. Toxicity of Moroccan actinobacterial isolates against *A. salina*. LT, low toxic isolate with a corrected mortality less than 50%; MT, moderate toxic isolate with a corrected mortality ranged from 50 to 80 %; HT, highly toxic isolate with a corrected mortality ranged from 80 to 100%.

Table 1. Lethal concentration LC_{50} (µg mL^{-1}) and time mortality TL_{50} (h) of the most active isolates using brine shrimp bioassay.

Isolate	LC_{50} (µg mL^{-1})	95% confidence limits		TL_{50} (h)
		Lower	Upper	
OS46	0.260	0.257	0.278	<1 h
37	0.342	0.293	0.445	5.20 ± 0.02
B62	0.840	0.811	0.932	<1 h
B56	1.094	1.043	1.256	2.29 ± 0.04
PH33	1.352	1.343	1.394	<1 h
AS1	2.604	2.550	2.799	3.59 ± 0.04
AS2	2.862	2.834	3.035	2.29 ± 0.04
B89	5.285	4.949	6.130	<1 h
CB33	7.168	6.859	8.131	6.66 ± 0.03
D51	10.893	10.429	12.358	7.94 ± 0.03
OS5	11.265	10.641	12,354	22.79 ± 0.11
B42	23.775	21.154	26.986	11.21 ± 0.06

RESULTS

Determination of anti-brine shrimp activity

The primary biological screening results show that out of 210 isolates, 22 (10.48%) were highly toxic against brine shrimp with a corrected mortality that ranged from 80 to 100%, 9 isolates (4.29%) were moderately toxic with a corrected mortality ranged from 50 to 80% while, 179 isolates (85.24%) have a corrected mortality less than 50% (Figure 1). Among the 22 highly active actinobacteria isolates, only 12 showed 100% brine shrimp mortality at the concentration of 100 µg mL^{-1}, and their corresponding lethal concentration (LC_{50}) and time mortality (TL_{50}) are given in Table 1. Thus, the 12 selected actinobacteria can be divided into two groups: (i) the highly toxic rapid group (HTR) including isolates which caused 50% of *A. salina* mortality in less than 1 h. Among these and except isolate B89 with a LC_{50} of 5.285 µg mL^{-1}, the others (OS46, B62 and PH33) were the most toxic with a LC_{50} of 0.260, 0.840 and 1.352 µg mL^{-1} respectively ; (ii) the highly toxic slow group (HTS) includes isolates 37, B56, AS1, AS2, 0S5, B42, D51 and CB33 which caused 50% of *Artemia salina* mortality in more than 1 h. Among this group, the isolates 37 and B56 were the most toxic with a respectively LC_{50} of

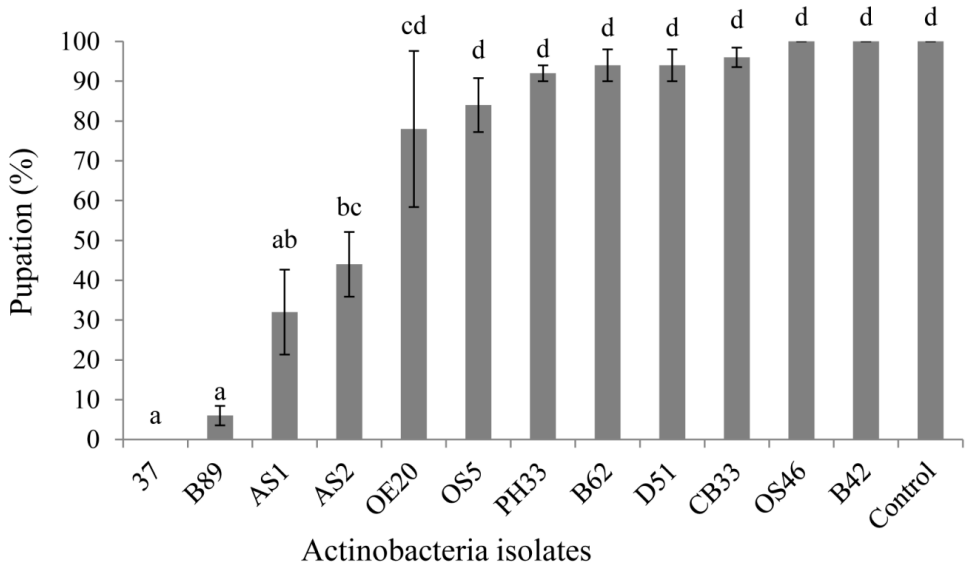

Figure 2. Insecticidal activity of the most active actinobacterial isolates on larval pupation of *Ceratitis capitata* after 7 days of exposure (means followed by different letters with in a column are significantly different; Tukeys HSD, $p < 0.05$).

Table 2. Chemical screening of the most active isolates on thin layer chromatography; using four specific insecticide reagents.

Isolate	TCL with specific insecticide reagents			
	Pyrethroids	Organophosphates	Organochlorines	Carbamates
37	-	-	R (9.33; 22.67)	-
B89	G (10.53; 22.37; 39.47)	G (12.68) Y (19.72; 32.39; 50.70)	R (30.88)	O (28.38; 56.76)
AS1	-	G (32.05)	-	-
OS5	-	G (25.64)	-	-

Color of reveled spot after staining with specific insecticide reagents are: G, grey; Y, yellow; R, red; O, orange; P, purple; -: no active spot; numbers in brackets represent the Rfx100.

0.342 and 1.094 µg mL^{-1}.

Insecticidal activity against *C. capitata*

The biological control against the first-instar larvae of *C. capitata* (Figure 2) showed that the percentage of pupation ranged from 0 to 100%, and only isolates 37, B89, AS1 and AS2 caused a significant reduction in larval pupation compared to control as determined by Tukey's Student range test ($p < 0.05$). No pupation was obtained from treated larvae with isolate 37. On the other hand, the remaining isolates showed no significant mortality in comparison to the control with no pupation reduction for isolates B42 and OS46.

Chemical screening

The thin layer chromatography (TLC) of crude extracts showed a diversity of the bioinsecticide compounds produced by the most promising active isolates against the first-instar larvae of *C. capitata* as indicated in Table 2. The variety of colored bands on TLC after staining with palladium (II) chloride, Fast Blue B, molybdatophosphoric acid and methyl yellow showed that the selected actinobacterial isolates gave at least one active spot on TLC plate. Thus, isolate 37 gave a red spot after staining with the methyl yellow indicating that these crude extracts could contain chlorinated insecticide compounds. Furthermore, the isolate AS1 gave a gray spot after spraying with the palladium chloride, a specific reagent for thiophosphate esters and other sulfur compounds. While, isolate B89 exhibits a wide chemical variety with at least one active spot for each specific reagent tested.

Acetylcholinesterase inhibitors

The screening of the most promising isolate as candidate

Table 3. Acetylcholinesterase inhibition bioassay of the crude extracts of the most active isolates at the concentration of 100 µg mL^{-1}.

Isolate	Inhibition of acetylcholinesterase
37	-
B89	+
AS1	+
OS5	+
Physostigmine	+
Methanol	-

+, Active spot; -, no active spot.

for the production of acetylcholinesterase inhibitors indicate that when the crude extract of the isolates AS1, OS5 and B89 was used in the TLC-bioautographic assay at the concentration of 100 µg mL^{-1}, a bright zones on a purple colored background were clearly observed. Conversely, both the isolates 37 and physostigmine failed to give an active spots on the TLC plates (Table 3).

DISCUSSION

Trend in insect pest control have been shifted from the reliance in conventional chemical pesticides to natural product such as microbial insecticide. The discovery of new active metabolites must be followed by adequate biological testing (Naine et al., 2012). In this study, we investigated the ability of Moroccan actinobacteria isolates to produce insecticidal compounds against *C. capitata* by combining several screening methods. From the 210 investigated Moroccan isolates, 5.71% showed 100% anti-brine shrimp activity. These isolated could be a potential candidate for the production of insecticidal compounds, and also a source of cytotoxic substances as described by many other authors (Xiong et al., 2004; Anibou et al., 2008; Deng et al., 2008). According to Meyer et al. (1982), LC_{50} value lower than 1000 µg mL^{-1} indicated significant cytotoxic potentials. In our bioassay, LC_{50} value of the twelve most active isolates ranged from 0.26 to 23.77 µg mL^{-1} indicating a high anti-brine shrimp activity. Similar toxicity values were obtained from actinobacteria by many other authors using *A. salina* as a model of screening bioinsecticides compounds from actinobacteria (Tantithanagorngul et al., 2011; Kekuda et al., 2012; Tanvir et al., 2014). However, the TL_{50} values obtained in our study were lower than those obtained by the same authors. The 12 selected actinobacteria isolates were there after tested against the Mediterranean fruit fly *C. capitata* and especially against the first instar which is considered to be, with the second instar, the most crops damaging. In fact, the most food intake is used to expand the cell and body size of first and second instars during the life cycle of this Diptera (Berni et al., 2009).

Our results show that the ingestion of freeze-dried fermentation of the isolates 37, B89, AS1 and AS2 caused a significant pupation reduction (P>0.05) compared to the control after 14 days. Many works have demonstrated that actinobacteria isolates are able to produce bioinsecticides compounds against *C. capitata* (Karthik et al., 2011; Deepika et al., 2012; Saurav et al., 2013). The fact that selected bioactive isolates seem to be differents regrading their morphological characteristics and fermentation (data not shown) suggested that they could produce different active compounds. Thus, a chemical characterization was conducted using thin layer chromatography (TLC) combined with chromogenic reagents; in order to assess the nature and chemical diversity of the produced bioinsecticide metabolites. The chemical screening of the acetic ester extracts by thin layer chromatography (TLC) helped to determine the diversity of the chemical constituents produced by the investigated isolate. The variety of the colored bands and their Rf obtained on TLC plates after staining with specific insecticide reagents represent secondary metabolites diversity in the crude extract and could indicate the mechanism of action of the respective actinobacterial isolates. Indeed, the isolate B89 produce a wide chemical variety of secondary metabolites, while the isolates 37, AS1 and OS5 exhibit less diversity. On the other hand, the production of organophosphate compounds could indicate a mechanism involving the enzyme acetylcholinesterase. Accordingly, only the isolates AS1, OS5 and B89 could act on this enzyme. We also notice that the isolate B89 produced compounds belonging to the four insecticide families investigated and could have therefore more than one mechanism of action. These finding were confirmed by a screening of acetylcholinesterase inhibitors. Indeed, the obtained result showed that except the isolate 37, all selected isolates inhibited the enzyme at the concentration of 100 µg mL^{-1}. In fact the organophosphates acted primarily by phosphorylation of the acetylcholinesterase enzyme at nerve endings (Moss and Henderson, 1999; Walker and Asher, 2005). Consequently, it allowed the accumulation of acetylcholine at parasympathetic neuroeffector junctions and caused insect poisoning (Waxman, 1998). It can be conferred from the study that Moroccan actinobacteria could be an important source of compounds with insecticidal activity. The isolates 37, B89, and AS1 showed a potential for controlling *C. capitata* and could be useful in integrated control against this Pest. The bioassay of selected isolates against the other stages of *C. capitata* and the taxonomic characterization of isolates with important larvicidal activity against the Medfly as well as isolation, purification and structural elucidation of the produced larvicidal bioactive metabolites are under investigation.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the excellence grant awarded by the Ministry of Higher Education of Morocco (N°d3/027) and the German Academic Exchange Service (DAAD) grant.

REFERENCES

Abbas N, Ali-Khan HA, Shad SA (2014). Cross-resistance, genetics, and realized heritability of resistance to fipronil in the house fly, *Musca domestica* (Diptera: Muscidae): a potential vector for disease transmission. Parasitol. Res. 113:1343-1352.

Abbott WS (1925). A method of computing the effectiveness of an insecticide. J. Am. Mosq. Control Assoc. 3:302-303.

Aboussaid H, Vidal-Quist JC, Oufdou K, El Messoussi S, Castañera P, González-Cabrera J (2011). Occurrence, characterization and insecticidal activity of *Bacillus thuringiensis* strains isolated from Argan fields in Morocco. Environ. Technol. 32:1383-1391.

Alaoui A, Imoulan A, Alaoui-Talibi ZE, Meziane AE (2010). Genetic structure of Mediterranean fruit fly (*Ceratitis capitata*) populations from Moroccan Endemic Forest of *Argania spinosa*. Int. J. Agric. Biol. 12:291-298.

Anibou M, Chait A, Zyad A, Taourirt M, Ouhdouch Y, Benherref A (2008). Actinomycetes from Moroccan habitats: isolation and screening for cytotoxic activities. World J. Microb. Biotechnol. 24:2019-2025.

Barakate M, Ouhdouch Y, Oufdou K, Beaulieu C (2002). Characterization of rhizospheric soil streptomycetes from Moroccan habitats and their antimicrobial activities. World J. Microb. Biotechnol. 18:49-54.

Barry JD, Vargas RI, Miller NW, Morse JG (2003). Feeding and foraging of wild and sterile Mediterranean fruit flies (Diptera: Tephritidae) in the presence of spinosad bait. J. Econ. Entomol. 96:1405-1411.

Berdy J (2005). Bioactive microbial metabolites. J. Antibiot. 58:1-26.

Berni J, Rabossi A, Pujol-Lereis L, Tolmasky D, Quesada-Allué L (2009). Phloxine B affects glycogen metabolism in larval stages of *Ceratitis capitata* (Diptera: Tephritidae). Pestic. Biochem. Physiol. 95:12-17.

Bolognesi C (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. Mutat. Res. Rev. Mutat. 543:251-272.

Chueca P, Garcerá C, Urbaneja A, Molto E (2013). A new mechanised cultural practice to reduce *Ceratitis capitata* Wied. populations in area-wide IPM. Span. J. Agric. Res. 11:1129-1136.

Croft BA (1990). Arthropod biological control agents and pesticides. Wiley-Interscience, New York. p. 723.

De Meyer M, Robertson MP, Peterson AT, Mansell MW (2008). Ecological niches and potential geographical distributions of Mediterranean fruit fly (*Ceratitis capitata*) and Natal fruit fly (*Ceratitis rosa*). J. Biogeogr. 35:270-281.

Deepika T, Kannabiran K, Khanna V, Rajakumar G, Jayaseelan C, Santhoshkumar T, Rahuman A (2012). Isolation and characterisation of acaricidal and larvicidal novel compound (2s,5r,6r)-2-hydroxy-3,5,6-trimethyloctan-4-one from *streptomyces* sp. against blood-sucking parasites. Parasitol. Res. 111:1151-1163.

Deng Y, Hu J, Liu L, Wang N, Wang S (2008). Screening for marine actinomycetes producing insecticidal metabolites. J. Biotechnol. 136: S533.

Dominiak BC, Ekman JH (2013). The rise and demise of control options for fruit fly in Australia. Crop Prot. 51:57-67.

Ferencz L, Balog A (2010). A pesticide survey in soil, water and foodstuffs from central Romania. Carpath. J. Earth. Environ. 5:111-118.

Herbert AK (2010). The spinosyn family of insecticides: realizing the potential of natural products research. J. Antibiot. 63:101-111.

Hsu J, Feng H (2006). Development of resistance to spinosad in oriental fruit fly (Diptera: Tephritidae) in laboratory selection and cross-resistance. J. Appl. Entomol. 99:931-936.

Jemâa JMB, Bachrouch O, Allimi E, Dhouibi M (2010). Field evaluation of Mediterranean fruit fly mass trapping with Tripack® as alternative to malathion bait-spraying in citrus orchards. Span. J. Agric. Res. 8:400-408.

Karthik L, Gaurav K, Rao K, Rajakumar G, Rahuman A (2011). Larvicidal, repellent, and ovicidal activity of marine actinobacteria extracts against *culex tritaeniorhynchus* and *culex gelidus*. Parasitol. Res. 108:1447-1455.

Kekuda PTR, Shobha KS, Onkarappa R, Goutham SA, Raghavendra HL (2012). Screening biological activities of a *Streptomyces* species isolated from soil of Agumbe, Karnataka, India. Int. J. Drug. Dev. Res. 4:104-114.

Koujiro F, Chie N, Yasufumi K, Hideki S, Ryozo O (1997). Application of high-performance thin-layer chromatography for the detection of organophosphorus insecticides in human serum after acute poisoning. J. Chromatogr. B. 704: 369-373.

Krzeminski LF, Landmann WA (1963). Methyl yellow as a spray reagent in the paper chromatography of chlorinated hydrocarbon pesticides. J. Chromatogr. B. 10: 515-516.

Kumari B, Madan V, Kathpal T (2008). Status of insecticide contamination of soil and water in Haryana, India. Environ. Monit. Assess. 136:239-244.

Lance DR, Woods WM, Stefan M (2014). Invasive insects in plant biosecurity: Case study - Mediterranean fruit fly. In: Gordh G, McKirdy S (Eds.), The Handbook of plant biosecurity. Springer, Netherlands. pp. 447-484.

Liou Q (2000). The research of new method of biomonitoring. Environmental Monitoring in China 16:38-40.

Liu H, Qin S, Wang Y, Li W, Zhang J (2008). Insecticidal action of Quinomycin A from a *Streptomyces* sp. KN-0647, isolated from a forest soil. World J. Microb. Biotechnol. 24:2243-2248.

Malacrida AR, Gomulski LM, Bonizzoni M, Bertin S, Gasperi G, Guglielmino CR (2007). Globalization and fruitfly invasion and expansion: the medfly paradigm. Genetica 131:1-9.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 45: 31-34.

Molina CA, Cana-Roca JF, Osuna A, Vilchez S (2010). Selection of a *Bacillus pumilus* strain highly active against *Ceratitis capitata* (Wiedemann) larvae. Appl. Environ. Microbiol. 76: 1320-1327.

Moss DW, Henderson AB (1999). Clinical enzymology. In: Burtis CA, Ashwood ER, Tietz NW (Eds.), Tietz Textbook of Clinical Chemistry. Saunders, Philadelphia. pp. 617-621.

Naine JS, Nasimunislam N, Vaishnavi B, Mohanasrinivasan V, Devi SC (2012). Isolation of soil Actinomycetes inhabiting Amrithi forest for the potential source of bioactive compounds. Asian J. Pharm. Clin. Res. 5: 189-192.

Oerke EC (2005). Crop losses to pests. J. Agric. Sci. 144:31.

Oerke EC, Dehne HW (2004). Safeguarding production - losses in major crops and the role of crop protection. Crop Prot. 23: 275-285.

Ogierman L, Silowiecki A (1981). Thin-layer and gas chromatographic separation of the stereoisomers of some synthetic pyrethroids. Chromatographia 14:459-461.

Omura S (2011). Microbial metabolites: 45 years of wandering, wondering and discovering. Tetrahedron 67: 6420-6459.

Osman KA (2011). Pesticides and human health. In: Stoytcheva M (Ed) Pesticides in the modern world - Effects of pesticides exposure. InTech, Rijeka. pp. 205-230.

Popp J, Peto K, Nagy J (2013). Pesticide productivity and food security - A review. Agron. Sustain. Dev. 33:243-255.

Preetha G, Stanley J, Suresh S, Samiyappan R (2010). Risk assessment of insecticides used in rice on miridbug, *Cyrtorhinus lividipennis* Reuter, the important predator of brown planthopper, *Nilaparvata lugens* (Stal.). Chemosphere 80:498-503.

Ravensberg WJ (2015). Commercialisation of Microbes: Present Situation and Future Prospects. In: Lugtenberg B (Ed) Principles of Plant-Microbe Interactions. Springer International Publishing. pp. 309-317.

Rivero A, Vézilier J, Weill M, Read AF, Gandon S (2010). Insecticide control of vector-borne diseases: When is insecticide resistance a problem. PLoS Pathog. 6: 1-9.

Ruberson JR, Nemoto H, Hirose Y (1998). Pesticides and conservation of natural enemies in pest management. In: Barbosa P (Ed) Conservation

Biological Control. Academic Press, San Diego CA. pp. 207-220.
Saurav K, Rajakumar G, Kannabiran K, Rahuman A, Velayutham K, Elango G, Kamaraj C, Zahir A (2013). Larvicidal activity of isolated compound 5-(2,4-dimethylbenzyl) pyrrolidin-2-one from marine *streptomyces* VITSVK5 sp. against *rhipicephalus (boophilus) microplus, anopheles stephensi*, and *culex tritaeniorhynchus*. Parasitol. Res. 112:215-226.
Sherma J, Fried B (2003). Handbook of thin layer chromatography. Marcel Dekker, New York-Basel. p. 703.
Shiomi K, Hatae K, Hatano H, Matsumoto A, Takahashi Y, Jiang CL, Tomoda H, Kobayashi S, Tanaka H, Ōmura S (2005). A new antibiotic, antimycin a9, produced by *streptomyces* sp. K01-0031. J. Antibiot. 58:74-78.
Stark JD, Vargas RI, Miller N (2004). Toxicity of sprinosad in protein bait to three economically important tephritid fruit fly species (Diptera: Tephritidae) and their parasitoids (Hymenoptera: Braconidae). J. Econ. Entomol. 97:911-915.
Su T, Cheng ML (2013). Laboratory selection of resistance to Spinosad in *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol. 51:297-488.
Tantithanagorngul W, Sujitwanit A, Piluk J, Tolieng V, Amorn P, Sangvanich P, Palaga T, Puthong S, Thamchaipenet A, Pinphanichakarn P (2011). Screening for brine shrimp larvicidal activity of *Streptomyces* isolated from soil and anti-tumor activity of the active isolates. Aust. J. Basic Appl. Sci. 5: 15-22.
Tanvir R, Sajid I, Hasnain S (2014). Biotechnological potential of endophytic actinomycetes associated with Asteraceae plants: isolation, biodiversity and bioactivities. Biotechnol. Lett. 36: 767-773.
Tewari SN, Singh R (1979). Identification and detection of carbamate pesticides in autopsy tissues using thin-layer chromatography. Fresenius. J. Anal. Chem. 294:287-287.
Vargas RI, Miller NW, Stark JD (2003). Field trials of spinosad as a replacement for Naled, DDVP, and malathion in methyl eugenol and cue-lure bucket traps to attract and kill male oriental fruit flies and melon flies (Diptera: Tephritidae) in Hawaii. J. Econ. Entomol. 96:1780-1785.
Walker JP, Asher SA (2005). Acetylcholinesterase-based organophosphate nerve agent sensing photonic crystal. Anal. Chem. 77:1596-1600.
Waxman MF (1998). The agrochemical and pesticides safety handbook. CRC Press, New Yok. p. 640.
Xiong L, Li J, Kong F (2004). *Streptomyces* sp. 173, an insecticidal micro-organism from marine. Lett. Appl. Microbiol. 38:32-37.
Zhiyu H, Sanzhen L, Hao H, Yaojian H, Wenjin S (2000). A rapid screening assay for insecticidal antibiotic produced by marine actinomycetes. Bull. Mar. Sci. 19:36-41.
Zhongduo Y, Xu Z, Dongzhu D, Zhuwen S, Mingjun Y, Shuo L (2009). Modified TLC bioautographic method for screening acetylcholinesterase inhibitors from plant extracts. J. Sep. Sci. 32:3257-3259.

academicJournals

Vol. 14(10), pp. 867-871, 11 March, 2015
DOI: 10.5897/AJB2014.14387
Article Number: 572AE7551260
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antibacterial activity of endophytic fungi isolated from conifer needles

Matjaž Ravnikar*, Matic Terčelj, Damjan Janeš, Borut Štrukelj and Samo Kreft

University of Ljubljana, Faculty of Pharmacy, Aškerčeva cesta 7, SI-1000 Ljubljana, Slovenia.

Received 24 December, 2014; Accepted 25 February, 2015

Fungi, in particular endophytes are a promising source of new antimicrobial compounds. The aim of this study was to screen the extracts of conifer needle fungal endophytes for antimicrobial activity and taxonomically place fungi producing ones to determined active metabolites. Seventy three strains of endophytic fungi were isolated from plant samples, mainly from needles of conifers, and cultured. Extracts of cultured endophytic strains were tested for antimicrobial properties using a microdilution assay. Their activity was compared to that of the antibiotic ampicillin. Samples that exhibited antimicrobial properties were further examined. Genomic DNA from five active fungal strains was isolated and species-specific DNA regions (ITS regions) were amplified and sequenced allowing us to determine the identity of the samples. Active endophytic fungi were two strains of *Lophodermium pinastri*, two strains of *Lophodermium seditiosum* and one of *Phoma herbarum*. All of these strains are known as parasitic and can be treated as endophytes only according to the lack of symptoms in their host tissue. This work demonstrates an interesting bottom-up approach to the discovery of new antimicrobial compounds.

Key words: Endophyte, antibiotic, parasitic, *Lophodermium*, antimicrobial, *Phoma*.

INTRODUCTION

The problem of drug-resistant pathogens and co-dependent infectious diseases is substantial and still growing. According to the World Health Organization (WHO) Global Burden of Disease report from the year 2013, infectious and parasitic diseases were still the second leading cause of death, causing 18.4% of all deaths worldwide (WHO, 2014). This brings the need for the search of new antibiotic compounds. Naturally, derived products remain the most important source of their discovery. Fungi are a versatile and precious source with an enormous pharmaceutical potential (Schulz et al., 2002; Vaz et al., 2009; Bhagobaty and Joshi, 2012; Wang et al., 2012). Recently, there have been an increasing number of publications regarding investigations of endophytic fungi producing antimicrobial substances (Janeš et al., 2007; Xiaoye et al., 2012). This niche should be meticulously examined and used as a base for sustainable research and development of new antibacterial substances that can both respond to current antimicrobial resistance and anticipate evolving resistance. Plant endophytic fungi have the ability to produce the same or similar compounds to those originating

*Corresponding author. E-mail: matjaz.ravnikar@ffa.uni-lj.si. Tel: +386 1 4769583. Fax: +386 1 4258031.

from their host plants (Erbert at al., 2012), as well as a great number of diverse bioactive compounds (Devaraju and Satish, 2010), which have been implicated in the protection of its host against pathogens and herbivores (Wicklow et al., 2005). Since then several new such substances were found (Xuanwei at al., 2010; Sumarah et al., 2011; Radić and Štrukelj, 2012). Metabolic interactions of endophytes with host can favor the synthesis of biologically active secondary metabolites (Owen and Hundley, 2004). Isolation of endophytes from their natural habitat can influence their metabolism (Raviraja et al., 2006). Certain factors can cause production of biologically active compounds in culture. Conditions like temperature, composition of culture medium, amount of nutrients and level of aeration influence growth and synthesis of secondary metabolites. Method of fermentation and extraction technique influences the amount and kind of compounds as well (Strobel and Daisy, 2003). In order to identify novel biologically active compounds; their biological activities necesicates testing by a variety of means. In the case of antibacterial activity it can be tested several different ways including disk diffusion methods, E test, and microdilution broth method. The last one, most common and available was used in our case. The most efficient method for determination of fungal species employs amplifying and sequencing of species-specific regions of fungal DNA. Among few such potential regions internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation.

MATERIALS AND METHODS

Bacteria

Escherichia coli, strain ER2738, were obtained from the Department of Pharmaceutical Biology, Faculty of Pharmacy, Ljubljana, Slovenia.

Fungi

Conifer needles were collected from several plant species (*Abies* sp*., Cedrus* sp*., Juniperus* sp*., Larix* sp*., Metasequoia* sp., *Picea* sp*., Pinus* sp*., Taxus* sp.), samples of leaves, stalks, roots and remaining plant parts of other plant species (*Sambucus* sp*., Calluna* sp*., Centaurea* sp.) were also collected from unpolluted habitats around Slovenia. Samples were kept in sterile test tubes or plastic bags at 4°C and transported to the laboratory as soon as possible. Once in the laboratory, samples were surface sterilized with 70% ethanol for 2 min and 1% sodium hypochlorite for 3 min in a sterile chamber with laminar flow of air to kill epiphytes (microorganisms on surface of samples) and cut in half longitudinally with a sterile razor. Internal surface was then exposed to a solid culture medium comprised of potato dextrose agar (PDA). Explicitly, the PDA medium was composed of potato extract, 0.4% (w/V); glucose, 2.0% (w/v); and agar, 1.5% (w/V) in water. After 2 to 5 weeks incubation it was determined when the fungi from the plant samples formed colonies, and every fungal colony was aseptically transferred onto 3 new PDA plates. Every Petri dish was wrapped with Parafilm and left in a chamber for cultivation of fungi at room temperature (22 to 25°C). Fungal cultures were re-inoculated to a new medium repeatedly every week for 1 to 3 times (Figure 1). Replicates were removed and only strains which differed in morphology (shape, patterns and color are distinguishing among different microorganisms) were kept and submitted to a subsequent antimicrobial test (Méndez et al., 2008). Solid PDA culture media overgrown with fungus (15 ml in 90 mm diameter Petri dish), were homogenized with an electric homogenizer to obtain a particle size of approximately 5 mm. The homogeneous mixture was transferred to a conical flask to which 70 ml of a solvent were added and closed with a glass stopper. The extraction solvents utilized were methanol, ethyl acetate or dichloromethane. The culture of every fungal strain was extracted in each of the three solvents at room temperature for 15 min in an ultrasonic bath, 24 h without ultrasound, and after that 15 min in the ultrasonic bath again. The mixture was filtered through a filter paper and washed three times with 10 ml of the chosen solvent. Samples were completely dried by rotary evaporation under reduced pressure to determine the weight of dry extracts. The extracts were then dissolved in methanol (50%) to a concentration of 1 mg/ml. The samples were stored in closed tubes at -20°C until further analysis.

Broth microdilution method

Broth microdilution test was performed according to the standard procedure (National Committee for Clinical Laboratory Standards, 2003). Bacteria from a freshly grown colony were transferred with a sterile sling and suspended in 10 mL of a sterile 0.9% solution of sodium chloride. The suspension was diluted with 0.9% of NaCl to obtain the absorbance of the final suspension between 0.08 and 0.1 at λ=625 nm. This corresponded to 10^8 CFU/mL of bacteria. 800 µL of that suspension were diluted with 25 ml of 0.02% sterile solution of Tween® 80 to obtain the bacterial suspension that was used as an inoculum in a microdilution test. Broth microdilution method was carried out in a 96-well microplate with round bottom wells and the volume of each well of 200 µL. Each well consisted of 90 µL of Mueller-Hinton broth, 10 µL of inoculum and 10 µL of tested extract (or control). All tests were carried out in duplicates. Controls and extracts were added in consecutive 2-fold dilutions. Sodium salt of ampicillin (Sigma-Aldrich, Germany) with a starting concentration 1 mg/mL was used as an efficiency comparison and an extraction solvent as a positive control. Starting concentration of fungal extracts added were 1 mg/mL. The microplate was then incubated for 24 h at 37°C before the results were evaluated.

Determination of endophyte species

Fungal samples were collected from permanent cultures (approximately 100 mg of fungal tissue) and homogenized using ultratorax homogenizer. QiagenDneasy plant mini kit was used to isolate fungal DNA. ITS regions were amplified with GeneAmp2700 Thermal cycler, using two sets of primers ITS F 5'-AGAAAGTCGTAACAAGGTTTCCGTAG-3', ITS R 5'-TTTTCCTCCGCTCATTGATATGCTT-3' and ITS-g F 5'-TCCGTAGGTGAACCTGCGG-3' (White et al., 1990), ITS-g R 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). The 30-cycle amplification program contained 30 s of denaturation (95ºC), 30 s of annealing (55ºC) and 1 min of elongation (72ºC). PCR products were analyzed with agarose gel electrophoresis, stained with Cybr gold stain and detected under UV light. Samples were extracted from gel and purified using Qiaex II Gel Extraction Kit. Purity of isolated DNA fragments was checked with spectrophotometer NanoDrop 1000. Purified samples were sequenced by GATC labs. Obtained sequences were analysed using NCBI BLAST software. Strains 2 and 35 demonstrating the highest antimicrobial activity

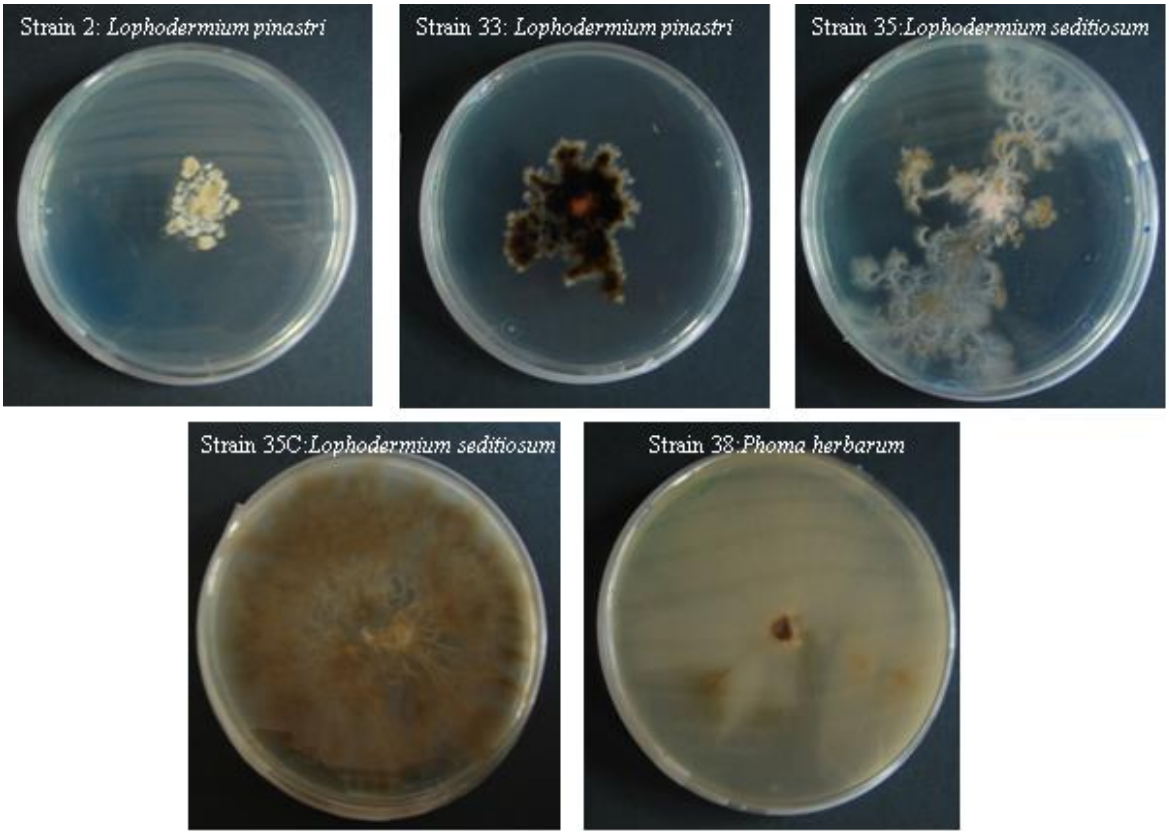

Figure 1. Fungal strains showing antibacterial activity growing on PDA plates.

Table 1. Results of the broth microdilution test with extracts of solid culture medium with mycelium with concentration 1 mg/ml and their dilutions in ratio 1:1.

Extract	1	2	3	4	5	6	7	8	9	10	11	12
Ampicillin Na	-	-	-	-	-	+	+	+	+	+	+	+
Dichloromethane extract 2	-	-	-	+	+	+	+	+	+	+	+	+
Dichloromethane extract 38	-	-	+	+	+	+	+	+	+	+	+	+
Dichloromethane extract 33	-	-	+	+	+	+	+	+	+	+	+	+
Dichloromethane extract 35	-	-	-	+	+	+	+	+	+	+	+	+
Dichloromethane extract 35C	-	+	+	+	+	+	+	+	+	+	+	+
Positive control	+	+	+	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-	-	-	-

(+) Opaque culture medium after incubation indicating bacterial growth; (-) clear culture medium after incubation, indicating bacterial growth inhibition. Numbers 1 to 12 represent consecutive 2-fold serial dilutions. Starting concentration of ampicillin/dichlormetane extracts in column 1 is 10 µg/well.

were also sent for morphological identification to CBS-KNAW Fungal Biodiversity Centre, Netherlands.

RESULTS

Seventy three (73) fungal strains were isolated from 73 plant samples. Extracts were prepared from the culture on the solid PDA medium of each of these strains. Microdilution tests (Table 1) showed 5 of them (extracts from strains marked: 2, 33, 35, 35C and 38) holding an antimicrobial activity against *Escherichia coli* ER2738 (Table 2). Only dichloromethane extract, but not methanol or ethyl acetate extracts, showed antibacterial activity. The second strongest antibiotic effect after ampicillin was

Table 2. Data about sampling of plant parts from host plants of endophytic fungi.

Sample number	Host	Herbal origins	Location
Lophodermium pinastri (2)	*Sambucus nigra* L. Black elder	Leaves	N 45° 39,224' E 15° 11,209'
Lophodermium pinastri (33)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,083' E 14° 46,491'
Lophodermium seditiosum (35)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,049' E 14° 46,510'
Lophodermium seditiosum (35C)	*Pinus nigra* Arnold European black pine	Needles	N 45° 32,049' E 14° 46,510'
Phoma herbarum (38)	*Pinus sylvestris* L. Scotch pine	Needles	N: 46° 02,383′ E: 14° 30,817′

demonstrated by the extracts of samples 2 and 35, followed by samples 33 and 38. Extract of sample 35C has shown weaker antibiotic effect. Fungal DNA was successfully extracted and amplified producing DNA fragments of anticipated lengths of around 600 BP corresponding to the ITS region. Sequencing of the fragments was performed in 2 parallels to ensure the correct nucleotide order. Sequences of all 5 samples were analysed with BlastN tool. All of our sequences had at least 99% identity to designated species and 92 to 98% identity to next related species. According to this analysis sample 2 and sample 33 are two different strains of *Lophodermium pinastri*, 35 and 35C are two different strains of *Lophodermium seditiosum* and sample 38 is *Phoma herbarum*. Morphological identification of samples 2 and 35 determined fungal species to be *Lophodermium pinastri* and *Lophodermium seditiosum* which matches the results of genetic species determination. All sequences were submitted to the Genebank database under the accession numbers: KC608049, KC608050, KC608051, KC608052 and KC608053.

DISCUSSION

Over 70 isolated endophytic strains revealed a very morphologically diverse coexistence of fungi in limited number of host plant samples. Fungal extracts were prepared with three solvents possessing different polarity in order to enable extraction of a broad spectrum of compounds. Dichloromethane as the least polar of them showed the best results indicating that active antibiotic compounds in the fungi are likely to be lipophilic. All of the five sample extracts that demonstrated antibiotic activity were less potent than ampicillin. However, active compounds are likely to be diluted in these extracts and could possess an even stronger antibiotic effect than ampicillin if purified. Interestingly, four of the five investigated fungal strains belonged to the same genus and only two species. Furthermore, *L. pinastri* and *L. seditiosum* are parasitic fungi rather than symbiotic and can be treated as endophytes only by certain measures (Rodriguez and Redman, 2008; Rodriguez et al., 2009). None of these fungal species has previously been known to produce antimicrobial substances. Small differences between ITS sequences of samples 2 and 33 and sequences of samples 35 and 35C showed that they belong to different strains. Macroscopic observation of the fungi growing on PDA plates also indicated that these strains are not duplicate isolates of the same strain. Further work towards isolation of active molecules from these extracts should be performed in the future. However, this study proves endophytic fungi to be an important source of potential new antimicrobial drugs and encourages the use of ITS region sequencing as a suitable method for endophytic fungi systematization.

Conclusions

According to the growing need for new antimicrobial compounds, endophytic fungi could represent a diverse and rich source of new compounds. This research resulted in discovery of five fungal strains that possess the potential for potent antibiotic compounds. These strains would be interesting candidates for upcoming isolation of active compounds.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Bhagobaty RK, Joshi SR (2012). Antimicrobial and antioxidant activity of endophytic fungi isolated from ethnomedicinal plants of the "Sacred forests" of Meghalaya, India. Med. Mycol. 19:5-11.

Devaraju R, Satish S (2010). Endophytic fungi: 'Trapped' or 'hidden' store houses of bioactive compounds within plants: A Review. J. Pharm. Res. 3:2986-2989.

Erbert C, Lopes AA, Yokoya NS, Furtado NAJC, Conti R, Pupo MT, Lopes JLC, Debonsi HM (2012). Antibacterial compound from the endophytic fungus Phomopsis longicolla isolated from the tropical red seaweed Bostrychia radicans. Botanica Marina 55:435-440.

Janeš D, Kreft S, Jurc M, Seme K, Štrukelj B (2007). Antibacterial Activity in Higher Fungi (Mushrooms) and Endophytic Fungi from Slovenia. Pharm. Biol. 45:700-706.

Méndez CC, Serrano MC, Valverde A, Martín-Mazuelos E, Pemán J, Almeida C (2008). Comparison of E-Test®, disk diffusion and a modified CLSI broth microdilution (M 38-A) method for in vitrotesting of itraconazole, fluconazole and voriconazole against dermatophytes. Med. Mycol. 46:119-123.

National Committee for Clinical Laboratory Standards (NCCLS) (2003). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard M7-A6, 6th ed. 20 (2), NCCLS, Wayne.

Owen NL, Hundley N (2004). Endophytes--the chemical synthesizers inside plants. Sci. Prog. 87:79-99.

Radić N, Štrukelj B (2012). Review: Endophytic fungi—The treasure chest of antibacterial substances. Phytomedicine 19:1270-1284.

Raviraja NS, Maria GL, Sridhar KR (2006). Antimicrobial Evaluation of Endophytic Fungi Inhabiting Medicinal Plants of the Western Ghats of India. Eng. Life Sci. 6:515-520.

Rodriguez R, Redman R (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J. Exp. Bot. 59:1109-1114.

Rodriguez RJ, White JF Jr, Arnold AE, Redman RS (2009). Fungal endophytes: diversity and functional roles. New Phytol. 182:314-330.

Schulz B, Boyle CD, Draeger S, Römmert A, Krohn K (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res. 106: 996-1004.

Strobel G, Daisy B (2003). Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol. Mol. Biol. Rev. 67:491-502.

Sumarah MW, Kesting JR, Sørensen D, Miller JD (2011). Antifungal metabolites from fungal endophytes of *Pinus strobus*. Phytochemistry 72:1833-1837.

Vaz ABM, Mota RC, Bomfim MRQ, Vieira MLA, Zani CL, Rosa CA, Rosa LH (2009). Antimicrobial activity of endophytic fungi associated with Orchidaceae in Brazil. Can. J. Microbiol. 55:1381-1391.

Wang LW, Xu BG, Wang JY, Su ZZ, Lin FC, Zhang CL, Kubicek C (2012). Bioactive metabolites from Phoma species, an endophytic fungus from the Chinese medicinal plant Arisaema erubescens. Appl. Microbiol. Biotechnol. 93:1231-1239.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols : A guide to Methods and Applications (ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White), Academic Press : San Diego, U.S.A. pp. 315-322.

WHO (2014). Disease and Injury Regional Mortality Estimates, 2000-2011 [Online]. Available at: http://www.who.int/healthinfo/global_burden_disease/estimates_regional/en/. Accessed on 13 May 2014.

Wicklow DT, Roth S, Deyrup ST, Gloer JB (2005). A protective endophyte of maize: Acremonium zeae antibiotics inhibitory to Aspergillus flavus and Fusarium verticillioides. Mycol. Res. 109:610–618.

Xiaoye S, Dongqi Z, Jian G, Chenglin H (2012). Isolation and evaluation of endophytic fungi with antimicrobial ability from Phyllostachys edulis. Bangladesh J. Pharmacol. 7:249-257.

Xuanwei Z, Huifang Z, Lu L, Lin J, Kexuan T (2010). A review: recent advances and future prospects of taxol-producing endophytic fungi. Appl. Microbiol. Biotechnol. 86:1707-1717.

academicJournals

Vol. 14(10), pp. 872-877, 11 March, 2015
DOI: 10.5897/AJB2014.14269
Article Number: 3DC419F51261
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Pathogenic, cultural, morphological and molecular variability among eight isolates of *Alternaria solani*, causing early blight of tomato

P. S. Nikam*, A. P. Suryawanshi and A. A. Chavan

Department of Plant Pathology, College of Agriculture, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani 431 402 (M.S.) India.

Received 25 October, 2014; Accepted 23 February, 2015

Among the fungal diseases infecting tomato crops, early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout is one of the most catastrophic disease causing accountable losses. Further, all of the tomato cultivars presently under cultivation have succumb more or less to early blight disease. Therefore, the present studies were undertaken for the pathogenic, cultural, morphological and molecular variability among the isolates of *A. solani*. The results reveal all of the eight isolates of *A. solani* as pathogenic to tomato (Cv. Pusa Ruby) and showed variability amongst them. The test isolates could grow better on the basic culture medium potato dextrose agar; however, highest mycelial growth was recorded on the isolate AsLt (88.50 mm), followed by AsBd (82.36 mm) and AsHl (78.40 mm), with excellent sporulation. All of the eight test isolates exhibited a wide range of variability in respect of their mycelial and conidial dimensions and septation. RAPD-PCR analysis of the four most virulent *A. solani* isolates, using 13 OPA primers revealed that the isolates AsBd (Beed) and AsLt (Latur) were closely related with 85% genetic similarity whereas, the isolates AsHl (Hingoli) and AsJl (Jalna) were closely related with 50% genetic similarity, but distinct from that of AsLt and AsBd isolates.

Key words: Tomato, *Alternaria solani*, isolates, pathogenic, molecular variability, virulent, primers.

INTRODUCTION

Tomato (*Solanum lycopersicon*) is one of the most popular vegetable crops grown throughout the world. India is one of the leading countries in tomato production with an area of 8.65 lakh ha and productivity of 19.5 metric tonnes/ha (Anonymous, 2011). Maharashtra, Bihar, Uttar Pradesh, Karnataka and West Bengal are the major tomato growing states in India. In Maharashtra it occupies an area of 5.20 thousand ha with the productivity of 14.2 metric tonnes/ha (Anonymous, 2011). Diseases are the major constraints in tomato cultivation causing qualitative as well as quantitative losses. Among the fungal diseases infecting tomato crop, early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout, is one of the most catastrophic disease. The disease induces the symptoms such as dark brown to black spots with concentric rings giving a target board

*Corresponding author. E-mail: psnikam123@gmail.com.

effect. The disease early blight have been reported to inflict the yield losses to the tune of 48 to 80% (Datar and Mayee, 1984; Mathur and Shekhawat, 1986; Pandey and Pandey, 2002). Under natural epiphytotics, the pathogen (*A. solani*) have been found to express a wide range of variability in disease symptoms expression depending upon the tomato cultivars, environmental conditions etc. Pathogenic, cultural, morphological and molecular variability in *A. solani* was also documented earlier by many workers (Kaul and Saxcena, 1988; Tong et al., 1994; Weir et al., 1998; Babu et al., 2000; Ahmad, 2002; Naik et al., 2010). The variability of *A. solani* isolates studied under greenhouse conditions of several isolates infecting 14 tomato genotypes has been reported earlier by Castro et al. (2000).

Therefore, the present investigations were attempted to explore the pathogenic, cultural, morphological and molecular variability among the eight isolates of *A. solani,* isolated from the tomato crop affected with early blight disease which were collected during survey in Marathwada region of the state of Maharashtra, India.

MATERIALS AND METHODS

Collection, isolation and maintenance of *A. solani* isolates

A roving survey of the tomato crop fields was undertaken during *Kharif* and *Rabi* seasons of 2010 to 2011. Tomato plant samples (leaves, fruits and branches) showing typical symptoms of early blight disease were collected from the eight districts *viz.,* Parbhani, Nanded, Hingoli, Latur, Osmanabad, Beed, Aurangabad and Jalna in Marathwada region of the state of Maharashtra. These diseased samples were brought to the laboratory washed thoroughly under running tap water, blot dried, cut into small bits (2 mm), surface sterilized (0.1% $HgCl_2$), washed in three sequential changes of sterile water, blot dried, aseptically subjected to tissue isolation, using autoclaved and cooled Potato dextrose agar (PDA) in sterile glass Petri plates (90 mm dia.) and incubated at 27±2°C. After a week of incubation, the culture growth was aseptically transferred, applying hyphal tip method onto fresh PDA plates and incubated at 27±2°C temperature. Further, sub-cultured and the pure cultures of each isolate thus obtained were maintained separately on PDA slants in glass test tubes and stored in refrigerator for further studies.

Pathogenicity test and pathogenic variability

The pathogenicity tests for *A. solani* eight isolates were attempted under screenhouse, applying Koch's postulates. For the purpose, 30 days old potted seedlings of susceptible tomato Cv. Pusa Ruby were spray inoculated with the spore suspension (5 x 10^6 spores / ml) of the test pathogen incubated in screen house and observed for the development of early blight. Disease incidence was rated as suggested by Pandey and Pandey (2002); symptoms expressed were studied and reisolated from the infected stem. The pathogenicity test as above was repeated twice to confirm results. Pathogenic variability of *A. solani* eight isolates was studied by applying detached leaf technique. The spore suspension (5 x 10^6 spores / ml) of each test isolate was separately prepared from 5 days old pure culture of the representative isolates. Healthy growing five tomato leaves of tomato susceptible Cv. Pusa Ruby per moist chamber per isolate were inoculated separately with the spore suspension (5 x 10^6 spores / ml) and incubated at room temperature. Observations on incubation period, number of spots, frequency, size of lesions and typical symptoms induced etc. were recorded and based on these characteristics, the test isolates were categorized as highly virulent, moderately virulent and a mildly virulent.

Cultural variability

All of the eight test isolates of *A. solani* were isolated aseptically on the PDA plates, incubated at 27±2°C for a week and their cultural characteristics were observed *viz*., colony diameter, colony colour / pigmentation, mycelial growth etc., and sporulation was recorded after 15 days of incubation, by observing under research microscope (Make : Labomed, 2000).

Morphological variability

Morphological characteristics *viz*., mycelial width, size of conidia, length of beak and number of transverse and longitudinal septa of all the eight isolates of *A. solani* were measured using ocular micrometer (Calibrated using stage micrometer) under 400 x magnification of research microscope (Make : Labomed, 2000).

Molecular variability

Based on cultural, morphological and pathogenic studies of the eight isolates of *A. solani,* only four highly virulent isolates (AsLt, AsBd, AsJl and AsHl) were selected for studying their molecular variability applying the following procedures and protocols. The genomic DNA of the four test isolates of *A. solani* was extracted using the protocol developed and standardized by Chakrabarty (2003).

PCR amplification

PCR was carried out in a final volume of 25 µl containing 0.1 to 0.5 µm of oligonucleotide primer (2.0 µl), 25 mM of each of the deoxynucleotide triphosphatases (0.2 µl), 50 mM $MgCl_2$ (1.5 µl), 0.3 µl of Tag DNA polymerase, 10 x PCR buffer (2.5 µl) and template DNA (100 ng / 0.5 µl). The reaction mixture was overlaid in PCR tubes except template DNA. Genomic DNA of each isolate was added to individual tube containing the water mix. Amplification was carried out in a thermocycler with initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min and primer elongation at 72°C for 2 min, followed by an extended elongation at 72°C for 10 min. In order to identify the most suitable RAPD primers for the study of molecular variations among the isolates, 13 random primers from OPA (Operon Technologies USA) series that is, OPA 1-13 were used for analyses of all the isolates. The amplified PCR products were separated on 1.2% agarose gel and visualized under illumination by staining with ethidium bromide and photographed by using Gel Doc. All amplifications were repeated at least twice and only reproducible bands were considered for analysis. The data was analyzed using the software NTSYS– pc to Plot Dendrogram.

RESULTS AND DISCUSSION

Pathogenicity test and pathogenic variability

Results of the pathogenicity tests for *A. solani* eight

Table 1. Cultural variability among the isolates of *A. solani,* causing early blight in tomato.

Isolates / districts	Colony diameter (mm)	Cultural characteristics	Colony colour/ pigmentation	Sporulation
AsPb (Parbhani)	76.25	Circular, smooth, without zonation	Black	+++
AsNd (Nanded)	65.80	Circular, smooth, with concentric zonation	Brownish black	++
AsHl (Hingoli)	78.40	Irregular, smooth, with concentric zonation	Dark grayish	++++
AsLt (Latur)	88.50	Irregular, smooth, with concentric zonation	Brownish black	++++
AsOb (Osmanabad)	75.60	Irregular, smooth, without zonation	Olivaceous black	++
AsBd (Beed)	82.35	Circular, smooth with concentric zonation	Pinkish red	++++
AsAb (Aurangabad)	70.42	Irregular, rough, without zonation	Light gray	++
AsJl (Jalna)	78.20	Circular smooth, with concentric zonation	Creamish white	++++

Sporulation: ++++, excellent; +++, good; ++, fair; +, poor. Cultural characteristics, colony colour and sporulation were also varied for the test isolates. Irregular smooth colonies, with concentric zonation, brownish black (AsLt isolate) and dark grayish (AsHl isolate), without zonation olivaceous black (AsOb isolate) and light grey (AsAb isolate) colonies were produced whereas, circular, smooth colonies with concentric zonation brownish black (AsNd isolate), Pinkish red (AsBd isolate) and creamish white (AsPb isolate) colonies were produced by the rest of the four isolates. The sporulation induced by all test isolates varied from poor (+) to excellent (++++). However, the isolates *viz*., AsLt, AsHl, AsJl and AsBd showed excellent (++++) sporulation whereas, it was good (+++) in AsPb and fair (++) in isolates *viz*., AsNd, AsOb and AsAb.

isolates conducted in screen house using susceptible tomato Cv. Pusa Ruby revealed that after a week of incubation period typical symptoms of early blight on the foliage similar to these observed on tomato foliage naturally affected with *A. solani* under field conditions were produced. Based on the observation and recorded data, four isolates (AsLt, AsBd, AsHl and AsJl) were found to be highly virulent due to short incubation period (4 to 5 days), maximum number of lesion size (6 to 8 / mm^2) and maximum average lesion size (12 to 15 / mm^2). Other isolates were rated as moderately virulent and a mildly virulent because these isolates were unable to cause symptoms of the disease in screenhouse experiments. Therefore, only four most virulent isolates (AsLt, AsBd, AsHl and AsJl) of *A. solani* were subjected for molecular variability study. The variation in pathogenicity of *A. solani* isolates was also established by Tong et al. (1994). The results are also in accordance with those of Castro et al. (2000) who demonstrated the variability of *A. solani* isolates on 14 tomato genotypes and Verma et al. (2007) who reported that the test isolates differed in the virulence pattern on ten tomato genotypes under screen house conditions.

Cultural variability

The results (Table 1) reveal that all the eight isolates of *A. solani* exhibited a great variability in respect of mycelial growth, colony color, colony diameter, colony zonation and sporulation. Maximum mycelial growth was recorded by AsLt (88.50 mm), isolate, followed by AsBd (82.35 mm), AsHl (78.40 mm), AsJl (78.20 mm), AsPb (76.25 mm), AsOb (75.60 mm), AsAb (70.42 mm) and AsNd (65.80 mm). Cultural characteristics, colony colour and sporulation were also varied for the test isolates. Irregular smooth colonies, with concentric zonation, brownish black (AsLt isolate) and dark grayish (AsHl isolate), without zonation olivaceous black (AsOb isolate) and light grey (AsAb isolate) colonies were produced whereas, circular, smooth colonies with concentric zonation brownish black (AsNd isolate), Pinkish red (AsBd isolate) and creamish white (AsPb isolate) colonies were produced by the rest of the four isolates. The sporulation induced by all test isolates varied from poor (+) to excellent (++++). However, the isolates viz., AsLt, AsHl, AsJl and AsBd showed excellent (++++) sporulation whereas, it was good (+++) in AsPb and fair (++) in isolates viz., AsNd, AsOb and AsAb. These results on cultural variability of *A. solani* isolates observed in the present study are similar to the findings of several earlier workers. Kaul and Saxcena (1988), based on cultural variability identified four isolates of *A. solani*. Similar cultural variability's among the isolated *A. solani* grown on PDA medium were reported earlier by several workers (Tong et al., 1994; Babu et al., 2000; Kumar et al., 2008; Naik et al., 2010).

Morphological variability

Studies on morphological characteristics of the eight test isolates of *A. solani* exhibited variations with respect to mycelial width, conidial size, beak length and septation (Table 2). Conidiophores were solitary or in small groups, straight or flexous, brown to olivaceous brown. The conidia were solitary, straight or slightly flexous, muriform and ellipsoidal tapering to a beak and pale or olivaceous brown. The mycelial width was found to be highest in AsJl (6.42 µm) isolate, followed by AsBd (6.20 µm), AsHl (4.50 µm) and AsLt (4.26 µm) isolates. Whereas, it was comparatively medium in the isolates *viz*., AsNd (3.73 µm), AsPb (3.64 µm) and AsAb (3.50 µm) while, least in AsOb (2.54 µm) isolate. The average conidial size (L x B)

Table 2. Morphological variability among the isolates of *A. solani,* causing early blight in tomato.

Isolate (district)	Mycelial width (µm)	Average size of conidia (µm)		Beak length (µm)	Number of septa	
		Length	Breath		Horizontal	Transverse
AsPb (Parbhani)	3.64	18.30	9.50	8.48	3-8	0-2
AsNd (Nanded)	3.73	22.20	9.91	10.34	4-7	1-2
AsHl (Hingoli)	4.50	28.35	12.90	12.21	4-8	1-3
AsLt (Latur)	4.26	37.57	13.21	12.81	5-9	1-3
AsOb (Osmanabad)	2.54	13.25	7.68	8.35	1-2	0-1
AsBd (Beed)	6.20	42.18	15.18	13.10	5-12	1-4
AsAb (Aurangabad)	3.50	18.28	8.82	9.10	3-5	0-2
AsJl (Jalna)	6.42	25.83	11.90	11.61	4-8	1-3

and their beak length were highest in the isolate AsBd (42.18 x 15.18 and 13.10 µm, respectively), followed by the isolated *viz*., AsLt (37.57 x 13.21 and 12.81 µm), AsHl (28.35 x 12.90 and 12.21 µm) and AsJl (25.83 x 11.90 and 11.61 µm) of conidial size and conidial beak length, respectively. Whereas, in rest of the three isolates AsAb, AsPb and AsNd, the conidial size and beak length were medium in the range of 18.28 x 8.82 to 22.20 x 9.91 µm and 9.10 to 10.34 µm, respectively, while, the isolate AsOb showed small sized conidia (13.25 x 7.68 µm) with short beak length (8.35 µm). The conidial septation (No. of horizontal and vertical septa) was also found to be varied among the test isolates. However, the septation was maximum in the isolate AsBd (5 to 12 and 1 to 4), followed by the isolates *viz*., AsLt (5 to 9 and 1 to 3), AsHl and AsHl (each 4 to 8 and 1 to 3), AsNd (4 to 7 and 1 to 2) and AsPb (3 to 8 and 0 to 2) and AsAb (3 to 5 and 0 to 2) with number of horizontal and vertical septa, respectively, while it was merge in the isolate AsOb.

These conidial features of *A. solani* test isolates observed in the study are in accordance with the *A. solani* spores characteristics originally described by Ellis and Ellis (1985) whereas, Ahmad (2002) studied the variations in conidial morphology of *A. solani,* causing tomato early blight disease and reported comparatively large sized (175 x 12.5 µm) conidia and their larger beak length (47 to 65 µm), which are contradictory to the conidial measurements found in present study. Verma et al. (2007), Kumar et al. (2008) and Naik et al. (2010) also reported similar variations in the conidial morphology of the isolates of *A. solani.* These variations may be due to different kind of *A. solani* isolates that prevailed there, season and tomato cultivars grown under the environmental conditions prevalent there.

Molecular variability

Based on amount of mycelial growth, pathogenic, cultural and morphological variability, only four most virulent *A. solani* isolates *viz*., AsLt, AsBd, AsJl and AsHl were subjected to molecular variability study, using RAPD-PCR analysis and 13 random primers of OPA series (OPA 1 to 13). The RAPD analysis (Figure 1) revealed genetic variability in all four test isolates of *A. solani*. Of the 13 primers tested, primers *viz*., OPA 4, OPA 7, OPA 9, OPA 11, OPA 12 and OPA 13 revealed amplifications. The primer OPA 4 and OPA 7 also generated multiple amplicons. Based on the efficiency of the individual primers to amplify polymorphic DNA fragment, six primers: OPA 4, OPA 7, OPA 9, OPA 11, OPA 12 and OPA 13 were selected for fingerprinting of all these species and provide representative profiles of isolates. The dendrogram constructed based on similarity coefficients grouped the isolates into two different groups at a similarity coefficient of 0.50 (Figure 2). Group I occupied by two isolates AsBd and AsLt and group II occupied by rest of two isolates AsHl and AsJl. The AsBd and AsLt were found more similar (85% similarity coefficient) to each other whereas, the isolates AsHl and AsJl were found closely related (50% similarity coefficient) but distinct from AsLt and AsBd. Thus, the present study based on RAPD-PCR analysis revealed that there may be chances of prevalence of the genetically variable populations amongst pathogen *A. solani,* of tomato crop grown in Marathwada region of Maharashtra State. However, isolates was found to be lower than the previous reports which may be attributed to less number of isolates used in this study.

Genetic variability in *A. solani,* causing early blight disease in tomato / potato crop grown in various regions was reported earlier by various workers (Weir et al., 1998; Morris et al., 2000; Wang and Zhang, 2003). These findings of the present study are in consonance with the earlier study of Verma et al. (2007), Kumar et al. (2008) and Naik et al. (2010). Naik et al. (2010) investigated genetic variations among four isolates of *A. solani* and indicated maximum similarity (73.78%) in isolates of Northern Karnataka region (ASR_5, ASD_4 and ASG_3) and isolate ASB2 from Southern Karnataka region shared only 45% genetic similarity indicating distinct polymorphism. Thus, from the foregoing results and discussion,

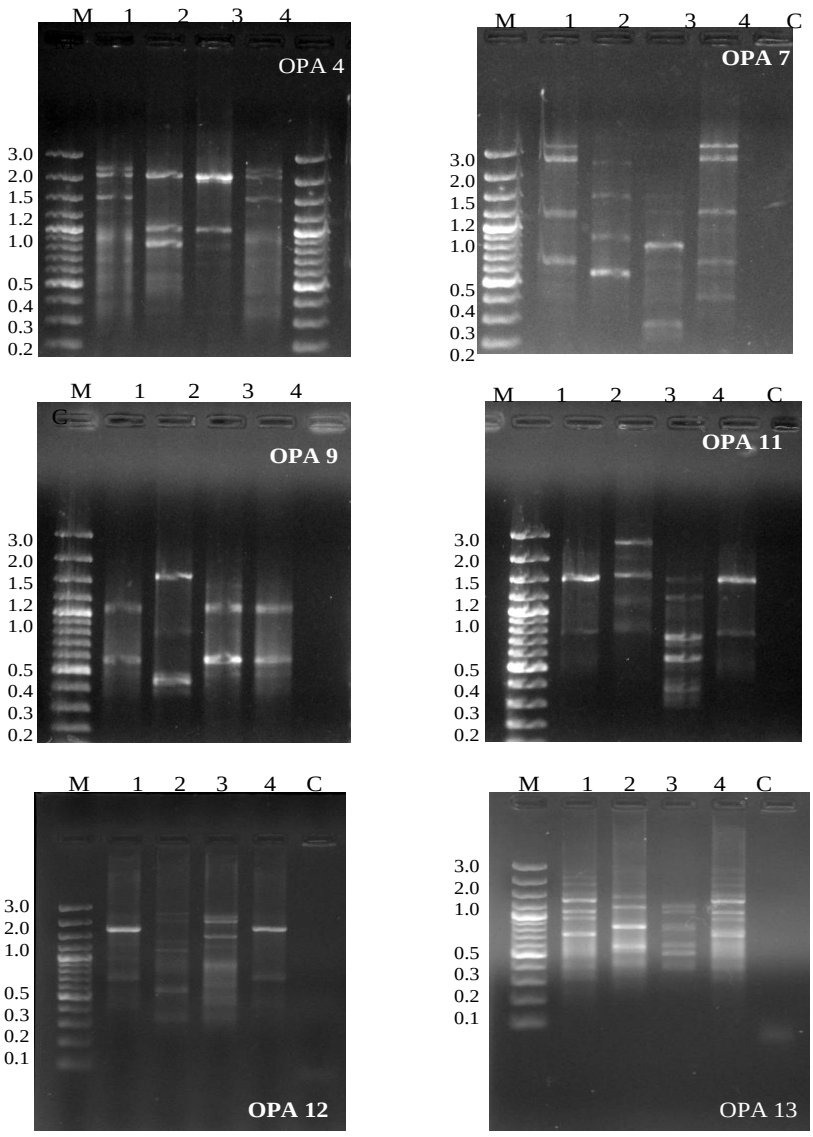

Figure 1. Molecular Variability of four virulent isolates of *A. solani*.

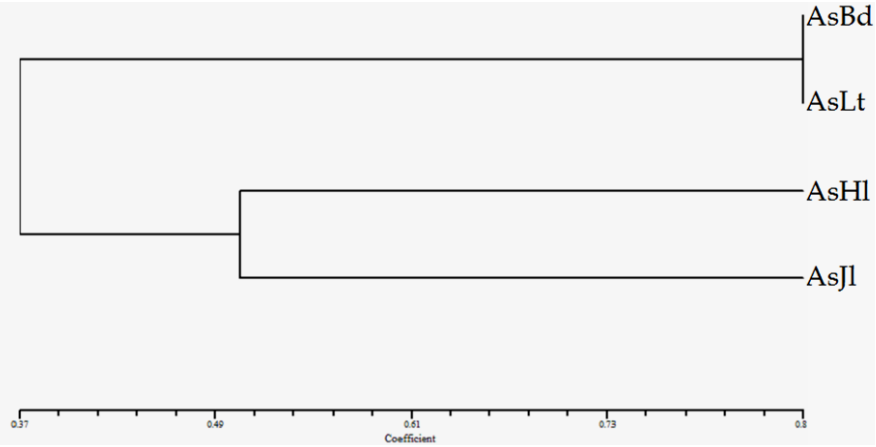

Figure 2. Dendrogram based on RAPD – PCR analysis depicting the relationship among the four fungal pathogens inciting early blight of tomato.

it could be concluded that there may be possibility of the existence of variability amongst *A. solani* isolates in the Marathwada region; however, its further confirmation is must. The future strategy could include collection of large number of samples from different parts of country from tomato, potato and other solanaceous hosts to get as many isolates as possible. This possibly aid in screening tomato and potato genotypes against broad spectrum of pathogen populations towards durable resistance.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We are thankful to Prof. R. L. Chavan, Department of Biotechnology, College of Agricultural Biotechnology, Latur for providing facilities and guidance for carrying the RAPD-PCR work.

REFERENCES

Ahmad S (2002). Conidial morphology of *Alternaria solani* and its variation in tomato. Ann. Agric. Res. 23(3):514-515.

Anonymous (2011). Cropwise area production under vegetables in India www.hortibiz.india.com.

Babu S, Seetaraman K, Nandkumar R, Johnson I (2000). Variability in cultural characteristics of tomato leaf blight pathogen. Plant Dis. Res. 15(1):121.

Castro MEA, Zambolim L, Chaves GM, Cruz CD, Matsuoka K (2000). Pathogenic variability of *Alternaria solani*, the causal agent of tomato early blight. Summa Phytopathol. 26:24-28.

Chakrabarty PK (2003). Techniques in Molecular Plant Pathology. Training manual. Central Inst. Cotton Res. Nagpur, India. p. 34.

Datar VV, Mayee CD (1984). Chemical management of early blight of tomato. J. Maha. Agric. Univ. 10(3):278-280.

Ellis MB, Ellis JP (1985). Microfungi on Land Plants. Croom Helm, Sydney, Australia, p. 425.

Kaul AK, Saxena HK (1988). Physiologic specialization in *A. solani* causing early blight of potato. Indian. J. Mycol. Plant Pathol. 18(2):128-132.

Kumar V, Haldar S, Pandey K, Singh R, Singh A, Singh PC (2008). Cultural, morphological, Pathogenic, and molecular variability amongst tomato isolates of *Alternaria solani* in India. World J. Microb. Biotechnol. 24(7):1003-1009.

Mathur K, Shekhawat KS (1986). Chemical control of early blight in *Kharif* Tomato. Indian J. Mycol. Plant Pathol. 16:235-238.

Morris PF, Connolly MS, St-Clair DA (2000) Genetic diversity of Alternaria alternate isolated from tomato in California assessed using RAPDs. Mycol. Res. 104:286-292.

Naik MK, Prasad Y, Bhat KV, Rani D (2010). Morphological, physiological, pathogenic and molecular variability among isolates of *Alternaria solani* from tomato. Indian Phytopathol. 63 (2): 168-173.

Pandey PK, Pandey KK (2002). Field screening of different tomato germplasm lines against *Septoria*, *Alternaria* and bacterial disease complex at seedling stage. J. Mycol. Plant Pathol. 32(2):234-235.

Tong YH, Liang JN, Xu JY (1994). Study on the biology and pathogenicity of *Alternaria solani* on tomato. J. Jiangsu Agric. Coll. 15:29-31.

Verma KP, Singh S, Gandhi SK (2007). Variability among *Alternaria solani* isolates causing early blight of tomato. Indian Phytopathol. 60(2):180-186.

Wang H, Zheng TY (2003). RAPD analysis on small spored *Alternaria* species. Mycosystema 22:35-41.

Weir TL, Huff DR, Christ BJ, Romaine CP (1998) RAPD-PCR analysis of genetic variations among isolates of *Alternaria solani,* and *Alternaria alternata* from potato and tomato. Mycologia 90:813-821.

academicJournals

Vol. 14(10), pp. 878-887, 11 March, 2015
DOI: 10.5897/AJB2014.14250
Article Number: 0761A2A51262
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Scanning electron microscopy-energy dispersive X-ray spectrometer (SEM-EDX) detection of arsenic and cadmium in himematsutake mushroom

Chun-Yen Liu[1], Chao-Jen Wang[1], Wen-Chuan Chung[2], Chien Chu[3], Wan-Yu Lin[1] and Wen-Hsin Chung[1]*

[1]Department of Plant Pathology, National Chung Hsing University, Taichung 40227, Taiwan.
[2]Taiwan Seed Improvement and Propagation Station, Hsinhse, Taichung 426, Taiwan.
[3]Taiwan Agricultural Chemicals and Toxic Substances Research Institute Council of Agriculture, Executive Yuan, Taichung 413, Taiwan.

Received 16 October, 2014; Accepted 23 February, 2015

The distribution of arsenic (As) and cadmium (Cd) in himematsutake was analyzed using scanning electron microscopy-energy dispersive X-ray spectrometer (SEM-EDX). The atomic percentage of the metals was confirmed by inductively coupled plasma-mass spectrometer (ICP-MS). Results show that the accumulation of As in pileus was higher than that in stipe by both SEM-EDX and ICP-MS analyses. The Cd level in pileus was higher than that in stipe by SEM-EDX analysis; whereas a higher level of Cd was found in stipe by ICP-MS analysis. Results of regression analysis showed that there was a positive correlation between the content of As and Cd in the piece samples by both SEM-EDX and ICP-MS methods. Results suggest that the SEM-EDX is one of the potential tools for rapid detection of metals, namely, As and Cd in himematsutake.

Key words: Arsenic (As), cadmium (Cd), scanning electron microscopy-energy dispersive X-ray spectrometer (SEM-EDX), coupled plasma-mass spectrometer (ICP-MS), himematsutake.

INTRODUCTION

Agaricus brasiliensis Wasser mushroom is a native species originated in the São Paulo State, Brazil (Wasser et al., 2002; Wasser, 2014). The name for *A. brasiliensis* mushroom include “Sun mushroom”, “God's mushroom” and “Almond Mushroom” in Brazil, “Himematsutake” and “Kawariharatake” in Japan, and Ji Song Rong in China (Largeteau et al., 2011; Wang, 2014; Wasser, 2014). Himematsutake is widely known as a medicinal mushroom in the world due to its abundant polysaccharide content, such as α-D-glucan, β-D-glucan, β-galactoglucan and xyloglucan (Largeteau et al., 2011; Firenzuoli et al., 2008; Mizuno and Nishitani, 2013).

*Corresponding author. E-mail: wenchung@dragon.nchu.edu.tw.

Abbreviations: SEM-EDX, Scanning electron microscopy-energy dispersive X-ray spectrometer; **As,** arsenic; **Cd,** cadmium; **ICP-MS,** inductively coupled plasma-mass spectrometer; **AAS,** atomic absorption spectrometry.

Clinical studies indicated that himematsutake has antitumor activity and hypocholesterolemic effect and can lower the diastolic and systolic blood pressure and improve the liver function (Firenzuoli et al., 2008; Wani et al., 2010; Wasser, 2011, Biedron et al., 2012). Because of these pharmacological activities, himematsutake has high market value with an annual production of 40 tons of dehydrated mushrooms in Brazil during 2006 to 2007 (Tomizawa et al., 2007). Previous studies indicated that certain mushrooms and macrofungi could easily accumulate heavy metals in its fruiting body (Falandysz et al., 2001; 2008; Soeroes et al., 2005; Tüzen, 2003; Tüzen et al., 1998). Kalač and Svoboda, reviewed the literatures on heavy metal accumulation in mushroms between 1970 and 2000 and indicated that *Agaricus campestris*, *Agaricus arvensis*, *Agaricus silvaticus*, *Agaricus silvicola* and other *Agaricus* spp., could accumulate cadmium (Cd), mercury (Hg), lead (Pb), copper (Cu) and silver (Ag) (Kalač, 2010; Kalač and Svoboda, 2000, Sun et al., 2012; Wang et al., 2014). Moreover, the metals accumulated in wild macrofungi were higher than those in farm grown macrofungi (Kalač and Svoboda, 2000). Thus, the ability to accumulate heavy metals in these macrofungi are associated with *Agaricus* species (Alonso et al., 2003; Svoboda et al., 2000), polluted areas (Soeroes et al., 2005; Tüzen et al., 1998), age of mycelium (Kalač and Svoboda, 2000), composition and pH of soil and of type of heavy metal (Baldrian, 2003; 2010).

Current analysis methods for detection of heavy metals in mushroom include atomic absorption spectrometry (AAS) (Tüzen, 2003; Kojo and Lodeniu, 1989; Behbahani et al., 2014), and inductively coupled plasma mass spectrometry (ICP-MS) (Falandysz et al., 2001; 2008; Chudzyński and Falandysz, 2008; Rabinovich et al., 2007). However, sample preparation and operation of AAS and ICP-MS analyses are time-consuming and costly. Recently, scanning electron microscopy modified with energy dispersive x-ray spectrometer (SEM-EDX) has been used to investigate heavy metals in mush-rooms, and found that Cd accumulates in *Pleurotus platypus* (Vimala and Das, 2011), the elements content in different strains of himematsutake (Jian et al., 2009), and the mechanism of cesium (Cs) accumulation in *Pleurotus ostreatus* (Sugiyama et al., 2000; 2008). Furthermore, Rumberger et al. (2005) used the SEM-EDX and ICP-MS to trace the metal elements in ectomycorrhiza from European beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.) forests in northern Brandenburg, and the results indicated that the amount of aluminum (Al), calcium (Ca), magnesium (Mg) and sulfur (S) analyzed by SEM-EDX were highly correlated with ICP-MS analysis. Thus, the SEM-EDX is one of the alternative equipment for the detection of the metal in bio-materials (Rumberger et al., 2005).

In Taiwan, himematsutake is popular for its medicinal activities (Wu, 2001). More than 35 tons are imported from different countries and the production area in Taiwan has recently been increased (www.ir.tari.gov.tw: 8080/bitstream/345210000/4825/2/ no155-all.pdf). However, the accumulation of Cd in himematsutake has also been reported in Taiwan and China, especially in the last few years (Hsu et al., 2009; Huang et al., 2008). For example, the Cd content exceeded the limit of 2 ppm based on the regulation set for the “Standard for the Tolerance of Heavy Metals in Edible Mushrooms” in Taiwan (Anonymous, 2007). Currently, a report showed that using sawdust bag-logs can reduce the heavy metals content (Wu, 2001). However, the safety is still doubtful due to the accumulation of heavy metals in edible parts of himematsutake, especially As and Cd (Chu, 2010; Chu et al., 2012). The high Cd content could damage the kidney and liver and cause diarrhea, nausea and vomit (Faroon et al., 2008; Das et al., 2010). The high As content could cause Blackfoot disease or cancer (Ng et al., 2003). In addition, the detection of heveay metals by AAS and ICP-MS is considerded a high-cost and time-consuming operation. The objectives of this study were to 1) analyze the distribution of As and Cd in pileus and stipe, 2) compare the detection efficacy of As and Cd with piece or powder specimens, and 3) evaluate whether SEM-EDX can be an althernative method for the detection of As and Cd in himematsutake.

MATERIALS AND METHODS

Collection of dried himematsutake fruiting body

The dried fruiting bodies of himematsutake were collected from three counties in Taiwan, namely Taichung, Changhua, Nantou, and China during 2010 to 2011. A total of six strains were evaluated in this study, including one USA strain collected from Taichung, two Japanese strains collected from Taichung and Changhua, one Taiwanese strain collected from Changhua, one unknown strain collected from Nantou and one strain collected from Guangdong in China (Table 1).

Analysis of arsenic (As) and cadmium (Cd) in piece and powder tissues of himematsutake by SEM-EDX

The SEM-EDX method was used to measure the percentage of As and Cd in pieces and powder of himematsutake. Each dried fruiting body was separated into pileus and stipe, and three fruiting bodies of each strain were selected for analysis. In the piece tissues analysis, three 15 × 3 × 2 mm^3 size of tissue samples were obtained from each pileus and stipe randomly, and then each tissue piece was cut into five sub-pieces (3 × 3 × 2 mm^3). The 45 tissue pieces were mounted on a cupper stub with carbon tape. On the other hand, the dried pileus and stipe tissues were milled, respectively by a sterile mortar, and each powder from the pileus or stipe was dusted moderately on the cupper stub with the carbon tape. The mounted pieces and powder samples were coated with platinum by a sputter coater (JEOL, JFC-1600, Japan) for 30 s at 20 mA and for 40 s at 10 mA, respectively. All samples were analyzed by SEM (JEOL, JFM-7401F, Japan) and EDX (Oxford, 7585, England) at 8 mm working distance with 15 kV and 500 X magnification. The atomic percentage of As and Cd was measured.

Table 1. Analysis of arsenic (As) and cadmium (Cd) in the piece of the pileus and the stipe of himematsutake strains collected from different areas by SEM-EDX method.

Himematsutake	Collected place	Tested part	Atomic percentage (%)[a]	
			As	Cd
USA strain	Taichung, Taiwan	Pileus	0.009±0.01*	0.013±0.02
		Stipe	0.008±0.01	0.014±0.02
Japanese strain	Taichung, Taiwan	Pileus	0.008±0.01*	0.009±0.02
		Stipe	0.006±0.01	0.010±0.02
Japanese strain	Changhua, Taiwan	Pileus	0.011±0.01	0.012±0.02
		Stipe	0.013±0.01*	0.015±0.02*
Taiwanese strain	Changhua, Taiwan	Pileus	0.013±0.02*	0.014±0.02
		Stipe	0.010±0.01	0.015±0.02
Unknown	Nantou, Taiwan	Pileus	0.015±0.02	0.015±0.02
		Stipe	0.013±0.02	0.025±0.03*
China	Guangdong	Pileus	0.015±0.02*	0.010±0.02
		Stipe	0.012±0.02	0.015±0.02*

[a]Three dried fruiting bodies were used for analysis. *Paired-Samples t test, P≤0.05

Analysis of As and Cd in himematsutake by ICP-MS

ICP-MS method was used to measure the concentrations of As and Cd in the pileus and the stipe of himematsutake. The milled sample of 0.25 g from pileus or stipe was put into polytetrafluoroethylene (PTFE) vessels (MarsX, CEM, Company, USA) followed by adding 6 ml of nitric acid (65%) as wetting material. The PTFE vessels were closed and micro-digested at 180°C for 15 min after pre-digested at 25°C to 28 for 30 min. Then, the mixture was cooled at 25 to 28°C for 20 min, and diluted with 25 ml of deionized water. The concetration of As and Cd was analyzed in the mixture by using ICP-MS (Agilent 7500 Series, Taiwan). The ICP-MS operation was accomplished by the following conditions (Radio frequency power: 1,500 W, RF matching: 1.75 V, Sample depth: 9 mm, Sample skimmer cones: Ni, Peristaltic pump: 0.10 rps, Argon flow rate Plasma: 15 L/min; Auxiliary: 0.32 L/min, Nebulizer: 0.87 L/min, Spray chamber temperature: 2°C, Integration time: 0.1 s, Mass monitored: As 75 m/z and Cd 111 m/z). There were three repeats for each treatment.

Statistical method

All sample analyses were run in quadruplicate. Statistical analysis was done using the SAS package (version 10.0) developed by SAS Institute Inc (SPSS Inc., Chicago, USA). Correlation analysis between atomic percentage from SEM-EDX and concetration from ICP-MS was conducted.

RESULTS

Analysis of As and Cd in pieces of the pelius and the stipe of himematsutake by SEM-EDX

Results of SEM-EDX analyses showed that the percentage of As in the pileus was significantly higher than the stipe pieces among the most strains tested (p=0.05). The percentage of As in the pileus was higher than that in the stipe (Table 1). The percentage of As in the pileus was high (0.015%) in the China strain and low (0.008%) in the Japanese strain from Taichung. Similarly, in the stipe, the percentage of As was high (0.012%) in the China strain and low (0.006%) in the Japanese strain from Taichung. On the other hand, the percentage of Cd in the stipe was significantly higher than the pileus among the Japanese strains from Changhua, the Taiwanese strain from Nantou, and the China strain (p=0.05). The percentage of Cd in the stipe was higher than that in the pileus (Table 1). The percentage of Cd in the stipe was 0.015% for the Japanese strain from Changhua, 0.015% for the China strain, and 0.025% for the Taiwanese strain from Nantou, respectively. However, the percentage of Cd in the pileus was 0.010% for the China strain, 0.012% for the Japanese strain from Changhua, and 0.015% for the Taiwanese strain from Nantou, respectively.

Analysis of As and Cd in the powder of pelius and stipe of himematsutake by SEM-EDX

The percentge of As and Cd in the powder samples, made from the dried fruiting bodies of himematsutake were examined by SEM-EDX. The results showed that the percentage of As in the pileus and stipe were significantly different in Japanese strain from Taichung and the China strain (Table 2). The percentage of As in the pileus was 0.016% for the Japanese strain from

Table 2. Analysis of arsenic (As) and cadmium (Cd) in the powder of the pileus and the stipe of himematsutake strains collected from different areas by SEM-EDX method.

Himematsutake	Collected place	Tested part	Atomic percentage (%)[a]	
			As	Cd
USA strain	Taichung, Taiwan	Pileus	0.016±0.02	0.022±0.03
		Stipe	0.013±0.02	0.026±0.03
Japanese strain	Taichung, Taiwan	Pileus	0.016±0.02*	0.023±0.02
		Stipe	0.009±0.01	0.020±0.02
Japanese strain	Changhua, Taiwan	Pileus	0.019±0.02	0.035±0.04
		Stipe	0.020±0.03	0.050±0.07*
Taiwanese strain	Changhua, Taiwan	Pileus	0.019±0.03	0.031±0.03
		Stipe	0.018±0.02	0.037±0.04*
Unknown	Nantou, Taiwan	Pileus	0.013±0.01	0.019±0.03
		Stipe	0.012±0.01	0.017±0.02
China	Guangdong	Pileus	0.021±0.02*	0.023±0.03
		Stipe	0.014±0.02	0.023±0.03

[a]Three dried fruiting bodies were used for analysis *Paired-Samples t test, P≤0.05

Table 3. Analysis of arsenic (As) and cadmium (Cd) in the pileus and the stipe of himematsutake strains collected from different areas by ICP-MS method.

Himematsutake	Collected place	Tested part	Atomic concentration (mg/kg)[a]	
			As	Cd
USA strain	Taichung, Taiwan	Pileus	1.440±0.86*	6.158±2.84*
		Stipe	0.555±0.36	1.480±0.72
Japanese strain	Taichung, Taiwan	Pileus	0.450±0.17	0.657±0.35
		Stipe	0.170±0.09	0.193±0.10
Japanese strain	Changhua, Taiwan	Pileus	2.270±0.74*	2.393±1.54*
		Stipe	0.938±0.41	0.818±0.42
Taiwanese strain	Changhua, Taiwan	Pileus	1.418±0.26*	19.530±4.33*
		Stipe	0.735±0.18	9.550±2.94
Unknown	Nantou, Taiwan	Pileus	8.251±3.27	40.838±21.84*
		Stipe	6.020±2.56	24.823±10.89
China	Guangdong	Pileus	1.570±0.84	0.663±0.33
		Stipe	0.850±0.47	0.348±0.36

[a]Three dried fruiting bodies were used for analysis. *Paired-samples t test, P≤0.05.

Taichung and 0.021% for the China strain, respectively. The percentage of Cd in the pileus and the stipe was significantly different in the Japanese strain and the Taiwanese strain from Changhua (Table 2). The Japanese strain showed higher Cd percentage in the stipe (0.05%) than that in the pileus (0.035%). In the Taiwanese strain, the Cd percentage in the stipe was 0.037%; whereas in the pileus, it was 0.031%.

Analysis of As and Cd in the pileus and the stipe of himematsutake by ICP-MS

The results indicated that As concentration in the pileus and the stipe were significantly different between the USA strain and the Taiwanese strain from Changhua. The concentration of As in the pileus was higher than that in the stipe (Table 3). The concentration of As in the pileus

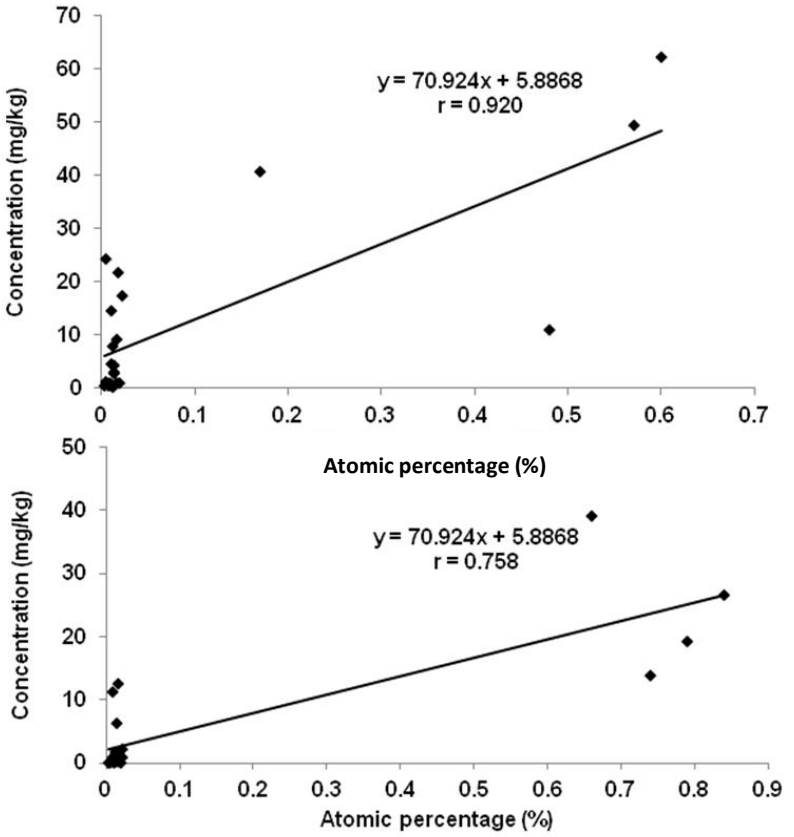

Figure 1. Correlation between the results of As in the pieces of the fruiting body obtained from the pileus (upper) and the stipe (down) examined by SEM-EDX and ICP-MS.

was 1.44 mg/kg for the USA strain and 0.450 mg/kg for the Japanese strain from Taichung. However, the As concentration in the stipe was 0.555 mg/kg for the USA strain and 0.170 mg/kg for the Japanese strain from Taichung. Similarly, the As concentration in the pileus (2.270 mg/kg) was higher than that in the stipe (0.938 mg/kg) for the Japanese strain from Changhua. In the Taiwan strain from Changhua, the As concentration in the pileus (1.418 mg/kg) was higher than that in the stipe (0.735 mg/kg). On the other hand, the Cd concentration in the pileus and the stipe were significantly different among the USA strain, the Taiwanese strains from Changhua and from Nantou. The Cd concentration in the pileus was higher than that in the stipe (Table 3). The Cd concentration in the pileus was 6.158 mg/kg for the USA strain and 2.393 mg/kg for the Japanese strain from Changhua. However, in the stipe, the Cd concentration was 1.480 mg/kg for the USA strain and 0.818 mg/kg for the Japanese strain from Changhua. The Taiwan strain from Changhua had higher Cd concentration in the pileus (19.530 mg/kg) than in the stipe (9.550 mg/kg). In the Taiwanese strain from Nantou, the Cd concentration in the pileus (40.838 mg/kg) was higher than that in the stipe (24.823 mg/kg).

The correlation between the results obtained by SEM-EDX and ICP-MS

In order to find the relationship between the results generated by SEM-EDX and ICP-MS, a correlation was prepared with STATISTICA software. For the piece samples, the results indicated that the atomic percentage of As and Cd in the pileus or the stipe samples by SEM-EDX analysis was positively related with the ICP-MS analysis. The correlation coefficient in terms of the atomic percentage of As and the concentration of As in the pileus and the stipe with pieces sampling were 0.920 and 0.758, respectively (Figure 1), and the correlation coefficient in terms of the atomic percentage of Cd and the concentration of Cd in the pileus and the stipe with pieces sampling were 0.771 and 0.835, respectively (Figure 2). However, when the powdery samples were examined, there was a low relationship between results generated from SEM-EDX and ICP-MS analyses. The correlation coefficient of the atomic percentage of As and the concentration of As in the pileus and the stipe were 0.277 and 0.205, respectively (Figure 3); meanwhile, the correlation coefficient of the atomic percentage of Cd and the concentration of Cd in the pileus and the stipe were

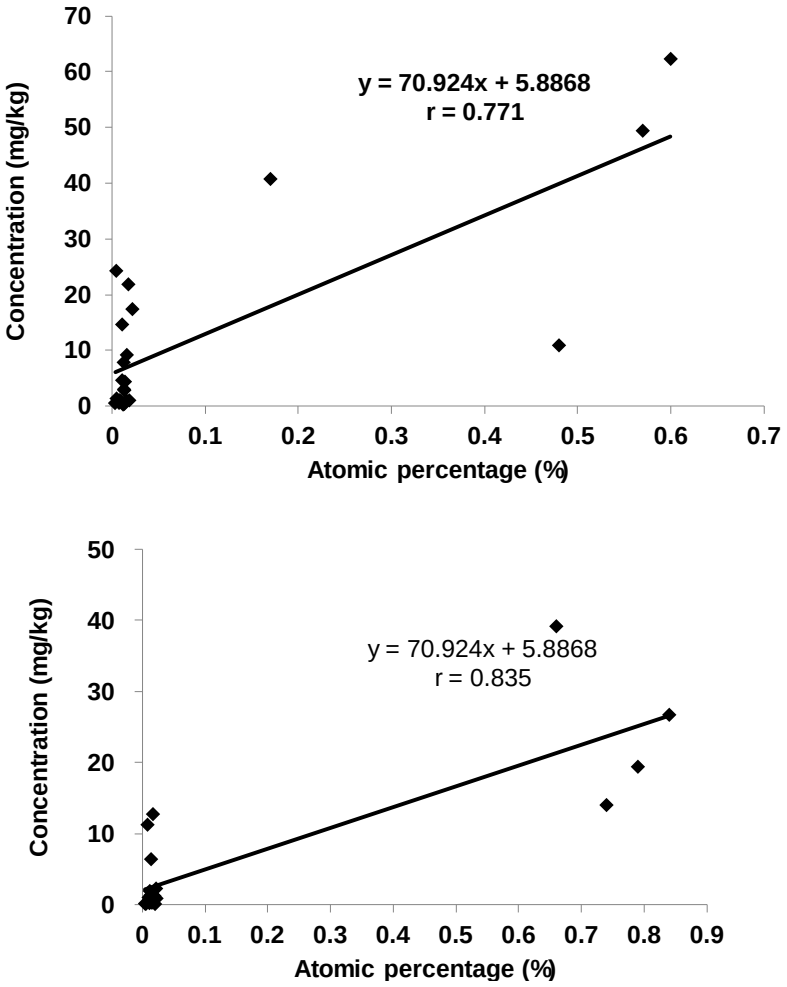

Figure 2. Correlation between the results of Cd in the pieces of the fruiting body obtained from the pileus (upper) and the stipe (down) examined by SEM-EDX and ICP-MS.

0.147 and -0.011, respectively (Figure 4).

DISCUSSION

The results indicate that the accumulation of As in the pileus was higher by SEM-EDX analysis while similar results were obtained by using ICP-MS analysis. Results of previous studies revealed that the process of metals accumulation started from the mycelial absorption of the substrates and then transitted to the stipe, the pileus or the gill (Kalač, 2010; Kalač and Svoboda, 2000; Thomet et al., 1999). Consequently, the distribution of the metals in different parts of the furiting body might show variations, particularly in the stipe and the pelius, the major accumulation sites (Chudzyński and Falandysz, 2008; Falandysz et al., 2003; Thomet et al., 1999). Our results demonstrated that the As more offent accumulated in the pelius of the himematsutake than the stipe did, which is in agreement with previous studies (Huang et al., 2007; Wang et al., 2009). Thus, the As can pass through the stipe easily and accumulate in the pileus after absorption from the substrate. In additon, the As content analyzed by ICP-MS showed similar result as did by SEM-EDX analysis. Brzostowski et al. (2011) indicated that the cultural substrate or environment could affect the accumulation of certain metals in the pileus or the stipe of *Paxillus involutus*, such as Ag (silver), Cd, Cr (chromium), Pb (lead) or Hg (mercury). In this study, the As content varied among different strains. We consider that culture substrates or production area could affect the As accumulation. However, the components and sources of the cultivate substrates used to culture the himematustake are commercial confidentiality among the growers, so the farmers would not provide them to us and the As content of the substrates could not be exmained.

In the Cd analysis, the results of SEM-EDX analysis were different from those of ICP-MS analysis. The results

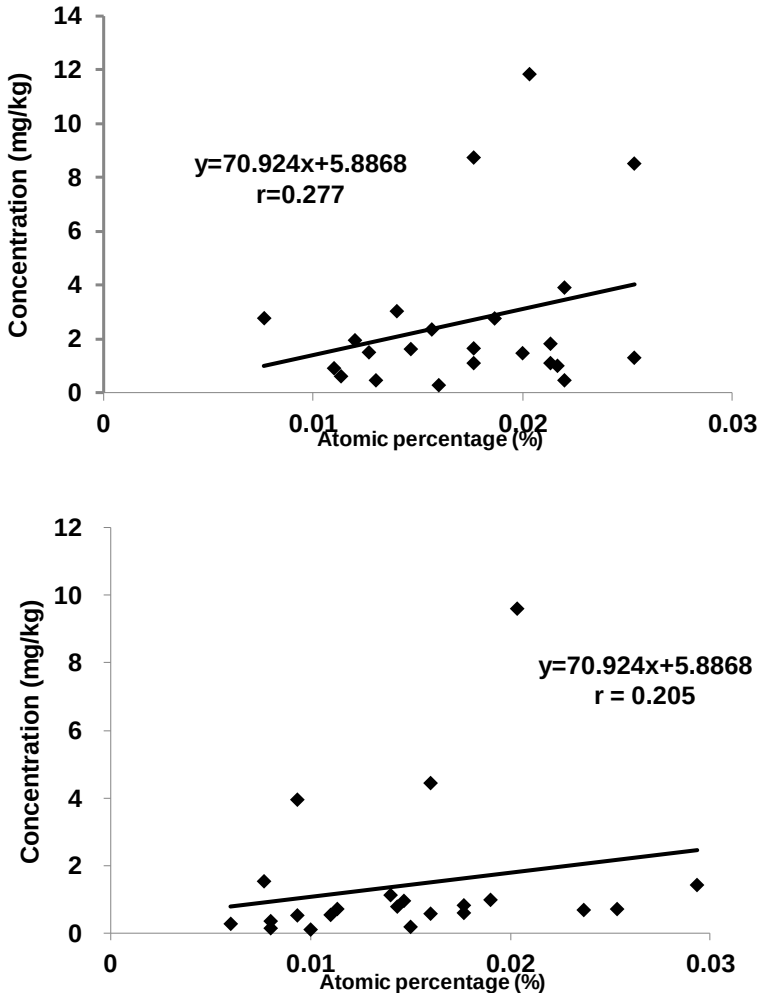

Figure 3. Correlation between the results of As in the powder of the fruiting bodies obtained from the pileus (upper) and the stipe (down) examined by SEM-EDX and ICP-MS.

of SEM-EDX analyses showed that the percentage of Cd in the stipe was higher than that in the pileus, while the Cd content in the pileus was higher than that in the stipe based on ICP-MS analysis. Results of previous studies indicated that the accumulation of Cd in the pileus of himematsutake was higher than that in the stipe (Huang et al., 2007; Wang et al., 2009). Moreover, Thomet et al. (1999) examined the Cd accumulation in the pileus, the stipe, and the gills of *A. macrosporus* and reported that the accumulation of Cd was the least in the stipe. In this study, the results of ICP-MS analyses showed that Cd could accumulate in the stipe of mushroom as reported in previous studies (Huang et al., 2007; Wang et al., 2009; Brzostowski et a., 2011; Thomet et al., 1999). Thus, the site of Cd accumulation in himematsutake is similar to As accumulation. We consider that Cd might not be distributed evenly over the pileus or the stipe and the random piece or powder samples could not represent the Cd content completely in himematsutake. Moreover, SEM-EDX is always used for qualitative analysis of bio- or nonbio-materials and not for quantitative analysis due to sampling limitation (Jian et al., 2009; Rumberger et al., 2005; Sugiyama et al., 2008; Vimala and Das, 2011).

The results of the analysis of the piece and the powder samples demonstrated that the atomic percentage in the piece samples using SEM-EDX was positively related to that with ICP-MS analysis. Results of previous studies indicated that the thickness of the specimen might interfere with the penetration depth of the electron beam and the surface electrochemistry of the specimens, and the standard sample size for quantitative analysis by EDX should be less than 0.1 μm (Goldstein et al., 2003). Although, the powder specimen was smaller than the piece specimen, the size of each powdery specimen used in this study was greater than 50 μm. Thus, the size of the powdery specimen is still too big for quantification analysis by SEM-EDX completely. Moreover, it is difficult to analyze each powdery specimen by SEM-EDX, and

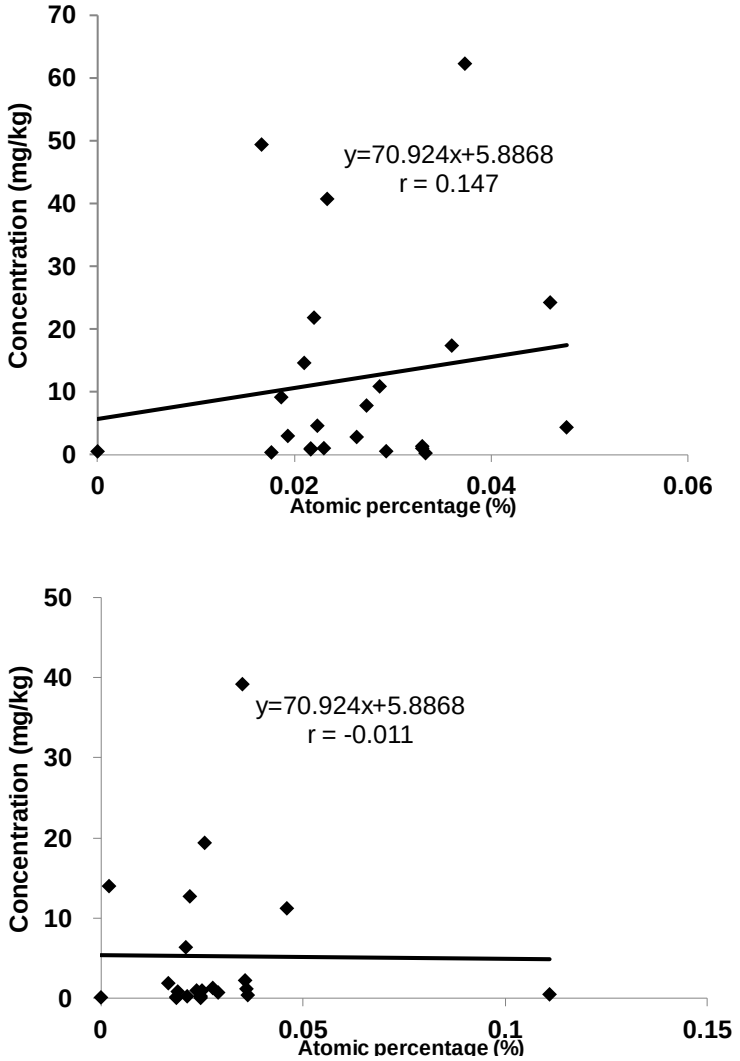

Figure 4. Correlation between the results of Cd in the powder of the fruiting bodies obtained from the pileus (upper) and the stipe (down) examined by SEM-EDX and ICP-MS.

the powdery specimens were selected randomly in this study. Previous study indicated that the element distribution in biological materials is not uniform (McCully et al., 2010), and the random selection of powdery specimens might cause the low correlation with ICP-MS analysis. In the other hand, each piece specimen could be observed in this study and the metals of As and Cd could be detected with high reproducibility. Thus, the piece samples will provide higher detection efficacy than the powdery samples for SEM-EDX examination. In this study, the results demonstrate that the accumulation of both As and Cd could be detected by SEM-EDS with the piece samples and the results represent the possible content in himematsutake. Thus, SEM-EDX can be a useful tool for the primary detection of metal in himematsutake rapidly. For increasing the accuracy and sensitivity of SEM-EDX, Tylko et al. (2004) suggested using the standard specimen with similar matrix to general specimen for the calibration of SEM-EDX. However, the preparation of the standard specimen, the stability and the accuracy of SEM-EDX technique warrant further study.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We sincerely thank Dr. C. J. Chang, Plant Pathologist, Department of Plant Pathology, University of Georgia, Griffin, GA, USA, Griffin, GA, USA, and Dr. C. P. Huang,

Donald C. Phillips Professor, Department of Civil and Environmental Engineering, University of Delaware, Newark, DE, USA, for their critical reading of the manuscript. Funding to support this research was provided by the Ministry of Education, Taiwan, ROC, under the ATU plan. This information is available free of charge via the Internet at http: //pubs.acs.org.

REFERENCES

Alonso J, García MA, Pérez-López M, Melgar MJ (2003). The concentrations and bioconcentration factors of copper and zinc in edible mushrooms. Arch. Environ. Con. Tox. 44:180-188.

Anonymous (2007). Standard for the Tolerance of Heavy Metals in Edible Mushrooms. Department of Health, Executive Yuan, Taipei, Taiwan ROC.

Baldrian P (2003). Interactions of heavy metals with white-rot fungi. Enzyme Microb. Technol. 32:78-91.

Baldrian P (2010). Effect of heavy metals on saprotrophic soil fungi. Soil Heavy Metals 19:263-279.

Behbahani M, Tapeh NA, Mahyari M, Pourali AR, Amin BG, Shaabani A (2014). Monitoring of trace amounts of heavy metals in different food and water samples by flame atomic absorption spectrophotometer after preconcentration by amine-functionalized graphene nanosheet. Environ. Monit. Assess. 186:7245-7257.

Biedron R, Tangen JM, Maresz K, Hetland G (2012). *Agaricus blazei* Murill – immunomodulatory properties and health benefits. Funct. Foods Health Dis. 2:428-447.

Brzostowski A, Falandysz J, Jarzyńska G, Zhang D (2011). Bioconcentration potential of metallic elements by Poison Pax (*Paxillus involutus*) mushroom. J. Environ. Sci. Health 46:378-393.

Chu CY (2010). Selection of beneficial microorganisms and their effects on fruiting body development of *Agaricus blazei* Murrill. Taichung: Doctoral Dissertation of National Chung Hsing University.

Chu JN, Young CC, Tan CC, Wu SP, Young LS (2012). Improvement of productivity and polysaccharide-protein complex in *Agaricus blazei*. Pesqui. Agropecu. Bras. 47:96-102.

Chudzyński K, Falandysz J (2008). Multivariate analysis of elements content of Larch Bolete (*Suillus grevillei*) mushroom. Chemosphere 73:1230-1239.

Das KK, Das SN, Dhundasi SA (2010). Nickel, its adverse health effects and oxidative stress. Indian J. Med. Res. 128:412-425.

Falandysz J, Brzostowski A, Kawano M, Kannan K, Puzyn T, Lipka K (2003). Concentrations of mercury in wild growing higher fungi and underlying substrate near Lake Wdzydze, Poland. Water Air Soil Pollut. 148:127-137.

Falandysz J, Kunito T, Kubota R, Gucia M, Mazur A, Falandysz JJ, Tanabe S (2008). Some mineral constituents of Parasol Mushroom (*Macrolepiota procera*). J. Environ. Sci. Health B. 43:187-192.

Falandysz J, Szymczyk K, Ichihashi H, Bielawski L, Gucia M, Frankowska A, Yamasaki S (2001). ICP/MS and ICP/AES elemental analysis (38 elements) of edible wild mushrooms growing in Poland. Food Addit. Contam. 18:503-513.

Faroon O, Ashizawa A, Wright S, Tucker P, Jenkins K, Ingerman L, Rudisill C (2008). Toxicological Profile for Cadimium, ed. Agency for Toxic Substrances and Disease Registry, Ed. Atlanta: Centers for Disease Control press.

Firenzuoli F, Gori L, Lombardo G (2008). The medicinal mushroom *Agaricus blazei* Murrill: review of literature and pharmaco-toxicological problems. Evid. Based Complement. Alternat. Med. 5:3-15.

Goldstein J, Newbury D, Joy D, Lyman C, Echlin P, Lifshin E, Sawyer L, Michael J (2003). Scanning electron microscopy and X-ray microanalysis. New York: Kluwer Academic Publishers.

Hsu CL, Chen SS, Chu CM, Lin YW, Kao YM (2009). Investigation of heavy metals (lead and cadmium) in *Agaricus blazei* Murill. Annual Report Bureau Food and Drug Administration. Taiwan R.O.C. 27:216-223.

Huang J, Ying Z, Yu Y, Li K (2007). Accumulation rule of heavy metal and the controlling technique by *Agaricus blazei* Murrill. Chin. Agric. Sci. Bull. 23:406-409.

Huang JC, Li KB, Yu YR, Wu H, Liu DL (2008). Cadmium accumulation in *Agaricus blazei* Murrill. J. Sci. Food Agric. 88:1369-1375.

Jian ZH, Weng BQ, Lei JG, Xiao SX, Wang YX, Tang XQ (2009). Cell biology identification and analysis on trace elements contents in different parts of pileus of *Agaricus blazei* Murril. J. Chin. Electron Microsc. Soc. 28:462-466.

Kalač P (2010). Trace element contents in European species of wild growing edible mushrooms: a review for the period 2000–2009. Food Chem. 122:2-15.

Kalač P, Svoboda L (2000). A review of trace element concentrations in edible mushrooms. Food Chem. 69:273-281.

Kojo MR, Lodeniu M (1989). Cadmium and mercury in macrofungi–mechanisms of transport and accumulation. Angew. Bot. 63:279-292.

Largeteau ML, Llarena-Hernández RC, Regnault-Roger C, Savoie JM (2011). The medicinal *Agaricus* mushroom cultivated in Brazil: biology, cultivation and non-medicinal valorisation. Appl. Microbiol. Biotechnol. 92:897-907.

McCully ME, Canny MJ, Huang CX, Miller C, Brink F (2010). Cryo-scanning electron microscopy (CSEM) in the advancement of functional plant biology: energy dispersive X-ray microanalysis (CEDX) applications. Funct. Plant Biol. 37:1011-1040.

Mizuno M, Nishitani Y (2013). Immunomodulating compounds in Basidiomycetes. J. Clin. Biochem. Nutr. 52: 202-207

Ng JC, Wang J, Shraim A (2003). A global health problem caused by arsenic from natural sources. Chemosphere 52:1353-1359.

Rabinovich M, Figlas D, Delmastro S, Curvetto N (2007). Copper- and zinc-enriched mycelium of *Agaricus blazei* Murrill: bioaccumulation and bioavailability. J. Med. Food 10:175-183.

Rumberger MD, Lentzsch P, Munzenberger B, Huttl RF (2005). Nutrient amounts of ectomycorrhizae analysed by EDX using ESEM and ICP. Mycorrhiza 15:307-312.

Soeroes CS, Kienzl N, Ipolyi I, Dernovics M, Fodor P, Kuehnelt D (2005). Arsenic uptake and arsenic compounds in cultivated *Agaricus bisporus*. Food Control 16:459-464.

Sugiyama H, Takahashi MN, Terada H, Kuwahara C, Maeda C, Anzai Y, Kato F (2008). Accumulation and localization of cesium in edible mushroom (*Pleurotus ostreatus*) mycelia. J. Agric. Food Chem. 56:9641-9646.

Sugiyama H, Terada H, Shibata H, Morita Y, Kato F (2000). Radiocesium concentrations in wild mushrooms and characteristics of cesium accumulation by the edible mushroom (*Pleurotus ostreatus*). J. Health Sci. 46:370-375.

Sun L, Liu G, Yang M, Zhuang Y (2012). Bioaccessibility of cadmium in fresh and cooked *Agaricus blazei* Murill assessed by in vitro biomimetic digestion system. Food Chem. Toxicol. 50:1729-1733.

Svoboda L, Zimmermannová K, Kalač P (2000). Concentrations of mercury, cadmium, lead and copper in fruiting bodies of edible mushrooms in an emission area of a copper smelter and a mercury smelter. Sci. Total Environ. 246:61-67.

Thomet U, Vogel E, Krähenbühl U (1999). The uptake of cadmium and zinc by mycelia and their accumulation in mycelia and fruiting bodies of edible mushrooms. Eur. Food Res. Technol. 209:317-324.

Tomizawa MM, Dias ES, Assis LJ, Gomide PHO, Santos JB (2007). Genetic variability of mushroom isolates *Agaricus blazei* using markers RAPD. Ciênc. Agrotec. 31:1242-1249.

Tüzen M (2003). Determination of heavy metals in soil, mushroom and plant samples by atomic absorption spectrometry. Microchem. J. 74:289-297.

Tüzen M, Özdemir M, Demirbaş A (1998). Heavy metal bioaccumulation by cultivated *Agaricus bisporus* from artificially enriched substrates. *Z. Lebensm.* Unters.–F. A 206:417-419.

Tylko G, Banach Z, Kilarski W (2004). PROZA and calibration curves for quantitative X-ray microanalysis of biological samples. Microchim. Acta 144:271-276

Vimala R, Das N (2011). Mechanism of Cd (II) adsorption by macrofungus *Pleurotus platypus*. J. Environ. Sci. 23:288-293.

Wang LL, Li HB, Wei HL, Wu XQ, Ke LQ (2014). Identification of cadmium-induced Agaricus blazei genes through suppression subtractive hybridization. Food Chem. Toxicol. 63:84-90.

Wang X, Li T, Li B (2009). A preliminary study on accumulation

characteristics of copper, zinc, silver, cadmium and mercury in *Agaricus blazei* Murill. Environ. Chem. 28:94-98.
Wani BA, Bodha RH, Wani AH (2010). Nutritional and medicinal importance of mushrooms. J. Med. Plants Res. 4:2598-2604.
Wasser SP (2011). Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. Appl. Microbiol. Biotechnol. 89:1323-1332.
Wasser SP (2014). Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. Biomed. J. 37:345-356.
Wasser SP, Didukh MY, de A. Amazonas MAL, Nevo E, Stamets P, da Eria AF (2002). Is a Widely Cultivated Culinary-Medicinal Royal Sun Agaricus (the Himematsutake Mushroom) Indeed *Agaricus blazei* Murrill? Int. J. Med. Mushrooms 4:267-290.
Wu KT (2001). Himematsutake (*Agaricus blazei*) cultivation on sawdust. J. Tech. Service 45:18-20.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals