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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.

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*Full Length Research Paper*

# Use of molecular biology techniques in the detection of fraud meat in the Egyptian market

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**Food safety and quality are major concerns and any case of food adulteration has a great impact on public opinion. Identification of animal species used in commercial meat products is important with respect to economic and sanitary issues. The aim of this research was to detect ruminant and equine species in minced meat and Egyptian sausage using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Species differentiation was performed by digestion of PCR products with Tsp509I and AluI restriction enzymes. Our results indicate that 4 (4%), 3 (3%) and 5 (5%) of examined samples (100) were contaminated with sheep, goat and donkey meat, respectively. These results indicate that 12% of examined samples were adulterated, although they were labeled as beef meat 100%. It can be concluded that molecular methods such as PCR and PCR-RFLP are potentially powerful and reliable techniques for detection of adulteration with different meat species in meat products.**

**Key words:** Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), *Cytochrome-b* gene, identification, adulteration, meat products.

## INTRODUCTION

Meat species specification is an area which needs specialized attention in the food quality management system. It is a vital field to ensure food safety (Singh and Neelam, 2011). In developing countries, there is an increasing demand for meat products (Delgado, 2003). There are different aspects that interfere in the selection of meat products including price, quality and nutritional attributes.

Nowadays consumers are increasingly aware of their health and are looking for more comprehensive information on the safety of the foods they consume (Verbeke and Ward, 2006). In spite of the food labeling

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regulations, the adulteration or misrepresentation of food products for more financial gain is a common practice all over the world (Shears, 2010; Doosti et al., 2014). Thus authentic testing of meat products avoids unfair market competition and protects consumers from fraudulent practices of meat adulteration.

Although historically, meat has not been widely associated with adulteration because of the recognizable joints (Nakyinsige et al., 2012), but in processed meat products, meat species substitution occurs more regularly (Ayaz et al., 2006). The substitution with cheaper species is very difficult to detect in such products by visual observation after grinding and/or heat processing (Abd El-Nasser et al., 2010). Also accidental cross contamination of meat products may occur during processing, due to improper handling and using shared equipments (Ilhak and Arslan, 2007).

Developments in molecular biology have facilitated identification of plant, bacteria, and animal species with high accuracy. Recently, the protein-based and DNA-based techniques were widely used to identify prohibited species in food products.

Protein-based technique includes the immunological (Lopez-Calleja et al., 2007), electrophoretical (Mayer, 2005), chromatography such as high performance liquid chromatography (HPLC) and DNA-based methods, including DNA hybridization (Hunt, 1997), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Mane et al., 2014), species-specific PCR (Haunshi et al., 2009), multiplex PCR (Ghovvati et al., 2009) and real-time PCR (Walker et al., 2013). The advantage of PCR based tests are higher accuracy, time-saving, high sensitivity and flexibility, compared to other methods.

PCR-RFLP is a two-step reaction to identify multiple species after restriction enzyme digestion of PCR amplified DNA sequence (Haider et al., 2012). Therefore, in this study, PCR and PCR-RFLP techniques were done for the detection of adulteration and identification of sheep, goat, donkey and horse's meat using species-specific oligonucleotide primers.

## MATERIALS AND METHODS

### Sample collection and preparation

One hundred (100) samples of beef meat (50 of minced meat and 50 of sausages) from popular retail markets in Cairo and Giza governorates were analyzed for detection of meat adulteration. The samples were stored at -20°C until used for DNA extraction in order to prevent the enzymatic degradation of DNA. The samples were prepared based on the method of Santaclara et al. (2007). Sausage samples were extracted by suspending in methanol-chloroform-water (2:1:0.8) solution for 2 h to prevent the oil disturbance in DNA extraction process.

Afterward, the supernatant was discarded and the samples were washed once using ultrapure (1 ml) water to eliminate the remnants of the used solution.

### DNA extraction

The extraction of mitochondrial DNA from all samples was performed using tissue mini kit (QIAGEN, Hilden, Germany). The procedure followed the manufacturer's instruction. Concentration and purity of DNA were also assessed by NanoDrop™ ND-2000 spectrophotometry (Thermo, Wilmington, USA).

### Species-specific primers and PCR amplification

The mitochondrial DNA segment (cytochrome-b gene) in cattle, sheep, goat, donkey and horse were amplified with the use of primer sequences as described in Table 1. Universal primers CB7u used in this study were targeted 195 bp fragment of variable region of mitochondrial cytochrome-b, while primers of the Donkey's and Horse's cytochrome-b were targeted 359 bp fragment of variable region of mitochondrial cytochrome-b (Doosti et al., 2014).

### PCR procedure

Amplification of species fragments was carried out in a total volume of 25 µL containing 12.5 µL of taq master mix (2x) (Invitrogen, California, USA), 0.1 mg/ml bovine serum albumin (BSA) (Roche, Mannhein, Germany), 10 pmol of each primer and 50 ng of template DNA. The volume was completed using DNase and RNase /free distilled water.

Amplification was performed in an Piometra personal thermal cycler with the following cycling conditions; after an initial heat denaturation step at 94°C for 2 min, 35 cycles were programmed as follows: 94°C for 1 min, 60°C (Universal primers CB7u) or 58°C (primers Donkey's and Horse's cytochrome-b) for 1 min, 72°C for 1 min and final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 2% agarose gel (Invitrogen, California, USA) run in TBE 1X Buffer for 80 min at 90 V and stained with ethidium bromide (10 µg/L) for 20 min.

### RFLP analysis

Five microliters of PCR product, 2 units of restriction enzyme Tsp509I or AluI restriction enzyme (Faenzyme,) (Fermentas, USA), 0.1 µL BSA (1 mg/ml) and 2 µL of 10X reaction buffer at the final volume of 20 µL were incubated for 2.5 h at 55°C and it was inactivated at 65°C for 20 min. Digestion products were separated by electrophoresis on non-denaturing 8% polyacrylamide gel stained with $AgNO_3$ and photographed by Fugi digital camera (Fine pix S5700, Japan).

### Irradiation process

Adulterated meat samples were subjected to three different doses of gamma irradiation (1.5, 3 and 5 kGy) at dose rate 3.49 kGy/ h using the "Indian Gamma Chamber 4000 A" with a $^{60}$Co source. The irradiation process was conducted at the National Center for Radiation Research and Technology (NCRRT), Nasr city, Cairo, Egypt. After irradiation, adulterated samples were kept at -20°C until used for DNA extraction repeating all previous steps.

## RESULTS AND DISCUSSION

Identifying meat species used in meat products is a critical point in the quality control measures. Molecular

**Table 1.** Primer sequences of species-specific DNA regions and their annealing temperatures.

| DNA regions | Primer sequence 5 to 3 | Annealing temperature (°C) |
|---|---|---|
| CB7u *cytochrome-b* | GCGTACGCAATCTTACGATCA<br>CTGGCCTCCAATTCATGTGAG | 60 |
| Donkey's and Horse's *cytochrome-b* | CCATCCAACATCTCAGCATGATGAAA<br>GCCCCTCAGAATGATATTTGTCCTCA | 58 |

**Table 2.** Fragments lengths for ruminant species, donkey and horse after digestion of the PCR products with restriction enzyme.

| Species | Amplicon (bp) | Restriction enzymes | Fragment length (bp) |
|---|---|---|---|
| Sheep | 193 | | 13, 75, 105 |
| Goat | 195 | Tsp509I | 13, 182 |
| Cattle | 185 | | 3, 68, 114 |
| Donkey and horse | 359 | AluI | - |
| Horse | 359 | | 74, 96, 189 |

**Table 3.** Number and % of adulterated minced meat and sausage samples (n= 50 each).

| Sample | Number of adulterated samples with | | | Percentage | | |
|---|---|---|---|---|---|---|
| | Sheep | Goat | Donkey | Sheep | Goat | Donkey |
| Minced meat (n=50) | 2 | 1 | 2 | 4 | 2 | 4 |
| Sausage (n=50) | 2 | 2 | 3 | 4 | 4 | 6 |

techniques have been used elsewhere for meat species identification. The PCR assays targets genomic, as well as, mitochondrial DNA for the purpose of meat species identification. The conventional techniques allow the qualitative detection of different species with a defined limit of detection. However, Real-time PCR generally offers greater sensitivity and specificity and is a quantitative method for identification of species (Walker et al., 2013). Species-specific PCR assay was found to be rapid and cost effective for identification of meat species due to specific detection of target sequence without the need of further sequencing or digestion of the PCR products with restriction enzymes (Rodriguez et al., 2004), but it cannot be designed when species are very closely related (Kelly et al., 2003). On the contrary, PCR-RFLP could differentiate closely related meat species (Amjadi et al., 2012; Jaayid, 2013; Mane et al., 2014).

The amplification of species-specific DNA segments and restriction fragment length polymorphisms in cattle, sheep, goat, horse and donkey are represented in Tables 2 and 3 as well as Figures 1and 2. The CB7u primer produced specific fragments of about 195 bp for ruminant species. The size of ruminant fragments depends on the number of deletions in each species sequence (Burger et al., 2002). In this study, Cyt-b gene sequence from mitochondrial DNA was used for ruminant and equine DNAs identification and detection in meat because, mitochondrial DNA has numerous copies per each cell and it can provide the sequence variety for identification of closely related species faster than genomic DNA (Bellis et al., 2003). The suitability of cyt *b* gene sequence analysis to verify the claimed origin of supplied meat on a routine basis was previously confirmed (Jaayid, 2013, Doosti et al., 2014). The PCR-RFLP technique was able to distinguish between meat of animals that belong to the same family or same species such as the bovine family or the equine family (Doosti et al., 2014). Discrimination between donkey and horse's meat was previously reported by several investigators (Abdel-Rahman et al., 2009; Jaayid, 2013), using *Alu*I restriction enzyme, three fragments (189-, 96- and 74-bp) from the amplified gene encoding *cytochrome-b* gene (359 bp) were obtained in horse, whereas in donkey meat samples no fragments were obtained.

Results of RFLP assay (Table 3) reveal that 2/50 (4%) (Figure 2 lane 2), 1/50 (2%) (Figure 1 lane 6), and 2/50 (4%) of minced meat samples were contaminated with sheep, goat and donkey, contrary to what was mentioned

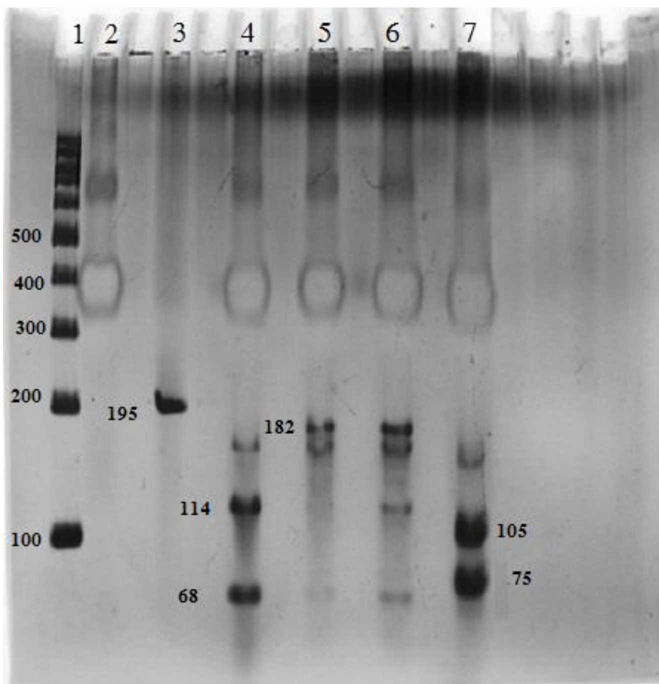


**Figure 1.** Polyacrylamide gel revealing restriction profiles of 195 bp Cyt-b PCR products obtained after treatment with Tsp509I. Lane 1 marker; lane 2, negative control; lane 3, positive control (undigested); lane 4, control cattle meat; lane 5, control goat meat; lane 6, commercial minced meat; Lane 7, control sheep meat.

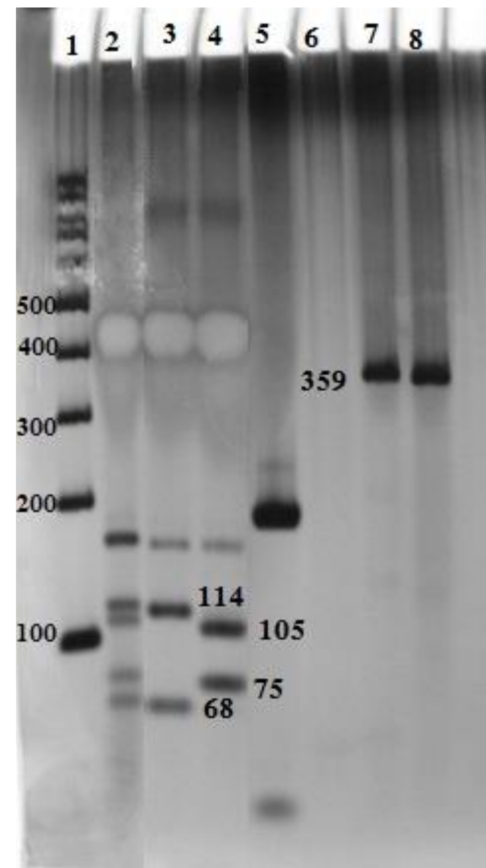


**Figure 2.** Polyacrylamide gel revealing restriction profiles of 195 bp Cyt-b PCR products obtained after treatment with Tsp509I for lanes 2-4. Lane 1, marker; lane 2, commercial minced meat; lane 3, control cattle meat; lane 4, control sheep meat; lane 5, positive control (undigested); lane 6, negative control; lane 7, control donkey meat; lane 8, PCR of primer of Donkey's and Horse's *cytochrome-b* after treatment with AluI restriction enzyme.

on their labels. Similarly 2/50 (4%), 2/50 (4%), 3/50 (6%) (Figure 2 lane 7, 8) of sausage samples were contaminated with sheep, goat and donkey. In total, 12% of the collected samples were contaminated with sheep, goat and donkey in contrast to what was labeled. Adulteration with donkey and horse meat was previously reported by several investigators (Jaayid, 2013; El-Shewy, 2007; Abd El-Nasser et al., 2010). The adulteration rate with donkey meat in our study was less than that reported in Assuit governorate in Egypt by Abd El-Nasser et al. (2010) in minced meat (7%) and sausage (8%); this may be due to the stringent control on food in Cairo, the Capital of Egypt. Donkey is not a species commercially used for human consumption. Its presence indicates adulteration for economic gain and so gives an idea that meat has been processed under non-sanitary conditions representing potential risks to human health. Adulteration of minced meat with sheep (60%) and goat (10%) was reported by Amjadi et al. (2012) with rate higher than that reported in our study.

Fraud in meat based products by adding low quality and cheap meat has always been an issue in food industries. The PCR-RFLP method used in this research, is cost-effective, reliable and very specific to be applied on food products that have endured different handling conditions during preparation process. It should play an important role in the food industry regulation and legal issues.

By irradiating adulterated meat with 1.5, 3 and 5 kGy, there was no change in the results of PCR and PCR-RELP. This finding was justified by the Council for Agricultural Science and Technology (CAST, 1989) by estimating that a dose of 1 kGy would break fewer than 10 chemical bonds for every ten million bonds present, an extremely small percentage. Cooking, or applying infrared radiation to foods, produces similar changes in chemical bonds.

It can be concluded from the findings of the present study that molecular methods such as PCR and PCR-RFLP are potentially reliable techniques for the detection of different meat species in meat products.

## Conflict of interests

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

*Full Length Research Paper*

# Forensic analysis of mitochondrial DNA hypervariable region HVII (encompassing nucleotide positions 37 to 340) and HVIII (encompassing nucleotide positions 438-574) and evaluation of the importance of these variable positions for forensic genetic purposes

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**The first objective of this study was the detection of mitochondrial hypervariable HVII and HVIII regions. Secondly, the study evaluates the importance of these positions for forensic genetic purposes and establishes the degree of variation characteristic of a fragment. Blood samples were collected from 270 healthy unrelated male living in Middle and South of Iraq. FTA® Technology was utilized to extract DNA. A portion of a noncoding region encompassing positions 37 to 340 for HVII and encompassing positions 438 to 574 for HVIII, was amplified in accordance with the Anderson reference sequence. By using EZ-10 spin column the PCR products were purified, sequenced and detected by using the ABI 3730*xL* Genetic Analyzer. New polymorphic positions 57, 63, 101, 469 and 482 are described that may be very important for forensic identification purpose in the future. This study shows the importance of the adoption of mitochondria in forensic medicine and criminal diagnosis and a private Iraqi society was discovered as the study sites. Further study on larger number of samples from different Iraqi ethics groups is suggested to confirm the results obtained by this study.**

**Keywords:** Forensic, frequency, HVII, HVIII, Iraq, polymorphism.

## INTRODUCTION

The introduction of DNA fingerprinting by an English scientist, Sir Alec Jeffreys in 1985 has had an enormous impact in forensic science (Jeffreys et al., 1985). Mammalian cells possess two different types and interdependent genomes, the nuclear genome and mitochondrial genome. Human DNA is basically composed of the coding and non-coding regions. The coding region only makes up about 3% of human genomic

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DNA. Mitochondria are semi-autonomously functioning organelles containing a resident genome that undergoes replication, translation and transcription of their own DNA. Mitochondrial DNA comprising of about 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs are a small circle of DNA (Helgason et al., 2003). MtDNA is only passed on from mother to child, it does not recombine and therefore there is no change between parent and child, unlike nuclear DNA (Ingman and Gyllensten, 2003; Ukhee et al., 2005; Imad et al., 2014a). There is more sequence divergence in mitochondrial than in nuclear DNA (Brown et al., 1993; Giulietta et al., 2000).

Each mitochondrion contains its own DNA, with many copies of the circular mitochondrial DNA in every cell. It is thought that each mitochondrion contains between 1 and 15, with an average of 4 to 5, copies of the DNA (Reynolds, 2000) and there are hundreds, sometimes thousands, of mitochondria per cell. The result is that there are many thousands of copies of the mitochondrial DNA in every cell. This compares with only two copies of nuclear DNA. The mitochondrion also has a strong protein coat that protects the mitochondrial DNA from degradation by bacterial enzymes. This compares to the nuclear envelope that is relatively weak and liable to degradation. DNA alterations (mutations) occur in a number of ways. One of the most common ways by which mutations occur is during DNA replication. An incorrect DNA base may be added; for example, a C is added instead of a G. This creates a single base change, or polymorphism, resulting in a new form. These single base mutations are rare but occur once every 1,200 bases in the human genome. The result is that the rate of change, or evolutionary rate, of mitochondrial DNA is about five times greater than nuclear DNA (Bar, 2000; Imad et al., 2014b). This is important in species testing, as even species thought to be closely related may in time accumulate differences in the mitochondrial DNA but show little difference in the nuclear DNA. A further reason for the use of mitochondrial DNA in species testing, and in forensic science, is its mode of inheritance. Mitochondria exist within the cytoplasm of cells, including the egg cells.

Spermatozoa do not normally pass on mitochondria and only pass on their nuclear DNA. The resulting embryo inherits all its mitochondria from its mother (Brown, 2002a,b; Tully, 2004; Imad et al., 2014c). This polymorphism allows scientists to compare mtDNA from crime scenes to mtDNA from given individuals to ascertain whether the tested individuals are within the maternal line (or another coincidentally matching maternal line) of people who could have been the source of the trace evidence.

Genetic studies of middle and south of Iraq by the use of molecular markers of mitochondrial DNA (mtDNA) have attracted the interest of population geneticists (Al-Zahery et al., 2003; Nadia et al., 2011). Sequence analysis of the HV1 and HV2 fragments of mitochondrial DNA (mtDNA) is today a routine method applied to forensic identification in cases where evidence specimens are not suitable for STR analysis.

## MATERIALS AND METHODS

### Population

Two hundred and seventy (270) healthy, randomly chosen individuals derived from the middle and south of Iraq provinces (Baghdad, Babil, Diwania, and Basrah). The number and ethicity of individuals were chosen in order to obtain a population sample to achieve the highest possible representation of the major ethno-religious and tribal groups of the Country living in these central and southern areas.

### DNA extraction and PCR primers

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 (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 100µl Whatman FTA purification reagent. Each wash was incubated for 5 minutes 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.

The primers were designed manually by using The Cambridge Reference Sequence. Each primer was diluted to a final concentration of 100 pm/µl and kept at -20°C for longer storage. A portion of a noncoding region encompassing positions from 37 to 340 for HVII amplified in accordance with the Anderson reference sequence (Anderson et al., 1981) GenBank: J01415. In MtDNA-HVII the portion of DNA was amplified in two primers: the first one is HVII-F (37-58) 5'-CATTCTCATAATCGCCCACGG-3' and the second HVII-R has a position (320-340) 5'-CCCCCCATCCTTACCACCCTC-3'. A portion of a noncoding region encompassing positions from 438 to 574 for HVIII was amplified in accordance with the Anderson reference sequence (Anderson et al., 1981) GenBank: J01415. In MtDNA- HVIII the portion of DNA was amplified in two primers: the first one is HVIII-F (438-459) 5'-CAACTAACACATTATTTTCCCC-3' and the second HVIII-R has position (574-555) 5'-AACCCCAAAGACACCCCCCA-3'. PCR reaction was done in 0.2 ml PCR tubes with the following mixtures: 1 µl of each forward and reverse primer (10 pm/µl), 2jl of DNA template (5 ng/41) and 46 µl of PCR ReddyRunTM Master Mix. The following PCR condition was used: 94°C for 5 min, 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 45 s and final extension step at 72°C for 7 min. PCR products were kept at 4°C in a separate fridge from the pre- PCR components to avoid contamination.

### Sequencing reaction of the PCR product

Purification and sequencing reaction of the PCR product was performed by EZ10-spin column DNA cleanup kit 100 prep EZ-10 spin column purification kits. PCR fragment was sequenced using ABI Prism Big Dye® Terminator Cycle Sequencing Kit on an ABI 377 sequencer. Each sequence obtained was then aligned with the Cambridge Reference Sequence.

**Table 1.** Hypervariable region (HVII) sequence variance and mtDNA haplotypes.

| Anderson | 39 | 41 | 42 | 46 | 49 | 53 | 56 | 57 | 63 | 69 | 70 | 71 | 78 | 81 | 101 | 105 | 127 | 141 | 179 | 196 | 208 | 216 | 220 | 222 | 234 | 275 | 322 | 327 | No. of Individual |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | C | T | T | A | G | A | T | T | G | G | G | C | G | G | C | T | C | T | T | T | T | T | C | A | G | G | C | |
| H1* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 126 |
| H2 | - | T | C | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H3 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | 3 |
| H4 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | 1 |
| H5 | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | G | - | - | - | - | 1 |
| H6 | T | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | 1 |
| H7 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | T | - | - | - | - | 1 |
| H8 | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H9 | - | - | - | - | - | - | T | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H10 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | C | - | - | - | - | - | - | 1 |
| H11 | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H12 | - | - | - | C | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H13 | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | 2 |
| H14 | T | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | C | - | - | - | - | - | - | 1 |
| H16 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | 4 |
| H17 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | C | - | - | - | - | - | - | - | 1 |

**Statistical analysis**

The pattern of inheritance had made statistical analysis of mtDNA type much easier than any other genetic marker. Since mtDNA is presented in each human being as haploid, determination of mtDNA type did not require the prerequisite of Hardy-Weinberg equilibrium for statistical analysis. Genetic diversity 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 et al., 2001). The probability of two randomly selected individuals was from a population having identical mtDNA types.

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

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

## RESULTS AND DISCUSSION

### Hypervariable region (HVII) sequence variance and mtDNA haplotypes

The study enabled identification of 95 different haplotypes and 28 polymorphic nucleotide positions in HVII Table 1. Among these 28 variations, there were 17(61%) variation between T and C and 11 variations (39%) between A and G. Seven polymorphic positions, 56, 63, 69, 81, 101, 208, and 222 have transverse substitution (Table 2). All the other substitutions determined during the analysis are transitions. The most frequent variant (H1) was consistent with the Anderson sequence (Brown et al., 1982; Guntheroth et al., 1986; Pastore, 1994; Yang and Yoder, 1999).

### Hypervariable region (HVIII) sequence variance and mtDNA haplotypes

The study enabled identification of 86 different haplotypes and 16 polymorphic nucleotide Positions in HVIII Table 3. Among these 16 variations, there were 11(69%) variation between T and C and 4 variations (25 %) between A and G. and just one position (6 %) between T and A. Three polymorphic positions, 447, 453, and 469

**Table 1.** Contd.

| Anderson | 39 | 41 | 42 | 46 | 49 | 53 | 56 | 57 | 63 | 69 | 70 | 71 | 78 | 81 | 101 | 105 | 127 | 141 | 179 | 196 | 208 | 216 | 220 | 222 | 234 | 275 | 322 | 327 | No. of Individual |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | C | T | T | A | G | A | T | T | G | G | G | C | G | G | C | T | C | T | T | T | T | T | C | A | G | G | C | |
| H18 | - | - | - | - | - | - | - | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H19 | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | C | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H20 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H21 | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | 1 |
| H22 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | 2 |
| H23 | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | T | 1 |
| H24 | - | - | - | - | - | - | - | - | - | - | A | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H25 | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | 1 |
| H26 | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H27 | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H28 | - | - | - | - | - | - | G | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | 1 |
| H29 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | C | - | - | - | - | A | - | 1 |
| H30 | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H31 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H32 | - | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | 1 |
| H33 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H34 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | T | - | - | - | - | 1 |
| H35 | - | - | C | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | 1 |
| H36 | T | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H37 | - | - | - | C | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H38 | - | - | - | - | - | A | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H39 | - | - | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | 2 |
| H40 | - | T | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H41 | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | 2 |
| H42 | - | - | C | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H43 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | 1 |
| H44 | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | 1 |
| H45 | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | 3 |
| H46 | - | - | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | 2 |
| H47 | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H48 | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H49 | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | 1 |
| H50 | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | G | - | - | - | 1 |
| H51 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | 2 |

**Table 1.** Contd.

| Anderson | 39 | 41 | 42 | 46 | 49 | 53 | 56 | 57 | 63 | 69 | 70 | 71 | 78 | 81 | 101 | 105 | 127 | 141 | 179 | 196 | 208 | 216 | 220 | 222 | 234 | 275 | 322 | 327 | No. of Individual |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | C | T | T | A | G | A | T | T | G | G | G | C | G | G | C | T | C | T | T | T | T | T | C | A | G | G | C | |
| H52 | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H53 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | 1 |
| H54 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | 2 |
| H55 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | T | 1 |
| H56 | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | C | - | - | - | - | - | 1 |
| H57 | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| H58 | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | A | - | - | 1 |
| H59 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | G | - | - | - | 3 |
| H60 | - | T | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H61 | - | - | - | - | - | A | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | 1 |
| H62 | - | - | - | - | - | - | - | - | - | - | A | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H63 | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | C | - | - | - | - | - | - | - | 1 |
| H64 | T | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H65 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | T | 2 |
| H66 | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H67 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | C | - | - | - | - | - | - | 1 |
| H68 | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | 1 |
| H69 | - | - | - | - | - | - | G | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H70 | - | T | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H89 | - | - | - | - | - | - | - | - | - | - | - | A | - | A | - | - | - | - | - | C | - | - | - | - | - | - | - | - | 1 |
| H90 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | T | - | - | - | - | 1 |
| H91 | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| H92 | - | - | - | - | - | - | - | - | - | - | - | - | T | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| H93 | - | - | - | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | - | - | - | T | 1 |
| H94 | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | T | - | - | - | - | - | - | - | A | - | - | 1 |
| H95 | - | - | - | - | G | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | - | - | - | - | - | - | - | - | 1 |
| Total | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 280 |

H*, Haplotype; G, guanine; T, thiamine; C, cytosine; A, adenine.

have transverse substitution (Table 4). Genetic diversity for the analysed DNA fragment was calculated according to the formula: D= $1-\sum p^2$ and recorded 0.950% and 0.965% for HVII and HVIII respectively. The relatively high gene diversity and a relatively low random match probability were observed in this study.

Comparative analysis of our results with previously published Iraq data (Pastore, 1994; Yang and Yoder, 1999; Muhanned et al., 2015), revealed significant differences in SNP patterns. Haplotypes detected in this study group have been

**Table 2.** Types of mutations in variable positions for HVII.

| Positions | Mutation | Type of mutation | Presence in Mitomap | Frequency | Frequency (%) |
|---|---|---|---|---|---|
| 39 | Transition | C-T | Presence | 0.047 | 4.70 |
| 41 | Transition | C-T | Presence | 0.053 | 5.30 |
| 42 | Transition | T-C | Presence | 0.042 | 4.20 |
| 46 | Transition | T-C | Presence | 0.031 | 3.10 |
| 49 | Transition | A-G | Presence | 0.049 | 4.90 |
| 53 | Transition | G-A | Presence | 0.046 | 4.60 |
| 56 | Transition | A-G | Presence | 0.036 | 3.60 |
| 56 | Transversion | A-T | Presence | | |
| 57 | Transition | T-C | Presence | 0.039 | 3.90 |
| 57 | Transition | T-G | New* | | |
| 63 | Transversion | T-A | Presence | 0.03 | 3 |
| 63 | Transition | T-C | New | | |
| 69 | Transversion | G-C | Presence | 0.044 | 4.40 |
| 69 | Transition | G-A | Presence | | |
| 70 | Transition | G-A | Presence | 0.04 | 4 |
| 71 | Transition | G-A | Presence | 0.026 | 2.60 |
| 78 | Transition | C-T | Presence | 0.046 | 4.60 |
| 81 | Transition | G-A | Presence | 0.026 | 2.60 |
| 81 | Transversion | G-C | Presence | | |
| 101 | Transition | G-A | New | 0.036 | 3.60 |
| 101 | Transversion | G-C | Presence | | |
| 105 | Transition | C-T | Presence | 0.036 | 3.60 |
| 127 | Transition | T-C | Presence | 0.035 | 3.50 |
| 141 | Transition | C-T | Presence | 0.025 | 2.50 |
| 179 | Transition | T-C | Presence | 0.027 | 2.70 |
| 196 | Transition | T-C | Presence | 0.035 | 3.50 |
| 208 | Transversion | T-A | Presence | 0.019 | 1.90 |
| 208 | Transition | T-C | Presence | | |
| 216 | Transition | T-C | Presence | 0.036 | 3.60 |
| 220 | Transition | T-C | Presence | 0.032 | 3.20 |
| 222 | Transition | C-T | Presence | 0.024 | 2.40 |
| 222 | Transversion | C-G | Presence | | |
| 234 | Transition | A-G | Presence | 0.023 | 2.30 |
| 275 | Transition | G-A | Presence | 0.023 | 2.30 |
| 322 | Transition | G-A | Presence | 0.012 | 1.20 |
| 327 | Transition | C-T | Presence | 0.045 | 4.50 |
| Genetic diversity* | $D= 1-\sum p^2 = 0.964 = 96.4$ % | | | | |

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

compared with other global populations: German (n = 200) (Lutz et al., 1998), US Caucasian (n = 604), Africa(n = 111), Malaysia (n = 195) (Budowle et al., 1999) and India (n = 98) (Mountain et al., 1995) (Table 5).

Walsh et al. (1991) and Tang (2002) show that the polymorphism of mtDNA coding area is less than that of mtDNA control region. Therefore, more efficient polymorphic sites should be used to provide an improved discrimination power for forensic mtDNA testing (Imad et al., 2014c).

However, mtDNA data on Iraqi population is very limited. This had limited the application of mtDNA in forensic cases and study of mtDNA population genetics in Iraq. In future, development of more multiplexes targeting mtDNA polymorphisms within the control and coding regions might reduce the matching probability of mtDNA type and increased the utility of mtDNA in forensic cases.

**Table 3.** Hypervariable region (HVIII) sequence variance and mtDNA haplotypes.

| Anderson | 444 | 447 | 449 | 453 | 456 | 458 | 469 | 471 | 482 | 485 | 493 | 494 | 504 | 508 | 533 | 534 | Number of Individual |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | C | T | T | C | C | C | T | T | T | A | C | T | A | A | C | |
| H1* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 157 |
| H2 | . | . | C | . | T | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H4 | . | . | . | . | T | T | . | . | . | . | . | . | . | . | . | . | 1 |
| H5 | G | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | 3 |
| H6 | . | . | . | . | . | T | . | . | . | . | . | T | . | . | . | . | 1 |
| H7 | . | G | . | A | . | | . | . | . | . | . | . | . | . | . | . | 1 |
| H8 | . | . | . | . | . | T | . | . | . | . | . | T | . | . | . | . | 1 |
| H9 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H10 | . | . | C | . | . | . | . | C | . | . | . | . | . | . | . | . | 6 |
| H11 | . | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H12 | . | . | . | . | . | . | . | C | . | . | . | . | . | . | G | . | 1 |
| H13 | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | 3 |
| H14 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H15 | . | . | . | . | T | . | . | . | . | C | . | . | . | . | . | . | 1 |
| H16 | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H17 | . | . | . | . | . | . | T | . | . | . | . | T | . | . | . | . | 1 |
| H18 | . | T | . | . | . | . | . | . | . | C | . | . | . | . | . | . | 3 |
| H19 | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | T | 1 |
| H20 | . | . | . | . | T | . | . | . | . | . | . | T | . | . | . | . | 1 |
| H21 | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | 2 |
| H22 | . | T | . | . | . | . | . | . | . | . | . | . | C | . | . | . | 3 |
| H23 | . | . | . | . | . | . | . | . | . | . | . | . | . | G | G | . | 1 |
| H24 | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | 1 |
| H25 | . | . | . | . | . | . | . | . | . | . | . | T | C | . | . | . | 1 |
| H26 | G | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | 1 |
| H27 | . | . | . | . | . | . | . | . | . | . | G | . | . | G | . | . | 1 |
| H28 | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | 1 |
| H29 | . | . | . | . | . | . | . | . | C | . | . | . | . | . | G | . | 1 |
| H30 | . | . | C | . | . | . | . | . | . | . | . | T | . | . | . | . | 1 |
| H31 | G | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H32 | . | . | . | . | . | . | T | C | . | . | . | . | . | . | . | . | 4 |
| H33 | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | 1 |
| H34 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H35 | . | . | . | . | . | . | . | . | . | . | . | . | C | . | G | . | 1 |
| H36 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H37 | . | . | . | . | . | . | . | . | . | C | . | T | . | . | . | . | 1 |
| H38 | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H39 | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H40 | . | T | . | . | . | . | . | . | . | . | G | . | . | . | . | T | 1 |
| H41 | . | . | . | . | T | . | . | . | . | . | G | . | . | . | . | . | 1 |
| H42 | . | . | . | . | . | T | . | . | C | . | . | . | . | . | . | . | 1 |
| H43 | . | . | C | . | . | . | . | . | . | . | . | T | . | . | . | T | 1 |
| H44 | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | 3 |
| H45 | . | T | . | . | . | . | . | . | C | . | . | . | . | . | . | . | 1 |
| H46 | G | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | 1 |
| H47 | . | . | . | C | . | . | . | . | C | . | . | . | . | . | . | T | 1 |
| H48 | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H49 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H50 | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | 1 |

**Table 3.** Contd.

| Anderson | 444 | 447 | 449 | 453 | 456 | 458 | 469 | 471 | 482 | 485 | 493 | 494 | 504 | 508 | 533 | 534 | Number of Individual |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | C | T | T | C | C | C | T | T | T | A | C | T | A | A | C | |
| H51 | . | . | . | . | . | T | . | . | C | . | . | . | . | . | . | . | 1 |
| H52 | . | . | C | . | . | . | G | . | . | . | . | . | . | . | . | . | 1 |
| H53 | . | . | . | . | . | . | . | C | . | . | G | . | . | . | . | . | 1 |
| H54 | . | T | . | . | . | . | . | . | . | C | . | . | . | . | . | . | 4 |
| H55 | . | T | . | . | . | . | . | C | . | . | . | . | . | . | G | . | 1 |
| H56 | . | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | 1 |
| H57 | . | . | . | . | . | . | T | . | . | . | G | . | . | . | . | . | 1 |
| H58 | . | . | . | . | . | . | T | . | . | . | . | T | . | . | . | . | 1 |
| H59 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H60 | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | T | 1 |
| H61 | . | . | . | . | . | . | . | . | . | . | G | . | C | . | . | . | 1 |
| H62 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | G | . | 1 |
| H63 | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | T | 1 |
| H64 | . | . | . | . | T | . | . | . | . | . | . | T | . | . | . | . | 1 |
| H65 | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | T | 1 |
| H66 | G | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | 1 |
| H67 | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | 1 |
| H68 | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H69 | . | . | . | C | . | . | . | . | . | C | . | . | . | . | . | . | 1 |
| H70 | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H71 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 |
| H72 | . | . | C | . | . | . | . | . | . | . | . | . | C | . | . | . | 1 |
| H73 | . | . | . | . | T | . | . | . | . | . | . | T | . | . | . | . | 1 |
| H74 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H75 | . | . | . | . | . | T | . | . | . | . | . | . | C | . | . | . | 1 |
| H76 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | T | 1 |
| H77 | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | 1 |
| H78 | . | . | . | . | . | . | . | . | . | . | . | . | C | G | . | . | 1 |
| H79 | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H80 | . | . | . | . | . | T | . | . | . | . | G | . | . | . | . | . | 1 |
| H81 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | T | 1 |
| H82 | . | . | . | . | . | . | . | C | . | . | . | . | . | G | . | . | 1 |
| H83 | . | . | . | . | . | . | . | . | C | . | . | . | . | . | G | . | 1 |
| H84 | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H85 | . | . | . | . | . | . | T | . | . | . | G | . | . | . | . | . | 1 |
| H86 | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | . | 1 |
| H87 | . | . | . | . | T | . | . | . | . | . | . | . | C | . | . | . | 1 |
| Total | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 275 |

H*, Haplotype; G, guanine; T, thiamine; C, cytosine; A, adenine.

## Conclusion

Sequence databases are the best source of information regarding the power of mtDNA for identity testing. Sequence analysis of the noncoding region of mtDNA (HVII) and HVIII conducted on a population of 270 unrelated individuals enabled identification of 34 different haplotypes in HVII and 86 different haplotypes in HVIII. New polymorphic positions 57, 63,101,469 and 482 are described that may in future, be suitable sources for genetic identification purposes. The ABI Prism Big Dye Terminator Cycle Sequencing kit used for sequencing of

**Table 4.** Types of mutations in variable positions for HVIII.

| Position | Mutation | Type of mutation | Presence in Mitomap | Frequency | Frequency (%) |
|---|---|---|---|---|---|
| 444 | Transition | A-G | Presence | 0.034 | 34 |
| 447 | Transversion | C-G | Presence | | |
| 447 | Transition | C-T | Presence | 0.066 | 6.6 |
| 449 | Transition | T-C | Presence | 0.055 | 5.5 |
| 453 | Transversion | T-A | Presence | 0.036 | 3.6 |
| 453 | Transition | T-C | Presence | | |
| 456 | Transition | C-T | Presence | 0.04 | 4 |
| 458 | Transition | C-T | Presence | 0.04 | 4 |
| 469 | Transition | C-T | Presence | 0.04 | 4 |
| 469 | Transversion | C-G | New* | | |
| 471 | Transition | T-C | Presence | 0.05 | 5 |
| 482 | Transition | T-C | New | 0.042 | 4.2 |
| 485 | Transition | T-C | Presence | 0.052 | 5.2 |
| 493 | Transition | A-G | Presence | 0.048 | 4.8 |
| 494 | Transition | C-T | Presence | 0.04 | 4 |
| 504 | Transition | T-C | Presence | 0.045 | 4.5 |
| 508 | Transition | A-G | Presence | 0.028 | 2.8 |
| 533 | Transition | A-G | Presence | 0.052 | 5.2 |
| 534 | Transition | C-T | Presence | 0.048 | 4.8 |
| Genetic diversity* D= $1-\sum p^2$ = 0.965 = 96.5% | | | | | |

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

**Table 5.** Comparisons of the characteristics across D-loop region in different human population groups.

| Population | Iraq[1] | India[2] | Malaysia[3] | Africa[4] | German[5] | US Caucasian[6] |
|---|---|---|---|---|---|---|
| Sample size | 280 | 98 | 195 | 111 | 200 | 604 |
| No. of variant sites | 44 | 83 | 149 | 97 | 153 | 233 |
| A→G | 64 | 233 | 473 | 323 | 330 | 1112 |
| G→A | 169 | 66 | 81 | 78 | 55 | 219 |
| T→C | 119 | 145 | 461 | 382 | 308 | 1007 |
| C→T | 51 | 117 | 321 | 486 | 199 | 688 |
| % transition | 96.9% | 94.85 | 92.16 | 95.77 | 95.61 | 97.61 |
| A→T | 1 | 1 | 2 | 0 | 4 | 2 |
| A→C | 0 | 23 | 81 | 15 | 5 | 47 |
| G→T | 0 | 0 | 0 | 18 | 0 | 1 |
| G→C | 2 | 0 | 3 | 0 | 1 | 6 |
| C→A | 0 | 0 | 30 | 17 | 11 | 12 |
| C→G | 4 | 4 | 1 | 6 | 19 | 6 |
| T→A | 2 | 7 | 5 | 0 | 1 | 0 |
| T→G | 0 | 0 | 3 | 0 | 0 | 0 |
| % transversion | 3% | 5.15 | 7.84 | 4.23 | 4.39 | 2.39 |
| Insertion | 0 | 168 | 322 | 140 | 291 | 983 |
| Deletion | 0 | 0 | 28 | 6 | 6 | 14 |

Note: % of transitions and transversions were calculated as number of observations divided by total substitution times. [1]This study, [2]Reference: [Mountain et al., 1995], [3]Reference: [Budowle et al., 1999], [4]Reference: [Budowle et al., 1999], [5]Reference: [Lutz et al., 1998], [6]Reference: [Budowle et al., 1999].

the amplified HVII and HVIII region had provided good quality of sequence for the purpose of this study.

## Conflict of interests

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# 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

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**The objectives of the study included the detection of genetic variation of 21 autosomal short tandem repeats (STRs) loci from random unrelated individuals in the middle and South Arab people of Iraq, the Forensic efficiency parameters of the autosomal 21 genetic loci using power plex21® kit and to evaluate the importance of these loci for forensic genetic purposes and the possibility to use the new kit in routine practical work. FTA® Technology was utilized to extract DNA from blood collected on FTA™ paper. Twenty one (21) STR loci including D3S1358, D13S317, PentaE, D16S539, D18S51, D2S1338, CSF1PO, Penta D, THO1, vWA, D21S11, D7S820, TPOX, D8S1179, FGA, D1S1656, D5S818, D6S1043, D12S391, D19S433 and Amelogenin were amplified by using power plex21® kit. PCR products were detected by genetic analyzer 3130xL then, the data processed and analyzed by PowerStatsV1.2 software. Several statistical parameters of genetic and forensic efficiencies based on allelic frequencies have been estimated. This includes the observed heterozygosity (Ho), expected heterozygosity (He), paternity index (PI), random match probability (RMP), power of discrimination (PD), chance of exclusion (CE), polymorphic information content (PIC) and P-value. The power of discrimination values for autosomal tested loci was from 75 to 96% therefore those loci can be safely used to establish a DNA-based database for Iraq population for identifical purpose. The high PIC values of the selected markers confirm their usefulness for genetic polymorphism studies and linkage mapping programs in human as well. The mean heterozygosity observed, expected and mean PIC values across the 20 loci were 0.79, 0.83 and 0.81, respectively indicating high gene diversity. A total of nine off-ladder alleles and one tri-allelic pattern were detected in this study.**

**Key words:** Autosomal STR, Iraq, off ladder, statistical parameters, tri-allelic pattern.

## INTRODUCTION

Microsatellites are a group of molecular markers chosen for a number of purposes including forensics individual identification and relatedness testing (Nakamura et al., 1987; Yamamoto et al., 1999). Microsatellites refer to DNA with varying numbers of short tandem repeats (Allor et al., 2005; Klintschar et al., 2005) between a unique sequence. DNA regions with repeat units that are 2 to 7 bp in length or most generally short tandem repeats (STRs) or simple

sequence repeats (SSRs) are generally known as micro-satellites (Ellegren, 2004). In the core repeated bases, long repeat units may contain several hundred to thousands (Butler and Hill, 2012; Silvia et al., 2009). DNA can be used to study human evolution using human genome analysis regions that are not subjected to selection pressure (Mats et al., 2007; Mohammed and Imad, 2013). Besides, information from DNA typing provides vital information in medico-legal with polymorphisms allowing for more biological studies (Walkinshaw et al., 1996). It has been found that microsatellites are evenly distributed in the genome on all chromosomes and all regions of the chromosome (Ensenberger et al., 2010). They can also be found inside gene coding regions, introns, and in the non-gene sequences. Most microsatellite loci are really small, ranging from a few to a few hundred repeats and this small size of microsatellite loci is important for PCR-facilitated genotyping. Basically, microsatellites containing a higher number of repeats are more polymorphic.

The number of repeats at a given locus can vary between individuals and there is a statistical probability that a given individual will have a set number of repeats at a particular STR locus (Moxon and Wills, 1999; Butler, 2005; Imad et al., 2014a; Muhanned et al., 2015). To process the results from each analysis, large database known as combined DNA index system (CODIS) has been set up. This database stores profiles from convicted offenders and unsolved casework. Similar databases have been set up in Europe, Japan, and other countries. The information in these databases can be used to detect and apprehend serial offenders by permitting rapid exchange of information between crime laboratories (Budowle et al., 2000; Ruitberg et al., 2001; Imad et al., 2014b).

Autosomal STRs locus information were evaluated and selected at The Cooperative Human Linkage Center. http://www.chlc.org evaluates the genetic markers and the loci are selected from there. Additional STR loci, chromosomal location and repeat sequence for each core STR locus are provided in Tables 1 and 2 (Klintschar et al., 2005). According to the International Society of Forensic Genetics (ISFG) recommendation, the repeat sequence motif was defined so that the first 5'-nucleotides on the Gene Bank forward strand define the repeat motif used; therefore, the repeat motif for each STR marker is listed based on this. A significant fact is that STR allele sizes are measured relative to an internal size standard during electrophoresis. This depends on the DNA strand that is labeled using a dye that may have a different apparent measured size. The PowerPlex® 21 System is compatible with automated PCR instrument and with the ABI PRISM® 3100, 3100-Avant, 3130, 3130xl, 3500 and 3500xL Applied Biosystems Genetic Analyzers. In the United States, Europe and Asia, the PowerPlex® 21 System is used, and it increases the discriminatory power and data-sharing possibilities by incorporating informative loci.

## MATERIALS and METHODS

### Preparation of blood stain samples

Blood samples were randomly collected from 400 healthy unrelated males and females living in the middle and south of Iraq **and** sent to the forensic genetic laboratories in (DNA Typing of Medico-Legal Institute, Baghdad, Iraq) and the same samples were sent to Department of Biomedical Science, University Putra Malaysia, Selangor, Malaysia to complete other tests.

### DNA extraction

DNA was extracted from all dried blood samples on FTA cards following the manufacturer's procedure as described in Whatman FTA Protocol BD01 (Mullen et al., 2009; Imad et al., 2014c). A 1.2 mm diameter disc was punched from each FTA card with a puncher and put directly in PCR tube.

### DNA amplification for Autosomal 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. Amplifications of 21 STR loci D3S1358, D13S317, PentaE , D16S539, D18S51, D2S1338, CSF1PO, Penta D, THO1, vWA, D21S11, D7S820, TPOX, D8S1179, FGA, D1S1656, D5S818, D6S1043, D12S391, D19S433 and Amelogenin were performed by using the PowerPlex®21 kit (Promega Corporation, Madison, WI 53711-5399 USA, © 2011,2012. All Rights Reserved. www.promega.com). The 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. The amplified samples were kept at -20°C in a light-protected box.

### PCR amplicon analysis (capillary electrophoresis)

The major application of capillary electrophoresis (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. Other related applications include the analysis of haploid markers in the Y chromosome and in mitochondrial DNA (mtDNA) (Imad et al., 2014). Nonhuman DNA testing can also be performed depending on the circumstances of the case. The techniques involved include genotyping, DNA sequencing and mutation detection. The analysis of STR loci in DNA is the most common method for the between two or more unrelated determination of human identity and can

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**Table 1.** Comparison of STR loci present in kits used in the United States.

| Locus | Power Plex®18D | Power Plex®21 | Power Plex®Fusion | Identifiler | Mini Filer | Extended[a] |
|---|---|---|---|---|---|---|
| TPOX | TPOX | TPOX | TPOX | TPOX | - | - |
| CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO |
| D5S818 | D5S818 | D5S818 | D5S818 | D5S818 | - | D5S818 |
| D7S820 | D7S820 | D7S820 | D7S820 | D7S820 | D7S820 | D7S820 |
| D13S317 | D13S317 | D13S317 | D13S317 | D13S317 | D13S317 | D13S317 |
| FGA | FGA | FGA | FGA | FGA | FGA | FGA |
| vWA | vWA | vWA | vWA | vWA | - | vWA |
| D3S1358 | D3S1358 | D3S1358 | D3S1358 | D3S1358 | - | D3S1358 |
| D8S1179 | D8S1179 | D8S1179 | D8S1179 | D8S1179 | - | D8S1179 |
| D18S51 | D18S51 | D18S51 | D18S51 | D18S51 | D18S51 | D18S51 |
| D21S11 | D21S11 | D21S11 | D21S11 | D21S11 | D21S11 | D21S11 |
| TH01 | TH01 | TH01 | TH01 | TH01 | - | TH01 |
| D16S539 | D16S539 | D16S539 | D16S539 | D16S539 | D16S539 | D16S539 |
| - | D2S1338 | D2S1338 | D2S1338 | D2S1338 | D2S1338 | D2S1338 |
| - | D19S433 | D19S433 | D19S433 | D19S433 | - | D19S433 |
| - | - | D12S391 | D12S391 | - | - | D12S391 |
| - | - | D1S1656 | D1S1656 | - | - | D1S1656 |
| - | D2S441 | - | - | - | - | D2S441 |
| - | - | - | D10S1248 | - | - | D10S1248 |
| - | - | D6S1043 | - | - | - | - |
| - | - | - | D22S1045 | - | - | - |
| Penta D | Penta D | Penta D | Penta D | - | - | - |
| Penta E | Penta E | Penta E | Penta E | - | - | Penta E |
| - | - | - | DYS391 | - | - | DYS391 |
| Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin |

[a](Lareu et al., 1996**)** for more information on the proposed U.S. extended core.

indisputably distinguish individuals if sufficient loci can be detected (Budowle et al., 2000).

Using the ABI Prism1 3730xl Genetic Analyzer, 16-capillary array system (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols, with POP-7™ Polymer and Data Collection Software, GeneMapper® V3.2 software (Applied Biosystems, Foster City, CA, USA) were done. 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. 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 (Weir, 1996; Butler et al., 2004).

**Statistical data analysis**

***Statistical analysis for autosomal STR***

The PowerStatsV1.2 (Promega, Madison, USA) was used to calculate the observed heterozygosity (Ho), power of discrimination (PD), probability of exclusion (PE) and polymorphism information content (PIC). Arlequin software program was used to conduct the exact test of population differentiation. In addition, Arlequin software program was used for the expected heterozygosity (He), and Hardy Weinberg Equilibrium (HWE). Where test results with *P*-values less than 0.05 were observed, the Bonferroni correction had to be applied to the data. The Bonferroni procedure (Weir, 1996) adjusted the rejection level for the smallest *P*-value at an overall level of α = 5 % to 0.05/x; where, x is equal to the number of tests conducted on the data. The Ho and He values were calculated by means of the same software program.

Following are the formulas used to compute various parameters for population data analyses:

***Expected heterozygosity***

Edwards et al. (1991) described the following formula for calculating an unbiased estimate of the expected heterozygosity:

$$\mathrm{H} = \frac{n}{(n-1)}\left[1 - \sum_{j=1}^{k}\left(n_j/n\right)^2\right] = \frac{n}{(n-1)}\left[1 - \sum_{j=1}^{k}\left(p_j\right)^2\right]$$

Where, *n1, n2, …, nk* are the allele counts of *K* alleles at a locus in a sample of *n* genes drawn from the population and *pj* is the allele frequency.

**Table 2.** Information on 21 autosomal STR loci present in The PowerPlex® 21 System kits Adapted from **(**Cotton et al., 2000 ; Wiegand et al., 1993) physical positions are from **(**Schneider et al., 1998**).**

| STR Locus[a n] | Label | Physical position | Chromosomal Location1 | Repeat Sequence (5´ 3´) |
|---|---|---|---|---|
| Amelogenin | Fluorescein | X and Y | Xp22.1–22.3 and Y | NA |
| D3S1358 | Fluorescein | Chr 3 (45.582 Mb) | 3p21.31 | TCTA Complex |
| D1S1656 | Fluorescein | Chr 1 (230.905 Mb) | 1q42 | TAGA Complex |
| D6S1043 | Fluorescein | Chr 6 (92.450 Mb) | 6q15 | AGAT |
| D13S317 | Fluorescein | Chr 13 (82.692 Mb) | 13q31.1 | TATC |
| Penta E | Fluorescein | Chr 15 (97.374 Mb) | 15q26.2 | AAAGA |
| D16S539 | JOE | Chr.16(86.386 Mb) | 16q24.1 | GATA |
| D18S51 | JOE | Chr 18 (60.949 Mb) | 18q21.33 | AGAA |
| D2S1338 | JOE | Chr 2 (218.879 Mb) | 2q35 | TGCC/TTCC |
| CSF1PO | JOE | Chr 5 (149.455 Mb) | 5q33.1 | AGAT |
| Penta D | JOE | Chr 21 (45.056 Mb) | 21q22.3 | AAAGA |
| TH01 | TMR-ET | Chr 11 (2.192 Mb) | 11p15.5 | AATG (19) |
| vWA | TMR-ET | Chr12(6.093 Mb) | 12p13.31 | TCTA Complex (19) |
| D21S11 | TMR-ET | Chr 21 (20.554 Mb) | 21q21.1 | TCTA Complex (19) |
| D7S820 | TMR-ET | Chr 7 (83.789 Mb) | 7q21.11 | GATA |
| D5S818 | TMR-ET | Chr 5 (123.111 Mb) | 5q23.2 | AGAT |
| TPOX | CXR-ET | Chr 2 (1.493 Mb) | 2p25.3 | AATG |
| D8S1179 | CXR-ET | Chr 8 (125.907 Mb) | 8q24.13 (125.976 Mb) | TCTA Complex (19) |
| D12S391 | CXR-ET | Chr 12 (12.450 Mb) | 12p12(12.341 Mb) | AGAT/AGAC Complex |
| D19S433 | CXR-ET | Chr19(30.416 Mb) | (35.109Mb) | AAGG Complex |
| FGA | CXR-ET | Chr 4 (155.509 Mb) | 4q28 (155.866Mb) | 4q28 (155.866Mb) |

*a*database of sequence-tagged sites (STSs) available on the NCBI website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists.
*n* The 13 CODIS core loci are highlighted in bold font.

***Match probability***

The probability of a match at locus *l* *PMl*, was first described from genotype data (Fisher, 1951):

$$PM_l = \sum_i \tilde{G}_{il}^2$$

Where, $\tilde{G}_i$ is the sample frequency of the *i*th genotype at locus *l*.

$$PM_l = \frac{\sum_{i=1}^{n} \tilde{G}_{il}^2 - 1/N_l}{1 - 1/N_l} \approx \sum_{i=1}^{n} \tilde{G}_{il}^2$$

Where, the first part of this equation is for a sample of size *Nl* at locus *l* (Jones, 1972).

***Power of discrimination***

Brenner and Morris (1990) described the following formula for calculating the power of discrimination:

$$1 - PM$$

**Polymorphism information content**

The PIC was also calculated using marker allelic frequencies using the following equation:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

Where, *n* is the number of alleles and *pi* is the allele probability of the *i*th allele (Botstein et al., 1980).

**Power of exclusion**

$$\text{Power of Exclusion (PE)} = H^2(1-(1-H)H^2)$$

Where, *H* = heterozygosity.

**Paternity index**

$$\text{Paternity Index (PI)} = \frac{H+h}{2h} = \frac{(1-h)+h}{2h} = \frac{1}{2h} = \frac{1}{2\sum_{i=1}^{n} p_i^2}$$

Where, *Pi* is the frequency of *ith* allele in a population of n samples; *h* = homozygosity and *H*= heterozygosity.

## RESULTS AND DISCUSSION

### Autosomal STRs

The short tandem repeats (STRs) are rich source of highly polymorphic markers in the human genome. They are relatively small in size, and can be studied with the polymerase chain reaction in a multiplex fashion (Butler, 2005). In this study, we used a commercial available multiplex STRs typing kit to study 21 STRs system (D3S1358, D13S317, D16S539, D18S51, D2S1338, CSF1PO, THO1, vWA, D21S11, D7S820, TPOX, D8S1179, FGA, D5S818, D19S433 and Amelogenin).

These STRs loci include five new nucleotide repeat STR loci: D12S391, PentaE, Penta D, D6S1043 and D1S1656.

### Allele frequency of common autosomal genetic loci

After the samples have been collected, DNA extracted and PCR amplified, they were genotyped for the 20 STR loci of interest. The genotyping information was then converted into allele frequencies by counting the number of times each allele was observed. Allele frequencies for each of the 20 STR loci in the Iraq population sample are shown in Tables 3 and 4. Since there are some alleles which were not sampled sufficiently and that an estimate of an allele frequency is uncertain if the allele is so rare that it is represented only once or a few times in a dataset, it is recommended that each allele be observed at least five times to be used in forensic calculations (Butler et al., 2009). The minimum allele frequency is 5/(2n); where, n is the number of individuals sampled and 2n is the number of chromosomes (as autosomes are in pairs due to inheritance of one chromosome from each parent). In the loci D5S818 (allele 13), the highest allele frequencies are found, and the lowest allele frequencies are at allele13 as seen in D3S1358 locus. D21S11 and D18S51 loci illustrate the largest number of different alleles. The following locations, the most common alleles at the 21 loci, were allele 13 for (D5S818, D8S1179 and D18S51), allele 10 for (D7S820 and Penta D), allele 16 for (D2S1338 and D2S1338), allele 11 for (TPOX, D16S539, PentaE, CSF1PO and D6S1043) loci, allele 12 for (D13S317 and D1S1656), allele 30.2 for D21S11 locus, allele 9 for THO1 locus, allele 13.2 for D21S11 locus, allele 6 for THO locus, alleles 17.3 and 16 for VWA locus, allele 30.2 for D19S433 locus, allele 17 for D3S1358 locus, allele 18 for D5S818 locus, allele 18 for D12S391 locus, allele 14.2 for D12S391 locus and allele 25 for FGA locus. The polymorphic nature of microsatellites (STR) makes them the markers of favorite in properties and genetic diversity studies (Ossmani et al., 2009; Chouery et al., 2010; Imad et al., 2014d). The best indicator of the genetic polymorphism within the sample is verified by the number of alleles and the expected heterozygosity found in the Iraq population. Basically the number of alleles is highly associated with the size of the sample. This is due to the presence of unique alleles in populations, which occur in low frequencies. The usefulness of the markers for genetic screening is verified by the number of alleles scored for each marker (Andreini et al., 2007).

The number of alleles and the expected heterozygosities detected in Iraq population are good indicators of the genetic polymorphism within the breed. Generally the number of alleles is highly dependent on the sample size because of the presence of unique alleles in populations, which occur in low frequencies and also because the number of observed alleles tends to increase with increases in population size. The number of alleles scored for each marker is an invaluable indicator of the future usefulness of the marker for genetic screening. Finding the same number of alleles for certain different loci in various populations (for example, Iran, Syrian, Emirates, Qatar and Egyptian populations) may indicate common ancestries (Reyhaneh and Sadeq, 2009; Alshamali et al., 2003; Ana et al., 2003; Clotilde et al., 2007). The frequency and the number of alleles, however, may be an indication for the degree of inbreeding within each population and thus reflects the homogeneity of the population. The analysis of STR polymorphisms by PCR-based method offers certain advantages over RFLP typing. In recent years, short tandem repeat (STR) systems have gained importance in forensic analysis of biological specimens as well as in paternity testing, as an alternative to the use of restriction fragment length polymorphism (RFLP) analysis (Alshamali et al., 2003; Ana et al., 2003; Clotilde et al., 2007; Hammond et al., 1994). The analysis of STR polymorphisms by PCR-based method offers certain advantages over RFLP typing: (1) STR loci can be typed with a high degree of specificity and sensitivity in a short time period, (2) these loci can be successfully amplified from a limited amount of DNA even if it is degraded, and (3) typing of multiple loci can be accomplished in a single multiplex reaction (Hochmeister et al., 1991; Lins et al., 1996).

### The amelogenin locus

The Amelogenin locus that occurs on both the X and Y chromosomes and enables sex typing (Sullivan et al., 1993) was also located within the reference human genome sequence. AMELX is located on the X chromosome at 10.676 Mb. AMELY is located on the Y chromosome at 6.441 Mb. Amplification of Amelogenin generates different length products from the X and Y-

**Table 3**. Allele frequencies and forensic efficiency parameters (D8S1179- D13S317) in Iraq.

| Allele | D8S1179 | D7S820 | D21S11 | THO1 | vWA | TPOX | D13S317 |
|---|---|---|---|---|---|---|---|
| 2.2 | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - |
| 6 | - | - | - | 0.0455 | - | - | - |
| 7 | - | 0.0265 | - | 0.1713 | - | - | - |
| 8 | 0.127 | 0.1896 | - | 0.1326 | - | 0.0197 | 0.2318 |
| 9 | 0.0064 | 0.1003 | - | 0.2362 | - | 0.0383 | 0.0373 |
| 9.3 | - | - | - | 0.1344 | - | - | - |
| 10 | 0.076 | 0.3304 | - | 0.18 | - | 0.1013 | 0.0546 |
| 10.2 | - | - | - | - | - | - | - |
| 11 | 0.1344 | 0.2054 | - | - | - | 0.2637 | 0.2248 |
| 12 | 0.1153 | 0.1115 | - | - | - | 0.2005 | 0.343 |
| 12.2 | - | - | - | - | - | - | - |
| 13 | 0.2153 | 0.0363 | - | - | - | 0.1985 | 0.0534 |
| 13.2 | - | - | - | - | - | - | - |
| 14 | 0.142 | - | - | - | 0.0917 | 0.178 | 0.0551 |
| 14.2 | - | - | - | - | - | - | - |
| 15 | 0.0549 | - | - | - | 0.0594 | - | - |
| 15.2 | - | - | - | - | - | - | - |
| 15.3 | - | - | - | - | - | - | - |
| 16 | 0.0181 | - | - | - | 0.2824 | - | - |
| 16.2 | - | - | - | - | - | - | - |
| 16.3 | - | - | - | - | - | - | - |
| 17 | - | - | - | - | 0.2806 | - | - |
| 18 | - | - | - | - | 0.0588 | - | - |
| 19 | - | - | - | - | 0.0212 | - | - |
| 20 | - | - | - | - | 0.2059 | - | - |
| 21.2 | - | - | - | - | - | - | - |
| 27 | - | - | - | - | - | - | - |
| 28 | - | - | 0.0614 | - | - | - | - |
| 28.2 | - | - | - | - | - | - | - |
| 29 | - | - | 0.1817 | - | - | - | - |
| 29.2 | - | - | - | - | - | - | - |
| 30 | - | - | 0.1393 | - | - | - | - |
| 30.2 | - | - | 0.1615 | - | - | - | - |
| 31 | - | - | 0.0596 | - | - | - | - |
| 31.2 | - | - | 0.0113 | - | - | - | - |
| 32 | - | - | 0.0485 | - | - | - | - |
| 32.2 | - | - | 0.0589 | - | - | - | - |
| 33 | - | - | 0.0589 | - | - | - | - |
| 33.2 | - | - | 0.0983 | - | - | - | - |
| 34 | - | - | 0.0029 | - | - | - | - |
| 34.2 | - | - | 0.1066 | - | - | - | - |
| 35 | - | - | 0.0112 | - | - | - | - |

chromosomes. Figure 1 shows the possible peaks for both genotypes X/X and X/Y represented as one and two peaks, respectively, in the GeneMapper electropherogram.

**New additional autosomal genetic loci**

To improve results with challenging DNA samples, a set of 20 autosomal STR loci was characterized in our

**Table 4**. Allele frequencies and forensic efficiency parameters (D19S433 - CSF1PO) in Iraq.

| Allele | D19S433 | D2S1338 | D18S51 | D16S539 | FGA | D5S818 | D3S1358 | CSF1PO |
|---|---|---|---|---|---|---|---|---|
| 2.2 | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - |
| 6 | - | - | - | - | - | - | - | - |
| 7 | 0.0023 | - | - | - | - | - | - | - |
| 8 | - | - | - | 0.1788 | - | - | - | 0.0158 |
| 9 | 0.0939 | - | - | 0.1012 | - | 0.0228 | - | 0.036 |
| 9.3 | - | - | - | - | - | - | - | - |
| 10 | 0.0082 | - | 0.0086 | 0.2185 | - | 0.0124 | - | 0.2636 |
| 10.2 | - | - | 0.0208 | | - | - | - | - |
| 11 | 0.1834 | - | 0.0454 | 0.251 | - | 0.0382 | - | 0.2858 |
| 12 | 0.157 | - | 0.1782 | 0.0987 | - | 0.0133 | - | 0.2633 |
| 12.2 | 0.1148 | - | - | - | - | - | - | - |
| 13 | 0.0726 | - | 0.2459 | 0.1003 | - | 0.4375 | 0.0037 | 0.0622 |
| 13.2 | 0.2169 | - | - | - | - | - | - | |
| 14 | 0.0745 | - | 0.17 | 0.0515 | - | 0.3588 | 0.04 | 0.011 |
| 14.2 | 0.0424 | - | | - | - | - | - | - |
| 15 | 0.0109 | 0.0583 | 0.1044 | - | - | 0.0208 | 0.2267 | 0.0633 |
| 15.2 | 0.0598 | - | - | - | - | - | - | - |
| 15.3 | - | - | - | - | - | - | - | - |
| 16 | - | 0.2015 | 0.1471 | - | - | 0.0407 | 0.2394 | - |
| 16.2 | - | - | - | - | - | - | 0.001 | - |
| 16.3 | - | - | - | - | - | - | 0.001 | - |
| 17 | - | 0.1383 | 0.0473 | - | - | - | 0.3471 | - |
| 18 | - | 0.0785 | 0.0111 | - | - | - | 0.1231 | - |
| 19 | - | 0.1513 | 0.0064 | - | - | - | 0.02 | - |
| 20 | - | 0.0587 | 0.0112 | - | 0.1014 | - | - | - |
| 20.2 | - | - | - | - | - | - | - | - |
| 21 | - | 0.0388 | 0.0112 | - | 0.0379 | - | - | - |
| 21.2 | - | - | - | - | - | - | - | - |
| 22 | - | 0.0612 | - | - | 0.1817 | - | - | - |
| 22.2 | - | - | - | - | 0.1389 | - | - | - |
| 23 | - | 0.0083 | - | - | 0.0787 | - | - | - |
| 23.2 | - | - | - | - | - | - | - | - |
| 24 | - | 0.0717 | - | - | 0.1373 | - | - | - |
| 24.2 | - | - | - | - | - | - | - | - |
| 25 | - | 0.0617 | - | - | 0.2429 | - | - | - |
| 26 | - | 0.0717 | - | - | 0.0812 | - | - | - |
| 27 | - | - | - | - | - | - | - | - |
| 28 | - | - | - | - | - | - | - | - |
| 28.2 | - | - | - | - | - | - | - | - |
| 30 | - | - | - | - | - | - | - | - |
| 30.2 | - | - | - | - | - | - | - | - |
| 31 | - | - | - | - | - | - | - | - |

laboratory at NIST (Hill et al., 2009). The efforts of the Human Genome Project have increased knowledge regarding the human genome, and hence there are many more STR loci available now than there were 10 years ago. In fact, more than 20,000 tetranucleotide STR loci have been characterized in the human genome (Collins

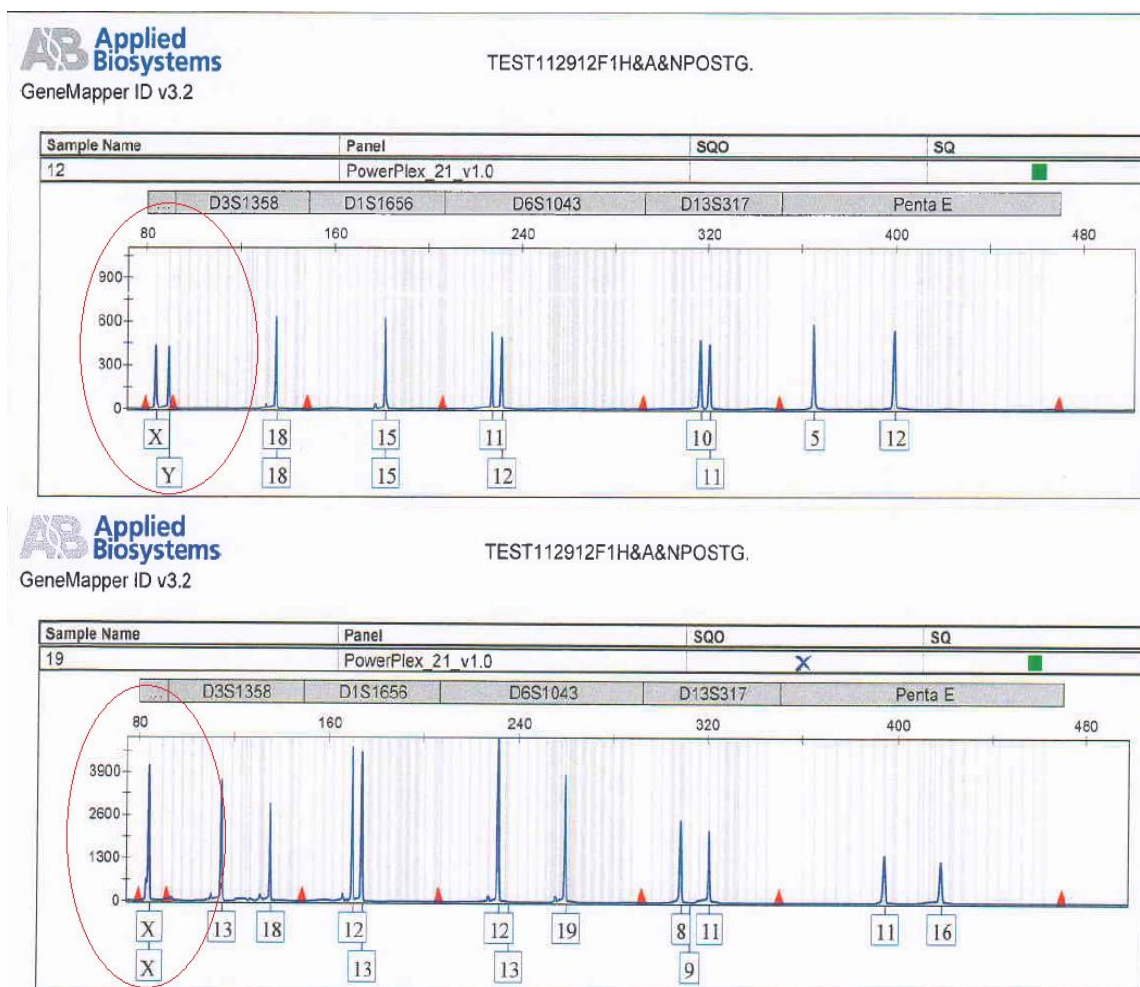


**Figure 1.** An electropherogram showing Amelogenin locus and its two possible genotypes obtained with PowerPlex®21 amplification and GeneMapper version 4.0.

et al., 2003) and there may be more than a million STR loci present depending on how they are counted (Ellegren, 2004). STR sequences account for approximately 3% of the total human genome (Landerand Linton, 2001).

## Allele frequency of D12S391 genetic loci

D12S391 is a highly polymorphic compoundtetranucleotide found on the short arm of chromosome 12 only 6.3 megabases from vWA. It possesses over 50 different alleles ranging from 13 to 27.2 repeats in length. A number of same-size, different-sequence alleles have been identified through sequence analysis. It is part of the extended European Standard Set and is present in NGM™ and NGM SElect™ kits from Applied Biosystems, the PowerPlex® ESI and ESX Systems from Promega, and ESSplex and ESSplex SE kits from Qiagen.

D12S391 is one of new nucleotide repeat STR loci and eight alleles were found in this study. As shown in Figure 2, the allele 18 was most frequent 0.359.

## Allele frequency of Penta D genetic loci

Penta D is a pentanucleotide repeat found on chromosome 21 about 25 Mb from D21S11. Alleles ranging from 1.1 to 19 repeats have been observed although some of the shorter alleles are likely due to flanking region deletions (Kline et al., 2011). Penta D is present in the PowerPlex® 16 and PowerPlex® 18D kits. Eleven (11) alleles were found in this study, and as shown in Figure 3, the allele 10 was most frequent 0.398.

## Allele frequency of penta E genetic loci

Penta E is a pentanucleotide repeat with very low stutterproduct formation that is located on the long arm of

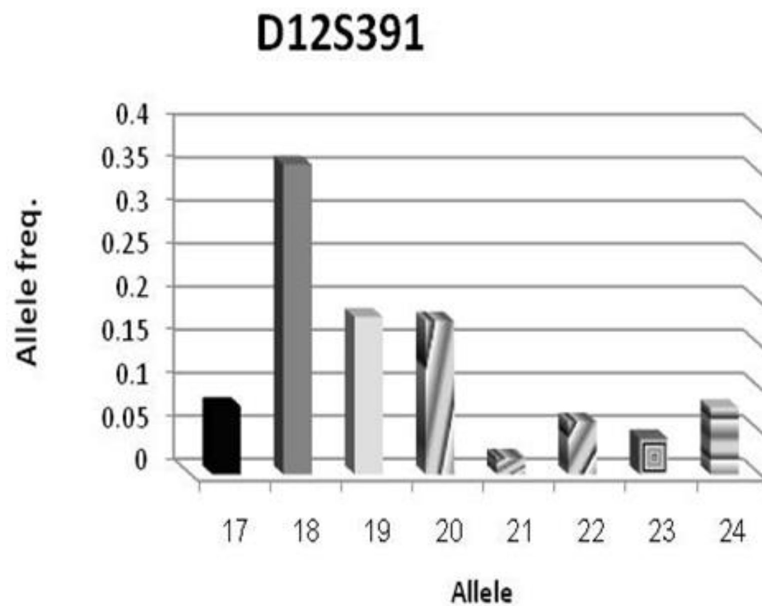


**Figure 2.** Allele frequency of D12S391 genetic locus.

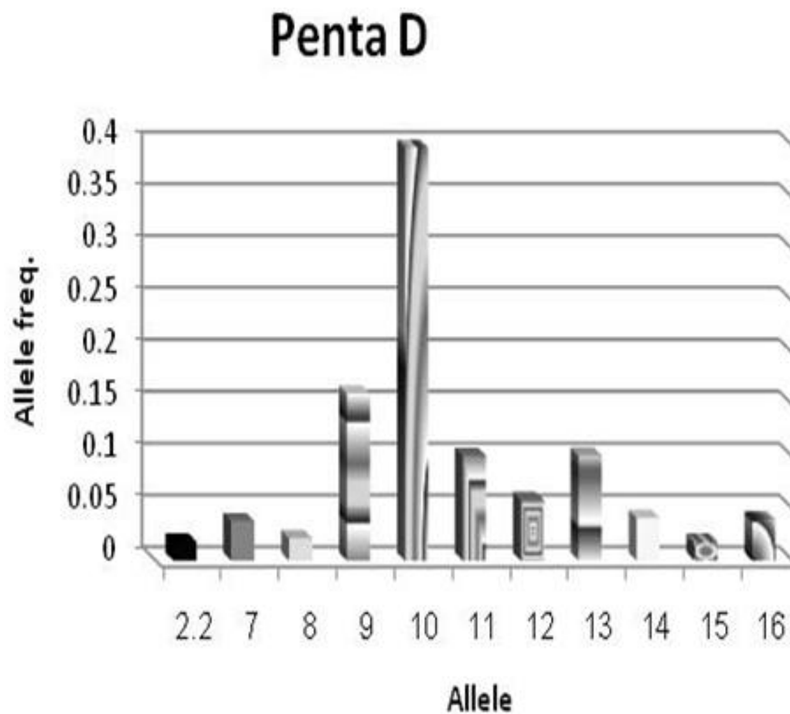


**Figure 3.** Allele frequency of Penta D genetic locus.

chromosome 15 with alleles ranging from 5 to 32 repeats. Penta E is highly polymorphic and is present in the PowerPlex® 16 and PowerPlex® 18D kits. The Penta E locus on chromosome 15 , one of the new pentanucleotide repeat STR loci examined in this study was highly polymorphism and 10 allele were found in this iraq population. As shown in Figure 4, the allele 11 was most frequent 0.241. The distribution was different from those reported in the Caucasian American and African American populations (Budowle et al., 2001; Levadokou

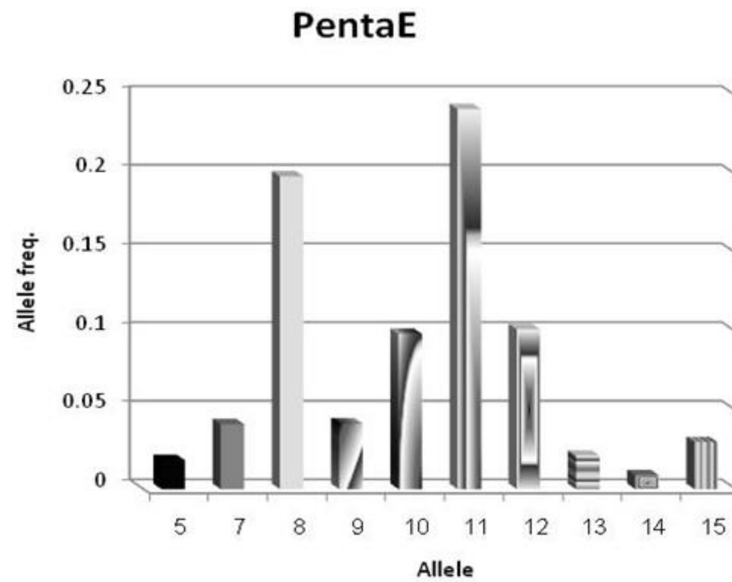


**Figure 4.** Allele frequency of Penta E genetic locus.

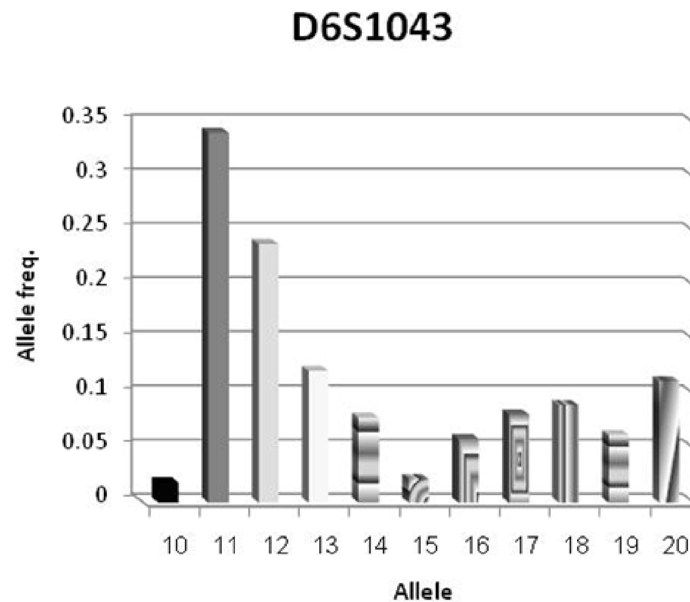


**Figure 5.** Allele frequency of D6S1043 genetic locus.

et al., 2001). As mentioned above, allele 13 and 14, which had not been detectrd in Caucasian and Africans, were found in the present Iraq population.

### Allele frequency of D6S1043 genetic loci

D6S1043 is a compound tetranucleotide repeat with alleles ranging from 8 to 25 AGAT or AGAC repeats. Some x.2 and x.3 alleles have been reported in population studies. D6S1043 is part of the Sinofiler™ kit and has been used to date almost exclusively in Chinese and other Asian population studies. D6S1043 is located less than 4 Mb from SE33 on the long arm of chromosome 6. Eleven (11) alleles were found in this study, and as shown in Figure 5, the allele 11 was most frequent 0.238.

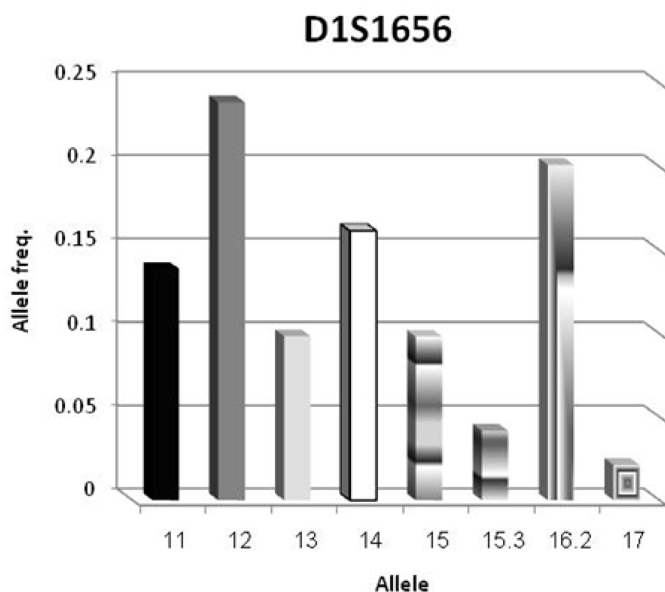


**Figure 6.** Allele frequency of D1S1656 genetic locus.

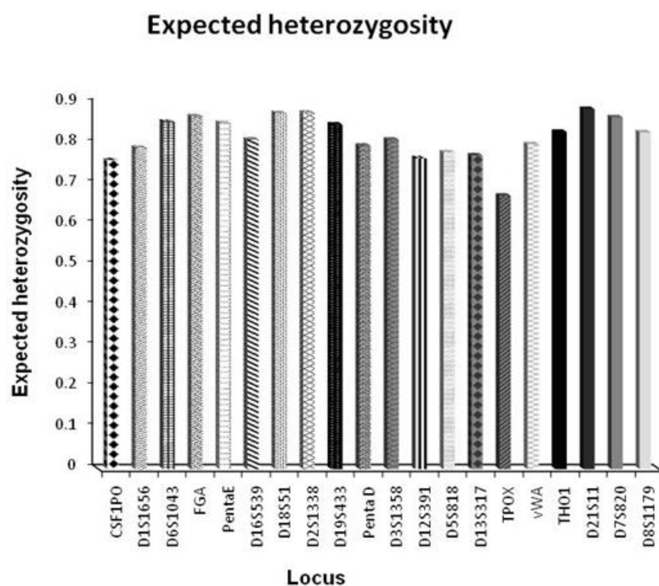


**Figure 8.** Expected heterozygosity of twenty autosomal genetic loci.

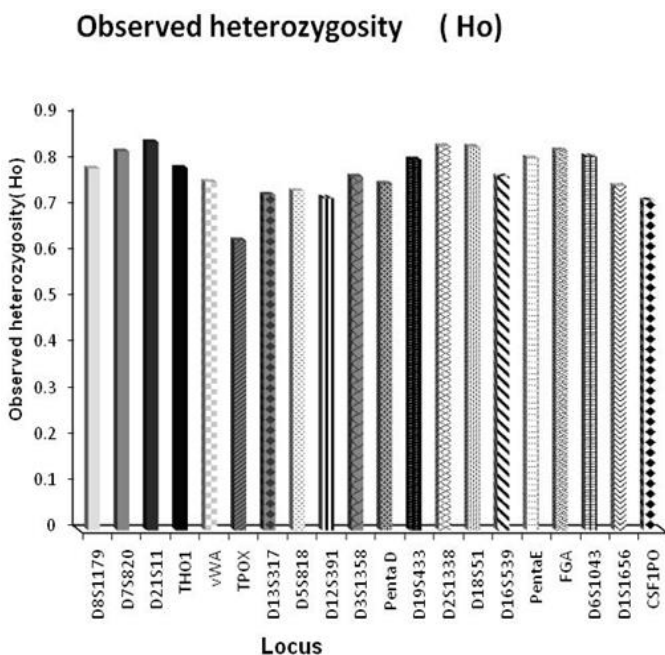


**Figure 7.** Observed Heterozygosity of twenty autosomal genetic loci.

## Allele frequency of D1S1656 genetic loci

D1S1656 is a tetranucleotide repeat found on the long arm of chromosome 1 with alleles ranging from 8 to 20.3 repeats. The x.3 alleles arise from a TGA insertion typically after four full TAGA repeats. It is part of the extended European Standard Set and is present in NGM™ and NGM SElect™ kits from Applied Biosystems, the PowerPlex® ESI and ESX Systems from Promega, and ESSplex and ESSplex SE kits from Qiagen. The D1S1656 is one of the new nucleotide repeat STR loci examined in this study which was highly polymorphic and eight alleles were found. As shown in Figure 6, the allele 12 was most frequent (0.238).

## Forensic efficiency parameters

Statistical analysis is used to interpret DNA results for genetic identity. In order to determine the significance of a match, it is necessary to support DNA typing results with statistical analysis. These analyses assign a value to the results obtained and enable easier resolution of forensic or paternity cases. Across all loci, the values for the matching probability, power of discrimination, power (chance) of exclusion, polymorphism information content and typical paternity index for the 20 STR loci of the Iraq population were determined and are indicated in Figures 7 to 14.

## Observed heterozygosity and expected heterozygosity

The two common ways to report heterozygosity are observed and expected heterozygosities. Observed heterozygosity is calculated by dividing the number of heterozygote individuals at a locus by the total number of all individuals at that locus and describes the heterozygosity actually observed in the members of the sampling group. Expected heterozygosity is calculated as

**Table 5.** The observed heterozygosity in different populations.

| Locus | Iraq | Turkey | Iran | Syria | Emirates | Qatar | Egypt | Gaza |
|---|---|---|---|---|---|---|---|---|
| D21S11 | 0.841 | - | - | 0.664 | - | - | 0.682 | 0.682 |
| TPOX | 0.628 | 0.567 | - | - | - | - | - | - |
| Penta E | - | - | - | 0.897 | - | - | - | - |
| FGA | - | - | - | - | - | - | 0.890 | |
| D18S51 | - | 0.790 | - | - | 0.130 | - | - | 0.912 |
| D2S1338 | - | - | - | - | - | 0.839 | - | - |
| vWA | - | - | - | - | - | 0.542 | - | - |
| LPL | - | - | 0.780 | - | - | - | - | - |
| F13A01 | - | - | 0.720 | - | - | - | - | - |
| D5S818 | - | - | - | - | 0.295 | - | - | - |

1 minus the homozygosity (the sum of squares of all allele frequencies at a locus) and represents the number of heterozygotes that would be expected under HWE based on the observed allele frequencies in the sampling group. A higher heterozygosity means that more allele diversity exists, and therefore there is less chance of a random sample matching. Observed heterozygosity and expected heterozygosity all over the 20 loci are presented in Figures 7 and 8, and the observed heterozygosity oscillated between studied populations as illustrated in Table 5.

The observed heterozygosity in a population relies on the number and the frequency of alleles of each locus. Moreover, the distribution of genotypes in a population sample may deviate from HWE expectation in a number of ways. These include the presence of an excess of homozygotes and a corresponding lack of heterozygotes or an excess (deficiency) of one or more classes of heterozygotes or a combination of those states. There are populations with low heterozygosity, lower than 65% in most tested loci. These populations are small, closed, inbred by cultural or geographical factors; one of those populations is the Qatari population where the levels of observed heterozygosity (*Ho)* oscillated, between 0.339 for D19S433 and 0.839 for D2S1338. Interestingly, *Ho* is lower than expected heterozygosity *(He) in* almost all the analyzed loci, with the unique exception of D5S818 locus. This fact was particularly conspicuous for the loci that deviated from the HWE expectations; the departures from HWE expectations detected in the Qatari population seem to be the result of excess of homozygotes over heterozygotes, which is likely to be the consequence of the high consanguinity rates reported for this population, which is 46% (Ana et al., 2006).

## Paternity index

The paternity index (PI) compares the likelihood that a genetic marker (allele) that the alleged father (AF) passed to the child to the probability that a randomly selected unrelated man of similar ethnic background could pass the allele to the child. The potential of a randomly selected man to pass the obligate gene is determined by using a database, which lists the frequency distribution of individual alleles within a given genetic system. Combined paternity index is an odd ratio that indicates how many times more likely it is that the alleged father is the biological father than a randomly selected unrelated man of similar ethnic background. The paternity index was high for all STR analyzed; it ranged from 2.651 (TPOX) to 2.864 (D21S11) (Figure 9).

## Random match probability

The match probability is the probability for a random match between two unrelated individuals drawn from the same population. It is the sum of the frequency squared of each genotype its that ranged from 0.011 to 0.168 (Figure 10).

A random match probability, is the probability that a person sampled randomly from the population, would have a particular STR profile. If assumptions can be made that (1) alleles within a locus are inherited independently and (2) that STR loci migrate independently of one another during meiosis, then it is possible to multiply the specific expected genotype frequencies (based on allele frequencies) for all the loci together to obtain the overall random match probability. This combination of all the loci match probabilities by multiplication is termed the product rule. The strength of a genetic match between a questioned sample and a known sample (rarity of a particular DNA profile) relies on the discriminative power of the product rule. Thus the assumptions of independence for alleles within a locus and independence among migrating loci are central to determining the strength of a match (Butler et al., 2005).

## Power of discrimination

Power of discrimination is defined as the probability that

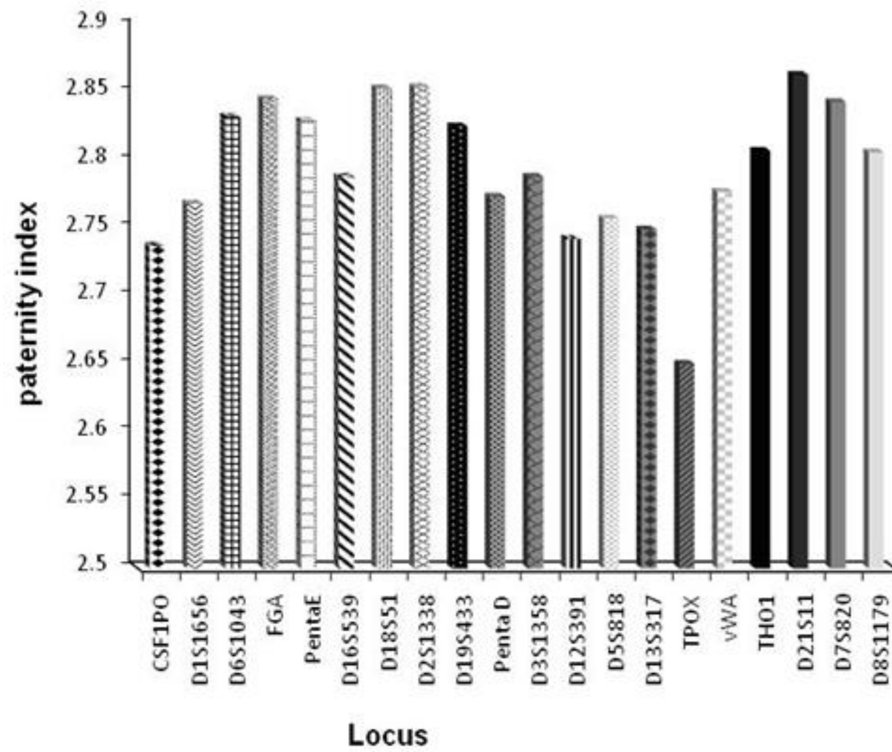


**Figure 9.** Paternity Index of twenty autosomal genetic loci.

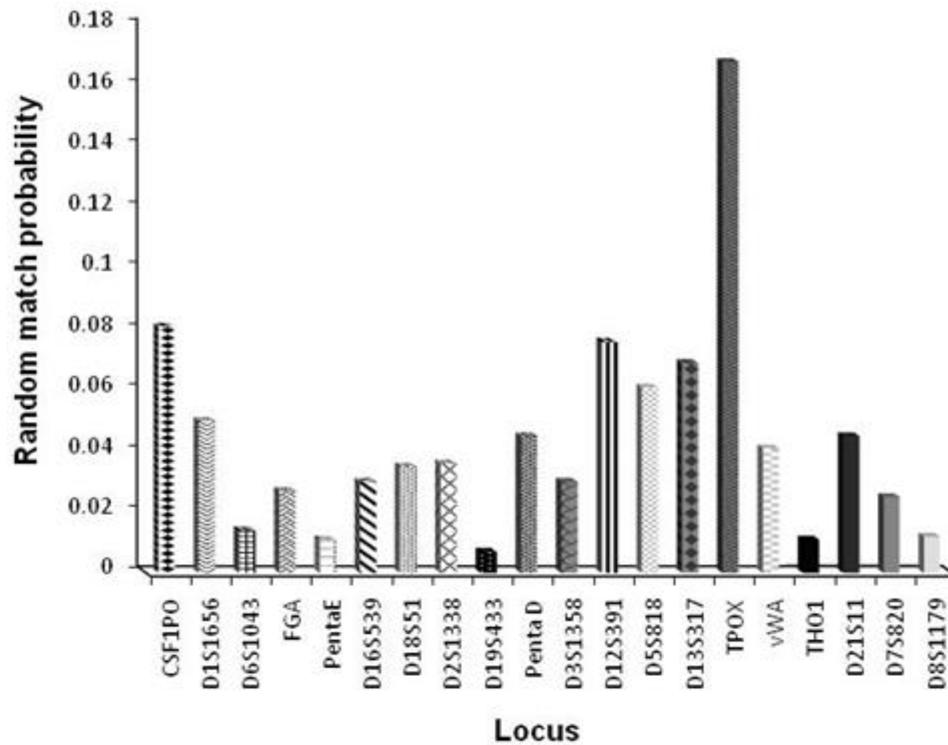


**Figure 10.** Random match probability of 20 autosomal genetic loci.

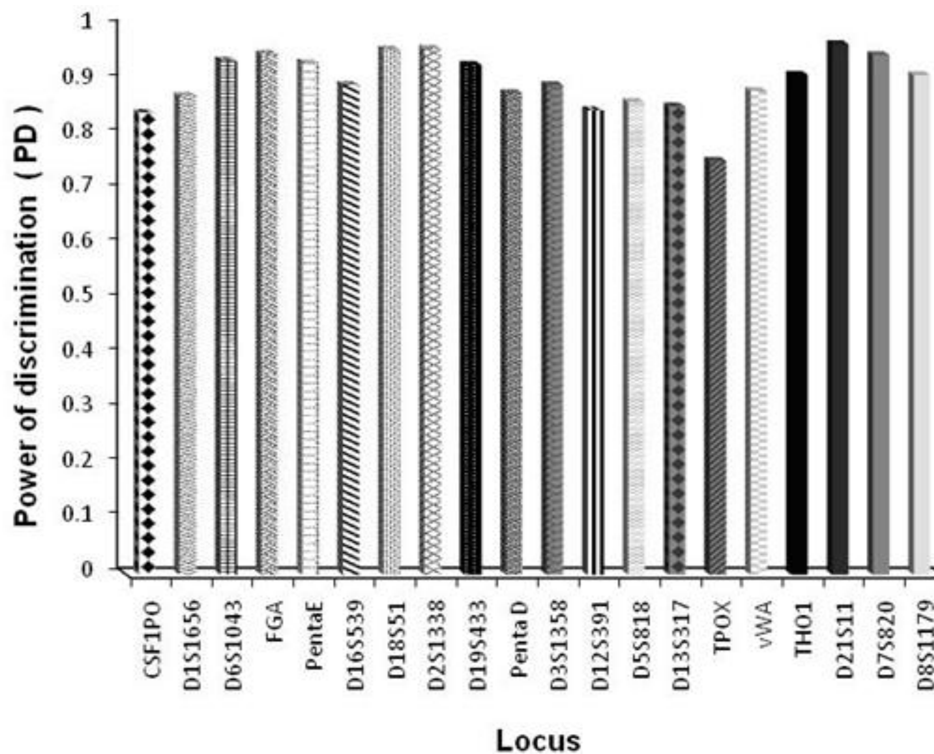


**Figure 11.** Power of discrimination of 20 autosomal genetic loci.

**Table 6.** The Power of discrimination in different populations.

| Locus | Iraq | Turkey | Iran | Syria | Emirates | Qatar | Egypt | Gaza |
|---|---|---|---|---|---|---|---|---|
| D21S11 | 0.969 | - | - | - | - | - | - | - |
| D2S1338 | 0.960 | - | - | - | - | 0.973 | - | - |
| D18S51 | 0.959 | 0.953 | - | 0.963 | 0.971 | - | - | 0.962 |
| TPOX | 0.759 | 0.772 | - | - | - | 0.855 | - | - |
| Penta E | - | - | - | 0.974 | - | - | - | 0.976 |
| Penta D | - | - | - | 0.951 | - | - | - | 0.961 |
| FGA | - | - | - | 0.957 | - | - | 0.973 | 0.967 |
| vWA | - | - | - | - | - | - | 0.937 | - |
| LPL | - | - | 0.924 | - | - | - | - | - |
| F13A01 | - | - | 0.922 | - | - | - | - | - |
| D5S818 | - | - | - | - | 0.889 | - | - | - |

two individuals selected at random from the population will not have an identical genotype at the locus. Power of discrimination (PD) for all the 20 loci is presented in Figure 11. Values for all tested loci was 75% for TPOX; ranged from 80 to 89% for the D3S1358, D13S317, D5S818, D12S391, vWA, Penta D, D16S539, D1S1656 and CSF1PO loci, and ranged from 91 to 96% for the rest of the loci. This infers that a DNA-based database for Iraq population can be safely used by using these loci. The possibility to find two persons with the same DNA profile if chosen at random in a population is defined as the matching possibility. It is impossible to find two individuals with the same genotype in the population because most common alleles for account of most common genotypes frequency and matching probability are not present. The highest PD observed in some populations is presented in Table 6.

The Penta E and Penta D loci included in the PowerPlex®21 PCR amplification kits were not typed in the Turkey, Emirates, Iran or Qatari populations because

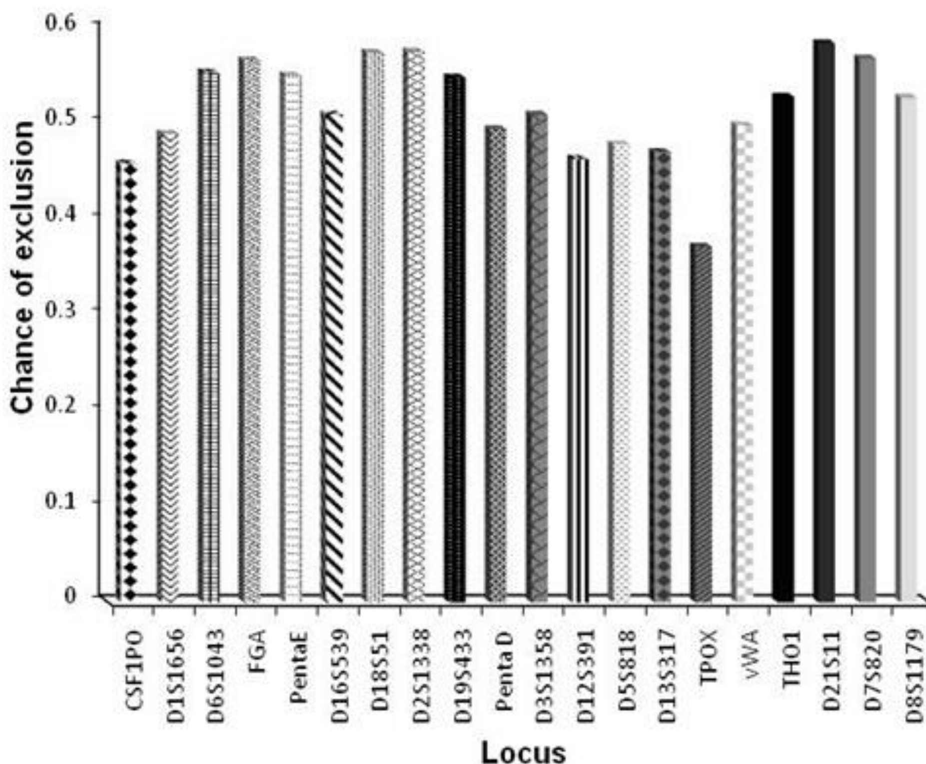


**Figure 12.** Chance of exclusion of twenty autosomal genetic loci.

**Table 7.** The power of exclusion in different populations.

| Locus | Iraq | Turkey | Iran | Syria | Emirates | Egypt | Gaza |
|---|---|---|---|---|---|---|---|
| D21S11 | 0.585 | - | - | - | - | - | - |
| D18S51 | - | 0.790 | - | - | 0.762 | - | 0.820 |
| TPOX | 0.372 | 0.258 | - | - | - | 0.326 | 0.364 |
| Penta E | - | - | - | 0.788 | - | - | - |
| CSF | - | - | - | 0.387 | - | - | - |
| FGA | - | - | - | - | - | 0.775 | - |
| LPL | - | - | 0.564 | - | - | - | - |
| F13A01 | - | - | 0.473 | - | - | - | - |
| D5S818 | - | - | - | - | 0.505 | - | - |

they used different kits in their genotyping studies. The combined discrimination power (CDP) for the Iraq population of middle and south of Iraq for the corresponding 20 STR loci used, has been calculated as 0.999999972. These results mean that those loci can be safely used to establish a DNA-based database for Iraq population.

## Chance of exclusion

Power of exclusion is the probability of excluding a random person as the contributor of an allele to a child at the locus. The power of exclusion (PE) can be calculated to express how rare it would be to find a random man who could not be excluded as the biological father of the child. In the present study and from the genotyping data, PE for every locus was calculated and presented in Figure 12. As expected, the power of exclusion was high for all the microsatellites analyzed; it ranged from 0.372 (TPOX) to 0.820 (D18S51). PE for different populations is given in Table 7. The combined power of exclusion, which is the exclusion probability considering all 20 loci,

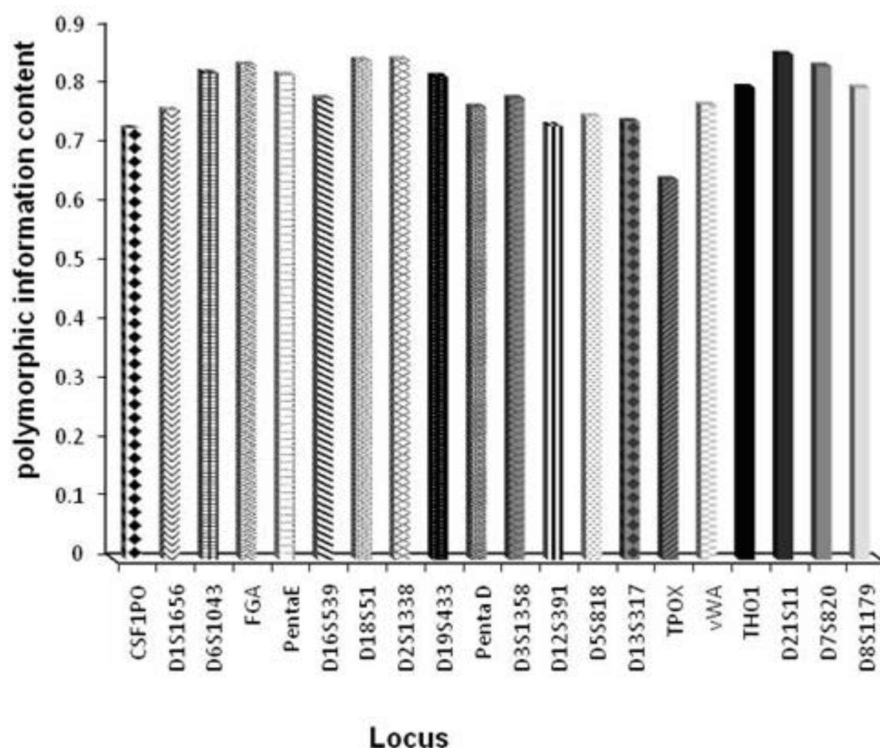


**Figure 13.** Polymorphic Information Content of twenty autosomal genetic loci.

**Table 8.** Polymorphic Information Content in different populations.

| Locus | Iraq | Turkey | Iran | Syria | Qatar | Egypt | Gaza |
|---|---|---|---|---|---|---|---|
| D21S11 | 0.859 | - | - | - | - | - | - |
| D18S51 | - | 0.840 | - | - | - | - | - |
| D2S1338 | - | - | - | - | 0.865 | - | - |
| TPOX | 0.646 | 0.540 | - | 0.640 | 0.638 | 0.610 | 0.650 |
| Penta E | - | - | - | 0.890 | - | - | 0.900 |
| FGA | - | - | - | - | - | 0.870 | - |
| LPL | - | - | 0.770 | - | - | - | - |
| F13A01 | - | - | 0.760 | - | - | - | - |
| D5S818 | - | - | - | - | - | - | - |

was greater than 99.989%, indicating that these loci are appropriate to determine parentage in Iraq population beyond any reasonable doubt.

## Polymorphic information content

Polymorphism information content is the probability that a given offspring of a parent possessing a rare allele at a locus will allow inferring of the parental genotype at the locus. The TPOX locus is the least polymorphic marker while D21S11 is the most polymorphic marker based on the degree of polymorphism of every marker, expressed in PIC terms (Figure 13). The usefulness of the findings of genetic polymorphism studies and linkage mapping programs in humans is confirmed by the high PIC values of the selected markers.

Similar degree of polymorphism was found in Syrian population, where the most polymorphic marker was found to be Penta E, and that the least polymorphic one was TPOX (Table 8). On the contrary, the Egyptian population showed that the FGA locus is the most polymorphic marker and that the TPOX locus, as observed in our population, is the least polymorphic marker. Even in other populations like the Iran, Qatar and Turkey, it was found

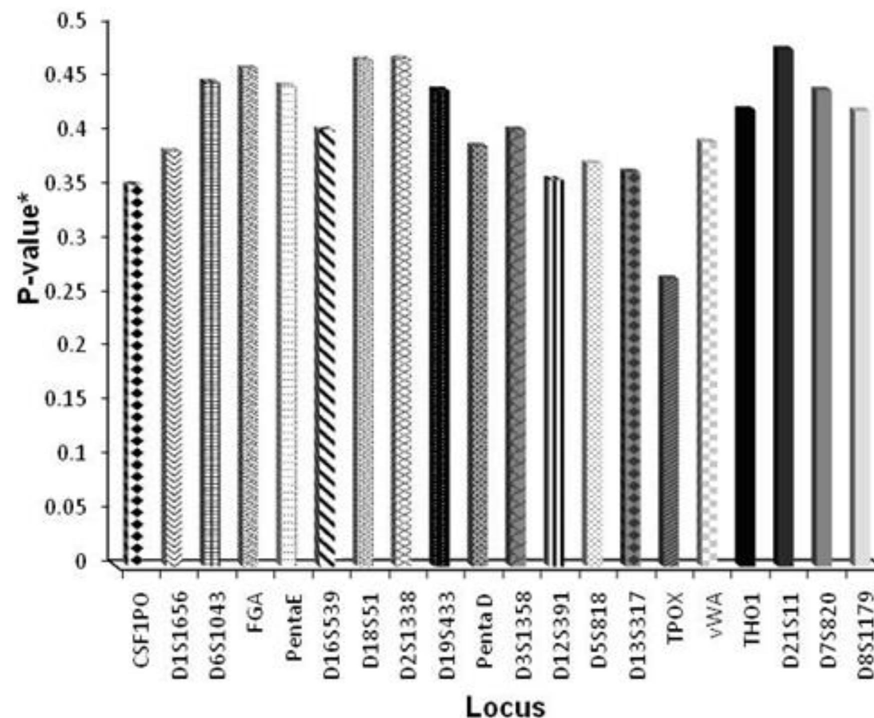


**Figure 14.** P-value of twenty autosomal genetic loci.

that the previously mentioned loci have a similar degree of polymorphism (Ana et al., 2006; Reyhaneh and Sadeq, 2009). The polymorphic nature of microsatellites makes them the markers of choice in characterization and genetic diversity studies. The high PIC values of the selected markers confirm their usefulness for genetic polymorphism studies and linkage mapping programs in human as well. The mean heterozygosity observed, expected and mean PIC values across the 20 loci were 0.77, 0.81 and 0.78, respectively, indicating high gene diversity.

## P-value: probability value of Chi-square test for Hardy Weinberg equilibrium

Checking for HWE is performed by taking the observed allele frequencies and calculating the expected genotype frequencies based on the allele frequencies. If the observed genotype frequencies are close to the expected genotype frequencies calculated from the observed allele frequencies, then the population is in Hardy–Weinberg equilibrium and allele combinations are likely to be independent of one another. The results obtained from the tests for HWE is shown in Figure 14. The null hypothesis states that all the STR loci tested are in HWE and any deviation from HWE expectations is due to sampling error (Hill et al., 2009). The alpha value was set at 0.05. The null hypothesis was rejected if the computed p values were below the alpha value of 0.05. Therefore having a p value above 0.05 to show that the STR alleles do not differ significantly from HWE and does not imply that the samples are in HWE. Butler (2005) states that “if a p-value of < 0.05 is observed with a set of alleles measured at a particular STR locus, it does not mean that a laboratory should avoid using this data because it ‘failed’ a test for Hardy-Weinberg equilibrium”

## Most common genotype frequencies

Another measure to reflect the usefulness of a particular set of DNA markers is to examine the frequencies of the most common genotypes, which would therefore be the least powerful in terms of being able to differentiate between two unrelated individuals (Edwards et al., 1991). The theoretically most common type can be calculated by considering a sample type that is heterozygous at all loci possessing the two most common alleles at each locus (Lander and Linton, 2001; Imad et al., 2014e). In Table 9, frequencies from the two most common alleles at each of the twenty loci were used to estimate a theoretical most common STR profile. A number of the newly available STR loci, such as D1S1659 and D6S1043, provide a better probability of identity than widely used loci such as D8S1179 and D2S1338. The “most common genotypes

**Table 9.** Most common genotype frequencies based on the two most common alleles found in a Iraq population.

| Locus | Allele 1 | Allele 2 | Allele1 Frequency (P) | Allele2 Frequency (q) | 2pq | Most common genotype frequency |
|---|---|---|---|---|---|---|
| D8S1179 | 13 | 14 | 0.2153 | 0.142 | 2pq | 0.0611 |
| D7S820 | 10 | 11 | 0.3304 | 0.2054 | 2pq | 0.1357 |
| D21S11 | 29 | 30.2 | 0.1817 | 0.1615 | 2pq | 0.0587 |
| THO1 | 9 | 10 | 0.2362 | 0.18 | 2pq | 0.0851 |
| vWA | 16 | 20 | 0.2824 | 0.2059 | 2pq | 0.1163 |
| TPOX | 11 | 12 | 0.2637 | 0.2005 | 2pq | 0.1057 |
| D13S317 | 8 | 12 | 0.2318 | 0.343 | 2pq | 0.1591 |
| D5S818 | 13 | 14 | 0.4375 | 0.3588 | 2pq | 0.3139 |
| D12S391 | 18 | 19 | 0.3597 | 0.1827 | 2pq | 0.1314 |
| D3S1358 | 16 | 17 | 0.2394 | 0.3471 | 2pq | 0.1662 |
| Penta D | 9 | 10 | 0.1614 | 0.3985 | 2pq | 0.1286 |
| D19S433 | 11 | 13.2 | 0.1834 | 0.2169 | 2pq | 0.0796 |
| D2S1338 | 16 | 19 | 0.2015 | 0.1513 | 2pq | 0.0609 |
| D18S51 | 12 | 13 | 0.1782 | 0.2459 | 2pq | 0.0876 |
| D16S539 | 8 | 11 | 0.1788 | 0.251 | 2pq | 0.0898 |
| PentaE | 8 | 11 | 0.1988 | 0.2415 | 2pq | 0.096 |
| FGA | 22 | 25 | 0.1817 | 0.2429 | 2pq | 0.0883 |
| D6S1043 | 11 | 12 | 0.3412 | 0.2358 | 2pq | 0.1609 |
| D1S1656 | 12 | 16.2 | 0.2387 | 0.2012 | 2pq | 0.2153 |
| CSF1PO | 10 | 11 | 0.2636 | 0.2858 | 2pq | 0.0961 |

frequency" results column can also be a useful metric to locus performance. For example, a comparison of D1S1659 and THO1 is instructive. These two loci both have 44 most common allele frequency, yet D1S1659 has 0.2153 common genotypes frequency while THO1 only has 0.0851. The greater number of genotypes formed with the different combinations of alleles in D1S1659 leads to better probability of identity (0.050 vs. 0.011) values. Furthermore, additional genotype combinations mean that D1S1659 will likely be more useful than THO1 for detecting contributors in DNA mixtures.

**New alleles are constantly being discovered that do not size exactly with the ladder alleles. These "off-ladder"**

STR typing is typically performed using size comparisons to standardized allelic ladders that possess the most common alleles, which have been sequenced to reveal the true number of repeats. Different STR kit manufacturers may supply allelic ladders with slightly different allele ranges. As more samples are run with STR loci, new alleles are constantly being discovered that do not size exactly with the ladder alleles. These "off-ladder" alleles can be variants with more or less of the core repeat unit than present in the common alleles found in the commercially available allelic ladder. Alternatively, these variant alleles may contain partial repeats or insertions/deletions in the nearby flanking region to the repeat. Insertion/deletion event that creates off-ladder alleles is found in new alleles can be discovered that occur outside the range defined by the commercially available allelic ladder. In many instances, these alleles are simply classified as greater than the largest allele as (or smaller than the smallest allele) in the ladder rather than attempting to extrapolate to a predicted number of repeats. Table 10 contains a list of variant or "off-ladder" alleles that have been reported to the NIST STRBase website as of April 2005. Off-ladder alleles are rare alleles that are not represented in the locus-specific allelic ladders. These off-ladder alleles do not fit within the 0.5 bp range of corresponding alleles in the allelic ladder. Since such alleles cannot be sized by direct comparisons to the reference alleles in the allelic ladders, genotyping software will often designate them as "off-ladder alleles". An off-ladder allele may occur between two alleles in the allelic ladder or it may be smaller (or larger) than the smallest (or largest) allele in the allelic ladder (Butler, 2005). To identify an off-ladder allele, the size (in base pairs) of the off-ladder allele is compared to the sizes of the two closest alleles in the allelic ladder.

A total of nine off-ladder alleles were detected in this study (Table 10). Three of them are located at the D1S1656 locus (Figure 15A, B and C); one is located in D12S391 (Figure 16). One occurred at the D19S433 locus (Figure 17). All of the samples containing off-ladder alleles were analyzed twice on the ABI 310 to confirm the data. Alleles' sizes in base pairs are all generated by the

**Table 10.** New variant or “off-ladder” discovered in this study and comparison with alleles reported in STRBase.

| STR Locus | Number Reported | STRBase | In our study | |
|---|---|---|---|---|
| | | Variant alleles reported as of Apr 2005 | STR Locus | Variant alleles |
| CSF1PO | 11 | 5, 7.3, 8.3, 9.1, 9.3, 10.1, 10.2, 10.3, 11.1, 12.1, 16 | - | - |
| D12S391 | 0 | None reported yet in STRBase | D12S391 | 18.3 |
| FGA | 69 | 12.2, 13.2, 14, 14.3, 15, 15.3, 16,16.1, 16.2, “<17”, 17, 17.2, 18.2,19.1, 19.2, 19.3, 20.1, 20.2, 20.3, 21.1, 21.2,21.3, 22.1, 22.2, 22.3, 23.1, 23.2, 23.3,24.1, 24.2, 24.3, 25.1, 25.2, 25.3, 26.1,26.2, 26.3, 27.3, 29.2, 30.2, 31, 31.2, 32.1,32.2, 33.1, 34.1, 34.2, 35.2, 41.1, 41.2, 42.1, 42.2, 43.1, 43.2, 44, 44.1, 44.2, 44.3, 45.1, 45.2, 46.1, 46.2,47.2, 48.2, 49, 49.1, 49.2, 50.2, 50.3 | - | - |
| TH01 | 7 | 4, 7.3, 8.3, 9.1, 10.3, 11, 13.3 | - | - |
| TPOX | 7 | 4, 5, 7.3, 13.1, 14, 15, 16 | - | - |
| VWA | 6 | 16.1, 18.3, 22, 23, 24, 25 | - | - |
| D3S1358 | 18 | 8, 8.3, 9, 10, 11, 15.1, 15.2, 15.3, 16.2, 17.1, 17.2, 18.1, 18.2, 18.3, “>19”, 20, 20.1, 21.1 | - | - |
| D5S818 | 5 | 10.1, 11.1, 12.3, 17, 18 | - | - |
| D7S820 | 22 | 5, 5.2, 6.3, 7.1, 7.3, 8.1, 8.2, 8.3, 9.1, 9.2, 9.3, 10.1, 10.3, 11.1, 11.3, 12.1, 12.2, 12.3, 13.1, 14.1, 15, 16 | - | - |
| D8S1179 | 4 | 7, 15.3, 18, 20 | - | - |
| D13S317 | 10 | 5, 6, 7, 7.1, 8.1, 11.1, 11.3, 13.3, 14.3, 16 | - | - |
| D16S539 | 10 | 6, 7, 9.3, 11.3, 12.1, 12.2, 13.1, 13.3, 14.3, 16 | - | - |
| D18S51 | 30 | 7, 8, 9, 11.2, 12.2, 12.3, 13.1, 13.3, 14.2, 15.1, 15.2, 16.1, 16.2, 16.3, 17.2,17.3, 18.1, 18.2, 19.2, 20.1, 20.2, 21.2, 22.1, 22.2, 23.2, 24.2, 27, 28.1, 28.3, 40 | - | - |
| D21S11 | 24 | 24.3, 25.1, 25.2, 25.3, 26.2, 27.1, 27.2, 28.1, 28.3, 29.1, 29.3, 30.3, 31.1, 31.3, 32.1, 33.1, 34.1, 34.3, 35.1, 36.1, 36.2, 37, 37.2, 39 | - | - |
| Penta D | 14 | 6, 6.4, 7.1, 7.4, 9.4, 10.3, 11.1, 11.2, 12.2, 12.4, 13.2, 13.4, 14.1, 14.4 | - | - |
| Penta E | 13 | 9.4, 11.4, 12.1, 12.2, 13.2, 14.4, 15.2, 15.4, 16.4, 17.4, 18.4, 19.4, 23.4 | - | - |
| D2S1338 | 3 | 13, 23.2, 23.3 | - | - |
| D19S433 | 11 | 6.2, 7, 8, “<9”, 11.1, 12.1, 13.2, 18, 18.2, 19.2, 20 | D19S433 | 16.2 |
| SE33 | 0 | None reported yet in STRBase | - | - |
| D6S1043 | 0 | None reported yet in STRBase | - | - |
| D1S1656 | 0 | None reported yet in STRBase | D1S1656 | 16.3 , 17.3 , 18.3 |

GeneScan® software. Complicated structures give rise to more variations (mutations) and thus a greater chance of finding microvariants as well as rarely seen full repeating-unit alleles. Microvariants differ from full repeat alleles by containing small sequence variations. The differences can be due to insertions, deletions, or point mutations (Butler, 2005). D7S820, can contain 8, 9, or 10 adjacent T nucleotides starting from 12 nucleotides downstream of the GATA repeat. This flanking region insertion/deletion gives rise to the 9.1, 9.3, 10.1, 10.3, etc. alleles observed in D7S820. An allelic ladder contains only alleles commonly found in the human population. Rare alleles and microvariants are, therefore, not often represented in the allelic ladder. Thus microvariants that fall between two alleles in an allelic ladder or alleles that fall outside of the allelic ladder range are labeled as off-ladder alleles. An FGA microvariant allele 16.1 was observed in sample M-101. A search on the STRBase website as of May 2006 showed that this particular allele was reported ten times. The repeat structure of FGA 16.1 is [TTTC]3TTTTTTCT[CTTT] 5T[CTTT]3CTCC [TTCC]2 [43]. Microvariant allele 24.3 was detected at D21S11of M-68. This allele was seen 9 times on the STRBase website in May 2006. The repeat sequence of D21S11

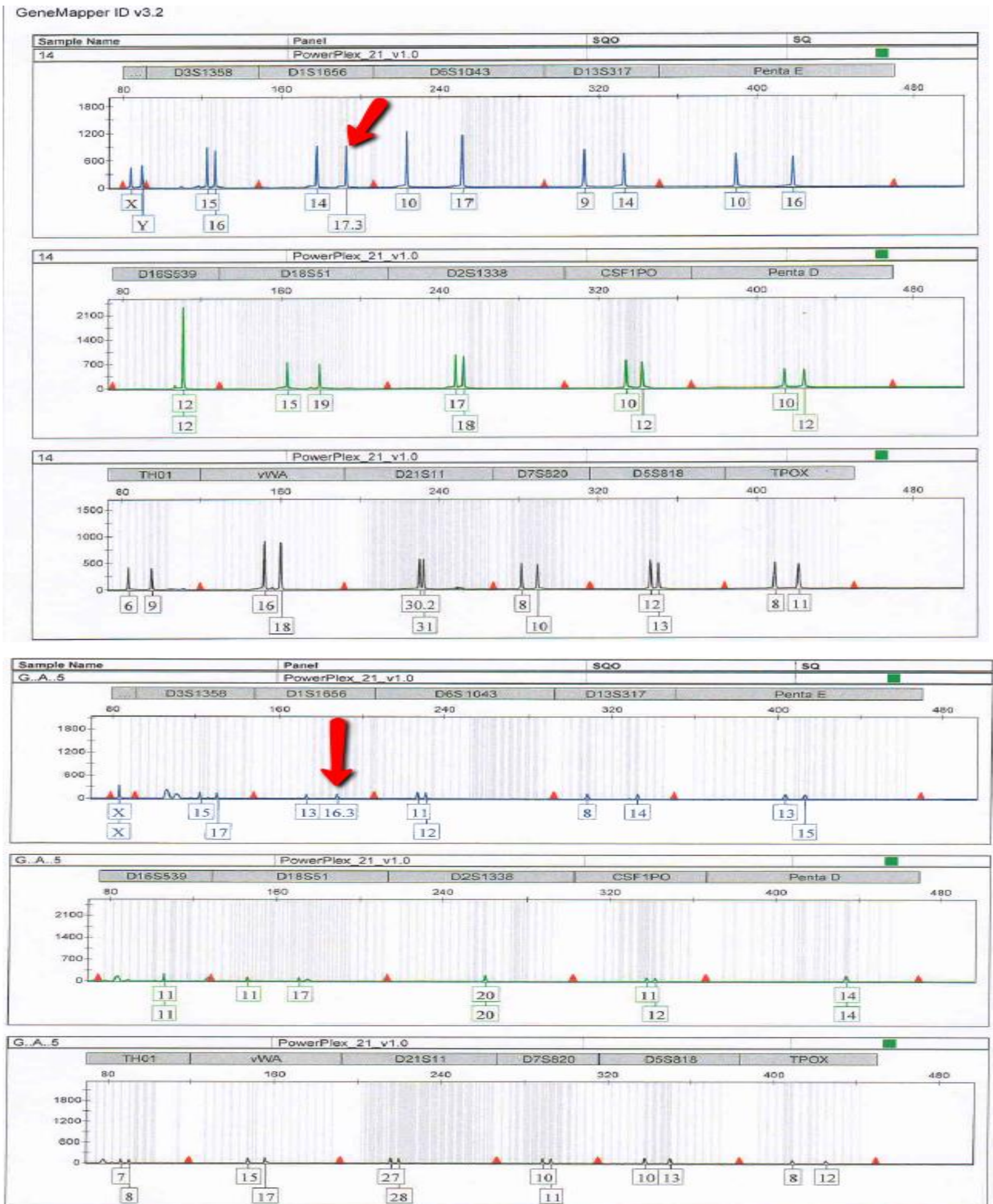


**Figure 15. A.** Off-ladder allele (17.3) located at the D1S1656 locus **. B.** Off-ladder allele (16.3) located at the D1S1656 locus. **C.** Off-ladder allele (18.3) located at the D1S1656 locus**.**

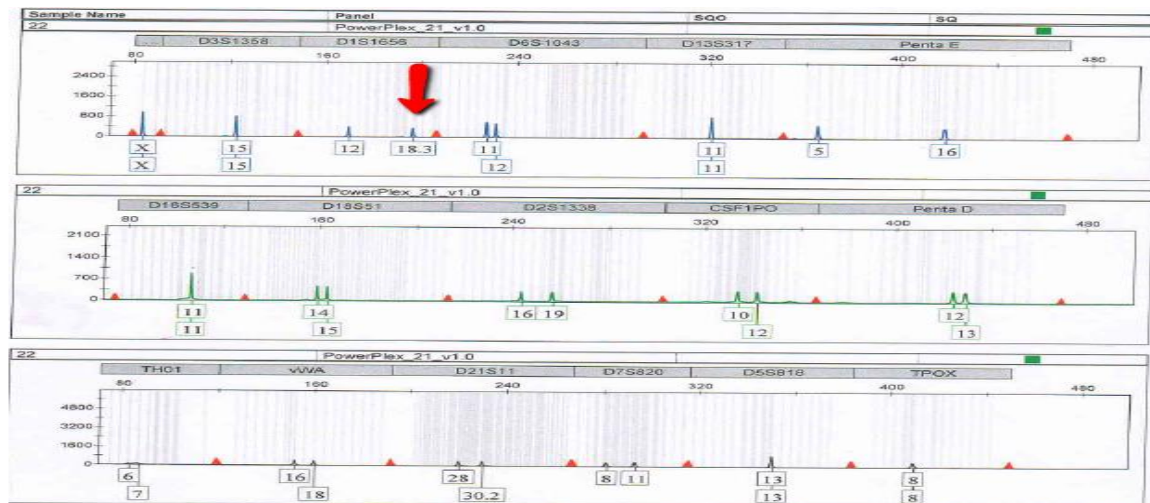


**Figure 15.** Contd.

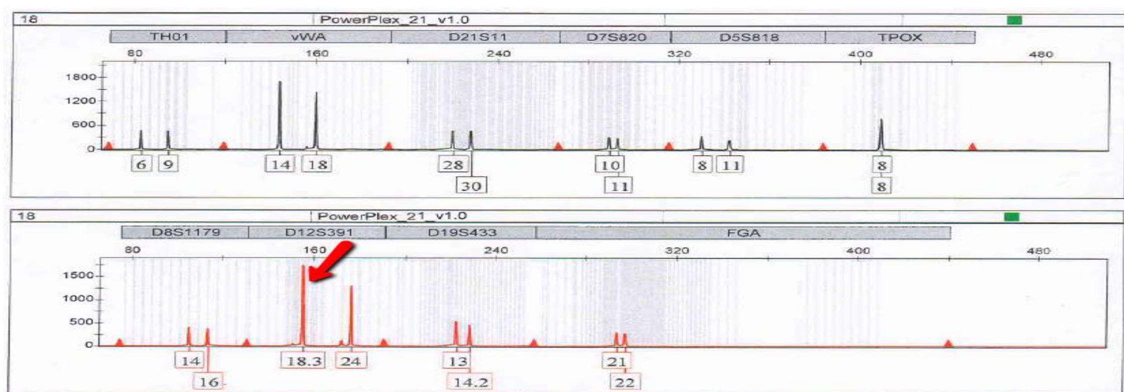


**Figure 16.** Off-ladder allele located at the D12S391 locus.

allele 24.3 is not published. However, the repeat structure is predicted to be similar to the form [TCTA]n[TCTG]n{[TCTA]3TA [TCTA]3TCA[TCTA]2TCCATA}[TCTA]nTATCTA (Butler, 2005). Off-ladder allele 40 was found at the D21S11 locus of samples M-77 and M-194. A search of the STRBase website (again in May 2006) does not turn up this particular allele and therefore no actual repeat sequence is given. However, the repeat motif is expected to have the similar D21S11 structure previously mentioned. The findings of off-ladder alleles at the FGA and D21S11 loci support the fact that off-ladder alleles are most often encountered at loci that have complex repeat structures.

**New Tri-allelic patterns have been observed for many of the core STR loci**

Tri-allelic patterns have been observed for many of the core STR loci and recorded on the NIST STRBase website. Clayton et al. (2004) described possible reasons for tri-allelic patterns, which can occur as an imbalance in amounts between the three alleles (type 1) or equal amounts of all three alleles (type 2). A type 1 tri-allelic pattern imbalance is typically a situation where the sum of the peak heights for two of the alleles is approximately equivalent to the third allele (Clayton et al., 2004). It is interesting to note that TPOX, which occurs closest to the tip of a chromosome, has the highest number of observed tri-allelic patterns—most of which are type 2 with equal intensity alleles. Thus, it is possible that this section of chromosome 2 is more likely to be duplicated in some individuals for telomere maintenance to keep the end of the chromosome intact (Chakhparonian and Wellinger, 2003; Louis and Vershinin, 2005). One triallelic pattern D16S539 observed in this study is shown in Table 11 and Figure 18. A number of explanations for these three-banded allele patterns have been suggested: (a) a

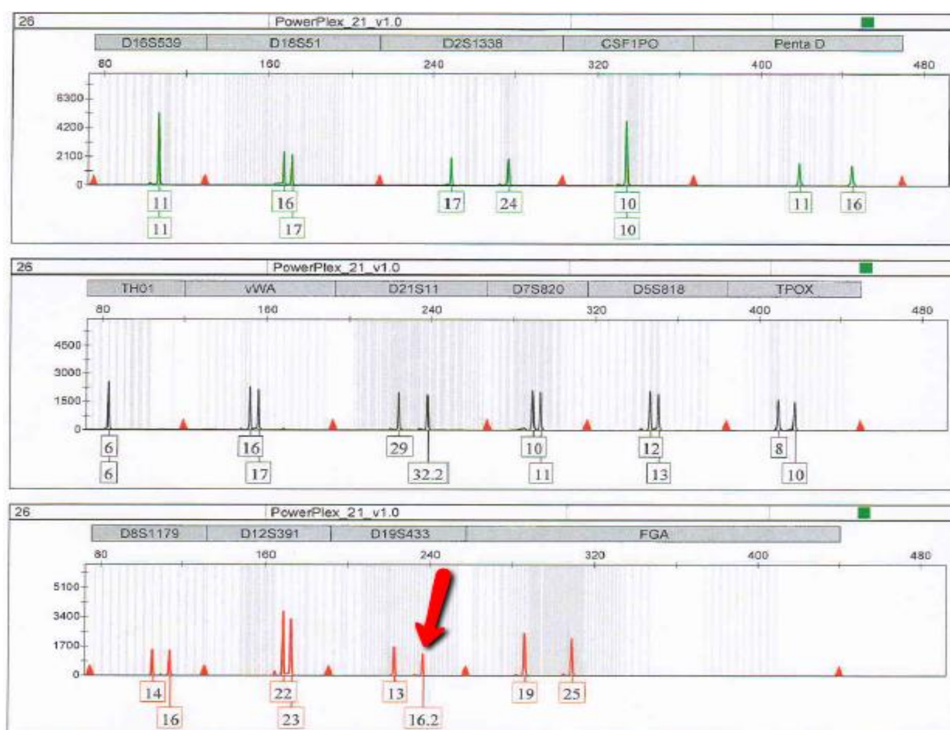


**Figure 17.** Off-ladder allele located at the D19S433 locus.

**Table 11.** A total tri-allelic pattern observed in our study and reported on STRBase.

| **STRBase** | | **In our study** | |
|---|---|---|---|
| STR Locus | Variant alleles | STR Locus | Variant alleles |
| CSF1PO | 9/11/12; 10/11/12 | - | - |
| FGA | 19/20/21; 19/22/23; 19/24/25; 20/21/22; 20/21/24; 20/23/24; 21/22/23; 21/25/26; 22/24/25; 22.2/23/23.2 | - | - |
| TH01 | 7/8/9 | - | - |
| TPOX | 6/8/10; 6/9/10; 6/10/11; 6/10/12; 7/9/10; 7/10/11; 8/9/10; 8/10/11; 8/10/12; 8/11/12; 9/10/11; 9/10/12; 10/11/12 | - | - |
| VWA | 11/16/17; 12/18/19; 14/15/17; 14/15/18; 14/16/18; 14/17/18; 15/16/17; 18/19/20 | - | - |
| D3S1358 | 15/16/17; 15/17/18; 16/17/19; 17/18/19 | - | - |
| D5S818 | 10/11/12; 11/12/13 | - | - |
| D7S820 | 8/9/12; 8/10/11 | - | - |
| D8S1179 | 10/12/13; 10/12/15; 12/13/14; 12/13/15; 13/15/16 | - | - |
| D13S317 | 8/11/12; 10/11/12; 10/12/13 | - | - |
| D16S539 | 12/13/14 | **D16S539** | **9/12/13** |
| D18S51 | 12/13/15; 12/14/15; 12/16/17; 14/15/22; 15/16/20; 16/17/20; 19/22.2/23.2 | - | - |
| D21S11 | 28/29/30; 28/30.2/31.2; 29/31/32; 30/30.2/31 | - | - |
| Penta D | None reported yet in STRBase | - | - |
| Penta E | None reported yet in STRBase | - | - |
| D2S1338 | None reported yet in STRBase | - | - |
| D19S433 | None reported yet in STRBase | - | - |
| SE33 | None reported yet in STRBase | - | - |
| D6S1043 | None reported yet in STRBase | - | - |
| D1S1656 | None reported yet in STRBase | - | - |

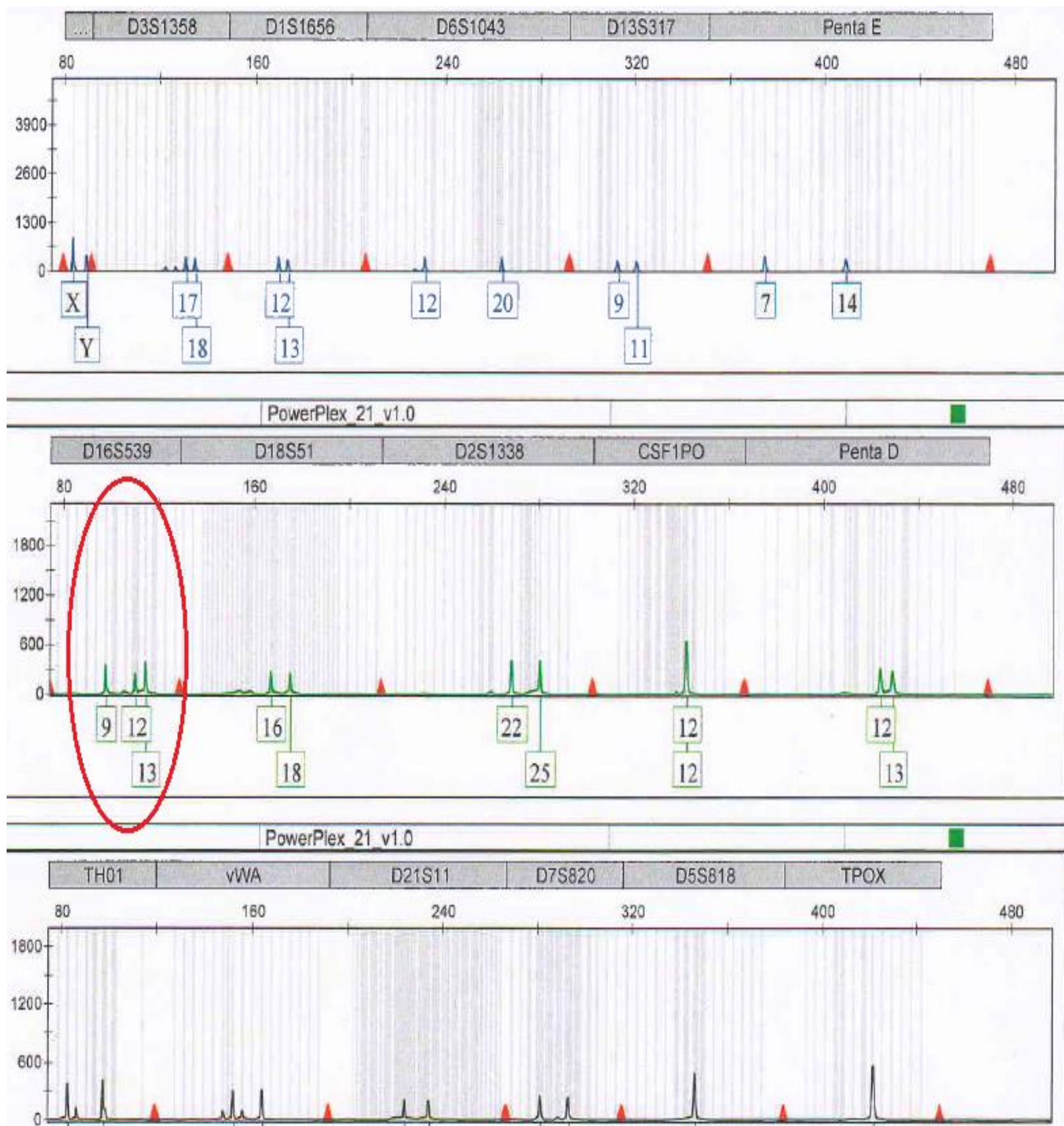


**Figure 18.** Tri-allelic pattern 9/12/13 observed in D16S539 genetic loci.

genetic duplication of a small chromosomal region containing the STR locus, (b) an improper segregation resulting from chromosomal meiotic or mitotic nondisjunction that leads to either true trisomy or to mosaicism, and (c) chimerism (Rubocki et al., 2001).

Although tri-allelic patterns have been reported by other researchers, it is unclear what their frequency is in the general population. The Ballard Laboratory has observed 10 in a database of individuals across the 15 Identifiler™ loci. However, in an extensive study by Crouse et al. (1999) individuals, only 19 tri-allelic patterns (18 at TPOX and 1 at CSF1PO) were observed (0.19% per locus). In this study, one tri-allelic pattern was observed at the TPOX locus of sample M-200. This sample was genotyped twice on the ABI 310 to verify the result. The TPOX tri-alleles are 8, 10, and 11. This sample was neither included in the STR allele frequencies study nor the HWE database validation. The presence of an extra peak at one locus out of all of the 15 loci tested indicates that this sample is not a mixture. Rather, the extra peak is a real

reproducible artifact of the sample. The sample was re-typed and the same tri-allelic pattern was obtained. The fact that the peak heights of all three alleles are similar suggests that the alleles are probably present in equal copy number (Egyed et al., 2000). Tri-allelic patterns appear to be common at the TPOX locus. A search of the STRBase website (Butler, 2006) as of May 2006 reveals 14 different TPOX tri-allelic patterns.

## Conclusion

The conclusions that have been drawn from the present study include: the results of the current study indicate these new autosomal STR Loci useful For DNA typing markers in Iraq can be used for establishment of a DNA database that will be beneficial for the population in terms of resolving social and moral disputes and will contribute to improvements in the justice system. The match probability is the probability for a random match between two unrelated individuals drawn from the same population; it ranged from 0.011 to 0.168. The mean heterozygosity observed, expected and mean PIC values across the 20 loci were 0.77, 0.81 and 0.78, respectively, indicating high gene diversity. A total of nine off-ladder alleles were detected in this study; four of them are located at the D1S1656 locus, three are located in D12S391, one occurred at the D19S433 locus and one discovered outside the range. One triallelic pattern observed in this study was 9/12/13 pattern.

## Conflict of interests

The authors did not declare any conflict of interests.

**Abbreviations: STRs,** Short tandem repeats; Ho**,** observed heterozygosity; **He,** expected heterozygosity; **PI,** paternity index; **RMP**, random match probability; **PD**, power of discrimination; **CE,** chance of exclusion; **PIC,** polymorphic information content.

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African Journal of Biotechnology

*Full Length Research Paper*

# Development of microsatellite markers for use in breeding catfish, *Rhamdia* sp.

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**Microsatellites markers for catfish, *Rhamdia sp.* were developed using Next Generation. A shotgun paired-end library was prepared according to standard protocol of Illumina Nextera DNA Library Kit with dual indexing, paired-end reads of 100 base pairs and grouped with other species. From a single race, five million readings obtained were analyzed with the program PAL_FINDER_v0.02.03. perl script was used to extract readings containing microsatellites with di-, tri-, tetra-, penta- and hexanucleotides from five million readings obtained in sequencing. Readings were grouped and used in Primer3 version 2.0.0 to design primers when GC content was greater than 30%, melting temperatures was within 58 to 65°C, with 2°C maximum difference between primers, the last 2 nucleotides in 3'extremity are G or C, and maximum poli-N of 4 nucleotides. When all criteria were met, a single pair of primers was selected according to the highest score in Primer3 and with greater amplification region of the repeated sequence. We identified 6,331 microsatellite loci potentially amplifiable (microsatellites) of which 4,755 were dinucleotide, 728 trinucleotide, 729 tetranucleotide, 117 pentanucleotídeo and 2 were hexanucleotídeo. A group of 12 loci microsatellite (including di- and tetranucleotides) has been sequenced, using Sanger's method, to obtain complete sequences for fragments between 140 and 200 pb and are currently being used to study the genetic diversity of catfish populations. The populations showed genetic variation with average number of alleles per locus of 6.14. Microsatellites acquired with Next-Generation Sequencing (NGS) are an efficient tool for obtaining highly polymorphic markers for non-model species.**

**Key words:** Next-generation sequencing, simple sequence repeats, *Rhamdia sp.*

## INTRODUCTION

The Brazilian aquaculture has expanded its production in the past years based on exotic species including the tilapia. However, recently there is an interest in incorporating native fish species of Brazil in this production system. As an example we can mention the tambaqui species (*Colossoma macropomum*) and cachara (*Pseudoplatystoma corruscans*) that are being targets of developments in strains Aquabrasil project. However, the species cited above are not applied for pisciculture of Rio Grande do Sul (RS), either restricted in legislation or even

environmental. It must be emphasized that the pisciculture of RS is based on exotic species, including carp (*Ctenopharyngodon idella*, *Cyprinus carpio*, *Hypoththalmichthys molitrix* and *Aristichthys nobilis*) and tilapia (*Oreochromis noloticus*) (Brazil, 2012). The catfish (*Rhamdia sp.*) is the native species most representative in the state of RS, by presenting a production more than 2,000,000 fingerlings (Brazil, 2012). Researches believe that the catfish is the most promising native species for intensive production in the state, because of its characteristics such as: easy to adapt in different environments, weather and artificial diets, the handling is very simple and have a good commercial acceptance (Baldisserotto, 2004; Pouey et al., 2011).

Although studies in cytogenetic will be developed for catfish (Huergo and Zaniboni-Filho, 2006; Silva et al., 2007; 2011), the genetics of the reproducers stock of *Rhamdia sp.* in the South region and Southeast of Brazil is currently not known. Knowledge of the genetic variability and standards of population structure are prerequisites for the strategies development for future genetic improvement programs. However, studies of genetic variability in catfish require the development of molecular markers. Among the currently available markers, the microsatellite markers (Simple Sequence Repeats, SSR) are a tool satisfactorily used in studies of population structure, species conservation and management of genetic resources (An et al., 2012).

Microsatellites present codominance and high polymorphism, being possible to studied the genetic differences between closely related populations (Na-Nakorn et al., 2010) are therefore considered a valuable tool for population genetics. The development of microsatellites markers coming from model species formerly required a very expensive technical effort, with lengthy and costly procedures. These proce-dures include techniques such as creating libraries enriched for SSR loci, cloning, hybridization to detect positive clones, plasmid isolation and sequencing of Sanger (Castoe et al., 2012). However advances in DNA sequencing technology has provided more efficient and cost effective methods to develop molecular markers for species that do not have available data (Buschiazzo and Gemmell, 2006), currently known as Next-Generation Sequencing (NGS).

Studies indicate that this new technology will replace the conventional protocols for isolation of microsatellites (Abdelkrim et al., 2009), and there are increasing reports employing NGS microsatellite markers in studies of species not models (Saarinen and Austin, 2010; Yu et al., 2011). This study has the objective to develop catfish microsatellites markers through NGS, aiming to understand the genetics of this species.

## MATERIALS AND METHODS

### Animals and DNA extraction

Blood samples were collected from catfishes from the Chasqueiro Pisciculture Station, located between the coordinates 32º02'15'' and 32º11'07'' of south latitude and 52º57'46'' and 53º11'18 '' of west longitude, belonging to the Federal University of Pelotas, in the municipality of Arroio Grande - RS, Brazil. For DNA extraction, the Blood Genomic DNA Miniprep Kit was used according to the manufacturer's instructions (Axygen Bioscience, USA). The quality of extraction was checked in 1% agarose gel, stained with Gelgreen (Biotium, USA) and visualized in white light transilluminator (Clare Chemical, USA). The total concentration of DNA was measured using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

### Preparation of genomic library

A single shotgun paired-end library was prepared from genomic DNA of catfish according to standard Illumina Nextera DNA Library Kit, Kit protocol with double index (Illumina, USA). A total of 200 ng of genomic DNA double was randomly fragmented. The sequencing library was conducted in a HiSeq sequencer (Illumina, USA) with paired-end reads of 100 base pairs and grouped with other species. From a single run ten million (five million-five million forward and reverse) from readings obtained were analyzed with the program PAL_FINDER_v0.02.03 to extraction of readings containing microsatellites tandem with dinucleotide (di 2), trinucleotide (3 tri) tetranucleotide (4 tetra) pentanucleotide (5 penta) and hexanecleotide (6 hexa). Once the extraction of readings were identified with PAL_FINDER, they were grouped together to a local subdirectory Primer3 software (version 2.0.0) (Rozen and Skaletsky, 2000) for drawing primers. To calculate the GC content, allocation of base ("N"), level of the duplicated sequences and quality of the sequences, the FASTQC v0.10.0 _ program was used.

### Drawing of primers

The following criteria for the primers design were used: 1) GC content higher than 30%, 2) melting temperatures of 58° - 65°C with a maximum of 2°C difference between the primers, 3) the last two nucleotides on the end 3' is G or C, and 4) Maximum poly-N of 4 nucleotides. If all other criteria are achieved, a single primer pair is chosen presenting the highest score assigned by Primer3, besides the larger size of the region of amplification of the repeated sequence. For each loci, primers had one of the incorporation of the M13 sequence (5 'TGT AAA ACG ACG GCC AGT 3'). The addition of this sequence allows the indirect identification of allele sizes facilitating the subsequent genotyping (Brownstein et al., 1996).

### PCR and SSR amplifications

From potentially amplifiables loci (PALs) obtained, a group of 12 loci was selected and amplified for subsequent microsatellite fragments obtaining by Sanger-type sequencing. Amplifications were performed in a total volume of 25 µl including 7.5 pmol of each primer, ~ 30 to 50 ng template DNA, 0.2 mM dNTP, 1 unit of Taq

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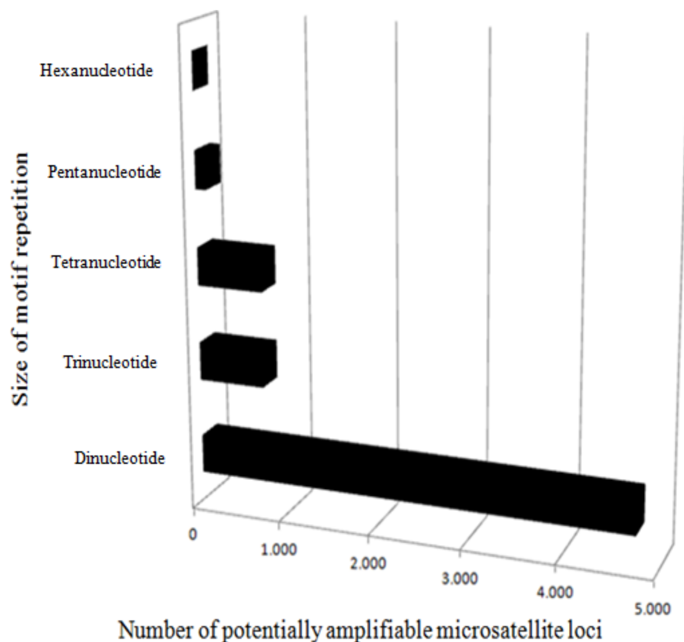


**Figure 1.** Potentially amplifiable microsatellite loci in catfish (*Rhamdia sp.*) according to the size of the motif. Results was based in five million read paired-end Illumina (100 to101 pb).

polymerase Dream (Fermentas), 1.5 mM $MgCl_2$, and 1 × PCR buffer. The annealing temperature was tested for each of the loci for gradient in one Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Germany). PCR products were verified by electrophoresis on 1% agarose gel and visualized by staining with GelGreen (Biotium, USA). The PCR products were sequenced using MegaBace sequencing kit (Amersham Biosciences, Uppsala, Sweden) in a capillary sequencer MegaBace 1000 (Amersham Biosciences, Uppsala, Sweden). Sequences were analyzed using Finch TV 1.4.1 software (Geospiza, Inc, USA). To confirm the polymorphism of microsatellite loci, four tetranucleotide (Rq68040, Rq137981, Rq164109 and Rq51373) and one dinucleotide (Rq91253) were chosen and tested in six populations of catfish reproducers, located in the cities of São João do Polêsine, Cruzeiro do Sul, Passo do Sobrado, Três de Maio, Seberi and Mato Leitão in the state of Rio Grande do Sul (RS). A total of one hundred and seventy-two animals (172) were genotyped. The samples were genotyped in 10% polyacrylamide gel for 3 h at 100 V/cm. DNA bands were stained with silver nitrate (Qu et al., 2005) and individual genotypes were defined according to the standards of the bands. Number of alleles of each loci, observed heterozygosity (Ho) and endogamy index (Fis) were analyzed using GENEPOP version 4.0 software (Rousset, 2008).

## RESULTS AND DISCUSSION

Large amounts of genomic sequences of species are possibly generated presently as a result of new technologies instead of genomic model. For catfish, native species and promising development of microsatellite markers allowed available large-scale data, assisting in the advancement of research for the species. Through the HiSeq sequencer (Illumina), five million paired reads (paired-end) were obtained, all quality scores by base showed values above Q28 and mean quality for reading was excellent; Q38. A number of 6,331 microsatellite loci potentially amplifiable (PALs) of the total readings were found in catfish, 4,755 of which were dinucleotide, 728 trinucleotide, 729 tetranucleotide, 117 pentanucleotídeo and 2 were hexanecleotídeo (Figure 1). In comparison with other genomes, catfish shows a number of microsatellite loci found in humans (5.264 microsatellites) (Dib et al., 1996) and discrepant of zebrafish, *Danio rerio* (116.915 microsatellites) (Rouchka, 2010) demonstrating the oscillation in microsatellites sequences. For *Henichorynchus siamensis* a freshwater teleost of great economic importance in the Mekong River Basin (Southeast Asia), 65.954 sequences were obtained with the Roche 454 GS-FLX platform, out of the total sequences obtained, 1.837 were SSRs (Iranawati et al., 2012). Although, it is a teleost, the number of sequences obtained was indeed smaller than those found in catfish, and the divergence may be due to size of the genome of each species or differences in the platforms used and its specific characteristics, such as coverage of the genome, sequence number and size of readings.

Iranawati et al. (2012) using the same platform for *Megalobrama Pellegrini*, a fish native to China, obtained 257.497 reads, with 49.811 PALs (Wang et al., 2012). Compared to the number obtained with the catfish, this is almost 20 times lower and almost nine times greater of PALs, relating these differences the distinctions between the two platforms. The GC content for each reading was equivalent to the expected theoretical distribution to catfish (41%), a positive result, because the unknown the actual GC content in the genome. A similar value has been found for *Parus major*, (40.7%) (Santure et al., 2011). However, for most animals, the percentage of GC varies, the values shown between 35 and 45% of the genome (Meglécz et al., 2012). The GC dinucleotide is rare present in all studied genomes (Tóth et al., 2000). The length of the sequences, the mean value expected to catfish was 100 bases. In these analyzes it was not observed that an error allocation base ("N") in any position reading, which is normal at the end of the readings occur in late positions. The level of duplicate sequences stayed around 9.56%, meaning that the library partial had a good coverage. From the total 6,331 PALs obtained, the most common motifs found for catfish (75.1%) were dinucleotide also obtained in the *Schizothorax biddulphi* (Luo et al., 2012) and *H. siamensis* (Iranawati et al., 2012), respectively, 77.08 and 74.41%. To *H. siamensis* 9.53% of the sequences were trinucleotide, 16.06% tetranucleotíde and repetitions type penta- and hexanucleotide were not detected (Iranawati et al., 2012). In the case of *S. biddulphi* similar to catfish, penta-and hexa presented themselves at low frequency (0.65 and 0.22%, respectively) (Luo et al., 2012).

In contrast, for *Raja pulchra* fish of total PALs obtained (312.236), 18% were dinucleotide repeats and 0.11% of trinucleotide type (Kang et al., 2012.). Although the

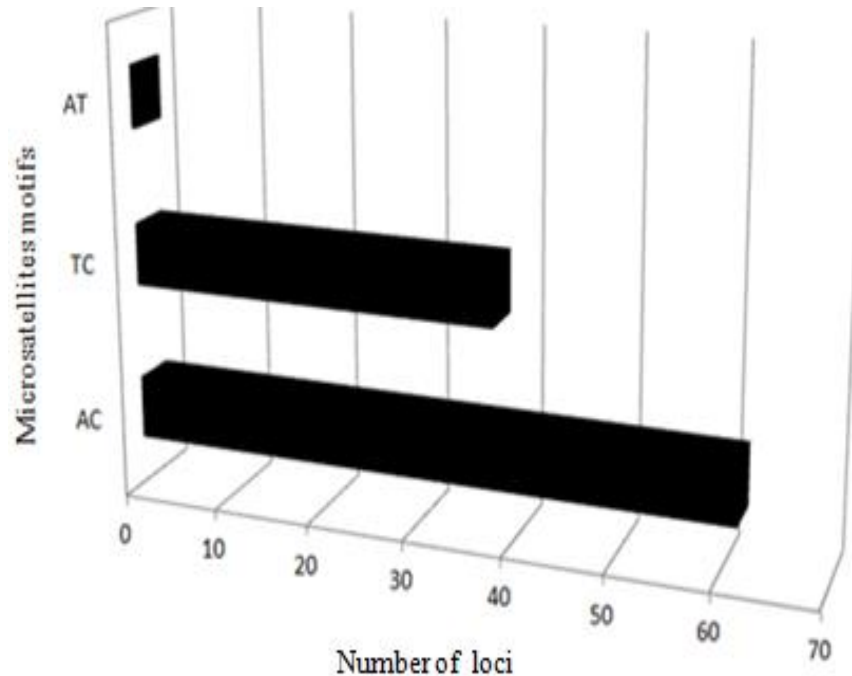


**Figure 2.** SSR motifs most common dinucleotide (2di) in *Rhamdia sp.* Results was based on five million read paired-end Illumina (100 to 101 bp).

authors have not submitted the results obtained for tetra-, penta-and hexanucleotide, it is possible to observe that the dinucleotide repeat type is not presented as the most frequent. The most frequent motif in dinucleotide repeats for catfish was TC and AC (Figure 2), similar to results obtained for *Fugu rubripes* (Edwardsa et al., 1998), *Ictalurus punctatus* (Somridhivej et al., 2008), *Etheostoma okaloosae* (Saarinen and Austin, 2010), *R. pulchra* (Kang et al., 2012), *Schizothorax biddulphi* (Luo et al., 2012), but in different parts of *Argopecten irradians* (TA) (Zhan et al., 2005), *C. carpio* (AC/TG) (Wang et al., 2007), *Crassostrea virginica* (Wang and Guo, 2007) and *Perca flavescens* (Zhan et al., 2009) (AG/TC). In the same context, according to Meglécz et al. (2012), the most common dinucleotide motifs in Chordata depending on species are AC and TC, which concurs with the results obtained for some fish, birds and plants. In relation to trinucleotide, the most frequent SSR motif found to catfish was ATT (Figure 3). Similarly, according to Calabuig et al. (2012) ATT motif was also more frequent in birds (*Coscoroba coscoroba*) and different to other fish as *R. pulchra* (AAT) (Kang et al., 2012), *C. carpio* (AAT/ATC) (Wang et al., 2007) and *Coreoperca whiteheadi* (CCT/GGA) (Tian et al., 2012). According to Meglécz et al. (2012) in studies with over 130 species of eukaryotes, the AAT trinucleotide motif was the most common. In spite of the catfish, also Chordata, did not show the same tendency, because some factors may influence the microsatellites composition for each species, such as mechanisms of mutation, types of microsatellites (allele length, repeat unit of length, composition), genomic context and natural selection (Buschiazzo and Gemmell, 2006).

For tetranucleotídeos, the most frequent SSR motif found was ATGG (Figure 4), unlike the motifs observed by Calabuig et al. (2012) with *C. coscoroba* and Castoe et al. (2012) with *Centrocercus minimus* and *Columbiana nucifraga*. According Meglécz et al. (2012), for Chordata in general the most common tetranucleotide motif is AGAT and in plants AAAT, different results in both studies and groups of species, suggests that the vast variation that can be found from among the repeats microsatellite loci. The number of repetitions of motifs with pentanucleotides type and hexanucleotides presented are relatively low (Figure 1), with variations. For the pentanucleotídeo 17 repetitions were the most common presenting motif ATAGG (Figure 5) and repetitions of hexanucleotides type were obtained only two different motifs ATGTGT and ATTAGG (Figure 6). The data obtained with more than 130 species of Chordata also demonstrate a low number of motifs of penta- and hexanucleotides, making it difficult to provide good estimation of its proportions (Meglécz et al., 2012). According to the authors no pattern appeared in the relative frequencies of motifs, both for plants as for the group of Chordata, and stressing that although these motifs it characterized GC-rich, there is no other relations between them. Two main mechanisms have been proposed to explain the formation of microsatellites (Buschiazzo and Gemmell, 2006): spontaneous formation

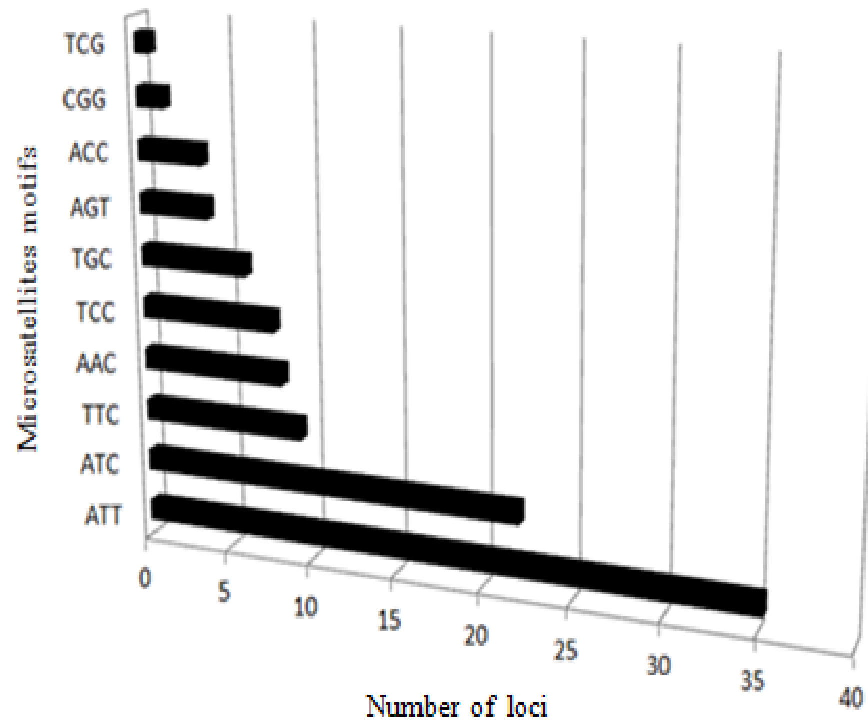


**Figure 3.** SSR motifs most common trinucleotide (3 tri) in *Rhamdia sp.* Results was based on five million read paired-end Illumina (100 to 101 pb).

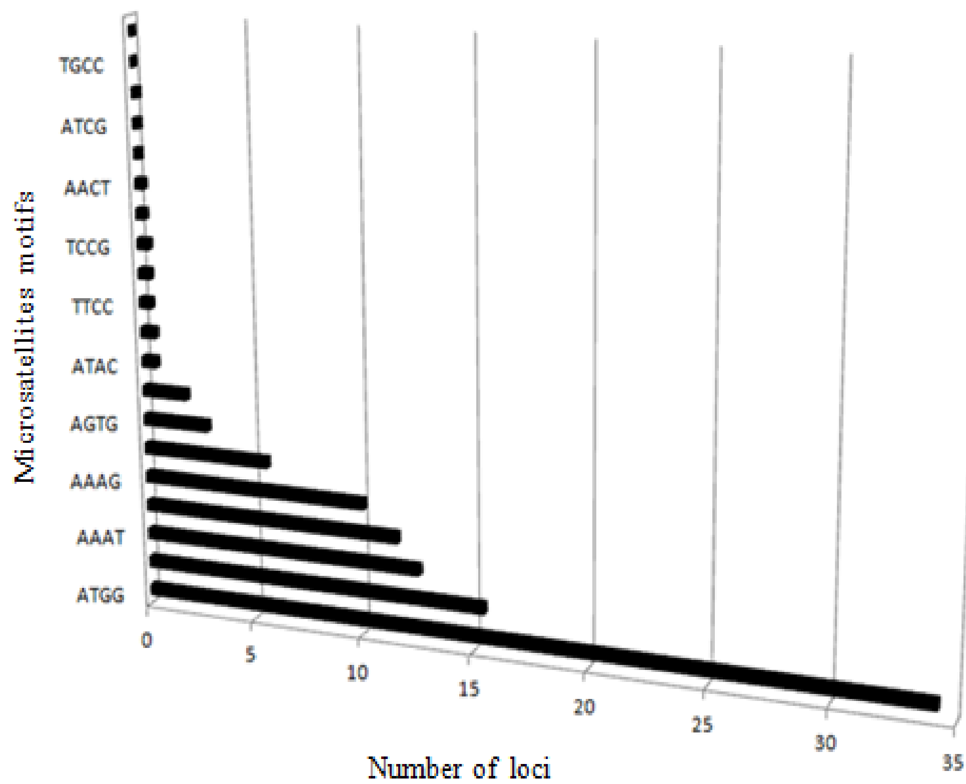


**Figure 4.** SSR motifs most common tetranucleotide (4 tetra) in *Rhamdia sp.* Results was based on five million read paired-end Illumina (100-101 pb).

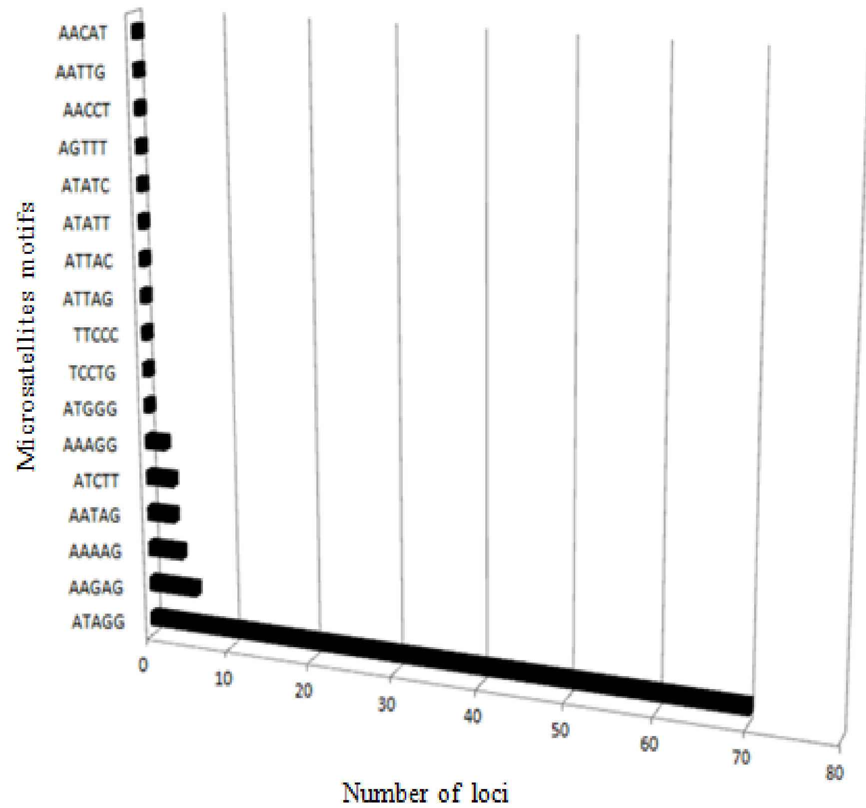


**Figure 5.** SSR motifs most common pentanucleotide (5 penta) in *Rhamdia sp.* Results was based on five million read paired-end Illumina (100 to 101 pb).

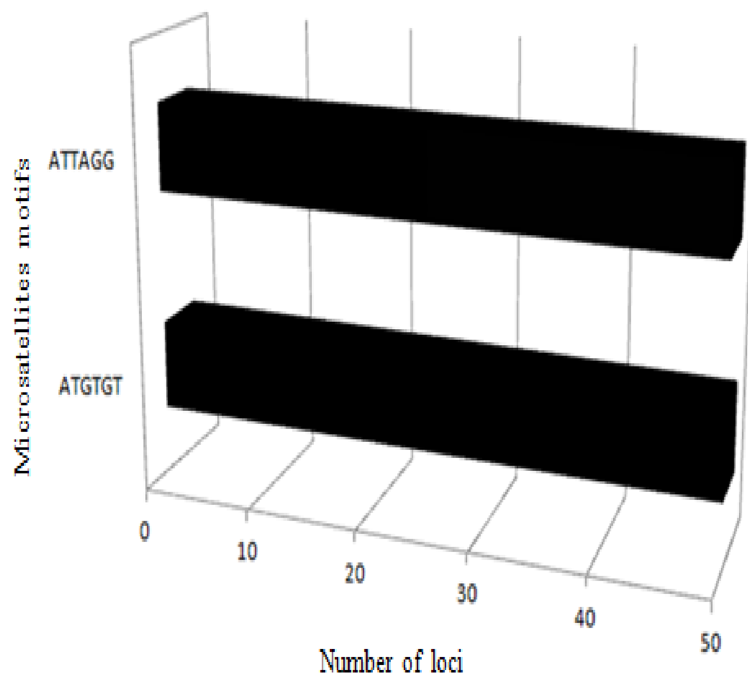


**Figure 6.** SSR motifs most common hexanucleotide (6 hexa) in *Rhamdia sp.* Results was based on five million read paired-end Illumina (100 to 101 pb).

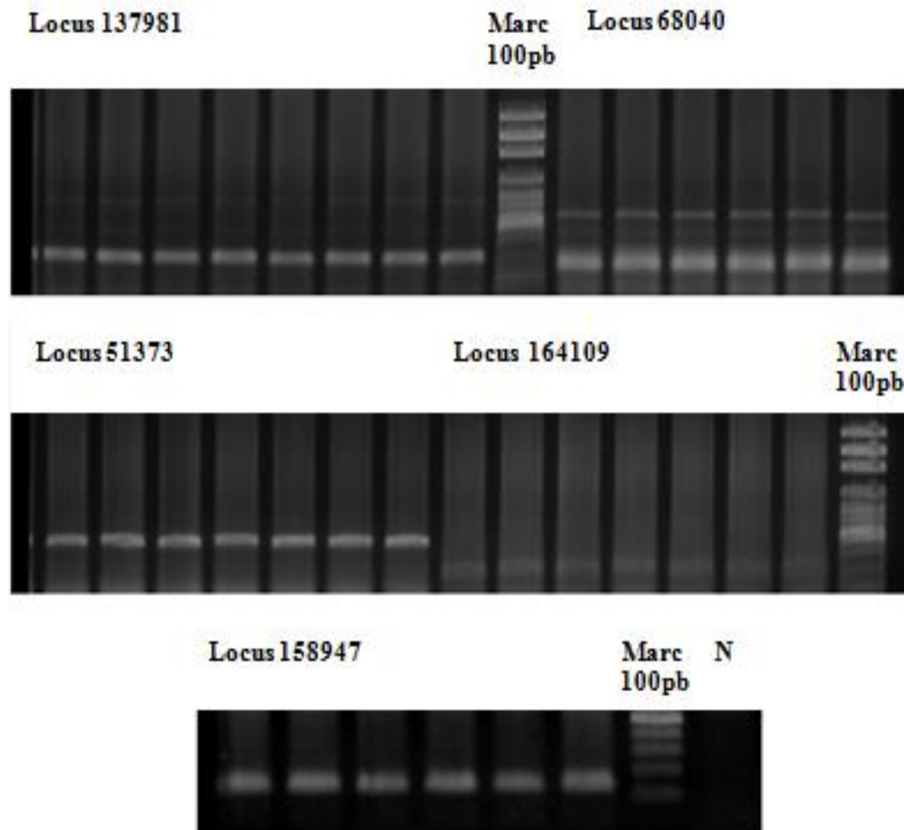


**Figure 7.** Amplifications of microsatellites developed for tetranucleotídeos *Rhamdia sp.* visualization in 1% agarose gel. Results was based on five million read paired-end Illumina (100-101 pb).

of unique sequences by substitution of insertion (Dieringer and Schlötterer, 2003) by creating the proto-microsatellite, then microsatellite of elongation or propagation proto- or complete or transposable elements (Wilder and Hollocher, 2001). The formation of proto-microsatellite, is less likely for lengthy motifs than for shorter (Meglécz et al., 2012), which would explain why dinucleotide motifs are the most frequents in most taxons, and because pentanucleotides and hexane-cleotides motifs are rare. Data demonstrated that beyond of variations in the frequency of microsatellite and types of repeats between taxon, the specificity can be explained, in part, by the interaction of evolutionary mechanisms through the differential selection in regions of the genome and in different species. This suggests that, motifs microsatellites can be specific and characteristic of the species and their supposed genomic evolution (Meglécz et al., 2012). Based on the 6,331 PALs obtained for catfish through the HiSeq platform (Illumina), a group of twelve (10 tetra and 2 dinucleotide) loci were selected with similar criteria Castoe et al. (2012), and specific primers are designed for obtaining fragments and subsequent sequencing. Among 12 loci, five tetranucleotídeos (Figure 7) and one dinucleotide (Figure 8) were amplified successfully in the initial evaluation of the primers. The remainder primers have not generated the desired amplification products under the PCR conditions tested. The sequences of the primers, locus name, motifs of repeats, annealing tem-perature and the size of the PCR product are summarized in Table 1.

For further analysis, PCR fragments (Polymerase Chain Reaction) obtained from the developed microsatellites loci were sequenced by the Sanger method, which allows the knowledge of the complete sequence of microsatellites resulting from next-generation sequencing through HiSeq platform (Illumina). Five loci tetranucleotídeos and one dinucleotide were tested; fragments purified with Miniprep PCR Clean-up Axygen kit (United States) were sequenced in triplicate. The amplifications obtained from PCR were sequenced with the sequencing MegaBace (Amersham Biosciences, Uppsala, Sweden) kit in one capillary sequencer MegaBace 1000 (Amersham Biosciences, Uppsala, Sweden). The microsatellites loci for catfish were named for subsequent publication in Gene Bank: Rq158947, Rq164109, Rq137981, Rq68040, Rq51373 and Rq91253. As shown in Figures 9 and 10, we can see that four of the five loci tetranucleotídeos and one of the loci dinucleotide chosen for analysis showed repeat sequences with the motifs obtained through

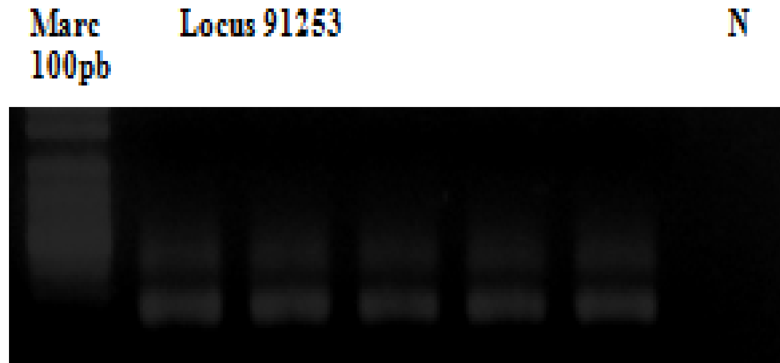


**Figure 8.** Amplifications of microsatellites developed for dinucleotides *Rhamdia sp.*, visualization in 1% agarose gel. Results was based on five million read paired-end Illumina (100 to 101 pb).

**Table 1.** Microsatellites primers designed for *Rhamdia sp.* (catfish), through the Primer3 software (version 2.0.0) (Rozen and Skaletsky, 2000).

| Primer | Sequence of *primer* (5' – 3') | Motif | $T_a$ (°C) | Product size (pb) |
|---|---|---|---|---|
| Rq 158947 | F: TGTAAAACGACGGCCAGTCCTGCACTGTGCCAGAAGG<br>R: ATCCATGCGTTTGTCCATGC | $(TCCA)_6$ | 54,6°C | 170 – 190 |
| Rq 164109 | F: TGTAAAACGACGGCCAGTTGATACACTGGTGCGAATCC<br>R: CAATGTCTTACATGCAGGTTCC | $(AAAC)_7$ | 56,4°C | 190 – 200 |
| Rq 137981 | F: TGACAATAAAGCAAGGTCATTTCG<br>R: TGTAAAACGACGGCCAGTGGGTCTGAATCACCAGTTGC | $(AAAT)_5$ | 56,4°C | 150-170 |
| Rq 68040 | F: TGTAAAACGACGGCCAGTGGTTAAAGTGAGCTCAGGCAGG<br>R: GGCGGAGGAGAGAAAGGG | $(AATG)_6$ | 68,5°C | 140 – 150 |
| Rq 91253 | F: ACA ATT AAC CCG GCT CAGTCC<br>R: CTG ACA GCA GCG GAA CGC | $(AC)_{13}$ | 64,2°C | 120 - 130 |
| Rq 51373 | F: TGTAAAACGACGGCCAGTCACTCCATTGCAGCTTCTTCC<br>R: ATC GAG TGA AAT GCA GCA GG | - | 52°C | - |
| Rq 43102 | F: TGTAAAACGACGGCCAGTCTCCCACTCACTCACACATACG<br>R: AACCGTTCCATGATGTTCCC | - | - | - |
| Rq 105923 | F: TGTAAAACGACGGCCAGTCACACGCAGATTTAATGAGGC<br>R: CACACTGGATCACCGACTTACC | - | - | - |
| Rq 23018 | F: TGTAAAACGACGGCCAGTAAGGAACCGTCTTGTGACCG<br>R: TTCATATGTAGAAACAACAACTATTGGG | - | - | - |
| Rq 155485 | F: TGTAAAACGACGGCCAGTCTTCATGGTCAGCTGTGAGG<br>R: GTGATGCGTTGCTTTCGG | - | - | - |
| Rq 193810 | F: TTACTGAATGATCGATATTATTGACG<br>R: TGTAAAACGACGGCCAGTAAAGGATGGATAGTCTCGCC | - | - | - |
| Rq 193399 | F: TGCTGAACTTCCAAACGTTCC<br>R: GACTAAAGCCGGGACCTTCC | - | - | - |

**Figure 9.** Tetranucleotídeos sequences obtained by Sanger sequencing from microsatellites loci developed for *Rhamdia sp.* (catfish) using next-generation sequencing.

**Figure 10.** Tetranucleotídeos sequences obtained by Sanger sequencing from microsatellites loci developed for *Rhamdia sp.* (catfish) using next-generation sequencing (jundiá) através de sequenciamento de próxima geração.

HiSeq platform (Illumina).

However, the locus Rq51373 showed a different result of the remaining sequenced loci tetranucleotides. The nucleotide sequence did not show any to microsatellite, however it is possible that the region where it was-repeat of sequence SSR the sequencing not carried out the reading (Figure 11). Further analysis should be conducted for this locus, in order to get more accurately verify results of the existence of the microsatellite. To evaluate the polymorphism of microsatellites loci, four tetranucleotides (Rq68040, Rq137981, Rq164109 and Rq51373) and one dinucleotide were chosen and tested in six populations of reproducers of catfish located in different regions of the state of Rio Grande do Sul. A total of 172 individuals were analyzed. The samples were submitted to genotyping in 10% polyacrylamide gel and stained with silver nitrate (Qu et al., 2005) (Figure 12). The mean number of alleles per locus was 6.14. The index of endogamy with positive values suggests a deficit of heterozygous in all loci analyzed in populations (Table 2). However, on locus Rq164109 two populations exhibited negative Fis values indicating that there was no occurrence of endogamy in these populations. From then on, it can be inferred that the populations showed genetic

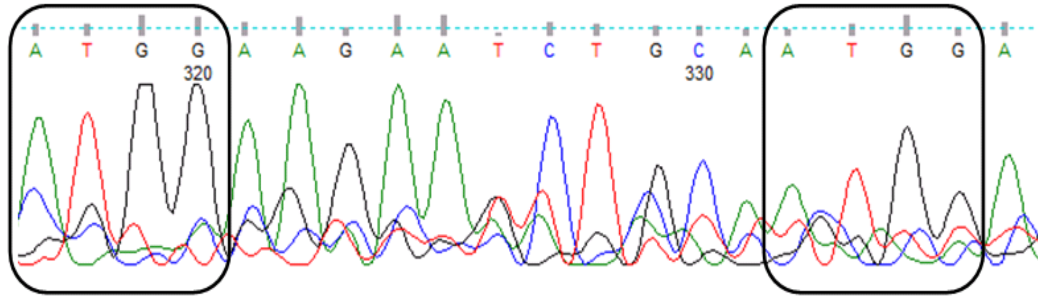


**Figure 11.** Locus Rq 51373. Tetranucleotídeos sequences obtained by Sanger sequencing from microsatellites loci developed for *Rhamdia sp.* (catfish) using next-generation sequencing.

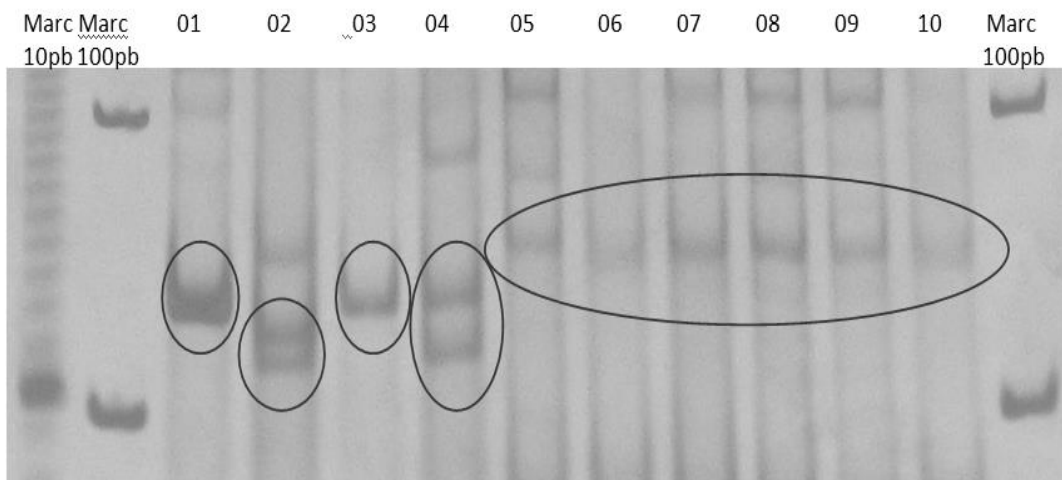


**Figure 12.** Banding patterns of microsatellites loci developed for *Rhamdia sp.* (catfish). 10% polyacrylamide gel stained with silver nitrate. Marker 10 bp, 100 bp marker; Standards bands of different loci (01 to 10).

variation and that the developed microsatellites markers are polymorphic (Figure 12). Although the both loci Rq51373 and Rq158947 has presented variations within populations analyzed, the banding patterns were not satisfactory. Thus, the locus Rq51373 in some samples could not be genotyped, resulting in a value of observed heterozygosity (Ho) equal to zero and one Fis equal to 1 (Table 2).

Analyzing genetic variability in two broodstocks catfish in Santa Catarina, Virmond et al. (2013) observed high level of polymorphism in three microsatellites loci, with 63 alleles genotyped in a total of 71 individuals. The endogamy coefficient with negative values in the two populations indicating that there was no occurrence of inbreeding and populations exhibited genic differentiation and genotypic significant. Differences between the values found by Virmond et al. (2013) and the data found in the populations of RS is mainly due to the fact of being broodstocks reunited from different regions of the state of Santa Catarina, with the objective of bringing together the greatest possible genetic variability to assemble families. In RS, according to the EMATER (Empresa de Assistência Técnica e Extensão Rural) and of the producers themselves, there are matrices exchange, that is, exchange of reproducers between the properties that produce catfish fingerlings, causing a probable occurrence of consanguinity among the populations and reducing the genetic variability.

## Conclusions

Through the data that were generated in this study, we can define that the sequencing strategy through library shotgun paired-end HiSeq platform (Illumina) is effective for catfish (*Rhamdia sp.*), generating 6,331 potentially amplifiable microsatellites loci. The microsatellite markers developed have variation within populations, so the next-generation sequencing presented as fast and inexpensive way to develop microsatellites markers for species

**Table 2.** Genetic characterization of the five microsatellites loci developed for *Rhamdia sp.* (catfish) in six populations.

| Locus | Repeat motif | Population | N | Na | Ho | Fis |
|---|---|---|---|---|---|---|
| Rq 164109 | $(AAAC)_7$ | 1 | 30 | 8 | 0.166 | 0.656 |
| | | 2 | 30 | 7 | 0.400 | 0.343 |
| | | 3 | 31 | 8 | 0.774 | -0.036 |
| | | 4 | 30 | 6 | 0.400 | 0.003 |
| | | 5 | 30 | 5 | 0.633 | -0.068 |
| | | 6 | 21 | 7 | 0.333 | 0.290 |
| Rq 137981 | $(AAAT)_5$ | 1 | 30 | 7 | 0.433 | 0.359 |
| | | 2 | 30 | 7 | 0.600 | 0.210 |
| | | 3 | 31 | 7 | 0.580 | 0.222 |
| | | 4 | 30 | 4 | 0.300 | 0.291 |
| | | 5 | 30 | 5 | 0.133 | 0.390 |
| | | 6 | 21 | 4 | 0.380 | 0.209 |
| Rq 68040 | $(AATG)_6$ | 1 | 30 | 5 | 0.200 | 0.647 |
| | | 2 | 30 | 4 | 0.000 | 1.000 |
| | | 3 | 31 | 6 | 0.645 | 0.063 |
| | | 4 | 30 | 5 | 0.100 | 0.625 |
| | | 5 | 30 | 3 | 0.166 | 0.444 |
| | | 6 | 21 | 6 | 0.095 | 0.629 |
| Rq 51373 | $(ATGG)_{24}$ | 1 | 30 | 4 | 0.000 | 1.000 |
| | | 2 | 31 | 5 | 0.000 | 1.000 |
| | | 3 | 30 | 1 | 0.000 | - |
| | | 4 | 21 | 4 | 0.190 | - |
| Rq 91253 | $(AC)_{13}$ | 1 | 30 | 10 | 0.133 | 0.794 |

Locus name, Repeat motif, N (number of individuals genotyped), Na (number of alleles), Ho (Observed heterozygosity), Fis (inbreeding coefficient).

not models, such as catfish, providing large-scale data, enabling the advancement of research for the species.

## Conflict of interest

Authors did not declare any conflict of interest.

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*Full Length Research Paper*

# Cultivation and bromatological analysis of the medicinal mushroom *Ganoderma lucidum* (Curt.: Fr.) P. Karst cultivated in agricultural waste

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**The objective of the study was to evaluate the production of two strains of *Ganoderma lucidum* on agricultural waste and carry out bromatological analyses of the basidiomata obtained from the cultivation. The experiment was carried out at the Mushroom Module at the School of Agronomic Sciences of the São Paulo State University (FCA/UNESP - Botucatu, SP, Brazil) and two strains were used (GLM-09/01 and GLM-10/02) which were cultivated on waste, oat straw, bean straw, brachiaria grass straw, Tifton grass straw and eucalyptus sawdust under two situations: with (20%) and without (0%) supplementation with wheat bran. All the waste was taken from dumps of agricultural activities in Botucatu-SP. Both treatments were carried out in 10 repetitions, totaling 200 packages. The mushrooms cultivation took 90 days. Next, the biological efficiency of the treatments and the bromatological analysis of the basidiomata were evaluated. The biological efficiency (BE) values (%) varied from 0.0 to 6.7%. In the mushroom bromatological analyses, the results ranged from 8.7 to 13.7%, from 2.0 to 6.7%, from 0.83 to 1.79% and from 38.8 to 54.5%, for total protein, ethereal extract, ash and crude fiber, respectively. Thus, we conclude that the substrates which presented the greater yield were the brachiaria straw, 20% in both strains tested (GLM-09/01 and GLM-10/02) and the bean straw, 20% in the strain GLM-10/02. The mushrooms showed high levels of ethereal extract, fibers and ashes and a low level of proteins.**

**Key words:** *Ganoderma lucidum*, bromatological analyses, mushroom.

## INTRODUCTION

The mushroom consumption is a millennial habit in many cultures. Such interest is explained by the fact that these macrofungi present several medicinal and nutritional characteristics. These beings were even considered as

deities by some civilizations, and were often used in religious ceremonies.

Nowadays, they have a guaranteed market in many places around the world, not only because of their importance in medicine, but also because they are obligatory elements in various cuisines, mainly in the Italian and French.

Among the most appreciated mushrooms in the world, *Lentinula edodes* (shiitake), *Pleurotus ostretatus* (shimeji, oyster mushroom, hiratake) and *Agaricus blazei* (Paris champignon) stand out mainly because of their nutritional characteristics and pleasant taste.

Among the mushrooms with a pharmacological value, the *Ganoderma lucidum,* (known as *orelha-de-pau*; *Reishi* by the Japanese and *Ling Zhi* by the Chinese) raised much interest on such potential, as they are reported mainly by their

medicinal power among their numerous properties, and they can also be used for preventing and treating various diseases, including cancer and AIDS (Russell and Paterson, 2006).

This species presents many pharmacologically active compounds, such as: triterpenoids, steroids, polysaccharides, proteins, alkaloids, nucleosides and nucleotides (Boh et al., 2007). They occur in different types of colors, such as Chinese red, bright yellow and white. The fruiting body is initially white; it becomes yellow with its ripening and acquires a brownish varnished aspect in its adult phase (Perumal et al., 2009).

At first, the mushrooms were extracted mainly from forests. Afterwards, they began to be cultivated in artificial environments. Several ways were employed to carry out such activity, such as the traditional cultivation (substrate sterilization through composting) and the axenic cultivation (sterilization at a temperature of 121º), which is considered the most common in many yields, as it is faster and more practical.

The substrates used in the cultivation are usually formulated with straw and sawdust, which allows the use of many agricultural and agroindustrial raw materials and are considered of low or none aggregate value. This practice is also an income option for growers who generate a great amount of waste, as it represents an efficient alternative to enable the use of organic material for bioconversion into products with a high added value: mushrooms. In addition, this raw material is rich in lignocellulosic fibers, which makes it a favorable substrate for the development of edible fungi.

Considering the great abundance of these residues generated by various segments (agricultural, agroindustrial, forest and wood) and the possibility of using them as substrate to cultivate edible fungi, we must also consider the several fungi species present in nature and their affinity with the residues. The *G. lucidum* species presents many distinct strains and also nutritional requirements which vary regarding the place where it is collected and the type of substrate. This study aimed to evaluate the production of two strains of *G. lucidum* on agricultural waste and carry out bromatological analyses of the initial and residual substrates and of the basidiomata obtained in cultivation.

## MATERIALS AND METHODS

The experiment was carried out at the Mushroom Module facilities at FCA/UNESP-Botucatu, SP, Department of Plant Production, Plant Defense, in a Dalsem Mushroom climatic chamber (“Reefer” container type with dimensions of 12 m in length X 2.25 in height and 2 m in width).

### Obtainment of strains

The strains of *G. lucidum* used in the experiment were GLM-09/01 and GLM-10/02, which are preserved in mineral oil at the Mushroom Module Bank matrix,+ Department of Plant Production, Plant Defense - FCA/UNESP, Botucatu.

### Residues used

The residues used in the experiment were: oat straw, bean straw, brachiaria grass straw, Tifton grass straw and eucalyptus sawdust. The eucalyptus sawdust was considered as testimony, once it constitutes a residue the mushrooms have affinity with, according to many studies observed in literature. All the waste was taken from dumps of agricultural activities from the city of Botucatu-SP.

### Residues processing

The residues of oat, bean, brachiaria grass and tifton grass straws were dried at environmental temperature and later stored in raffia bags until their use. The same procedure was applied to the eucalyptus sawdust.

### Inoculum “Spawn” preparation

Sorghum grains, which were used to prepare the inoculums, were cooked for 40 min in boiling water. Afterwards, they were left resting for 40 min to drain the excess of water. Next, 20 g $Kg^{-1}$ of calcitic limestone and 160 g $Kg^{-1}$ of plaster were added, considering the weight of the cooked grains when wet. The homogenization was carried out in a construction mixer (capacity of 420 L).

After that, 250 g of substrate were transferred to the HDP bags. Later, the secondary matrix of the two strains of *G. lucidum* (GLM-

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**Table 1.** Treatments used to evaluate the yield of *Ganoderma lucidum* in substrates based on agricultural waste and supplemented with wheat bran (proportion based on dry matter).

| Strain | Substrates |
|---|---|
| GLM-10/02 and GLM-09/01 | Tyfton straw 0% of wheat bran |
| | Tyfton straw 20% of wheat bran |
| | Brachiaria straw 0% of wheat bran |
| | Brachiaria straw 20% of wheat bran |
| | Oat straw 0% of wheat bran |
| | Oat straw 20% of wheat bran |
| | Bean straw 0% of wheat bran |
| | Bean straw 20% of wheat bran |
| | Eucalyptus sawdust 0% of wheat bran |
| | Eucalyptus sawdust 20% of wheat bran |

10/02 and GLM- 09/01) grown in petri dishes containing malt agar medium were divided into 8 triangular fragments with the same size. Each package received a fragment of inoculum and was sealed using heat sealer and incubated at 25°C until they were totally colonized by the fungi.

#### Substrate preparation

The experiment was entirely randomized in a 2 × 10 factorial scheme, corresponding to two strains and 10 types of substrates. Both treatments were carried out in 10 repetitions, totaling 200 packages.

The substrates were prepared according to their formulations (Table 1). The residue was inserted into a construction mixer with the $CaCO_3$ and the supplement of wheat bran. Next, water was added until it reached 65% of humidity. The mixture was homogenized until acquiring uniformity.

Afterwards, 1 kg of the mixture was added to high-density polyethylene (HDP) bags. The properly identified packages were sealed in heat sealer and sterilized at a temperature of 121°C for 180 min.

#### Inoculation

After the sterilization period, the packages were taken to an inoculation room, where they were maintained under environmental temperature and inoculated with their referred strains, according to the treatments. For so, side cuts were made in each bag with a pair of scissors sterilized with alcohol; the scissors were also sterilized in the flame of a Bunsen burner at every cut. 12 g of inoculum seed previously prepared was inserted in each substrate through these openings. The packages were sealed in heat sealer, identified and distributed in a room which was adapted for incubation with adjusted temperature of 25°C and humidity of 75%, until the full colonization of the substrate.

Thirty (30) days after incubation, the substrates had been completely colonized by the fungi and were taken to the Dalsem Mushroom climatic chamber, where they were kept until the end of the cultivation cycle, by keeping the same temperature and humidity used in the previous incubation.

The primordia began to appear after 85 days of incubation and the first harvests were initiated ten days later. The harvest was carried out manually in the stage prior to sporulation by removing all the basidiomata.

#### Basidiomata processing

The mushrooms collected were put in aluminum foil and dehydrated in a greenhouse with forced ventilation and temperature adjusted to 40°C until they reached a constant weight. Afterwards, the samples were stored in high-density polyethylene (HDP) bags, properly identified according to each treatment. The grinding was carried out in a knife mill with a 30-mesh sieve. Nest, all samples were packed in properly sealed plastic bags and identified until their use in the later analyses.

#### Variables analyzed

##### *Biological efficiency (BE)*

The yield was expressed by biological efficiency (BE), which represents the percentage of conversion of the substrate into fungi biomass (mushrooms).

$$BE\ (\%) = \frac{\text{Total dry mass of mushrooms (g)}}{\text{Dry mass of the initial substrate (g)}} \times 100$$

#### Chemical and bromatological characterization

The ground samples of the substrates (initial and residual) and mushrooms were taken to the Bromatology Laboratory of the Faculdade de Medicina Veterinária e Zootecnia - FMVZ/UNESP, Botucatu, SP, Brazil, to be analyzed for crude protein, ash, ethereal extract and crude fiber, according to the methodology recommended by Silva and Queiroz (2002). The conversion factor of 4.38 (Furlani and Godoy, 2007) was used to determine the proteins level in the mushrooms.

#### Statistical analysis

The experiment data were submitted to the ANOVA variance

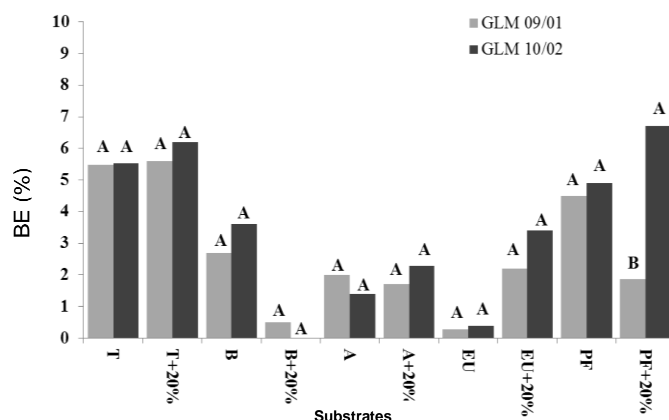


**Figure 1.** Biological Efficiency (EB), % of the strains GLM-09/01 and GLM-10/02 of *Ganoderma lucidum,* cultivated on substrates based on agroindustrial residues. T, Tifton straw without supplementation; T + 20%,Tyfton straw supplemented with 20% of wheat bran; B, Brachiaria straw without supplementation; B + 20%,Brachiaria straw supplemented with 20% of wheat bran; A, oat straw without supplementation; A + 20%,oat straw supplemented with 20% of wheat bran; EU, eucalyptus sawdust without supplementation; EU + 20%, eucalyptus sawdust supplemented with 20% of wheat bran; PF, bean straw without supplementation; PF+ 20%, bean straw supplemented with 20% of wheat bran. The means followed by the same letters in each treatment do not differ from each other (Tukey, 5%) (mean of two repetitions). CV (%), 58.89.

analysis using the SISVAR 4.2 statistics software, which was developed by the Departamento de Ciências Exatas at the Universidade Federal de Lavras, Minas Gerais, Brazil (UFLA). The means were compared by the Tukey's test at 5% probability.

## RESULTS AND DISCUSSION

The biological efficiency is a parameter used to measure the yield of a substrate in the cultivation of mushrooms. After 90 days of cultivation, it was possible to estimate these values expressed as percentages. According to the data obtained in the production of two strains of *G. lucidum*, it is possible to observe that the values of BE (%) varied from 0.0 to 6.7%, and the best results were obtained in the substrates T + 0% (BE - 5.5%) in the two strains, T + 20% in both strains (GLM-09/01 - BE 5.6% and GLM-10/02 - BE 6.2%), and BS + 20% (Figure 1), only for the strain GLM-10/02 - (BE - 6.7%).

Similar results to the highest values obtained in this experiment were found by González-Matute et al. (2002), who reported a biological efficiency of 7.6% using sunflower seed supplemented with 2.5% of malt extract as substrate for the cultivation of *G. lucidum*. In the same experiment, using other substrates [sunflower seed (100%); sunflower seed + 5% of malt; sunflower seed + 2.5% of wheat bran and sunflower seed + 5% of malt] the authors obtained higher values of BE (8.8, 9.9, 8.6 and 10%, respectively). Gurung et al. (2012) also found similar values to the experiment (BE 7.81%) using *Aulnus nepalensis* sawdust without any supplementation in the cultivation.

Higher percentages than the referred experiment were observed by Erkel (2009) when he used poplar sawdust (a European tree used in the paper industry) supplemented with gluten and sugar cane molasses in the proportions of 1, 2 and 3% as substrate in the cultivation of *G. lucidum*. Higher results were obtained and the most satisfactory were verified in the treatment sawdust + sugar cane molasses 1% (BE 20.3%), followed by the treatment sawdust + gluten 1% (BE 19%). The treatments with the highest supplementation levels (2 and 3%) obtained lower values of BE (%), showing that the ideal supplementation was 1% for both supplements. For Aysun and Gokcen (2009), who used substrates based on sawdust supplemented with residues of green tea in the proportions of 75:25, 80:20, 85:15 and 90:10, the highest results were obtained in the proportions 80:20, (BE 34.90%) and 75:25 (BE 31%).

Triratana et al. (1991) obtained a biological efficiency of 17% when using rubber tree sawdust *(Hevea brasiliensis)* supplemented with rice bran in the cultivation of *G. lucidum.* Similar data were observed by Veena and Pandey (2006), who used sawdust supplemented with rice bran in the proportion of 9:1 obtaining indices of BE equivalent to 20%.

Rolim et al. (2014) obtained higher results than the others (BE - 72%) by cultivating *G. lucidum* in substrate based on elephant grass + mango tree sawdust, supplemented with 10% of wheat bran and 10% of crushed sugar cane. Percentages close to the lowest result obtained in the experiment were reached by Gurung et al. (2012), who cultivated this experiment fungus in substrate based on Sal sawdust (*Shorea robusta*) supplemented with 10% of wheat bran and Sal sawdust + 10% of rice bran, and obtained BE of 0.0 and 0.81%, respectively. The authors also obtained 0% of BE when they used mango tree sawdust supplemented with 20% of wheat bran as substrate for the cultivation.

The biological efficiency obtained in the experiment was not satisfactory regarding most data observed in literature. Such fact might probably be associated with the nitrogen sources present in the substrate (Table 2). According to Hsieh and Yang (2004), the species *G. lucidum* requires a 70:1 to 80:1 C/N ratio for a satisfactory growth and the average C/N ratio in this experiment was of 53:1.

The substrates based on eucalyptus sawdust without supplementation and brachiaria straw with 20% of supplementation obtained a lower BE value (%) (Figure 1). It was possible to verify by means of the analyses carried out in the initial substrate (Table 2) that such substrate presented a lower concentration of nitrogen and a broader C/N ratio (121:1 and 35:1, respectively),

**Table 2.** Centesimal composition, pH and C:N ratio of initial substrates (raw material supplemented with wheat bran.

| Treatment | C (%) | N (%) | C:N | pH |
|---|---|---|---|---|
| T | 17.52 | 0.50 | 35/1 | 6.5 |
| T+20% | 19.93 | 0.60 | 34/1 | 5.8 |
| B | 19.22 | 0.47 | 41/1 | 7.1 |
| B+20% | 20.50 | 0.59 | 35/1 | 6.3 |
| A | 18.64 | 0.30 | 63/1 | 6.8 |
| A+20% | 19.49 | 0.42 | 48/1 | 5.7 |
| EU | 21.77 | 0.18 | 121/1 | 3.5 |
| EU+20% | 19.83 | 0.35 | 58/1 | 4.4 |
| PF | 20.10 | 0.47 | 43/1 | 6.9 |
| PF+20% | 20.32 | 0.44 | 47/1 | 6.9 |

T, Tifton straw without supplementation; T + 20%,Tyfton straw supplemented with 20% of wheat bran; B, Brachiaria straw without supplementation; B + 20%,Brachiaria straw supplemented with 20% of wheat bran; A, oat straw without supplementation; A + 20%,oat straw supplemented with 20% of wheat bran; EU, eucalyptus sawdust without supplementation; EU + 20%, eucalyptus sawdust supplemented with 20% of wheat bran; PF, bean straw without supplementation; PF+ 20%, bean straw supplemented with 20% of wheat bran.

**Table 3.** Bromatological values of *G. lucidum* cultivated on agricultural waste.

| Substrate | Bromatological parameters | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Protein (%) | | Ethereal extract (%) | | Crude fiber (%) | | Ash (%) | |
| | Strain | | | | | | | |
| | GLM 09/01 | GLM 10/02 | GLM 09/01 | GLM 10/02 | GLM 09/01 | GLM 10/02 | GLM 09/01 | GLM 10/02 |
| T | 12.5A* | 12.4 A | 4.8A | 2.9B | 48.2A | 48.4A | 1.6A | 1.4A |
| T+20% | 15.2A | 13.8B | 3.5B | 4.9A | 51.7A | 51.4A | 1.8A | 1.7A |
| B | 11.6A | 12.2A | 4.7A | 4.5A | 48.8A | 50.2A | 1.3A | 1.6A |
| B+20% | 13.8A | 13.6A | 6.7A | 6.7A | 48.8A | 48.1A | 1.8A | 1.7A |
| A | 9.9A | 9.3A | 5.2A | 5.4A | 56.2A | 50.7B | 1.0A | 0.8A |
| A+20% | 10.6B | 12.5A | 4.8A | 4.8A | 57.7A | 47.8B | 1.3A | 1.5A |
| EU | 9.6A | 8.7B | 5.7A | 5.8A | 41.2A | 38.8B | 1.1A | 0.8A |
| EU+20% | 11.9A | 11.2B | 3.1A | 2.0B | 50.7A | 51.2A | 1.5A | 1.6A |
| PF | 12.3A | 11.6B | 4.5A | 4.9A | 57.3A | 51.3B | 1.4A | 1.4A |
| PF+20% | 14.8A | 12.5B | 4.8A | 2.7B | 50.1B | 54.3A | 1.8A | 1.2B |

*Averages followed by equal letters in each line and among strains, within each bromatological parameter, are not statistically different among each other (Tukey, 5%). T - Tifton straw without supplementation; T + 20% - Tyfton straw supplemented with 20% of wheat bran; B - Brachiaria straw without supplementation; B + 20% - Brachiaria straw supplemented with 20% of wheat bran; A - oat straw without supplementation; A + 20% - oat straw supplemented with 20% of wheat bran; EU - eucalyptus sawdust without supplementation; EU + 20% - eucalyptus sawdust supplemented with 20% of wheat bran; PF - bean straw without supplementation; PF + 20% - bean straw supplemented with 20% of wheat bran.

far from the one recommended for the cultivation of *G. lucidum*.

It was also observed that the substrates which provided a lower biological efficiency (EU and EU + 20%) were the ones that presented pH of 3.5 and 4.4 respectively, considered inappropriate for the cultivation of *G. lucidum* as it requires 5.0 to 6.9 for its development (Nawawi and Ho, 1990; Gurung et al., 2012; Kamara and Bhatt, 2013).

The nutritional values of the basidiomata of the two species of *G. lucidum* (GLM-09/01 and GLM-10/02) cultivated in substrates based on agricultural waste can be verified in Table 3. In the comparison of the total protein

levels present in the basidiomata of strains GLM-09/01 and GLM-10/02, the obtained values varied from 8.7 (EU) to 13.7% (T+20%) for the strain GLM-10/02 and from 9.5 (EU) to 15.1% (T+20%) for the strain GLM-09/01.

Significant differences between the two strains were not observed in the treatments T, B, O and B + 20%. In the treatments T + 20%, EU, EU + 20%, BS, BS + 20%, the basidiomata of the strain GLM-09/01 presented the highest levels of total proteins, whereas the basidiomata of the strain GLM-10/02 obtained the highest percentages of total proteins in the treatment O + 20%. Higher results were obtained by Rawat et al. (2012), who obtained 20.6% of total proteins in the basidiomata of *G. lucidum* collected in the Himalaya forest.

By comparing the highest protein value obtained with the nitrogen values of the substrates used, we observed that the residues which provided mushrooms rich in proteins (T + 20%) presented the highest N levels (0.60%). According to Silva et al. (2002), the level of crude protein of the basidiomata is influenced by the level of nitrogen present in the initial substrate. Those authors cultivated *P. sajor caju* on substrates supplemented with different concentrations of nitrogen and noticed that the greater the concentration, the greater the amounts of proteins present in the mushrooms. Substrates with higher N levels were not colonized by the fungus. The values of ethereal extract for the basidiomata of the strain GLM-09/01 varied from 2.0 (EU + 20%) to 6.75% (B + 20%).

By analyzing the strains separately in relation to 09/01, the treatments T, EU + 20%, BS + 20% WB presented a higher level of ethereal extract. Regarding the strain GLM-10/02, the highest values were obtained from the treatment T + 20%. Lower results than the ones obtained in this experiment (1.7%) were observed in *G. lucidum* samples sold in markets in Vietnam (Hung and Nhi, 2012). Ogbe and Obeka (2013) also obtained lower results (1.5%) in *G. lucidum* collected in Nigerian forests. Aremu et al. (2009) carried out the bromatological analyses of the fruiting body of *G. lucidum* also collected in Nigerian forests, but found results similar to those of this experiment (6.9%).

The crude fiber values present in the basidiomata of *G. lucidum* varied from 38.8 (EU) to 54.5% (BS+20%) in the strain GLM-10/02 and from 41% (EU) to 57.7% (O+20%) in the GLM-09/01. When analyzing each strain separately in relation to the GLM-09/01, the highest concentrations of fibers were obtained in the basidiomata provided from the treatments O (56.2%), O + 20% (57.7%), BS (57.3%) and EU (41.2%). BS+20% was the treatment which provided a higher result (54.5%) for strain GLM-10/02.

All the results observed in literature were lower than the ones obtained in this experiment, such as Ogbe and Obeka (2013), who analyzed mushrooms *G. lucidum* collected in the campus of the University of Nassarawa-Nigeria and found 7.7% of crude fiber present in the basidiomata. However, Aremu et al (2009), who also collected *G. lucidum* in Nigerian forests, obtained 6.9% of fibers in the analyses. Nagaraj et al. (2013) obtained 14.4% of fibers in mushrooms *G. lucidum* found in Indian forests.

Considering the ash levels present in the basidiomata of the strains GLM-09/01 and GLM-10/02 in each specific substrate in comparison with GLM-09/01, we observed that the greater concentrations of ash were originated from treatments BS + 20% WB (1.8%). The two strains did not present significant differences among each other in the other treatments. The lowest values in both strains were verified in the treatments O and EU.

The values oscillated from 0.83 (O) to 1.79% (T + 20%) for the strain GLM-10/02 and from 1.0% (O) to 1.8% (B+20%) for the strain GLM-09/01. The ash values corresponded to about 5 to 12% of dry matter of the basidiomata and it estimates the amount of micro and macro elements present (Kalac, 2009).

Similar results were observed by Hung and Nhi (2012) in *G. lucidum* mushrooms sold in Vietnamese markets (1.4%). Higher values than those of the experiment were observed by Singh et al. (2014) analyzing basidiomata of *G. lucidum* (8.3%) and *G. philipii* (7.6%) found in Indian forests. Higher results were also obtained by Ogbe and Obeka (2013) and Aramu et al. (2009) in *G. lucidum* mushrooms collected in Nigerian forests, who found ash values of 8.4 and 7.8%, respectively. Higher results to those obtained in the experiment were also observed by Ragunathan and Swaminathan (2003), by cultivating *P. citrinopileatus* on residues of coconut husk and corn cob (6.10 to 6.30%) and Sales-Campos (2008), by using wood and agroindustrial residues in the cultivation of *P. ostreatus.*

## Conclusions

Out of the substrates analyzed, the ones based on 20% of brachiaria straw presented the greater yield in both strains tested (GLM-09/01 and GLM-10/02) and the substrate based on 20% of bean straw presented the greater yield in the strain GLM-10/02. The mushrooms showed high levels of ethereal extract, fibers and ash and a low level of proteins. The analyzed residues are within the standard observed in the literature, regarding both the yield and the bromatological analyses of the basidiomata obtained.

## Conflict of interests

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# Use of multivariate analysis to evaluate the effect of sucrose on *in vitro* cassava conservation

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**The aim of this work was to evaluate the effect of sucrose to reduce the *in vitro* growth of cassava plants using multivariate statistical tests. Cassava conservation has a relevant role as an auxiliary strategy for preservation and genetic breeding. Micro-cuttings of cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 from the Active Germplasm Bank of the Embrapa Cassava and Fruits were tested with five different concentrations of sucrose (0, 14.6, 29.2, 43.8, 58.5 mM) and the following variables were evaluated: plant height (cm), total number of leaves, number of senescent leaves, number of micro-cuttings, size of callus, number of roots and plant vigor. The data were submitted to analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA). According to the criterion of Singh, the variable that contributed most to explain the behavior of cassava plants under the conditions studied was the number of leaves, with 36.52%, while the vigor of the plants showed the lowest contribution (0.66 %). The best concentration for *in vitro* cassava conservation was 58.45 mM of sucrose, based on the number of viable plants after incubation.**

**Key words:** Biotechnology, germplasm conservation, tissue culture, *Manihot esculenta*, genetic resources.

## INTRODUCTION

The genetic diversity of cassava in Brazil provides a wide genetic base for breeding programs in the tropics. This variability covers a group of genes that confer resistance to major pests and diseases that affect the crop and enables adaptation to different edaphoclimatic conditions (Albuquerque et al., 2009).

Genetic erosion in cassava is mainly caused by biotic and abiotic stresses, which along with the expansion into new agricultural frontiers is a fact that should not be ignored in genetic breeding program, to avoid jeopardizing present and future actions. Therefore, efforts are required to conserve this germplasm (Fukuda et al., 2002; Rival and Mckey, 2008).

*In vitro* conservation comprises maintenance of micro controlling different growing conditions, such as temperature, photosynthetically active radiation, photoperiod and

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**Table 1.** List of cassava accessions, common name, origin and collection site.

| Accessions | Common Name | Origin | Collection Site |
|---|---|---|---|
| BGM 264 | Cigana | EAUFBA | Amargosa-BA, Brazil |
| BGM 265 | Panguá | IPEAL | Santa Teresinha-BA, Brazil |
| BGM 1037 | Casco de Burro | CENARGEN | Aurora do Norte-TO, Brazil |
| BGM 1282 | Macaxeira-Cara | CENARGEN/CNPMF | Ouricuri-PE, Brazil |

Source: *Embrapa Cassava and Fruits* (2003).

concentrations of osmotic retardants and hormones in the culture medium (Canto et al., 2004), therefore extending the interval between subcultures. This reduces the need for labor and reagents and as a result decreases the risks of fungal and/or bacterial contamination (Lemos et al., 2002).

Plants kept *in vitro* do not perform photosynthesis enough to guarantee the development of explants, it is necessary to add a carbon source such as a sugar to the culture medium. The sugar most commonly used in nutritive media is sucrose because it is easily metabolized by many species (De Conti et al., 2011). Reducing the amount of sucrose in the culture medium results in lower explant growth.

Usually, univariate statistical methods are used in studies of *in vitro* conservation to evaluate the effectiveness of treatments. However, studying the variables separately may not be enough to model biological phenomena, since important information is lost by disregarding the existing correlations between variables. Multivariate analysis involves considering all relevant variables at the same time in the theoretical interpretation of data (Neto, 2004). This enables evaluating a set of characteristics by taking into account their correlations, leading to inferences about the set of effects of characteristics at a set level of significance (Johnson and Wichern, 1992). However, multivariate analysis techniques have only infrequently been used in studies involving *in vitro* conservation (Carvalho, 2014; Franco et al., 2007).

The aim of this work was to evaluate the effect of sucrose in reducing the *in vitro* growth of cassava plants using multivariate statistical tests, aimed at more efficient study of *in vitro* conservation of cassava germplasm.

## MATERIALS AND METHODS

### Plant material

Four cassava cultivars were selected from different regions of Brazil, from experimental fields of the Active Germplasm Bank of the Embrapa Cassava and Fruits. Table 1 shows the accessions, common name, origin and collection site.

### *In vitro* plant growth

The plants of the accessions from Table 1 were used as initial explants for conservation experiments. Excised meristems from plants kept in a greenhouse were decontaminated and inoculated in 4S medium containing MS (Murashige and Skoog, 1962) salts supplemented with 1 mg $L^{-1}$ of thiamine, 100 mg $L^{-1}$ of inositol, 0.02 mg $L^{-1}$ of naphthalene acetic acid (NAA), 0.04 mg $L^{-1}$ of benzylamino-purine (BAP), 0.05 mg $L^{-1}$ of gibberellic acid ($GA_3$), 20 g $L^{-1}$ of sucrose, solidified with 2.4 g $L^{-1}$ of Phytagel® (SIGMA, USA), with pH adjusted to 5.8, at a temperature of 27 ± 1ºC, 16 h photoperiod and photon flux density 30 µmol $m^{-2}s^{-1}$ for 30 days (establishment phase).

The multiplication phase was conducted with three subcultures at intervals of 45 days using as explants microcuttings of 1.0 cm inoculated in 17 N medium (CIAT, 1984), composed of 1/3 of the macro and micronutrients of MS medium supplemented with 0.35 mg $L^{-1}$ of thiamine, 35 mg $L^{-1}$ of inositol, 0.01 mg $L^{-1}$ of NAA, 0.01 mg $L^{-1}$ of BAP, 0.01 mg $L^{-1}$ of GA3, 20 g $L^{-1}$ of sucrose, 2.4 g $L^{-1}$ Phytagel ®, with pH adjusted to 5.8. The incubation was carried out in a growth room with 16-h photoperiod, temperature of 26 ± 1°C and photon flux density of 30 µmol.$m^{-2}$.$s^{-1}$.

### *In vitro* conservation

Microcuttings (1.0 cm length) from plants obtained in the previous step were inoculated in basic culture medium “8S” (CIAT, 1984). The medium consisted of mineral salts and vitamins from "MS" supplemented with NAA (0.01 mg $L^{-1}$), BAP (0.02 mg $L^{-1}$), $GA_3$ (0.1 mg $L^{-1}$), with pH adjusted between 5.7 and 5.8, solidified with 2 g $L^{-1}$ of Phytagel®. The plants were stored in a slow growth room with a temperature of approximately 22°C, having a 12-hphotoperiod and photon flux density of 30 µmol $m^{-2}s^{-1}$. The sucrose concentrations used were based on the results obtained by Macia (2011).

The effect of four sucrose concentrations was evaluated against a control treatment without sucrose in the culture medium. The evaluated concentrations were 14.6, 29.2, 43.8 and 58.5 mmol $L^{-1}$. Evaluations were performed at 30, 60, 120 and 330 days after incubation. Then after 330 days in the preservation medium, the surviving plants were transplanted and cultured in 17 N growth medium. The plant viability was assessed by calculating the regeneration rate (%) per accession and treatment.

### Variables

The following variables were evaluated: plant height (PH) in cm, total number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC), number of roots (NR), root length (RL) in cm, size of callus (CS), using the scale 0 (absent), 1 (small), 2 (medium) and 3 (large), and plant vigor (V), using the scale 3 (totally green plant), 2 (slightly yellowish plant), 1 (very yellowish plant) and 0 (dead plant).

### Statistical analysis

A completely randomized model was used in split plots in time to

**Table 2.** Summary of analysis of variance (ANOVA) for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), callus size (CS), number of microcuttings (NMC), number of roots (NR), root length (RL) and vigor (V) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282.

| SV | DF | MS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PH | NL | NSL | CS | NMC | NR | RL | V |
| Acession | 3 | 223.74** | 23.41** | 17.20** | 4.29** | 5.77** | 9.87** | 73.14$^{ns}$ | 1.28$^{ns}$ |
| Sucrose | 4 | 641.62** | 43.26** | 31.73** | 3.40** | 24.86** | 28.76** | 318.74** | 0.18$^{ns}$ |
| Access*Suc | 12 | 127.84** | 7.90** | 5.62** | 2.37** | 4.44** | 4.04** | 57.47$^{ns}$ | 0.41$^{ns}$ |
| Error A | 76 | 14.91 | 1.93 | 1.07 | 0.30 | 0.52 | 0.64 | 22.30 | 0.19 |
| Days | 4 | 468.71** | 62.03* | 116.36** | 3.04* | 20.90** | 38.35* | 224.34** | 25.36* |
| Error B | 16 | 2.31 | 0.25 | 0.23 | 0.06 | 0.12 | 0.16 | 3.86 | 0.07 |
| Access*Days | 12 | 28.94** | 1.41$^{ns}$ | 2.50** | 0.08$^{ns}$ | 0.57** | 1.03** | 24.78** | 0.27$^{ns}$ |
| Suc*Days | 16 | 35.52** | 2.25** | 2.60** | 0.06$^{ns}$ | 1.30** | 3.13** | 30.76** | 0.13$^{ns}$ |
| Access*Suc*Daysem | 48 | 9.78** | 0.94** | 1.02** | 0.12** | 0.34** | 0.51** | 10.79$^{ns}$ | 0.19** |
| Error C | 1054 | 4.00 | 0.44 | 0.32 | 0.05 | 0.14 | 0.25 | 5.91 | 0.08 |
| CV (%) | | 41.97 | 30.52 | 32.60 | 14.99 | 19.93 | 36.04 | 22.07 | 19.24 |
| Mean | | 4.76 | 5.51 | 3.72 | 2.08 | 3.62 | 2.11 | 11.01 | 1.99 |

*significant at 5% by the F-test. $^{ns}$not significant at 5%; SV, source of variation; DF, degree of freedom; MS, mean square.

**Table 3.** Relative contribution of the variables to diversity according to the criterion of Singh (1981) for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), callus size (CS), number of microcuttings (NMC), number of roots (NR) and vigor (V) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 for different concentrations of sucrose.

| Variable | Sij | Sij (%) |
|---|---|---|
| PH | 6846.15 | 7.12 |
| NL | 35132.47 | 36.52 |
| NSL | 32344.24 | 33.62 |
| CS | 631.84 | 0.66 |
| NMC | 12385.41 | 12.87 |
| NR | 8657.44 | 9.00 |
| V | 207.87 | 0.22 |

Sij, Measure of the relative importance of character j in canonical variables based on the Mahalanobis distance i.

perform analysis of variance. In the plots, four accessions and five concentrations of sucrose were analyzed, while in the subplots four evaluation times were considered (30, 60, 120 and 330 days after incubation) along with their respective interactions with the plots factors. Five replications per treatment were used, each consisting of three plants. The experimental plot was formed by a test tube containing one microcutting. The data on number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC) and number of roots (NR) were transformed to $(x+0.5)^{1/2}$ to satisfy the assumptions for the analysis of variance. All the assumptions of ANOVA were tested. In ANOVA, F-test criterion was used to test the significance of the treatments. The analyses were performed with the aid of the SAS program (SAS Institute Inc., 2000).

Multivariate analysis of variance (MANOVA) was also performed to check the effect of the treatments regarding the variables. According to Johnson and Wichern (1992), the Wilks criterion was used to test the significance of the treatments. Based on the matrix of the sums of squares and products obtained from MANOVA, the partial correlation coefficients were calculated, and multicollinearity diagnosis was performed according to the criterion of Montgomery and Peck (1981). For the calculation of the relative contribution of each variable in the multivariate analysis, the criterion of Singh (1981) was used.

## RESULTS AND DISCUSSION

The analysis of variance (ANOVA) indicated different treatment effects of sucrose, accession and evaluation time on the dependent variable, according to the treatment and the variable considered (Table 2).

It is necessary to emphasize that, since the evaluations are made in the same experimental plot, it was necessary to include two new components in the model (errors a and b). This is because, of course, there is no randomization of the evaluation times in different repetitions, that is, measures were taken at the same time on the same plots. Consequently, there is no independence of measurements taken over time. Thus, it is suggested to perform analysis of variance with three experimental errors (Ramalho et al., 2000).

As indicated by the criterion of Singh (1981), the variables that contributed most to explain the behavior of cassava plants under the established conditions were the number of leaves (NL) and number of senescent leaves (NSL), with 36.52 and 33.62%, respectively, while callus size (CS) and plant vigor (V) showed the lowest contribution with 0.66% and 0.22%, respectively (Table 3).

This analysis allows the selection of variables that are important for *in vitro* conservation studies, enabling the management of data and subsequent analyses.

Table 4 shows that partial correlation coefficients, obtained by multivariate analysis (MANOVA), allowed an

**Table 4.** Partial correlation coefficients for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC) and number of roots (NR) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 for different concentrations of sucrose.

| Variables | NL | NSL | NMC | NR |
|---|---|---|---|---|
| PH | 0.65** | 0.62** | 0.78** | 0.52** |
| NL | | 0.80** | 0.79** | 0.59** |
| NSL | | | 0.71** | 0.53** |
| NMC | | | | 0.57** |

** Significant at 1% by the t-test.

even more detailed analysis, as was performed with the most important variables of the study (PH, NL, NSL and NMC) according to the criterion of Singh (1981). The highest correlations were obtained between NL and NSL (0.80**), NL and NMC (0.79**) and PH and NMC (0.78**), involving conservation impact variables that express the development of plants under the established conditions. The partial correlation coefficient shows how the behavior of a variable is related to another and in some cases allows the elimination of one of them, since their behavior can be predicted by the other. In cases where the measurement of a given variable is laborious or can be destructive, the high correlation with another variable may eliminate the need for these measurements.

The condition number of Montgomery and Peck (1981) refers to the ratio between the highest and lowest values obtained from the principal component analysis; if the condition number is less than 100, it is refer to a low multicollinearity. The condition number from the multi-collinearity diagnosis obtained in this study was 51.54, classified as weak multicollinearity in the correlation matrix according to the classification of Montgomery and Peck (1981), so it was possible to obtain a reliable estimate in biological terms. In this work, NL, NSL and PH showed high correlation with the number of microcuttings (NMC), facilitating evaluation of the results obtained on the efficiency of treatments in reducing the metabolism of plants and their subsequent viability. These variables are considered to be of high relevance to *in vitro* conservation studies of cassava and the statistical tools used in this work confirmed this importance.

An advantage of multivariate extension is the possibility to estimate the partial correlation matrix obtained from the sum of squares and the product of residuals when compared with the traditional univariate method. The partial correlation establishes the degree of association between two variables, eliminating the effect of the treatments. When the number of repetitions is large, Ito and Schull (1964) and Korin (1972) observed that the tests used in multivariate analysis of variance are not greatly influenced by the heterogeneity of variance and covariance matrices. Mardia (1971) concluded that multivariate analysis of variance is robust and its ability to deal with non-normality makes it a more suitable tool for tissue culture work.

In the search for a suitable condition that can accommodate the largest possible number of *in vitro* cassava accessions with the most standardized behavior possible, the elimination of two variables (CS and V) implies a significant reduction of work.

In a given study, the importance of a variable will depend on the species and the objective. For example, in citrus conservation the number of microcuttings is not a determining factor, as evidenced by studies by Carvalho, (2014). In cassava, this variable is essential because several microcuttings are obtained from the same plant and the increase in the number of microcuttings will depend on the treatment. It is quite complicated to break the apical dominance of cassava *in vitro* and the replication depends on the number of microcuttings. Vidal (2009), studying *in vitro* cassava cultivation, found a strong positive correlation (0.71), similar to the one obtained in this work (0.78), between plant height and number of microcuttings. Also, Macia (2011) suggests that these two variables may present good correlation for *in vitro* cassava conservation.

The results obtained in this work are consistent with those obtained by Londe et al. (2012), when evaluating sucrose's effect on growth rate. They noted that the 58 mM concentration produced greater plant height. Macia (2011) tested different sucrose concentrations on *in vitro* cassava conservation (29, 58, 116 and 232 mM) and observed the best results in the same concentration. The plants showed a reduction of cellular metabolism without compromising their viability after the conservation period.

Regarding the difference in behavior among the four accessions, BGM 1282 presented the lowest values for all variables. The other three cultivars (BGM 264, BGM 265 and BGM 1037) showed similar performance for PH, NL, NSL and NMC variables in the concentration of 43.8 mM (15 $gL^{-1}$). Regarding the management of *in vitro* germplasm banks, this result can be considered encouraging since one of the major difficulties found is exactly the different behaviors observed among the

**Table 5.** Number of plants surviving after 330 days (NPSO), number of cultured microcuttings (NMC), number of viable plants (NVP) and plant viability (PV) of different cassava accessions after preservation in different sucrose concentrations.

| Sucrose (mM) | NPSO | NMC | NVP | PV% |
|---|---|---|---|---|
| 0.0 | 10 | 10 | 0 | 0 |
| 14.6 | 8 | 8 | 0 | 0 |
| 29.2 | 2 | 2 | 0 | 0 |
| 43.8 | 7 | 19 | 5 | 26 |
| 58.5 | 5 | 25 | 22 | 88 |
| **Accessions** | | | | |
| BGM 264 | 4 | 26 | 5 | 19 |
| BGM 265 | 17 | 24 | 18 | 75 |
| BGM 1037 | 2 | 6 | 4 | 67 |
| BGM 1282 | 6 | 8 | 0 | 0 |

genotypes preserved.

The maintenance of plant viability after the incubation period is one of the most important aspects of *in vitro* conservation. For this purpose, the metabolism reduction cannot cause a loss of viability. This work showed that the loss of plants in BGM 264 begins from 120 days of culture, similar to BGM 265, regardless of sucrose concentration. The first dead plants of BGM 1037 and BGM 1282 appeared at 30 days of conservation, confirming one of the problems for *in vitro* cassava conservation.

Table 5 shows, in general, the results obtained separately by treatment and accessions, confirming that the survival rates are directly related to the best treatment for the conservation of these four accessions. Despite the encouraging results with the concentration of 43.8 mM, only one accession (BGM 265) presented viable plants at the end of 330 days of cultivation, with a 39% viability rate. For BGM 264 and BGM 1037, viable plants were obtained after the same conservation period with the higher sucrose concentration, 90% and 100% in that order, although there were only four BGM 1037 plants tested. These results can be considered promising since for cassava the maximum time of conservation is 270 days (IITA, 2002).

Table 5 shows the drastic reduction in metabolism observed in accession BGM 1282, which made renewed growth after incubation impossible, making clear the need for adjustments in the conservation of this material with higher concentrations of sugar. Once again, these results emphasize the strong genotype-dependence observed in the *in vitro* behavior of cassava varieties and how limiting and determining the carbohydrate source can be.

## Conclusion

Multivariate analysis can be considered an efficient tool for studies of *in vitro* cassava conservation, and the sucrose concentrations of 14.6 and 29.2 mM reduced the metabolism of cassava plants *in vitro*, but made it impossible to regenerate them. The best concentration for *in vitro* cassava conservation is 58.5 mM of sucrose.

## Conflict of interests

The authors did not declare any conflict of interest.

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**Abbreviations: PH,** Plant height; **NL,** total number of leaves; **NSL,** number of senescent leaves; **NMC,** number of micro-cuttings; **NR,** number of roots; **RL,** root length; **CS,** size of callus; **V,** plant vigor.

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# Inducible secretion of phytate-degrading enzymes from bacteria associated with the medical plant *Rosa damascena* cv. Taifi using rice bran

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**More than 320 bacteria were isolated from the soil (Rhizosphere, endophyte, flowers and leaves) of *Rosa damascena* cv. Taifi and screened for phytase activity. Phytase activity was checked for 24 isolates in *Bacillus* broth media supplemented with and without rice bran. Twelve (12) isolates were found with detectable phytase activities. Among them, four selective bacterial strains were active (BAFA.Taifi94, BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117). The phytase activity of strain BAFA.Taifi111, which was grown in the *Bacillus* broth media and supplemented without rice bran showed 19.7 units/ml initial growths whereas, with an induction of rice bran 37.7 units/ml was observed after 10 days in the shaker at 30°C/150 rpm. The optimum temperature for BAFA.Taifi94 and BAFA.Taifi103 was 60°C, whereas it was 70°C for BAFA.Taifi111. The stability at 80°C was exhibited by BAFA.Taifi117. The optimum pH range was pH 5-6.5 at 60°C. The obtained *Bacillus* species for phytase production have been induced using rice bran and their physical properties such as temperature optima, pH optima and thermo stability were found similar to the previously characterized and published or commercially available *Bacillus* phytases.**

**Key words:** *Bacillus sp.,* phytase activities, soil bacteria, *Bacillus* broth, *Bacillus* broth.

## INTRODUCTION

Phytate occurs in cereals such as corn, rice bran, wheat bran and in seeds of cotton and rape, as well as in legume soybean (*Glycine max*) with different concentrations from 0.4 to 6.4%. The phytic acid composition of rice bran varies from 0.14 to 0.99% on dry basis weight. The phytase enzyme hydrolyzes phytate to *myo*-inositol and phosphate (Konietzny and Gernier, 2002). Many bacteria, yeast and fungi have phytase activities (Pandey

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et al., 2001). Phytases were detected in various bacteria, e.g. *Aerobacter aerogenes* (Greaves et al., 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Lactobacillus* (Angelis et al., 2003), *Aspergillus (*Casey and Walsh, 2003), *Rhizopus* (Sutardi and Buckle, 1988), *Bacillus sp. (*Choi et al., 2001), *Bacillus subtilis* (Powar and Jagannathan,1982), *Klebsiella* sp (Shah and Parekh,1990), *B. subtilis* (natto) (Shimizu, 1992), *Escherichia coli* (Greiner et al., 1993), *Enterobacter* sp. (Yoon et al., 1996) and *Bacillus* sp. DS 11 (later designated as *Bacillus amyloliquefaciens*) (Kim et al., 1998). The genera *Bacillus* and *Enterobacter* produced extracellular phytases. The *E. coli* phytase is a periplasmic enzyme. Muddy soils containing the majority of natural bacterial strains are capable to produce phosphatase and phytase. Nineteen *Bacillus* bacterial strains isolated from maize root had phytase activity and produce favorable enzyme when 1% of rice bran was supplemented in the growth media as reported (Hussin et al., 2007).

Phytase, a well-known industrial enzyme is an object of extensive research. Bacterial and fungal (*Aspergillus* and *Penicillium*) enzymes conquered many applications in manufacturing sectors (Alva et al., 2007; Krootdilaganandh, 2000) and used in biofertilizers (Farouk et al., 2013). Many phytases were revealed, characterized in the last 10 years and are commercially produced. Phytase market volume exceeds US$250 million and is growing at around 10% every year. Commercial Phytases from four different sources, *Peniophora lycii* (6-phytase of fungal origin); *Aspergillus niger* (3-phytase of fungal origin, HAP); *E. coli* (6-phytase of bacterial origin, HAP); *Penicillium funiculosum* (3-phytase of fungal origin, HAP) are available. Storage stability of commercial pure phytases (OptiPhos, Phyzyme and Ronozyme) at 23°C was more than 91% for 30 days and up to 180 days approximately, more than 85% were in retaining activity. Pure phytases were more stable than in pre-mixes. Coated phytases are more stable than the non-coated (Sulabo et al., 2011). Extracellular phytase production was optimized at 39.7°C, at an initial pH of 7.1, supplementation with 13.6% rice bran, 320 rpm of agitation and 0 vvm of aeration by *Enterobacter sakazakii* ASUIA279 and it was purified (Farouk et al., 2012). A recombinant *E. coli* DH5α that had shown higher phytase activity after 17 h incubation than *E. sakazakii* ASUIA279 after 5 days of incubation was reported (Ariff et al*.,* 2013). A novel phytase gene (phyMS) from *Mycobacterium smegmatis* showed specific activity of 233.51 U/mg, optimal pH of 3 and 7 at 60°C was reported by Tamrin et al. (2014).

*B. subtilis,* a widely used food microorganism, generally recognized safe (GRAS) by the American Food and Drug Administration. Researches were carried out on *B. subtilis* from soil and other sources*. B. subtilis* CF92, an isolate from cattle feces, produces phytase with an exhibited optimal activity at 60°C at pH 7.0 and stable over a pH range of 4.0 to 8.0 (Hong et al., 2011). Phytase production by *B. subtilis* US417 (112 U/g of wheat bran) with a higher productivity in (SF) Submerged [2.3 U/(g×h)] than in (SSF) Solid State Fermentation [1.2 U/(g×h)] was reported (Kammoun et al*.,* 2012). Bacterial phytases have considerable potential in commercial applications (Ursula and Griner, 2004). Among different substrates used (wheat bran, rice bran, bengal gram bran, red gram bran, groundnut oil cake, sesame oil cake, coconut oil cake, cotton oil cake, soya bean meal, oatmeal, corn meal and barley meal) for *B. subtilis*, wheat bran was observed as the best substrate for phytase yield. Combination of wheat bran with rice bran and groundnut oil cake in 1:1 ratio resulted in better phytase production (0.50 and 0.79 U/ml respectively) than using wheat bran only (Sreedevi and Reddy, 2012). A *B. subtilis* strain (BPTK4) isolated from boggy water sample produced phytase during the 48th h of incubation at 32°C with the pH of 6.5 was reported (Shamna et al., 2012). A high temperature withstanding neutral phytase isolated from *Bacillus nealsonii* ZJ0702 presented in soil was sequentially purified to homogeneity by ammonium sulfate precipitation (Yu and Chen, 2013). From poultry waste, an extracellular phytase producing bacteria was isolated and phytase was purified by ammonium sulphate fractionation of protein and dialysis followed by SDS-PAGE. The DNA from *Bacillus megaterium* was isolated in the 60th h at 37°C, pH 6.5 and 200 rpm shaking (Dhiraj et al., 2013).

Phytase producing bacteria (21 isolates) were isolated from Soil samples of Bt Rhizosphere, which were collected from NBt cotton growing area of Andhra Pradesh, India. An isolate NBtRS6 yielded phytase more than the other isolates (Ushasri et al., 2013). A strain (DR6) amid the 32 phytase producing bacteria confirmed a 39 mm clear zone on phytase specific medium (PSM-pH 5.5, Temperature 50°C with Glucose and Sucrose as Carbon source and Yeast extract as Nitrogen source) and was identified as *B. subtilis* with an enzymatic activity 378 U/mL as reported (Singh et al., 2013). Phytase activity of 22.165 U/ml was reported for *P. aeruginosa* isolated from rhizospere soil samples (Sasirekha et al., 2012). The initial pH of cultivation media and incubation temperature play a vital role in the phytase productions of the bacterial strains. According to the source of the bacteria isolated, their optimum temperature also varies; high phytase production was at pH 6.5 and then lowered to pH 4.0 after incubation for two days at 37°C. The optimal temperature for many phytases produced by microorganisms is between 25 to 37°C (Choi et al., 1999). Most of the phytases have their pH optima in the range of pH 4.0 to 5.6. Phytase from *Bacillus sp.* normally have optimum pH at 6.5 to 7.5 (Satyanarayana and Vohra, 2001) with a temperature range of 36 to 48°C. Better production was achieved at 37°C with the pH 7; *B. subtilis* (pH 6.0 to 6.5 and 60°C) (Shimizu, 1992), *B. amyloliquefaciens* (pH 7.0 to 8.0 and 70°C) (Kim et al.,

**Table 1.** The colonies (77 to 171) from leaf, root and flower extracts at different temperature.

| ***Bacillus*** **agar plates with incubation temperature (°C)** | **Colonies from leaves** | **Colonies from roots** | **Colonies from roots** |
|---|---|---|---|
| BAS 30 | 77-98 | 133-148 | 149-171 |
| BAS 40 | 99-120 | - | - |
| BAS 50 | 121-132 | - | - |

1998) and *E. coli* (pH 5.0 and 70°C) as reported (Choi et al., 1999). The six bacterial strains among 30 strains which were isolated from Malaysian maize plantation were grown in Luria Bertani (LB) and Luria Bertani+Rice Bran (LBRB) media for five days produced highest phytase activity was also reported (Hussin et al., 2009). The aim of the current work is to isolate bacteria from soil of *Rosa damascena* plant using rice bran and study the phytase activity along with the temperature and pH optimization.

## MATERIALS AND METHODS

### Materials, chemicals and rose extract

Chemicals used were of analytical grade and commercially available. Glycerol (from Scharlab, S.L. Barcelona, Spain); phytic acid, citric acid (from Sigma-Aldrich, U.S.A); sodium acetate 100 mM (from Loba Chemie Pvt. Ltd, Mumbai), and liquid nitrogen (from Air liquid, Jeddah).

### Preparation of the rose extract

Initially, water was sterilized by autoclaving at 121°C at 15 psig for 20 min for the extraction. The Taif roses, *R. damascena* cv. Taifi with a delicate and passionate fragrance from the rose pot in early morning hours were plucked and collected. The plant itself, the green leaves, flowers and roots were washed with sterilized distilled water and then with ethyl alcohol. All the parts which were taken in mortar and pestle were homogenized to fine powder separately, with liquid nitrogen and extracted using sterilized distilled water. The rose extract collected was kept in a shaking incubator at 30°C for 2 h and then stored at -80°C until further use. This rose extract was used for further study of phytase activity.

### Media preparations and screening

Initially, the mud of *Rosa damascena cv. Taifi* was collected from the cleaned root and it was washed with 3.5 L of sterilized distilled water (Farouk et al., 2014b). The supernatant was collected after filtration using Whatman filter paper No.1. The different nutrients media such as *Bacillus* agar, Nutrient agar, Tryptic soya agar and Potato dextrose agar were made by autoclaving at 121°C at 15 psig for 20 min.

The sand water filtrate was poured into the Bacillus agar, potato dextrose agar and nutrient agar plates and kept in incubation temperatures at 30, 40 and 50°C. The growth in *Bacillus* agar at 50°C was predominant. An aliquot of extracts from flowers, leaves, and roots were smeared in *Bacillus* agar, potato dextrose agar and nutrient agar and kept at 30°C for 24 h. All the plates containing a total of 321 colonies were marked. Colonies were picked out from the plates and transferred to sterilized potato dextrose agar, nutrient agar and peptone broths which were prepared by autoclaving at 121°C at 15 psig for 20 min. All the 321 tubes containing the colonies were organized and kept at -80°C. From the 321 tubes, colonies were inoculated in *Bacillus* agar plates. The colonies (77-171) from leaf, root and flower extracts at different temperature are shown in Table 1.

The *Bacillus* agar medium was prepared using the premix from Fluka's Hichrome *Bacillus* agar (49.2 g of premix was added to 1l of distilled deionized water, the pH was adjusted to 7.1 and the solution was autoclaved at 12°C for 15 min) and freshly prepared extracts were used during this project .

The *Bacillus* broth medium was prepared using the premix from Fluka's Hichrome *Bacillus* agar (49.2g of premix was added to 1l of distilled deionized water, the pH was adjusted to 7.1 and the solution was autoclaved at 121°C for 15 min. (The agar was removed by filtering using Whatman filter paper to get clear broth). The *Bacillus* broth was poured into sterilized 20 ml bottles.

The Hichrome *Bacillus* agar and broth media contained Peptic digest animal tissue (10 g/l); meat extract (1 g/l); D-Mannitol (10 g/l); Nacl (10 g/L); phenol red (0.025 g/l) and agar (15 g/l) as composition. Final pH was (at 25°C) 7.4±0.2. The solutes were shaken until they dissolved and the pH was adjusted to 7.4 using 1 N NaOH (8 ml). The volume was then adjusted to 1 L by adding deionized water. The sterilization was done by autoclaving at 121°C for 20 min at 15 psi (1.05 kg/cm) on liquid cycle. The composition of Hichrome *Bacillus* broth media was the same as Hichrome agar media except the agar.

The Potato dextrose agar media with a composition of 24 g/l potato dextrose broth from Fluka, (Sigma Aldrich, U.S.A.) and 28 g nutrient agar from Himedia, India was used. The pH was adjusted to 7.4 and the solution was autoclaved for 20 min at 15 psi (1.05 kg/cm) at 121°C on liquid cycle.

The Tryptic soya agar media with a composition of 40 g/L Tryptic soya agar from Fluka, (Sigma Aldrich, U.S.A.) in distilled water was adjusted to pH 7.4 and the solution was autoclaved for 20 min at 15 psi (1.05 kg/cm) at 121°C on liquid cycle.

The agar plates were made by weighing 28 g of nutrient agar obtained from Himedia, India, and dissolving it in 1l of sterilized distilled water. The pH was adjusted to 7.4 and the solution was autoclaved for 20 min at 15 psi (1.05 kg/cm) at 121°C on liquid cycle. After autoclaving, the medium was swirled gently to distribute the melted agar evenly throughout the solution. It was allowed to cool down to 50 to 60°C. Then, 20 ml of this medium was poured on to 90 mm Petri dishes under sterile conditions and allowed to cool. After the medium strengthens completely, they were inverted and stored at 4°C. They were removed from storage 1 to 2 h prior to use (Sambrook and Russell, 2001**)**. Suspension of the bacterial cultures were covered completely on the agar plates and allowed to dry. The agar plates were then inverted and incubated for 24 h at 37°C. After incubation, growth appeared. The colonies were marked.

### Sample preparation for enzyme assays

The production of enzymes was carried out in the production medium

without addition of agar using the shaken flask fermentation method. The inoculum of the selected strain was produced using sterile *Bacillus* broth in 20 ml sterilized sample bottles. Five percent of inoculums were inoculated on 20 ml of production medium. The bottles were incubated at 30°C for 48 h with shaking at 150 rpm for better aeration and growth of organism.

The samples of enzyme activity assays were prepared by centrifuging 1.5 ml bacterial culture for 1 min at 13,000 rpm (Idriss et al., 2002). The supernatant was used for assay. The amount of phytase produced was assayed using chemical assay using ammonium molybdenum method (Heinonen and Lahti, 1981).

#### Induction of phytate degrading enzymes using rice bran

Production of phytate-degrading enzyme from Malaysian soil bacteria using rice bran containing media was reported (Hussin et al., 2010). In this study, the autoclaved 5% rice bran was supplemented to the production medium and incubated at 30°C for 48 h at shaker conditions at 150 rpm for better aeration and growth of organism. The amount of phytase produced was assayed again using ammonium molybdenum method (Heinonen and Lahti, 1981). The 5% rice bran was prepared by mixing in distilled water and the pH was adjusted to 6.42. The solution was autoclaved for 15 min at 15psi (1.05 kg/cm$^2$) on liquid cycle.

#### Phytase activity assay

Phytase measurements were carried out at 28°C. The reaction was initiated with the addition of phytase enzyme day after a day. After 30 min incubation, the liberated inorganic phosphate was measured using a modification of the ammonium molybdate method (Heinonen and Lahti, 1981). A freshly prepared solution of acetone: 5 N sulfuric acid: 10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 µl of 1.0 M citric acid were added to 400 µl of the phytase assay mixture. The cloudiness was removed by centrifugation at 10,000 rpm for 10 min prior to the measurement of absorbance at 355 nm in a UV double beam spectrophotometer. In order to quantify the phosphate released; a calibration curve was constructed within the range of 5 to 1200 mM phosphate**.**

The control was prepared by adding the stop solution prior to adding the enzyme into the assay mixture. The phytase activity was calculated from the average of at least three phytase assay measurements. One unit (U) of phytase activity was defined as the concentration of inorganic phosphate, in µmol, released per min per mL of enzyme preparation (U/ml). The activity was calculated using the slope of the straight line resulting from the assay with the sample (absorbance/minute) and the slope of the straight line arising from the standard curve (absorbance/µmol of P).

The phytase activity of BAFA.Taifi strains in *Bacillus* media supplemented with rice bran and in the absence of rice bran was carried out. The culture filtrates were measured for phytase activity in microplate reader MR96A (Shenzhen Mindray Bio-Medical electronics Co., Ltd.) at a primary wavelength of 405 nm and a secondary wavelength of 450 nm.

#### Optimization of phytase production

In order to determine the effect of temperature on phytase production, the selected bacterial isolate was grown in production media and incubated at different temperatures: 4°C, ROOM Temperature 25, 30, 40, 50, 60, 70, 80 and 90°C for 1 h at pH 6.5. Culture filtrates were later measured for phytase activity in microplate reader MR96A (Shenzhen Mindray Bio-Medical electronics Co., Ltd.) at a primary wavelength of 405 nm and a secondary wavelength of 450 nm. Bacterial strains used were BAFA.Taifi94, BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117.

The effect of initial media pH on phytase production was conducted by adjusting the production media to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 and 8.5 before bacterial inoculation. After 1 h of incubation at 50°C, culture filtrates were measured for phytase activity in spectro UV–Vis double beam PC 8 scanning auto cell spectrometer (Labomed, Inc; U.S.A) at wavelength 630 nm. Bacterial strains used were BAFA.Taifi94, BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117.

The induction of phytase degrading enzymes using rice bran was determined by adding the production media with 5% rice bran and inoculated with test organisms. The media was incubated for 24 h at 37°C. Culture filtrates were later measured for phytase activity in microplate reader MR96A at a primary wavelength of 450 nm and a secondary wavelength of 630 nm.

## RESULTS

### Phytase activity of bacterial strains

A total of 321 bacterial strains were isolated from the mud sample of *Rosa damascena cv.Taifi*, among them, four strains were found to be positive for phytase production by their zone forming ability in CMC containing media (Farouk et al., 2014a). Among 48 bacterial isolates from *Bacillus* agar plates, an isolate BAFA.Taifi117 exhibited the highest enzyme activity.

Since the growth study was essential for the production of extracellular enzymes, it was studied by using the shaken flask fermentation method. The stationary phase of growth was reached after about 48 h. The production of phytase was detected after 36 h of cultivation.

The production of phytase was considerably low before 36th hours and after 48th hours of production. It was considered as the log phase and its variation also depends on the nutrient present in the medium and the cultural condition of the organism. The environmental parameter also influences the maintenance time of the bacteria. The plate assay and chemical assay confirmed the production of phytase. Although, the production of phytase was detected after 36 h of cultivation, it increased during growth and reached maximum level (37.7 U/ml) in rice bran medium after ten days as shown in Figure 1.

The isolated *Bacillus* strains in *Bacillus* broth medium supplemented with rice bran in shaken flask at 150 rpm produced significant amount of phytase during the 48th hour of incubation at 30°C with pH of 6.5. In *Bacillus* broth medium supplemented with rice bran in shaken flask at 150 rpm, water was used as the source for the isolation of phytase producing bacteria.

Figure 2 shows a comparative study of phytase activity of different BAFA strains with the supplementation of rice bran in media. Increasing moderate phytase activity was observed by the induction of rice bran after ten days for BAFA.Taifi111.

### Effect of temperature and pH

In the study of the *Rosa damascena*, the optimum

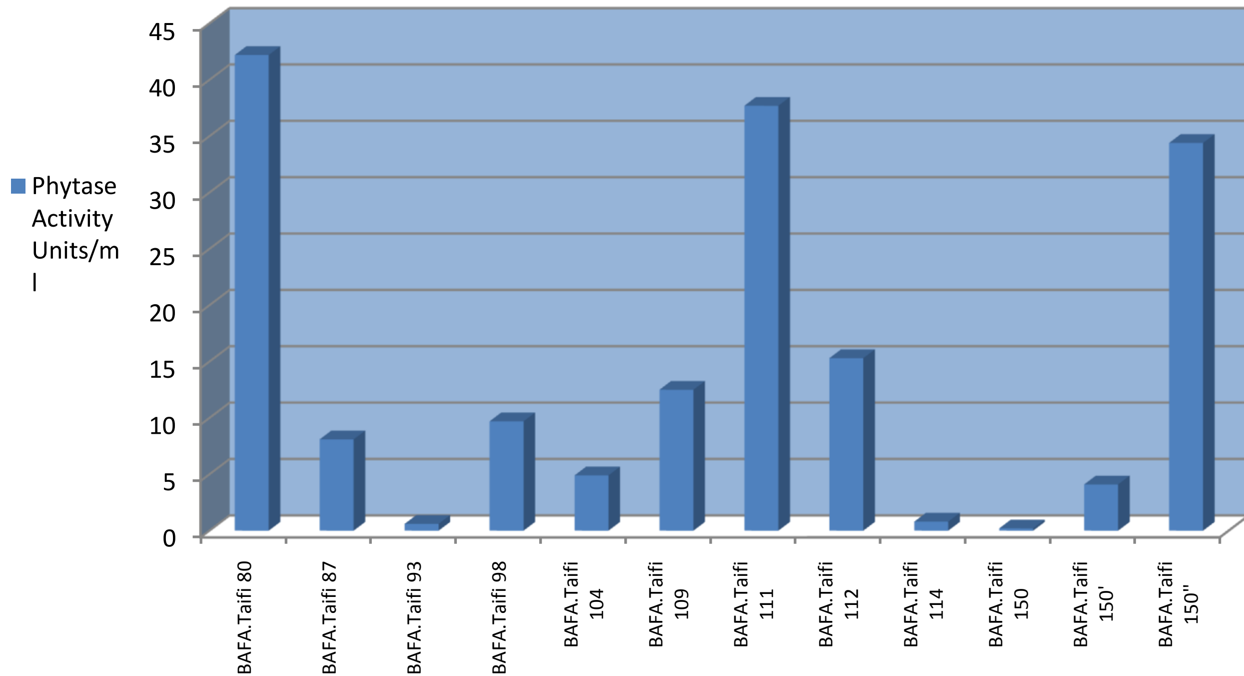


**Figure 1.** The phytase activity of selected bacterial strains in *Bacillus* broth and rice bran.

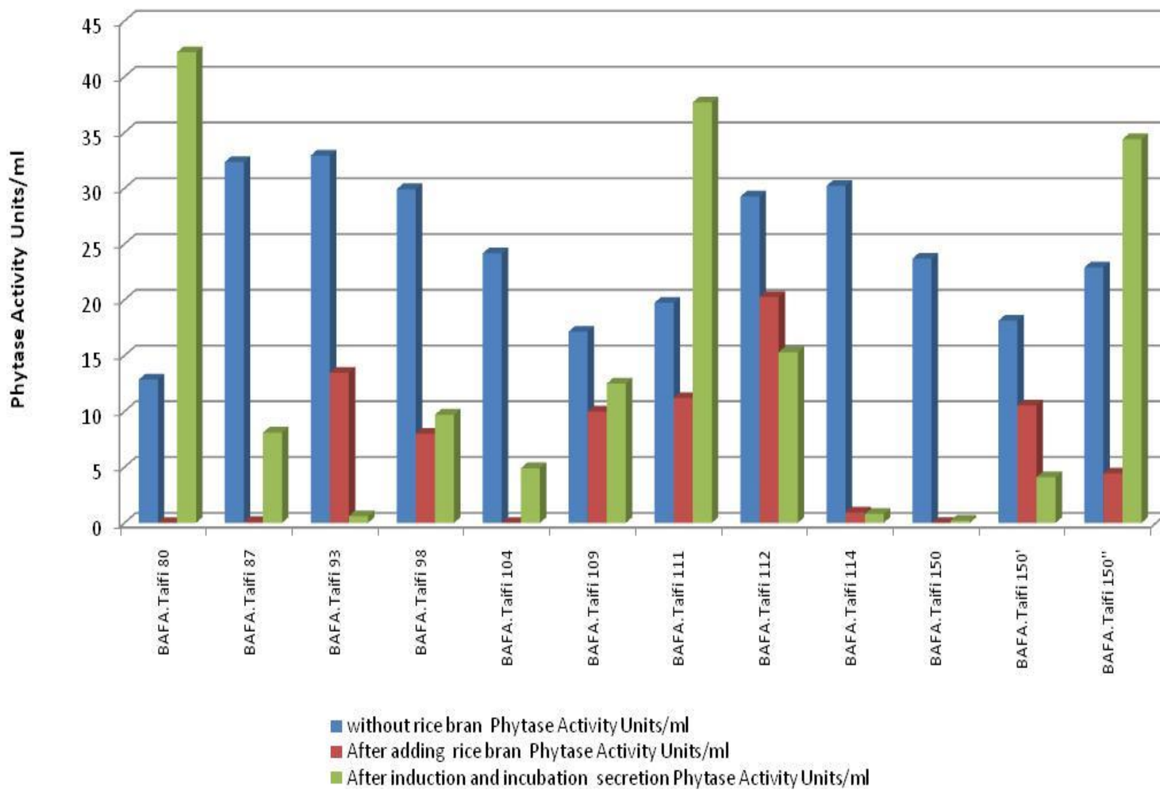


**Figure 2.** Comparative phytase activity study of BAFA.Taifi strains with and without rice bran.

temperature for phytase production was found to be different for the selected strains. The BAFA.Taifi94 and BAFA.Taifi103 have an optimum phytase activity at 60°C; The BAFA.Taifi111 has an optimum phytase activity at

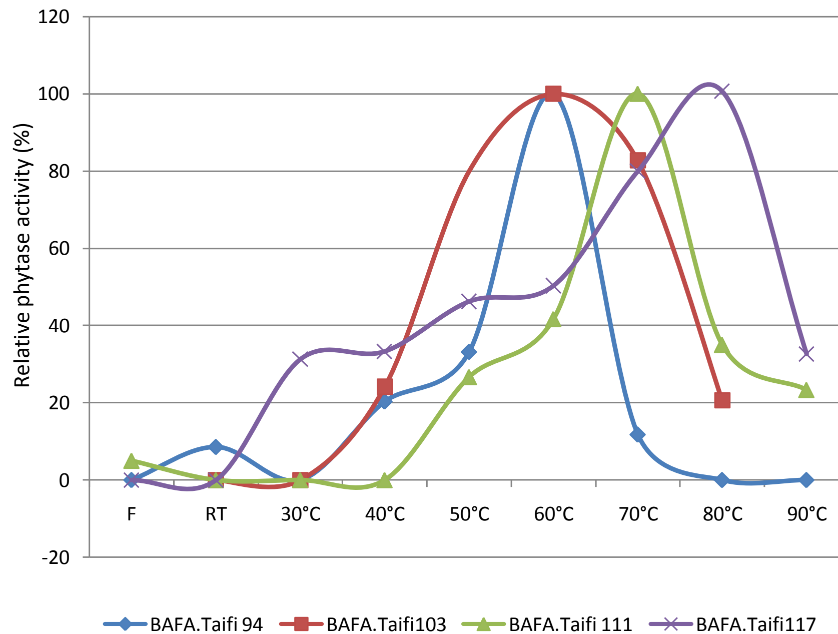


**Figure 3.** Temperature optimization curve for bacterial strains BAFA.Taifi94, BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117. F represents fridge temperatrue (4°C) and RT is room temperature (25°C).

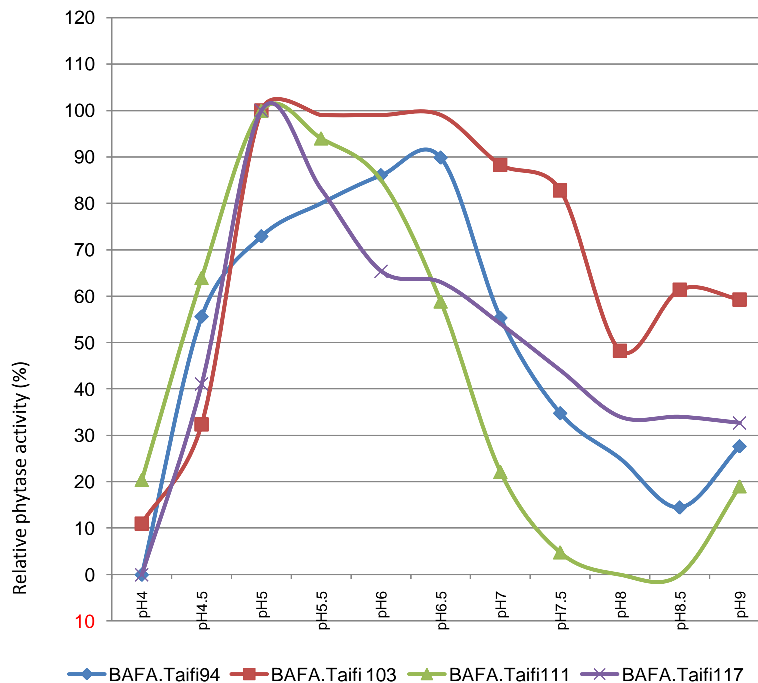


**Figure 4.** The pH Optimization curve for bacterial strains BAFA.Taifi94, BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117.

70°C and the BAFA.Taifi117 has an optimum phytase activity at 80°C as shown in Figure 3.

Besides temperature, pH is found to be one of the most important factors for phytase detection. The significant production of phytase was observed at the pH of 6.5 (100 U/ml) (Figure 4). It was found that the strain BAFA.Taifi117 requires a slightly acidic pH for phytase production.

**Table 2.** The selected bacterial strains with phytase activity at pH 6.5.

| Bacterial strains | Optimum temperature (°C) | Phytase activity (U/ml) |
|---|---|---|
| BAFA.Taifi94 | 60 | 100 |
| BAFA .Taifi103 | 60 | 100 |
| BAFA.Taifi111 | 70 | 100 |
| BAFA.Taifi117 | 80 | 101 |

**Table 3.** The selected bacterial strains with phytase activity at 37ºC.

| Bacterial strains | Optimum pH | Phytase activity at 37ºC (U/ml) |
|---|---|---|
| BAFA.Taifi94 | 6.5 | 90 |
| BAFA .Taifi103 | 5 | 100 |
| BAFA.Taifi111 | 5 | 100 |
| BAFA.Taifi117 | 5 | 101 |

The optimum pH on phytase production was found to be at pH 5 and pH 6.5 at 37°C for the selected strains. The BAFA.Taifi103, BAFA.Taifi111 and BAFA.Taifi117 have an optimum phytase activity at pH 5 at 37°C and the BAFA.Taifi94 has an optimum phytase activity at pH 6.5 at 37°C as shown in Figure 4.

Tables 2 and 3 represent the selected Bacterial strains with phytase activity at constant pH 6.5 and temperature at 37°C respectively.

By testing ten BAFA. Taifi strains, it was found that the phytase activity of the BAFA.Taifi strain (BAFA.Taifi80) without rice bran was 12.5 units/ml after one day, but with the induction of rice bran, it was observed to be 42.2 units/ml after 10 days in a shaker at 30°C/150 rpm.

The phytase activity of the BAFA.Taifi strain (BAFA.Taifi111) without rice bran was 19.7 units/ml after one day, but with the induction of rice bran it was observed to be 37.7 units/ml after 10 days in a shaker at 30°C/150 rpm.

The phytase activity of the BAFA strain (BAFA.Taifi150) without rice bran was 22.9 units/ml after one day, but with the induction of rice bran, it was observed to be 34.4 units/ml after 10 days in a shaker at 30°C/150 rpm.

The phytase activity of both strains BAFA.Taifi109 and BAFA.Taifi112 without rice bran after one day was 17.15 and 29.63units/ml. But after 10 days, the phytase activity with the induction of rice bran showed a moderate reduction of 12.5 and 15.3 units/ml, respectively.

The phytase activity of the BAFA.Taifi strains without rice bran was 32.9 units/ml for BAFA.Taifi93; 30.2 units/ml for BAFA.Taifi114; and 32.2 units/ml for BAFA.Taifi87. A drastic reduction in the phytase activity was observed with the induction of rice bran and it was 0.8, 0.6 and 8.1 units/ml respectively after 10 days in a shaker at 30°C/150 rpm.

This study revealed that the addition of 5% of rice bran stimulates the expression of phytase by the bacterial strains of the *Rosa damascena* cv.Taifi. The considerable amount of phytase activity reveals that the bacterial sp. can be an effective source of phytase.

## DISCUSSION

Phytase in rice bran is present as a less soluble K-Mg salt; normally it is enclosed by starch or combined with protein, which leads to a lower rate of hydrolysis (Wang et al., 1999). So, by using rice bran as a known carbon source, phytase production was favoured continuously in the shaker conditions in the presence of phytate (Konietzny and Greiner, 2002). Rice bran has been used for the stimulation of phytase secretion in *Bacillus subtilis* (Agalya et al., 2013).

However, inducible secretion of phytase-degrading enzymes from bacteria associated with *Rosa damascena* cv.Taifi using rice bran had shown an excellent result against 5% rice bran induction. Rice bran plays a major role in phytase production as observed in certain bacterial strains (Hussin et. al., 2010). The characterization of secreted phytase from BAFA.Taifi strains has confirmed features of previously studied *Bacillus* phytases in our laboratory (Igbasan et al., 2000). The real and beneficial production of phytase using cheap natural media such as rice bran is to be studied further as one of the best known media for phytase production (Hussin et al., 2007). Most of BAFA.Taifi strains taken for this study behaved differently with time and their stimulation for the secretion of phytase in liquid media contains rice bran in their ingredients with various concentrations.

The role of various cereal substrates like sugar cane baggage and wheat bran on phytase production can be studied in the future for the bacilli strains obtained from *Rosa damascena* and other Taif rose varieties. Further

study is to be done for cloning to confirm the purification of phytase degrading enzymes and their specific activity.

## Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

*Full Length Research Paper*

# Sero-prevalence of infectious bursal disease in backyard chickens around Mekelle, Northern Ethiopia

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**A cross sectional study was conducted from January to June 2012 to determine the sero-prevalence of Infectious bursal disease (IBD) in chicken reared under backyard poultry production systems around Mekelle town, Tigray regional state. During the study period blood samples were collected from 384 unvaccinated backyard chickens from three different areas. A commercial indirect enzyme linked immuno sorbent assay (ELISA) was used to test the sera for IBD anitbodies kit. The overall sero-prevalence of infectious bursal disease virus (IBDV) antibody in chickens was found to be 45.05% (173/384). There was a significant difference (P < 0.05) in the sero-prevalence of IBDV among/between the different age groups, sex and origin of chickens. The result of this study indicates that IBD is prevalent in the study area. The prevalence of IBDV antibody in unvaccinated backyard chickens might be due to field exposure of chickens to the disease and indicated the importance of further study on the epidemiology of the disease and the sero-type of the IBDV that are circulating in the country to design appropriate control measures.**

**Key words:** Infectious bursal disease (IBD), chickens, sero-prevalence, Mekele, indirect enzyme linked immuno sorbent assay (ELISA).

## INTRODUCTION

Livestock plays an important role in the agricultural economy of Africa. Poultry occupies a unique position in terms of its contribution to the provision of high quality food protein to rural small holder farming families in Africa. Both poultry meat and eggs enrich and contribute to a well-balanced diet of young children in sub-Saharan Africa (Tadelle et al., 2003). Despite these facts, the contribution of poultry production to the small holder farmers and the country economy is still restricted by various factors like low inputs of feeding, poor management, infectious diseases and lack of appropriate selection and breeding practice (Alemu, 1995; Tadelle and Ogle, 2001; Halima et al., 2007).

Infectious diseases such as newcastle disease and infectious bursal disease are reported to be the major health and production constraints of chickens (Alamargot, 1987; Zeleke et al., 2002; Zeleke et al., 2005b). Infectious bursal disease (IBD) is an acute highly contagious globally occurring viral poultry disease. The causal virus belongs to the family Birnaviridae of the genus

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Avibirnavirus. Two serotypes of the virus are known. Serotype 1 virus is pathogenic to chickens. Serotype 2 virus is non-pathogenic to chickens but has been isolated from both chickens and Turkeys. Serotype 1 viruses can be further categorized into four groups on the basis of their pathogenicity, as classical, variant, attenuated and very virulent strains (Lim et al., 1999).

Infection with infectious bursal disease virus (IBDV) is presents in two clinical forms: Acute onset high mortality in chickens (up to 20%), usually in birds around 3 to 4 weeks old; immune-suppressive disease as a result of infection at an early age, predisposing birds to secondary infections such as gangrenous dermatitis, inclusion body hepatitis, anaemia syndrome, and *Escherichia coli* infections (Lukert and Saif, 2003). Older birds show the subclinical form of IBD depending on the strain and amount of the infecting virus, age and breed of birds, route of inoculation, and presence and absence of neutralizing antibodies (Muller et al., 2003). A previous study in Ethiopia indicated that the mortality rate of IBD ranges from 45 to 50%. The overall seroprevalence of IBD antibody recorded in different part of the country and different poultry production systems reached up to 93.3% (Zeleke et al., 2005a). This study was conducted to determine the Sero-prevalence of IBD in unvaccinated backyard chickens around Mekelle town, Northern Ethiopia.

## MATERIALS AND METHODS

### Study area

The study was conducted in chicken around Mekelle, the capital city of Tigray regional state, Ethiopia. Three areas around the city namely, Chelekot, Adigudom and Wukro were selected for this study. Mekelle is located at a latitude of 30° 29`N and a longitude of 39° 28` E with an elevation of 2084 m above sea level (CSA, 2005). The mean annual rainfall ranges from 11.3 to 39.1 mm and the temperature varies from 12 (in November and December) to 27°C (in January and March). Mekelle and its surrounding have humid and hot climate (MoM, 1998).

### Sample size determination and study type

A cross-sectional study was undertaken from January 2012 to June 2012 to determine the sero-prevalence and risk factors of IBD infection in non-vaccinated backyard chickens. The sample size was determined using the formula described by Thrusfield (1995). A total of 384 was collected for this study by considering an expected prevalence of 50% and an absolute precision of 5% with 95% confidence level.

### Study animals and sample collection

Apparently, healthy chickens reared in a backyard poultry production system were selected randomly from each study areas. The chickens were categorized into three age groups (< 6 months, 6 to 12 months and ≥ 12 months). Blood samples (2.5 ml) were collected from the brachial (wing) vein of chicken using 5 mL sterile disposable syringe with 22 gauge and 1¼ needle size (Alcorn, The blood samples were allowed to clot for 24 h at room temperature to allow serum separation. Sera samples were separated into labeled sterile cryovial tube and stored at -20°C until tested. All sampled chickens were not-vaccinated against IBDV.

### Enzyme linked immuno sorbent assay (ELISA) test

Sera samples were tested for IBDV specific antibodies using a commercial IBDV-ELISA kit (proFLOK®PLUS IBD, Synbiotics Corporation, Frontera San Diego, CA, USA) in National Veterinary Institute, Debre zeit, Ethiopia, following the manufacturer's directions. Serum, diluted in dilution buffer, is added to an IBD antigen coated plate. Specific IBD antibodies in the serum form an antibody-antigen complex with the IBD antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate was added to each well and the formed antibody-antigen complex binds to the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate which contains a chromogen (ABTS) is added to each well. Chromagen color change from clear to green blue occurs in the presence of peroxidase enzyme. The relative intensity of color, developed in 15 min (compared to the controls), is directly proportional to the level of IBD antibody in the serum. After the substrate has incubated, a stop solution was added to each well to terminate the reaction and the plate was read using an ELISA plate reader at 405 to 410 nm. The test sample was found to be positive when the SP >0.299 and titration > 0 according to the following formula:

$$\text{Sample to be positive (SP)} = \frac{\text{Sample absorbance - Average normal control absorbance}}{\text{Corrected positive control absorbance}}$$

An IBD ELISA titer value was calculated by the equation given as below:

$$\log_{10}\text{Titer} = (1.172 \times \log_{10}\text{SP}) + 3.614$$

$$\text{Titer} = \text{Antilog of } \log_{10}\text{Titer}$$

## Data analysis

The data was entered into Microsoft excel spread sheet and coded appropriately. For data analysis, SPSS version 16, was used. Descriptive statistics were used to determine the sero-prevalence of IBD in chicken. The Chi-square test was used to determine the association between the disease and the considered risk factors such as origin, age and sex. In all cases, 95% confidence intervals and P<0.05 were set for significance.

## RESULTS AND DISCUSSION

Of the 384 examined backyard chickens sampled, 45.05% (n=173) were positive for IBD antibodies. The sero-prevalence of IBD in Wukro, Adigudom and Chelekot was 60.77, 43.08 and 30.65%, respectively. The sero-prevalence of IBD recorded in female and male chickens was 54.18 and 27.82%, respectively. The sero-prevalence of IBD in chicken aged above 12 months (64.57%) was relatively higher than both chickens aged

**Table 1.** Sero-prevalence of IBD between/among the different areas and the sex and age groups.

| Risk factors | Category level | Number of collected samples | Number of positive samples | Percentage (%) | *p-value* |
|---|---|---|---|---|---|
| Origin | Chelekot | 124 | 38 | 30.65 | 0.041 |
| | Wukro | 130 | 79 | 60.77 | |
| | Adigudom | 130 | 56 | 43.08 | |
| | Total | 384 | 173 | 45.05 | |
| Sex | Male | 133 | 37 | 27.82 | 0.047 |
| | Female | 251 | 136 | 54.18 | |
| | Total | 384 | 173 | 45.05 | |
| Age | < 6 month | 152 | 33 | 21.71 | 0.039 |
| | 6 to12 months | 105 | 58 | 55.24 | |
| | >12 months | 127 | 82 | 64.57 | |
| | Total | 384 | 173 | 45.05 | |

six to 12 months (55.24%) and below six months of age (21.71%). In general, there was statistically significant difference (*p- value* < 0.05) in the sero-prevalence of IBD between/among the different areas and the sex and age groups (Table 1).

The overall prevalence of IBD in this study was 45.05%. This sera-prevalence is lower than the findings of Degefu et al. (2010) in South West and West Shoa, Abrar (2007) in selected areas of East Showa Zone and Nigussie (2007) in Addis Ababa and Adami Tulu areas, who reported a prevalence of 76.64, 76.3 and 65.9%, respectively, in non-vaccinated backyard chickens. Kassa and Molla (2012) also reported IBD prevalence of 75% in West Gojjam and 72% in North Gondar, in unvaccinated local breed backyard chickens. However, our finding of this study (45.05%) was higher than that of Reta (2008) in unvaccinated backyard chickens in East Shoa Zone and Hailu et al. (2009) in North West Ethiopia in village chickens who reported a prevalence of 39.2 and 38.9%, respectively. The variations in the sero-prevalence of IBD might be attributed to differences in the sensitivity and the specificity of the tests used by the authors. In addition, differences in the breed used and the agro-ecological zones of the studied area, the availability of the veterinary service and the awareness of the public towards the control and treatment of the disease, and the breed difference between these study areas could also influence the sero-prevalence results.

The prevalence of IBD among/between the different age and sex groups and origins was statistically significant (*p-value* < 0.05). The highest sero-prevalence of IBD was in Wukro (60.77%), as compared to other cities showing low sero-prevalences, like Adigudom (43.08%) and Chelekot (30.65%). This difference in sero-prevelance may be due to the variation in geographic and climatic conditions of the area, and husbandry and hygienic condition of the production systems. With regard to sex, the sero-prevalence of IBD was higher in female (54.18%) than male (27.82%). This difference might be due to physiological and immunological difference between the two sexes. Moreover, the reproductive demands placed on females may increase the risk of infection as compared to male. It is also possible that there are some other unmeasured risk factors in common, such as different male and female behaviours, which increases exposure to pathogens (Bettridge et al., 2014). The sero-prevalence of IBD was higher in chickens aged above 12 month (64.57%) than those aged 6 to 12 months (55.24%) or below 6 month (21.71%). However, this finding is contrary to that reported by Singh and Dhawedkar (1992) and Saif et al. (2000) showing higher sero-prevalence of IBD in chicken aged below 12 weeks of old.

The results of this study would suggest that Infectious Bursal Disease virus is prevalent in the study areas and the sero-prevalence of IBDV antibody in backyard chickens is most probably due to field exposure to these viruses. A further study on the epidemiology, possible risk factors of IBDV exposure e and strains of the IBDV circulating in the country would be valuable in helping to develop control strategies to prevent clinical disease.

## Conflict of interests

The authors did not declare any conflict of interest.

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African Journal of Biotechnology

*Short Communication*

# Analysis of polymorphisms in the mitochondrial *ND5* gene in Pantaneira and Creole breeds of sheep

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**The aim of this study was to assess genetic variation between a population of Pantaneira sheep in the Brazilian state of Mato Grosso do Sul, and Creole sheep from the south of the country by molecular analysis of the *ND5* gene in mitochondrial DNA. The analysis revealed the presence of 16 haplotypes with all Pantaneira sheep grouped together carrying a single haplotype, and there was no grouping with any of the Creole sheep. The $F_{ST}$ value was 0.44, indicating that there is a genetic difference between the two breeds, which may indicate that both breeds underwent differentiation.**

**Key words:** *Ovis aries, ND5 gene*, genetic diversity, phylogeny, breed differentiation.

## INTRODUCTION

Brazil has many types of domestic animals, including sheep, which originated from breeds brought by colonisers after the discovery. The first steps towards obtaining funding for the improvement, management and conservation of Brazilian naturalised sheep are characterising the diversity of naturalised races, the genetic relationship between them and understanding their origins (Mariante and Cavalcante, 2006). The Creole sheep has been reared for centuries in the Brazilian states of Rio Grande do Sul and Santa Catarina, where there are two known varieties, Fronteira and Serrana (Gonçalves et al., 2010). Considering the geographic distribution of sheep in Brazil and phenotypic similarities between the animals, it is thought that Pantaneira sheep originated from the Creole sheep, and research has been carried out to determine whether the difference between the groups is sufficient for the Pantaneira sheep to be recognised as a separate breed (Paiva et al., 2008). NADH dehydrogenase is one of the main enzymes found in respiratory complexes in mammals. It has 42 polypeptide chains, seven of which are encoded by the mitochondrial genome. The subunit five (*ND5*) was used by Tserenbataa et al. (2004) and by Gonçalves et al. (2010) to study sheep diversity. The former study searched for subspecies of *Ovis ammon* in Mongolia by sequencing this region, and the results suggested the existence of two subspecies (*O. ammon*

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**Table 1.** DNA sequence comparisons of the different haplotypes found with the *ND5* gene.

| | 11825 | 11832 | 11834 | 11839 | 11846 | 11849 | 11869 | 11882 | 11915 | 11921 | 11939 | 11978 | 12023 | 12050 | 12067 | 12074 | 12077 | 12086 | 12101 | 12117 | 12125 | 12148 | 12149 | 12152 | 12154 | 12171 | 12179 | 12180 | 12182 | 12185 | 12191 | 12197 | 12214 | 12226 | 12229 | 12230 | 12236 | 12238 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ref | T | C | T | T | T | T | C | T | C | C | T | A | T | T | T | C | G | C | G | C | T | T | C | T | C | C | T | G | C | A | A | A | T | G | A | T | G | C | |
| H1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 19 |
| H2 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 |
| H3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | G | . | . | . | . | . | . | . | . | . | T | . | . | . | 2 |
| H4 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H5 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | 1 |
| H6 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | T | . | . | . | . | T | . | G | . | . | C | . | T | . | . | . | 1 |
| H7 | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | G | . | . | . | . | . | . | . | . | T | . | . | . | . | 1 |
| H8 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | G | . | . | . | G | . | . | . | . | . | T | . | . | . | 1 |
| H9 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | T | . | G | A | . | . | . | . | . | . | . | . | T | . | . | . | 1 |
| H10 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | A | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H11 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | A | . | . | T | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H12 | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | A | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H13 | . | . | C | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 |
| H14 | . | . | C | . | C | . | . | . | . | . | . | . | C | . | C | . | . | . | . | A | . | . | . | . | G | . | . | . | . | . | . | . | . | . | T | . | . | . | 1 |
| H15 | . | . | C | . | C | . | . | . | . | . | . | . | C | . | . | . | . | . | . | A | . | . | . | . | G | . | . | . | . | . | . | . | . | . | T | G | . | . | 1 |
| H16 | . | . | C | . | C | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | G | . | . | . | T | A | . | A | 1 |

*ammon* and *O. ammon darwini*). In the study by Gonçalves et al. (2010), genetic differentiation was found between animals of the Creole sheep in the south of Brazil belonging to the varieties Serrana and Fronteira. Genetic polymorphisms in mitochondrial DNA (mtDNA) reveal haplotype diversity within species, and are therefore, a useful tool for establishing phylogenetic relationships at the species level (Avise et al., 1987). Therefore, the aim of this study was to assess the variation between a population of Pantaneira sheep in the state of Mato Grosso do Sul and Creole sheep in the south of Brazil through molecular analysis of the mtDNA *ND5* region.

## MATERIALS AND METHODS

Blood samples from 19 Pantaneira sheep from the state of Mato Grosso do Sul were collected and stored at 4°C. DNA was extracted in the Animal Biotechnology Laboratory (Faculty of Agrarian Sciences at Federal University of Grande Dourados) using a total blood DNA extraction protocol as described by Crispim et al. (2012). An 657-bp region of the mitochondrial *ND5* gene was amplified using the primers ND5 F 5'-AATAGTTTATCCAGTTGGTCTTAGG-3' and ND5 R 5'-AAGATTTGTTGGAGATCTCAGGTG-3' (Tserenbataa et al., 2004). Polymerase Chain Reaction (PCR) was performed in a final volume of 25 µL and the amplification mix was made with 7.5 µL of ultra pure water, 1.5 µL of each primer (10 pmoles), 12,5 µL of PCR Master Mix (Fermentas®) and 2.0 µL of DNA (10 to 20 ng). The reaction protocol was 95°C (5 min) one cycle; 95°C (1 min), 52°C (1 min), 72°C (1 min 30 s) 36 cycles and 72°C (10 min) one cycle. The amplified fragments were purified and sequenced by an automatic sequencer ABI 3730 XL (Applied Biosystems). Sequence editing and alignment was performed with the reference sequence AF010406 (Hiendleder et al., 1998) by using the program DNA Alignment (Fluxus Technology Ltd). To compare sequence variability between Pantaneira and Creole breeds, the sequences of 17 Creole sheep were obtained from GenBank with accession numbers EU854593 to EU854607 and EU854609 to EU854610 (Gonçalves et al., 2010). The analysis of molecular variance (AMOVA), the Wright's F-statistics ($F_{ST}$), haplotype and nucleotide diversities were calculated using the program Arlequin 3.5 (Excoffier and Lischer, 2010). The relationships among the haplotypes obtained were analysed by the construction of networks using the program Network version 4.1.1.2 (Fluxus Technology Ltd) by the Median-Joining method (Bandelt et al., 1999).

## RESULTS

A total of 36 sequences were analysed using the mtDNA *ND5* gene. Sixteen haplotypes were found, and all animals of the Pantaneira breed were grouped together with a single haplotype. Table 1 shows DNA sequence comparisons of the different haplotypes. AMOVA revealed that the percentage of variance between the analysed populations was 44.44%, and within populations was 55.56% ($p < 0.05$). The $F_{ST}$ value found was 0.44, a value that indicates genetic difference according to Hartl and Clark (2010). A significant difference ($p < 0.05$) was also found between animals of

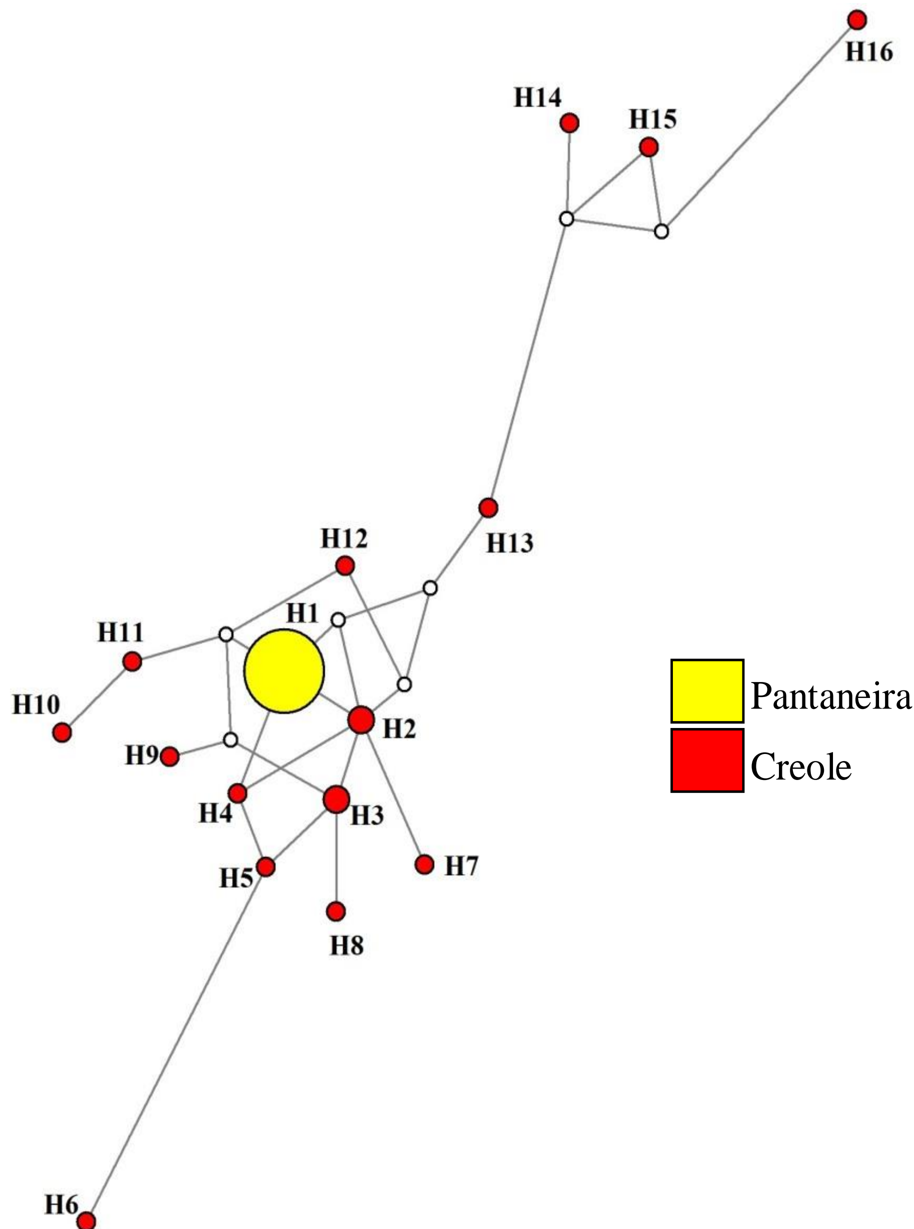


**Figure 1.** Network constructed by the median-joining method (Bandelt et al., 1999) showing the 16 haplotypes found using the mtDNA *ND5* gene for individuals from the Pantaneira and Creole sheep. The area of the circles for each haplotype is proportional to its frequency. The lengths of the lines are proportional to the mutational steps that separate each haplotype. The white dots are median vectors that represent hypothetical haplotypes introduced by the algorithm used.

the Pantaneira breed and in two varieties of Creole sheep, with the distance between breeds being 0.44, according to the distance method pairwise difference based on $F_{ST}$ values calculated with AMOVA. Figure 1 shows that the haplotype networks constructed for Pantaneira and Creole sheep, based on mutation points present in the sequences, demonstrate the relationship between the different haplotypes. Table 2 shows the haplotype and nucleotide diversities calculated with Arlequin 3.5 for the *ND5* gene.

## DISCUSSION

The $F_{ST}$ value found in this study with the *ND5* gene, calculated with AMOVA, was 0.44 and according to Hartl and Clark (2010), $F_{ST}$ values above 0.25 indicate a genetic difference. Therefore, we may say that there was differentiation between Pantaneira and Creole breeds when analyzed with this marker. In addition, Holsinger and Weir (2009) say that if the $F_{ST}$ value is high the allele

**Table 2.** Haplotype (H) and nucleotide (π) diversities for Pantaneira and Creole sheep.

| Breed | H | π |
|---|---|---|
| Pantaneira | 1.000±0.017 | 0.000±0.000 |
| Creole | 1.000±0.020 | 0.010±0.006 |

frequency is different indicating difference between populations, which once again shows that there could be differentiation between the populations studied. The network (Figure 1) revealed that Creole sheep have a different haplotype than Pantaneira sheep since they did not group together, suggesting that differentiation has occurred between these groups, therefore more research would be necessary to know if this would be enough so that the Pantaneira sheep could be acknowledged as a different breed, a suggestion also made by Paiva et al. (2008). Furthermore, several haplotypes in the Creole sheep were close to the one formed by the Pantaneira breed animals which may indicate that, although these animals do not share the same haplotype, they belong to the same haplogroup. The fact that all animals of the Pantaneira breed grouped together in one single haplotype explains the values found for haplotype and nucleotide diversity (Table 2). The geographical region where these animals are found today belonged previously to Paraguay, so it is possible that the Pantaneira breed has been influenced by Paraguayan breeds and the analysis of its mitochondrial genome might confirm this assumption, by showing their genetic distance from the Creole. Thus, the significant differences identified for the mtDNA *ND5* gene between Pantaneira and Creole sheep may indicate that differentiation has occurred in both races; however, further research using other markers is required to investigate this further.

## Conflict of interest

The authors have no conflict of interests to declare.

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