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Review

Resistance by *Enterobacter* spp. towards several antimicrobial drugs and heavy metals: A review

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Antimicrobial resistance developed in several pathogens poses an increasing threat to human health across the world. No country can escape from the medical and economic impacts from this serious problem. Although the antibiotic resistance is not a new phenomenon, the current magnitude and speed with which it is developing is a cause for the global concern including in India. There are so many common diseases resulting from the microorganisms such as blood stream infections, urinary tract infections, post-operative wound infections and intra-abdominal infections. In this review the antimicrobial susceptibility or resistance of *Enterobacter* towards antimicrobial agents and heavy metals, viz. ceftazidime, moxifloxacin, nalidixic acid, sulfamethaxazole, and nickel and lead is discussed briefly along with other antimicrobials and heavy metals. The mechanisms behind the resistance by *Enterobacter* was analyzed and evaluated by many workers after using currently employed susceptibility testing methods for *Enterobacter* spp. There are some factors influencing mode of action of fluoroquinolones, quinolones and sulfamethaxazole. History, classification, identification, clinical features and treatment of infections and the epidemiology of antimicrobials (drugs and heavy metals) resistance by the *Enterobacter* spp. is included in this review. Now a day, antimicrobial resistance is common in hospitals where acquired infections can be perilous. This situation compels scientists to synthesize new antibiotics and treatment modalities. *Enterobacter* causes nosocomial infections. It is ubiquitous and can survive on skin and dry surfaces and replicate in contaminated fluids. Numerous outbreaks have been described. Various mechanisms have been adapted by microorganisms to resist toxicity of antimicrobials. Antimicrobial drugs may be rendered inactive or ineffective by the major ways such as barrier to antibiotic entry into the bacterial cell, prevention of the antibiotic from reaching the target, often by extrusion, alteration of the target of the drug and inactivation of the antibiotic by modification or destruction. In addition, bacteria may be able to bypass the metabolic pathway affected by a particular drug or may be able to overproduce an enzyme that is inhibited by the drug action, more than one mechanism may operate at any given time

Key words: Enterobacter, resistance, mechanism, ceftazidime, moxifloxacin, nalidixic acid, nickel, lead.

INTRODUCTION

Microbes, man and environment have intrinsic correlationship that exists since time immemorial. They have evolved umpteen number of mechanisms to survive and keep them fit in nature. Resistance is a complex phenomenon that involves the microorganism, the environment and the patient-separately and interactively. Resistance may be a characteristic of the microbe before exposure to a given drug or may arise as a consequence

of therapy. Resistance usually involves gradation, rather than being an “all or none” phenomenon. Antimicrobial resistance is defined as a property of bacteria that confers the capacity to inactivate or exclude antimicrobials, or a mechanism that blocks the inhibitory or killing effects of antimicrobials, leading to survival despite exposure to antimicrobials (Bagde, 2014). Simply put, microbial resistance may be defined as the ability of a specific microorganism to withstand a drug that interferes with its growth functions (Meyers, 1987).

The consequence of resistance affects not only patient’s lives but also reaches far beyond the individual patient, affecting health care system and society across the world (Giske et al., 2008). The ongoing pandemic spread of resistance bacteria illustrates that the problem can only be addressed through international cooperation. We may very well be forced with unimaginable setbacks medically, socially, and economically within just few years.

Scope of resistance phenomenon is expanding every day. Microbes initially were resistance to common antimicrobials have now developing resistance to any antimicrobial that is antagonistic to its survival that includes drugs, antibiotics, heavy metals and other antimicrobials such as preservatives and other chemicals (Bagde, 2014). Nowadays, antimicrobial resistance which is spreading rapidly is of great concern, because it is common in hospitals where acquired infections can be perilous. This situation compels scientists to synthesize new antibiotics and treatment modalities. The reason of microbial resistance can be due to increased misuse of antibiotics in foods (livestock, poultry and agriculture). A number of significant factors, such as organism identification, antibiotic sensitivity testing and host factor situations, should be taken into account in order to treat various infections effectively

Microbial resistance to antimicrobials may be present since immemorial time. It is known that resistance genes and plasmids were present in bacteria even before the advent of antibiotics. It is likely that in their struggle for survival in nature the bacteria may have developed resistance genes.

Although the discovery of antibiotics creates a new era in the treatment of infectious diseases, the bacterial evolutionary response exerts a selective pressure on it. An antibiotic kills the susceptible bacteria and favors the overgrowth of these bacteria that carry a resistant gene. Thus these antimicrobial agents induce the development of resistance by multiplication and spread resistance. Examples of resistance to chemotherapy have been noted in all categories of microorganisms including bacteria, fungi, viruses and parasites. To date, resistance in bacteria is most prevalent (Richman, 1995; Dixon et al., 1996).

The present scenario is such that the available antibiotics have become ineffective in diseases of proven bacterial etiology especially in a hospital setting. To add to this previously harmless species, are now being reported to be posing a serious therapeutic challenge, for example; recently, a study by Prakash et al. (2005) reported high-level aminoglycoside and β- lactam resistance among unusual (non-faecalis and non-faecium enterococci) and atypical (biochemical variant) species of enterococci at a hospital (JIPMER) in South India.

Phenomenon of microbial resistance

Antimicrobial drugs may be rendered inactive or ineffective in the following major ways: (1) Barrier to antibiotic entry into the bacterial cell; (2) Prevention of the antibiotic from reaching the target, often by extrusion; (3) Alteration of the target of the drug and (4) Inactivation of the antibiotic by modification or destruction (Berkowitz, 1995; Neu, 1995). In addition, bacteria may be able to bypass the metabolic pathway affected by a particular drug or may be able to overproduce an enzyme that is inhibited by the drug action, such as occurs in resistance to the folate antagonists. More than one mechanism may operate at any given time (Berkowitz, 1995).

Mechanism of microbial resistance towards antimicrobial agent

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to more than 1one class of antimicrobial agents. First of all, the organisms destroy the antibacterial agent before it can have an effect. In this microorganism may undergo gene acquisition encoding *Β*-lactamases enzyme. Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls lacking the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes. Thus, normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. The last event may occur through one of several genetic mechanisms, including transformation, conjugation, or transduction. Through

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genetic exchange mechanisms, many bacteria developed multidrug resistance (Tenover, 2006).

Due to spontaneous mutation, there may be: (1) Alteration of target protein which binds to antibacterial agent. This happens because of modification or elimination of binding site (e.g., change in penicillin-binding protein 2b in *pneumococci*); (2) Up regulation of the production of enzymes that inactivate the antimicrobial agent (e.g., erythromycin ribosomal methylase in *staphylococci*); (3) Downregulation or alteration of outer membrane protein channel that the drug requires for cell entry (e.g., OmpF in *E. coli*), or (4) Upregulation of pumps that expel the drug from the cell (efflux of fluoroquinolones in *S. aureus*) (McManus, 1997).

In all of these cases, transposons may facilitate the transfer and incorporation of the acquired resistance genes into the host's genome or into plasmids through pillus in two different organisms. In gram-positive bacteria there is production of sex pheromones by the mating pair, allowing exchange of DNA. During transduction resistance genes are transferred via bacteriophage. In transformation the bacteria releases their DNA complement after cell lysis, can move resistance gene in to susceptible strain. Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is termed *horizontal evolution*, and may occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation, Conjugation facilitate the clumping of donor and recipient organisms, allowing the exchange of DNA (McManus, 1997).

Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antibacterial agents into their environment. Although a single mutation in a key bacterial gene may only slightly reduce the susceptibility of the host bacteria to that antibacterial agent, it may be just enough to allow its initial survival until it acquires additional mutations or additional genetic information resulting in full-fledged resistance to the antibacterial agent (McManus, 1997). However, in rare cases, a single mutation may be sufficient to confer high-level, clinically significant resistance upon an organism (e.g., high-level rifampin resistance in *S. aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni*).

Antibiotic resistance occurs when plasmids coding antibiotic resistance are present in *Salmonella typhi.* Allied to the problem of resistance is the transfer of resistance plasmid that has been observed to code for multidrug resistance (Daughri et al., 2005). Drug resistance of pathogen is a serious medical problem, because of very fast arise and spread of mutant strains that are insusceptible to medical treatment. Microorganisms use various mechanisms to acquire drug resistance viz. horizontal gene transfer through plasmid, transposons and bacteriophage, recombination of foreign DNA in bacterial chromosome, and mutation in different chromosomal locus (Klemm et al., 2006).

Brown et al. (2003) have reported that the horizontal gene transfer is the factor in occurrence of antibiotic resistance in clinical isolates and suggested that the high prevalence of resistance to a particular antibiotic does not always reflect antibiotic consumption as suggested by Nwanze et al. (2007). In a report from India it is stated that certain virulence factors such as heamolysin production and presence of fimbriae in *E. coli* may be associated with urovirulance. Moreover the differences in sensitivity patterns of isolates could be attributed to time differences between two studies or environmental factors such as practices of self-medication, the drug abuse and indiscriminate misuse of antibiotics among the general population which has favored the emergence of resistance of resistant strains (Mandal et al., 2003).

According to Dever and Dermody (1991) reduced antibiotic penetration was also a resistance mechanism for several classes of antibiotics, including the beta-lactam drugs, the aminoglycosides, chloramphenicol and the quinolones. Piddock et al. (1986) reported resistance to nalidixic acid against Enterobacteraceae at 30 µg/ml. The R-factor (Resistance factor) is a plasmid having two components, transfer factor called the resistance transfer factor (RTF) which is responsible for conjugational transfer. The resistance determinants (r) represents for each of the several drugs. These two components can exist as separate plasmids, in such cases though the host cell remain drug resistant the resistance is not transmissible, this infers that resistance transfer factor is required for transferable/infectious drug resistance. Resistance to the sulphonamides may be mutational or plasmid mediated and may involve more than one mechanism, like alteration in metabolic pathway. Some sulfonamide resistant bacteria do not require extracellular Para Amino Benzoic Acid (PABA) and utilize preformed folic acid (Bagde, 2014).

Mechanism of heavy metals action

Guha and Mookerjee (1979) in an attempt to understand the mechanism of action of nickel on macromolecular synthesis showed that $NiCl_2$ affected it indirectly by inhibiting the activity of dehydrogenases. As a result, due to limitation of energy generating compounds, after a brief lag, synthesis of macromolecules ceased. Martinez et al. (1991) reported inhibition of other enzymes of TCA cycle aconitase and fumerase by nitric oxide in *Rhodobacter capsulatus* under light anaerobic or dark aerobic condition. The activity of these enzymes was also found to be inhibited in *E. coli* (Wimpenny and Cole, 1967) and *Aerobactor aerogenes* (Wimpenny and Warmsley, 1968) when grown anaerobically in presence of nitrate. Bagde and Varma (1983) have also reported similar site of action of chromium and lead.

Mechanism of microbial resistance towards the heavy metals

Metal resistance is inherited by plasmids in many bacteria (Silver and Phung, 1996, Ryan and Colleran, 2002).Mitsuhashi et al. (1963) observed reduced MICs up to 10 fold and complete loss of resistance in some strains of *E. coli*, after treatment with the curing agent acriflavine. Similar observations have been reported by Ghosh et al. (2001).

In studies on mechanisms of resistance to heavy metals, the chemical form of a metal determines its solubility, mobility, and toxicity towards an organism. Therefore, it also affects the MIC value. Inorganic heavy metals that occur as water-soluble salts exert greater toxicities, than water-insoluble forms of the same metals. Foster (1983) and Silver (1992) reported plasmid mediated reduced accumulation of heavy metals. Resistance of heavy metals due to reduced accumulation has also been reported by Belliveau et al. (1991) and Cooksey (1994). Resistance to heavy metals, pollutants, UV light and other antimicrobial substances such as antibiotics was due to extrachromosomal DNA (Foster, 1983).

When organisms are exposed to metal salts, it is first taken up by the cell and then the metal is localized in different parts of the cell where it exerts its toxic effect on those parts of the cells. Three main fractions of the cells from which the localization of heavy metals could be estimated are: Cell wall, cell membrane, and cytoplasm.

Unlike antibiotic resistance, there are no universally acceptable metal ion concentrations which are used to designate microbial metal tolerance (Calomiris et al., 1984). Bagde and Varma (1983) reported that chromium and lead inhibited the synthesis of protein, DNA and RNA almost equally in *E. coli* and *A. aerogenes*. Bagde and Salvi (1994) reported that cobalt and nickel inhibited protein, DNA and RNA synthesis of *S. paratyphi B* and *Shigella flexineri*.

The mer, chr, czc, and ncc genes that are responsible for resistance to heavy metals, were shown to be present in these bacteria by using RT-PCR. In the study it is observed that both gram-positive and gram-negative bacteria showed the metal tolerance against Cd^{2+} and Co^{2+}. In addition, gram-positive isolates (*Staphylococcus aureus*, *Bacillus subtilis*, *and Bacillus cereus*) showed higher expression levels of *czc*D and *ncc*A genes than *mer*A and *chr*B genes in comparison to gram-negative bacteria isolates. However, cobalt-zinc-cadmium (*czc*D) and nickel-cobalt-cadmium (*ncc*A) gene were up-regulated in the all strain of bacteria treated with Co^{2+} and Cd^{2+}. Therefore, Co^{2+} and Cd^{2+} resistance genes are widely distributed in both gram-positive and gram-negative isolates obtained from different samples of Egyptian soils (Laila et al., 2011).

This review summarizes and evaluates the effects of some specific antimicrobials and heavy metals upon the *Enterobacter* species.

This study have been drawn up so as to be useful for a wide range of healthcare professionals, such as specialist physicians and other healthcare workers (infectious diseases, microbiology, surgery, intensive care), public health officers, infection control professionals, administrative personnel in hospitals, and epidemiologists.

Antimicrobial resistance in the various pathogens has been reported since decades by scientists but not well understood in some instances till the date. Some pathogenic species shows same mechanisms and some species shows varied types of mechanisms in conflict with a single or multiple drug resistance. There are same mechanisms in the same or different species to resist different group of antimicrobials. Somewhere it is observed that there are different mechanisms of resistance existing for different type of antimicrobials among same or different species. The mechanism of resistance to some selected drug and heavy metals is reveled as plasmid mediated resistance.

Some heavy metals are important and essential trace elements, but at high concentrations, most can be toxic to microbes. Microbes have adapted to tolerate the presence of metals or can even use them to grow. Thus, a number of interactions between microbes and metals have important environmental and health implications (Spain and Alm, 2003).

Enterobacter spp. and *Serratia* spp. (particularly *Enterobacter cloacae* and *Serratia marcescens*) are found important as nosocomial pathogens and outbreaks caused by these organisms have been documented. Cross-transmission via transient contamination of Health Care Workers' hands has also been well documented in epidemic and endemic situations (Yu et al., 1999).

The concurrence of high antibiotic consumption, critically ill patients and a permanent influx of pathogenic species within the healthcare setting nurtures the development of resistance and provides an ideal scenario for the dissemination of resistant microorganisms and horizontal transfer of resistance genes. Therefore the management of Multi Drug Resistant microorganisms (MDR) in healthcare facilities has become a key issue. The degree of antimicrobial resistance in these surroundings depends on intrinsic factors related to the particular idiosyncrasies of each centre as well as on external factors such as the influx of resistant pathogens that originate in the community. Intrinsic differences in resistance rates between hospitals can be attributed to use of individual rooms vs. two or three bedrooms or open units, staffing, antibiotic stewardship, environmental cleaning, adherence to hand hygiene precautions and infection control programs (Tacconelli et al., 2014).

THE GENUS *ENTEROBACTER*

Enterobacter is gram-negative, facultatively anaerobic,

rod-shaped, non-spore-forming, motile aerobic gram negative bacilli of the family Enterobacteriaceae. They are oxidase-negative, indole-negative, and urease-negative. The genus *Enterobacter* ferments lactose with gas production during a 48 h incubation at 35 to 37°C in the presence of bile salts and detergents. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection. The genus *Enterobacter* is a member of the coliform group of bacteria. It does not belong to the fecal coliforms (or thermotolerant coliforms) group of bacteria, as does *E. coli,* because it is incapable of growth at 44.5°C in the presence of bile salts. Two clinically important species from this genus are *Enterobacter aerogenes* and *Enterobacter cloacae* (https://en.wikipedia.org/wiki/Enterobacter; Maki et al., 1976).

The history of *Enterobacter*

They first achieved wide notoriety as pathogens in 1976 following a nationwide outbreak of septicemia in 378 patients at 25 hospitals resulting from contaminated intravenous solutions (Maki et al., 1976). Because they can replicate in glucose-containing parental fluids, they continue to cause sporadic outbreaks of this type. Free-living *Enterobacter* are capable of nitrogen fixation. Certain species, notably *E. cloacae*, are involved in symbiotic nitrogen fixation in plants and have been isolated from the root nodules of certain crops, such as wheat and sorghum, and from the rhizospheres of rice (https://www.britannica.com/science/Enterobacter).

Classification and taxonomy

The isolate *Enterobacter cloacae* PRE9 is gram-negative rod, circular, soft, and cream-colored colony on medium. It is motile, gas producing bacteria which are catalase, citrate, Voges-Proskauer (VP) positive but indole, oxidase, and methyl red test (MR) negative. Stiles and Ng. (1981) isolated Enterobacteriaceae (86%) from different meat samples. The percentages of positive biochemical and identifying characteristics of seven member of Enterobacteriaceae were described. Among all, characteristics of *Enterobacter cloacae* 89.2% were positive for motility, 95.2% for VP, 92.2% for citrate, 100% were positive for acid and 98.8% for gas production from glucose.

The average guanine-plus-cytosine contents for the defined species and of the new classes are: *Enterobacter cloacae* (10 strains), 54.5 mol% (standard deviation, 1.32); *Enterobacter agglomerans* (syn. *Erwinia herbicola)* (1 strain), 52.4 mol% (standard deviation, 0.41); *Enterobacter aerogenes* (1 strain), 53.5 mol% (standard deviation, 0.29). The importance of the guanine-plus cytosine contents for discriminating defined species and new classes is discussed earlier (Izard et al., 1978).

Epidemiology

A landmark study in 1987 by Flynn et al. (1987) highlighted the importance of *Enterobacter* arising from a patient's endogenous gut flora causing subsequent infection. In this study of 87 patients undergoing cardiac surgery, all patients underwent surveillance cultures before and after surgery. Of 12 nosocomial infections due to *Enterobacter* in this group of patients, 9 were due to strains detected colonizing the gut preoperatively.

Enterobacter may also spread from patient to patient due to inadequate attention to infection control measures, especially hand-washing. In a study employing a consensus PCR technique for molecular typing of strains, Davin-Regli et al. (1996) studied 185 clinical isolates of *E. aerogenes* collected from two Intensive Care Unit's over a one-year period from a hospital in France. A ubiquitous clone was found to be responsible for two-thirds of epidemiologically related transmissions in these units.

According to data collected between 1992 and 1999 by National Nosocomial Infection Surveillance (NNIS) survey from the Centers for Disease Control (CDC), increased frequency of *Enterobacter* infections, particularly ICUs was observed (Archibald et al., 1997). Fridkin (2001) reported that *Enterobacter* was the fifth leading cause of ICU infections in the United States and third most common cause of nosocomial pneumonia overall. *Enterobacter* species is an opportunistic pathogen to humans and causes nosocomical infections. It carries seven operons with heavy metal resistant gene which make them capable to survive in heavy metal rich environment.

Enterobacter causes nosocomial infections. It is ubiquitous and can survive on skin and dry surfaces and replicate in contaminated fluids. Numerous outbreaks have been described, including infections due to contaminated enteral feedings (Simmons et al., 1989), humidifiers and respiratory therapy equipment (Wang et al., 1991) and hydrotherapy water in a burn unit (Mayhall et al., 1979).

Diagnostics, isolation, identification and species determination

This organism is easy to isolate from clinical specimens and biochemical tests readily separate it from other members of the Enterobacteraceae family.

In a report of 33,869 gram-negative isolates (16.1% *Enterobacter*) from 396 ICUs in the United States

sampled between 1990 and 1993, the Intensive Care Unit Surveillance Study, emerging resistance to extended-spectrum cephalosporins was a problem in both *Enterobacter* and *Klebsiella* (Itokazu et al., 1996). A follow-up study from the same investigators analyzed 35,790 isolates from ICUs in the United States sampled between 1994 and 2000 (Neuhauser et al., 2000). In this later collection of organisms, the prevalence of resistance to third generation cephalosporins in *Enterobacter* was quite stable at 37%. Likewise, resistance to aminoglycosides and carbapenems remained infrequent. The important observation from the more recent data set was a significant increase in the prevalence of resistance to fluoroquinolones in *Enterobacter*, *Klebsiella* and *Pseudomonas aeruginosa*. An analysis of antibiotic usage data was supplied by IMS Healthcare Inc., Westport, CT. This data revealed a highly significant association between the use of fluoroquinolones and resistance to quinolones in gram-negative rods, particularly in the case of *Pseudomonas*, but also for *Klebsiella* and *Enterobacter*. There was significant cross-resistance noted in *Enterobacter*, Pseudomonas *aeruginosa* and *Klebsiella pneumoniae* isolates in this survey. In all three organisms, ciprofloxacin resistant strains were significantly more likely to be resistant to gentamicin, amikacin, ceftazidime and imipenem as compared to susceptible stains. This cross-resistance complicates selection of appropriate empiric therapy of multiresistant strains.

A clear gradient of increasing ceftazidime resistance rates was noted. The prevalence of ceftazidime resistance was 12% among community isolates, versus 26% among nosocomial isolates. Within these hospitals, resistance rates were consistently higher among ICU isolates (36% versus 26%) (Archibald et al., 1997).

Clinical features and treatment of infections

E. cloacae showed higher MIC values for heavy metals and a larger range of antibiotic resistance than B. cereus (Qing et al., 2007). *Enterobacter* species causes nosocomial infections, including lungs, urinary tract, intraabdominal cavity and intravascular devices. *E. sakazakii* causes neonatal sepsis with meningitis (Bar-Oz et al., 2001; Nazarowec and Farber, 1997).

The susceptibility of *Enterobacter* isolates to Trimethoprim-sulfamethoxazole (TMP-SMX) was examined (Fung-Tomc et al., 1989, Wang et al., 1991). These reports have found susceptibility rates in excess of 90%. A report of *Enterobacter* bacteremia among pediatric patients emphasized the importance of central venous catheters as a portal of entry (63% of cases) and the excellent activity of TMP-SMX (91% susceptible). This agent is used infrequently in the treatment of *Enterobacter* infections (Andresen et al., 1994).

Drug of choice

The occurrence of nosocomial infections due to *Enterobacter* is rising and broad resistance to third generation cephalosporins, penicillins and quinolones is a rising problem. A number of agents remain effective for treatment. Aminoglycosides retain good activity but usually require combination with another agent. Quinolones are highly active against most strains, but emerging resistance is a major concern. TMP-SMX is under-utilized as therapy of *Enterobacter* infections. Among the beta-lactams, the fourth generation cephalosporins and carbapenems are the most attractive options (Archibald et al., 1997).

CLASSIFICATION OF CEPHALOSPORIN, FLUOROQUINOLONES, QUINOLONES, SULFONAMIDES

Cephalosporins

Cephalosporins is a derivative of 7-aminocephalosporanic acid, for example, cephalexin, ceftazidime, cephoxitin, ceftriaxone.

Sulfonamides

Sulfonamides is derived from sulfanilamide, the first successful antibacterial e.g., sulfadiazine, sulfamethoxazole. Trimethoprim is used to "potentiate" the sulfonamides.

Quinolones

Quinolones are synthetic, antibacterial agents with broad-spectrum activity. They inhibit the enzyme topoisomerase II, a DNA gyrase that is necessary for the replication of the microorganism, further developed in new generation as Fluoroquinolone.

Fluoroquinolones

This form a group of broad-spectrum antibiotics that are derived from nalidixic acid, e. g., ciprofloxacin, and norfloxacin.

ANTIMICROBIAL RESISTANCE

It has been shown that a link exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and metals) may be closely located on the same plasmid

in bacteria and the presence of organisms that acquire specific mechanisms to resistance to heavy metals increases destruction or transformation of toxic substances in the natural environment (Philip et al., 2001). Consequently, the range of genes carried on these plasmids (frequently associated with these heavy metal resistant determinants) was shown to extend far beyond those coding for antibiotic resistance (Tsai, 2006). Bacterial resistance to antibiotics and other antimicrobial agents is an increasing problem in today's society. Resistance to antibiotics is acquired by a change in the genetic makeup of a bacterium, which can occur by either a genetic mutation or by transfer of antibiotic resistance genes between bacteria in the environment (American Academy of Microbiology, 2000).

HEAVY METAL RESISTANCE

The experimental findings showed that high level plasmid mediated Cd^{2+} and Zn^{2+} resistance in the *Enterobacter sp.* BN4 strain is due to decreased Cd^{2+} and/or Zn^{2+} uptake/accumulation by resistance strain. Based on the fact that subsequent plasmid curing experiments demonstrated the ability to grow in presence of Cd^{2+} and/or Zn^{2+} was encoded by the 98 kb plasmid, whereas the ability to grow in presence of Pb^{2+} was found to be encoded by the chromosome. The Cd^{2+} and Zn^{2+} removal capacity of the respective metal resistant strain (pBN4) were about 36 and 45 µg g^{-1} DW respectively, while the removal capacity of the both metal by sensitive variant showed a significant high Cd^{2+} and Zn^{2+} removal capacity of 153 and 228 µg g^{-1} DW respectively. The order of the metals toxicity to the bacterium was found to be plasmid DNA that was determined by plasmid curing and conjugation experiments. The isolated endophytic Enterobacter was not only tolerant to heavy metals, but also bound considerable amount of heavy metals from the growth medium. The biosorbed order of the metals by parental strain and its cured derivatives strain based on the cell dry weight was found to be in the order of Pb^{2+}> Zn^{2+}>Cd^{2+} (Bahig El-Deeb, 2009).

In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999). To have a toxic effect, however, heavy metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems; one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient, it results in an influx of a wider variety of heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies and Silver, 1995).

Correlation of metal tolerance and antibiotic resistance

Because our current antibiotics are becoming less useful but are used more heavily against antibiotic resistant pathogenic bacteria, infectious diseases are becoming more difficult and more expensive to treat. The increased use of antibiotics in health care, as well as in agriculture and animal husbandry, is in turn contributing to the growing problem of antibiotic resistant bacteria. Products such as disinfectants, sterilants, and heavy metals used in industry and in household products are, along with antibiotics, creating a selective pressure in the environment that leads to the mutations in microorganisms that will allow them better to survive and multiply (Baquero et al., 1998). According to Lawrence's (2000a) discussion of the Selfish Operon Theory, clustering of genes on a plasmid, if both or all genes clustered are useful to the organism, is beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation. Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable, in terms of survival, for a bacterium to acquire resistance to both stresses. In plasmid mediated resistance, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance at survival. During such a situation, one may suggest an association with antibiotic resistance and metal tolerance. For example, Calomiris et al. (1984) studied bacteria isolated from drinking water and found that a high percent of bacteria that were tolerant to metals were also antibiotic resistant.

The selection of heavy metal resistance by microorganisms often promotes for antibiotic resistance in the environment. The resistance of these organisms to both metals and antibiotics could cause hazard into the environment and present very serious health implication because of the ability of these organisms to pass these resistant genes which may be transferred together via plasmids to other microbial cell around and will affect a whole bacterial population thereby complicating treatment (Sulaimon et al., 2015).

MULTIDRUG RESISTANCE IN ENTEROBACTER *SPECIES*

The saga of *Enterobacter* as a nosocomial pathogen is closely linked to the logarithmic increase in the use of extended-spectrum cephalosporins in the 1980's. A series of reports emphasized the proclivity of members of

this genus to acquire broad beta-lactam resistance during therapy with extended-spectrum cephalosporin's (Chow et al., 1997; Sanders, 1992). Enterobacteriacea protect them by secreting various enzymes for inactivating antibiotics, modifications in their targeting molecules, and use of antibiotics efflux pump systems. Subsequent studies have shown that prophylaxis with second and third generation cephalosporins has been associated with selection of multiresistant *Enterobacter (*Flynn et al., 1987*)*.

Quinolones

A cautionary note is raised by the report of Davin-Regli et al. (1997). These authors reported an outbreak of *Enterobacter hormachei* infections among patients in a French hospital who had been treated with quinolones. Twenty-one resistant isolates were detected over a one-year period. All were clonally related by the random amplification of DNA technique. Quinolone resistance in Enterobacter is usually due to chromosomal genes that may upregulate efflux pumps (Nikaido, 2001) or confer resistance due to altered DNA gyrase (Dekitsch et al., 1999).

In the ISS survey of 5451 Enterobacter isolates from 396 American ICUs collected between 1990 and 1993, ciprofloxacin was effective against 96% of strains. The prevalence of resistance to quinolones in Enterobacter grew significantly between 1994 and 2000, although 90% of strains remained susceptible in 2000. The newer quinolones such as moxifloxacin and gatifloxacin have greater activity against gram-positive pathogens than the older members of this class, but have no greater activity against gram-negative rods in general and *Enterobacter* in particular. It is reasonable to anticipate that quinolone resistance rates will continue to increase over time as these agents are increasingly employed in the treatment of serious *Enterobacter* infections.

Beta-lactams and extended spectrum cephalosporins

All of the so-called "third generation" cephalosporins and the monobactams (e.g. aztreonam) have approximately the same risk of emergence of resistance during treatment of *Enterobacter* infections. The data on preventing this type of resistance by employing concomitant aminoglycoside therapy is mixed. Jacobson et al. (1995) found a lower incidence of emergence of resistance to extended-spectrum cephalosporins among patients treated with concomitant aminoglycoside therapy, while Chow et al. (1991) did not.

A newer group of broad spectrum cephalosporins, the so-called "fourth generation" compounds, (e.g. cefepime and cefpirome) usually retain their activity against *Enterobacter* strains resistant to third generation cephalosporins (Segreti and Levin, 1996). The basis for this retained activity is (1) faster penetration through outer membrane porin proteins, (2) superior stability to chromosomal beta-lactamases, and (3) enhanced binding to critical penicillin-binding proteins in *Enterobacter* as compared to older cephalosporins (Bellido et al., 1991a, b; Fung-Tomc et al., 1989).

Sanders (1992) described successful therapy with cefepime of 17 infections due to *Enterobacter* strains resistant to third generation cephalosporins. These patients had infections at a variety of sites. All patients responded clinically and bacteriologic eradication was documented in 88%. Cefpirome is structurally similar to cefepime and has roughly comparable activity against Enterobacter strains, including those displaying resistance to third generation cephalosporins (Jones, 2001). There is less data available on clinical efficacy of this agent against multiresistant gram-negative pathogens.

Broad spectrum penicillins

Piperacillin is slightly less active than extended-spectrum cephalosporins against Enterobacter; in the ISS study, 63% were susceptible to ceftazidime vs 60% to piperacillin. In the Chow study, no patient receiving piperacillin experienced treatment failure due to emergence of resistance (Evans et al., 1998). In contrast, the work of Jacobson and colleagues reported a statistically significant association of prior piperacillin therapy with broad beta-lactam resistance.

Carbapenems

Carbapenems exhibit excellent activity against a wide variety of enteric gram negative pathogens, including *Enterobacter* (Norrby, 1995). Resistance to carbapenems in Enterobacter is rare (1% of NNIS isolates in 1999) (Fridkin, 2001), presumably because Enterobacter isolates require two separate mutations to acquire carbapenem resistance: loss of porin proteins plus hyperproduction of beta-lactamase (Livermore, 1991). Carbapenem resistance among *Enterobacter* isolates does not appear to be increasing over time.

In the series of Chow et al. (1991) none of seventeen patients receiving imipenem for Enterobacter bacteremia had resistant organisms emerge during therapy. Meropenem has activity comparable to imipenem against Enterobacter and found to be effective in the therapy (Colardyn and Faulkner, 1996; Edwards, 1995). There are a number of new carbapenem and penem agents in development that have excellent activity against Enterobacter. Ertapenem is a new carbapenem

with an extended serum half-life that has superb activity against enteric pathogens including *Enterobacter*. But this agent has limited activity against nonfermenters like *Pseudomonas aeruginosa* and *Acinetobacter* (Livermore et al., 2001).

Aminoglycosides

Aminoglycoside resistance in Enterobacter is usually due to plasmid-mediated aminoglycoside modifying enzymes. 4999 isolates of Enterobacter collected from 396 ICUs in the United States between 1994 and 2000, 98% were susceptible to amikacin and 92% were susceptible to gentamicin and tobramycin. These rates were steady over this time period. In the Chow study only one of 89 patients receiving aminoglycoside therapy failed treatment due to emergence of resistance during therapy (Chow, 1991).

Mechanisms of action of ceftazidime, moxifloxacin, nalidixic acid and sulfamethaxazole

Nucleic acid synthesis requires DNA gyrase enzyme and topoisomerase that removes the positive supertwists by nicking and then sealing phosphodiester bonds in DNA backbone. In addition, DNA gyrase can actively introduce negative super twists into close circular DNA at the expense of ATP hydrolysis. These negative supertwists promote parental strand separation at the replication fork. For example, all quinolones and fluoroquinolones inhibit microbial DNA synthesis by blocking DNA gyrase (Garrod et al., 1981; Piddock and Zhei, 1991).

Sulfonamides can enter into the reaction in place of Para amino acid benzoic acid (PABA) and compete for the active center of the enzyme. As a result, nonfunctional analogs of folic acid are formed, preventing further growth of bacterial cells. The inhibiting action sulfonamides on bacterial growth can be counteracted by an excess of PABA in the environment (competitive inhibition).

Resistance to some penicillins and cephalosporins may be a function of the loss or alteration of penicillin binding proteins (PBP) in *N. gonorrhoeae* and in resistant strain of *S. pneumonia* in South Africa (Domanski et al., 1997).

Resistance mechanisms towards ceftazidime, moxifloxacin, nalidixic acid and sulfamethaxazole

The plasmid mediated ESβLs confer resistance to oxyimino-cephalosporins, such as cefotaxime, ceftazidime and ceftriaxone (Vercauteren et al., 1997). Plasmid mediated β-lactamases TEM-1, which has a broad activity range against penicillins and cephalosporins, is carried on a transposon (Tn4). β-Lactamases are enzymes that act on the β-lactam bond of certain antimicrobials, such as the penicillins and cephalosporins, thus inactivating them (Berkowitz, 1995; Fraimow and Abrutyn, 1995).

Resistance to sulphonamides and trimethoprim is mediated by metabolic bypass, in this case due to synthesis of altered dihydropteroate synthetase and dihydropteroate reductase (Gibreel and Skold, 1999). The major cause of sulfonamide resistance is the plasmid-mediated production of an altered dihydropteroatesynthetase, which is 1000 times less sensitive to the drug than mild type enzyme (Satoskar et al., 1999). Tran and Jacoby (2002) reported a multi resistance plasmid that encodes transferable resistance to quinolones. Cooper et al. (1990) reported the cross-resistance among the quinolones. The absence of β lactamase enzyme in resistant *Enterobacter* was reported previously by Nirbhavane and Bagde (2015).

NICKEL AND LEAD RESISTANCE IN ENTEROBACTER SPECIES

E. cloacae showed scanty growth in Pb even at 0.5 mg/L but stop growing at 0.75 mg/L, while Enterococcus cloacae had MIC of 0.5 mg/L of Ni (Sulaimon et al., 2014).

Enterobacter aerogenes has an ability to completely degrade 0.6 mM lead concentration in 60 h while the MIC of lead for the strain was observed to be 3.6 mM. Also it had an optimum pH and temperature of 7.5 and 37°C, exhibited multiple metal tolerances and showed an improved reduction rate of Pb in presence of glucose in the medium (Macklin, 2013). Nirbhavane and Bagde (2016a) observed that the resistance to the heavy metal nickel and lead were found to be 200 and 300 ppm by the resistant *Enterobacter,* respectively.

In an investigation reported by Banerjee et al. (2015), the isolation and characterization of a potent heavy metal accumulating bacterial strain *E. cloacae* B1 from polluted soil at Ghaziabad, India was carried out. The minimum inhibitory concentration of the selected bacterial strain was recorded to be 1100 ppm for lead, 900 ppm for cadmium, and 700 ppm for nickel.

Mechanisms of heavy metal resistance

The high value of lead and copper may be due to the fact that lead and copper are used by bacteria cells in small quantities in cellular enzyme as reported by (Ansari and Malik, 2007; Sulaimon et al., 2014) also copper and lead appear to bind to material on the cell surface. Similar results were obtained from the study of Aiking et al. (1985), Roane et al. (2001) and Ansari and Malik (2007). Another implication of heavy metal tolerance in the environment is that it may also select antibiotic resistance

genes (Lawrence, 2000b).

Many bacterial heavy metal resistances have been studied for many years especially in contaminated regions. Resistance to toxic heavy metals has been found in bacteria from clinical and environmental origins. The genetic determinants of resistance are frequently located on plasmids or transposons (Cervantes et al., 1991).

Lee et al. (2006) isolated a nickel-resistant bacterium from soil in order to identify a novel nickel resistance determinant. Using 16S rRNA gene sequencing, an isolate was identified as *Enterobacter* sp. Ni15. This species showed a medium-level (resistant to up to 10 mM) nickel resistance in nutrient-rich media.

All the different isolates including *Enterobacter* spp. were tested for the presence of plasmids, showed three distinct bands of open circular, linear and supercoiled plasmid on 1% agarose gel (Gandhi et al., 2015).

The capacity of *the Enterobacter* BN4 to survive in different sources of pollution with elevated heavy metal levels (Schulz et al., 2006).

In a study (Nirbhavane and Bagde, 2016), resistant *Enterobacter* spp. to heavy metals nickel and lead was screened, and presence of the plasmid mediated heavy metal resistance was determined. In this study sensitive *Enterobacter* showed no growth in 50 ppm of nickel and 100 ppm of lead, while resistant species of Enterobacter tolerated 200 ppm of nickel and 300 ppm of lead. Komura and Lazaki (1971) reported that the MIC of two different metals could be same for any particular microorganism.

Detection of antimicrobial resistance in *Enterobacter* species

After the screening of pathogens and determination of MIC, selection of a pair of *Enterobacter* spp. which contain a sensitive and a resistant species towards all the antimicrobials and heavy metals was done. The resistant species of *Enterobacter* found to be resistant to different classes of antimicrobials, that is, third generation cephalosporin (ceftazidime), sulfa drug (sulfamethoxazole) Quinolone (moxifloxacin), and fluoroquinolone (nalidixic acid). The resistant *Enterobacter* has showed resistance to the all above mentioned antimicrobials and heavy metals, while the sensitive *Enterobacter* showed susceptibility toward these antimicrobials and heavy metals collectively. This pair was selected amongst the total 121 isolates of 15 different pathogens which were obtained from two different hospitals and a microbiology laboratory (Nirbhavane and Bagde, 2015).

Current trends in resistance research

Antimicrobial resistance has become, a serious public health concern with economic and social implications throughout the world, be it community acquired infections like *Streptococcal* infections, pneumonia, typhoid fever, etc., or hospital acquired infections due to methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *enterococci* (VRE), vancomycin intermediate *Staphylococcus aureus* (VISA) or extended spectrum β-lactamase (ESβL) enzyme producing gram-negative bacteria (Dancer, 2001).These infections lead to higher rates of hospitalization, longer hospital stay, and increase in the cost of treatment and thus, increased economic burden on the community.

Nature is abounding with metals in elemental form or in complexed forms. Around 75% of the naturally known elements in the biosphere are heavy metals and out of 90 elements 53 are heavy metals (Weast, 1984) which mostly persist in the environment. Some of them are most hazardous to human health. Microbes develop various mechanisms of resistance to survive in metal polluted sites. Resistance genes may be present on chromosomes or extra chromosomal plastids or transposons. Mechanisms employed by microorganisms to resist metal may be one or more combination of mechanisms, like intracellular sequestration, extracellular sequestration of metals, detoxification or exclusion of metals to resist metal toxicity.

The greatest contributions to medicine in the 20th century were the discovery of potent antimicrobials. Unfortunately, the emergence of antimicrobial resistant bacteria now threatens these advances and is today, one of the greatest concerns with regard to the use of antimicrobials. Increased antimicrobial resistance presents a major threat to public health because; it reduces the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality, and health care expenditure (Smith et al., 2002). This is of particular concern to hospitalized patients, with more and more hospitals worldwide, facing the crisis of the upsurge and dissemination of antimicrobial resistant bacteria; particularly those bacteria which cause nosocomial infections. Today the numbers of antimicrobial resistant bacteria are on the rise and development of new antimicrobials, has not kept pace with.

In studies involving mechanisms of resistance to antimicrobials, preliminary screening is necessary and always an essential part of the study as it assists in identifying and understanding trends in resistance (Jones, 2001).

Certain bacteria such as multidrug resistant gram-negative bacteria are particularly worrisome (Giske et al., 2008). In the US, two thirds deaths due to bacterial infections are caused by gram-negative bacteria (Foster, 2010). Multidrug resistant organisms are defined as microorganisms that are resistant to one or more classes of antimicrobial agents e.g. ESBL, MRSA, VRE etc. These highly resistant organisms deserve special attentions in healthcare facilities as they are associated

with increased length of stay, costs, and mortality (Siegal et al., 2006).

It was observed that when effect of ciprofloxacin on was studied, the growth of sensitive Shigella dysenteriae was completely inhibited at 1µg/ml concentration of ciprofloxacin, while the resistant strain tolerated even 10 µg/ml concentration of ciprofloxacin mechanism of resistance was found to be presence of plasmid (Lankeshwar and Bagde, 2013). When effect of Nalidixic acid and ciprofloxacin was studied, the growth of sensitive *Pseudomonas aeruginosa* was completely inhibited at 1 µg/ml nalidixic acid and 0.9 µg/ml concentration of ciprofloxacin, while the resistant strain tolerated even 60 µg/ml concentration of nalidixic acid and 4 µg/ml concentration of ciprofloxacin. and mechanism of resistance was found to be the presence of permeability barrier (Lankeshwar and Bagde, 2004). When effect of ciprofloxacin and sparfloxacin on *Staphylococcus aureus* was studied, the growth of sensitive *Staphylococcus aureus* was completely inhibited at 0.5 µg/ml of ciprofloxacin and 1 µg/ml concentration of sparfloxacin, while the resistant strain tolerated even 5 µg/ml ciprofloxacin and 50 µg/ml concentration of sparfloxacin and mechanism of resistance was found to be the presence of plasmid (Lankeshwar and Bagde, 2008).

In another study, when the growth of sensitive *Klebsiella pneumoniae* was completely inhibited at 8 µg/ml concentration, the resistant strain tolerated even 256 µg/ml concentration of ceftazidime. Mechanism of resistance was found to be the production of extended Spectrum of Beta-lactamase (Tahur, 2006). The growth of sensitive *Pseudomonas aeruginosa* was completely inhibited at 16µg/ml concentration, while resistant strain tolerated even 128 µg/ml concentration of sulphamethoxazole. Mechanism of resistance was found to be the presence of plasmid. When effect of nickel on *St. aureus* was studied, the growth of sensitive *S. aureus* was completely inhibited at 30 ppm concentration, while resistant strain tolerated even 180 ppm concentration of nickel. Mechanism of resistance was found to be the presence of plasmid. In strains showing presence of plasmid, curing with ethidium bromide yielded 80 to 90% elimination of resistance (Tahur, 2006).

Generations of new antibiotics

When the next generations of antibiotics were developed to overcome the problems of resistance against available antibiotics, bacteria developed mechanisms to resist the newer antimicrobial also. The 3rd and 4th generations of cephalosporins were introduced which were not destroyed by the β-lactamases produced by the gram-negative bacteria (Gotoh et al., 1998).

Use of ceftazidime with cefepime in hospitals

Recognizing that exposure to third-generation cephalosporins plays a role in the selection of multidrug-resistant organisms, a number of studies have evaluated the effect of substitution of the fourth-generation cephalosporin cefepime for third-generation cephalosporins on susceptibility patterns. Empey et al. (2002) reviewed antibiotic use and antimicrobial resistance before and after a university hospital formulary change that was aimed at reducing utilization of third-generation cephalosporins. After the formulary change to cefepime, the use of ceftazidime and cefotaxime underwent a combined decrease of 89%. Cefepime use was associated with a significant decrease in infections due to ceftazidime-resistant *K. pneumoniae* and *P. aeruginosa.*

The use of a ceftazidime-glycopeptide combination as initial empirical therapy for neutropenic fever resulted in a 75% reduced rate of susceptibility to ceftazidime among Enterobacteriaceae with AmpC b-lactamase-mediated resistance (Mebis et al., 1998).

Elimination, prevention and control of microbial resistance

Prevention is better than elimination, cure and control of any problem. However multifaceted approach is more advisable when the problem is blown out of proportion like resistance problem. Many guidelines, strategies and plans have been developed to deal with resistant microbial infections either generally or for specific organisms (Goldmann et al., 1996; Hospital infection control practices advisory committee, 1996; Department of Health and Human Services and department of labor, 1995).

Bacterial plasmids have genes that confer highly specific resistances to As, Bi, Cd, Cu, Cr, Hg, Zn, and other toxic heavy metals. For each toxic cation and anion, generally a different resistance system exists, and these systems may be linked together on multiple resistance plasmids (Silver et al., 1989).

Tahur (2006) and Lankeshwar and Bagde (2008, 2013) successfully cured plasmid by using various methods such as acrylamide method, acridine orange method and SDS treatment and showed that the resistance species became as sensitive ones to those antimicrobials that showed no activity towards the microorganisms before the treatment in plasmid elimination,

Plasmid was isolated and cured successfully which is a big evidence to reveal the resistance mechanism in these studies. In a study by Akhavan et al. (2015), the plasmid DNA was isolated from *K. pneumonia* with approximate size of 4.9 kb. Curing of plasmid was carried out with SDS. Plasmid curing was achieved by growing the strain treated with SDS. A plasmid isolated from *Klebsiella spp.* was treated with 10% SDS that leads to loss of a plasmid. A Strain of *Enterobacter* (Ent- 5) tolerated high concentrations of copper (23 mM), nickel (16 mM),

chromium (8 mM) and cadmium (14 mM). Cured Ent-5 was not able to grow on Ni and Cu. The sensitivities of the plasmid cured Ent-5 to nickel and copper indicated that copper and nickel resistance is correlated with plasmids (Unaldi Coral et al., 2005).

Nirbhavane and Bagde (2015) showed that *Enterobacter* carrying resistance plasmid was treated with 2 to 10% concentration of sodium dodecyl sulphate. The treatment was found to be effective to turn the resistant cells in to susceptible ones. The resistance was lost and the resistant species completely changed in to the sensitive species. This was very much in agreement to earlier studies of plasmid elimination using SDS by other workers (Tomoeda et al., 1968; Pan-Hau et al., 1981; Lankeshwar and Bagde, 2008, 2013). Reportedly also acriflavine was used for elimination of resistance to penicillin in *S. aureus* (Hashimoto et al., 1964).

Elimination of microbial resistance by various means and methods could be an important step in prevention and control of resistant microorganisms. There are many methods available which could be applied for this purpose. Different workers have used different reagents for elimination of resistance factor. Jacob et al. (1965) reported action of SDS in elimination of resistance factor. It may destroy the cell wall first and then cell membrane completely or partially, resistance factor (R) and fertility (F) factor associated more closely with the cell membrane as smaller replicons which then be damaged more easily by SDS.

To find out the location (chromosomal or extra chromosomal) of gene(s) responsible for the resistance to drugs, when plasmid curing experiments were performed using both physical and chemical agents; it was observed that, curing was most effective with ethidium bromide, followed by acridine orange (Hahn and Ciak, 1976; Tahur, 2006).

The reaction of plasmid in development of resistance was studied by Pan-Hou et al. (1981) in *Enterobacter aerogens* against mercury. Tran and Jacoby (2002) reported a multi resistance plasmid that encodes transferable resistance to quinolones.

Overall, the association of antibiotic use with the development of multidrug resistance underscores the fact that making suboptimal antimicrobial choices has global implications. The use of a particular antimicrobial agent may not only select for overgrowth of bacterial strains with innate resistance, but also may select for the development of diverse genetic vectors that encode and are capable of disseminating resistance mechanisms (O'Brien, 2002). Genetic elements of this kind may spread widely through the world's bacterial populations.

Challenges of microbial resistance

The number of drug resistant bacteria is on the rise but development of new treatment options have not kept pace. With bacteria replicating as often as once every 20 min, combined with their remarkable ability to change their physical and chemical makeup, resistant strains can evolve with amazing speed. Even greater concern is the fact that bacteria are able to easily transfer genetic information from one strain to another, there by passing on their resistance. This leads to increasing morbidity and mortality and an overall increase in health care costs. The dramatic reduction in development of new antibiotics active against these multi drug resistant pathogens has further complicated the therapeutic dilemma.

To survive under metal-stress conditions, bacteria have developed a variety of resistance mechanisms to counteract heavy metal stress (Spain and Alm, 2003). Though the levels of antibiotic resistance are rising inexorably, yet it has taken a long time to realize the extent of the problem, and there is still much that we need to learn about the mechanisms (Burnet et al., 2000). Despite efforts in the search for new antibiotics as well as the improvement of existing antibiotic performance, bacterial resistance to antimicrobial agents remains a problem in the treatment of infections.

Remedies for resistance crisis

The importance of adhering to the recommendations must be recognized by clinicians to prevent antimicrobial resistance in the healthcare setting (Salgado et al., 2005). Strict policies must be compared against the cost of management of high-level resistance (Vriens et al., 2002). The program optimization of therapy, implementing, teaching and monitoring treatment guidelines can have a major impact on patient care. It is observed that judicious use of antibiotics is essential considering growth of antimicrobial resistance and escalating costs in health care. Specific measures must include the revision of isolation guidelines. In addition, campaigns designed to educate the public and the health care community about the dangers of antimicrobial resistance and what may be done to control it. These campaigns include the "Get Smart" program, which primarily focuses on outpatients (Centers for Disease Control and Prevention, 2005) and the 12-step Campaign to Prevent Antimicrobial Resistance in Healthcare Settings.

The foreseen decline in effectiveness explains the needs for data to inform the public health agenda about the magnitude and evolution of antibiotic resistance as a serious threat to human health and development. Opportunistic pathogens are the cause of the community and hospital acquired infections worldwide.

It is today not possible to present a full picture of the spread of antibiotic resistance, and its health and economic burden due to the lack of global data. Some countries and regions do have surveillance system in place: others have no system at all for collecting the data. Hence the worldwide data on antimicrobial resistance

studies and its burden makes it almost impossible to track and contain emerging outbreaks in particular and challenges in general. It also makes impossible to evaluate the effect of national and regional initiatives to contain antibiotic resistance.

Infectious diseases continue to be a leading cause of mortality the world and more so in developing countries with low access of health services (World Health Organization Report, 2007). ICU is high antibiotic research area; therefore a correlation was simultaneously sought between antibiotic resistance and susceptibility pattern. A positive correlation was observed with carbapenem (imipenem and meripenem) and cefoperazone-sulbactum usage and development of resistance in pathogens. However piperacillin-tazobactum showed a positive correlation with *Acinetobacter* but a negative correlation with Pseudomonas (Jaggi et al., 2012).

There is the need to pursue detailed studies on antibiotic resistance in various areas which may lead to the better understanding of the magnitude of antibiotic resistance. Number of antibiotics used in the empirical treatments needs to be re-evaluated. Also further research is needed regarding rapid diagnosis of infection, accurate presumptive identification of patients, and development of new antimicrobials for drug resistance. No doubt that physician will eventually need the next generation of novel antibiotics to prevent and treat infections (Curtis, 2005).

The national, state and hospital level programs of surveillance and intervention must be strengthened to prevent the continued emergence of multidrug resistant pathogens and to limit their spread in to other communities or other institutions (Mathai et al., 2002). Continuous local monitoring of resistance patterns is necessary for the appropriate selection of empirical antimicrobial therapy. The frequency of resistance among S*higella* isolates has increased substantially between 2000-2002 and 2006-2009 and the spectrum of resistance has widened. The option for antimicrobial therapy in shigellosis in Andman is limited to a small number of drugs (Bhattacharya et al., 2012). Widespread selective pressure and efficient dissemination channels for multidrug resistant organisms are major factor that might have contributed to the rapid emergence and spread of the resistant organisms (Okeke et al., 2005).

The emergence of resistance to several new drugs such as fluroquinolones, 3rd generation cephalosporins, and macrolides is cause of concern not only at local level but at regional level also. A comprehensive strategy for resistance control involving regulation of drug availability, antimicrobial drug quality assurance, and adequate surveillance and discouraging the culture of antimicrobial abuse needs to evolved (Okeke et al., 2005).

A network of laboratories for real time monitoring of antibiotic resistance among enteric pathogens and timely dissemination of such information is essential immediately.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Colostrum immune quality of local sow breed in Benin: Growth, survival and acquisition of passive immunity in new-born piglet

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The objective of this study was to evaluate husbandry practices of pregnant sows, immunoglobulin G (IgG) concentration in colostrum of sow from local breed of Benin, their transfer to litter and the survival and growth of piglets until 42 days of age within traditional farms pig. Results showed that sows were raised according to traditional practices characterized by sustainable or precarious housing without any hygiene measures. They were fed with forages and by-products arising from traditional and industrial food-processing. Reproductive performance allowed by these farming practices remained weak. The litter size obtained was 5.5 piglets and was a feature of the small less prolific West African local pig. The observed number of piglets weaned at 42 days of age was 5 and the average mortality rate from birth to 42 days of life was 15%. The piglets were characterized by low average birth weight at 439 g, low average intra-litter weight gain at 24 h (38 g), body weight at 42 days of 2119 g, an Average Daily Gain (ADG) of 40 g/day and a Relative ADG (RADG) of 30 g/day.kg. The mortality was negatively associated ($P < 0.05$) with the birth weight and the weight gain at 24 h. The weight gain within 0-42 days was positively associated with the birth weight ($r = 0.59$; $P < 0.001$). The ADG from 0 to 42 days and from 1-42 days were positively correlated respectively with the birth weight ($r = 0.55$; $P < 0.001$) and the weight gain at 24 h ($r = 0.41$; $P < 0.005$). Despite weak growth performance of the piglets before weaning, the sows were characterized by adequate IgG concentration in the colostrum collected at 24 h after the onset of parturition (22 mg/ml). However, the serum IgG levels at 7 days of life of the piglets were low (2 mg/ml). Finally, the study demonstrated that sows from local breed produce colostrum with adequate IgG concentration. However, transfer of these antibodies to piglets was low. Feeding of sows with forages may be beneficial in improving the immunological quality of colostrum in local pig breed. Finally, both weight gain at 24 h and birth weight were important to predict survival and growth of suckling piglets.

Key words: Sow, colostrum, Immunoglobulin G, local breed, Benin, piglets.

INTRODUCTION

The local pig breed of Benin is the one found in several West African countries. It is raised mainly in traditional farms and could ensure sustainable incomes for small farmers. Both mortality and growth of suckling piglets are important for profitability and survival of traditional farms. The traditional husbandry practices of these animals often lead to low survival and growth performance of piglets under sow.

Ingestion of adequate amounts of colostrum components (nutrients, immune factors and growth promoters) soon after birth is crucial for survival and growth performances of new-born piglets (Quesnel et al., 2012; Ferrari et al., 2014). Thus, colostrum production may be one of the causes of low reproduction performances. A priori in African indigenous pig, this hypothesis is not based on literature data, because to our knowledge, there is a lack of information concerning colostrum production and acquisition of passive immunity in this breed. By contrast, several studies on colostrum are available on improved pig breeds. Furthermore, little data exist on growth performance of suckling piglets from local breed of Benin in traditional breeding. It would be worth obtaining data on colostrum from local sow breed and its impact on survival and growth performance of piglet in traditional farms in Benin and consequently, to explore opportunities to improve viability of these farms. This study aims to evaluate traditional husbandry practices of pregnant sows, survival and growth of suckling pig for 42 days of age, immunoglobulins G (IgG) concentration in colostrum and their transfer to the piglets from local breed.

MATERIALS AND METHODS

This study has been approved by the Committee for Animal Experiments of the Department of Animal Production and Health of Polytechnic School of University of Abomey-Calavi of Benin. The methods used in this protocol involving animals were in accordance with the ethical standards of this institution. So, all applicable guidelines for the care and use of animals of this institution were followed during this study.

Animals

The study was performed from September 2014 to March 2015 in nine (9) traditional pig farms at south of Benin with twelve (12) local sows breed (parity1) followed from pregnancy to 42 days of lactation. During this follow up, the mode of animal management (feeding, housing) was inventoried. Boar used for servicing was generally bought or borrowed. At birth, piglets were identified, weighed and returned to the sow. They were weighed thereafter at 24 h and then weekly until 42 days of age. Their survival was also followed under the sow from birth to 42 days of life. In total, fifty-five (55) piglets born alive from the remaining 10 sows (2 sows died with loss of their piglets) were observed from birth to 42 days of age.

Sample collection and analyses

On 10 of the 12 sows, a sample of colostrum obtained from all teats was collected manually 24 h after the onset of farrowing. Colostrum ejection was induced with intravenous injection of 1 ml of oxytocin per sow. Colostrum was filtered through gauze tissue and stored in a continuous chain of cold. Three (03) piglets per litter were randomly selected for blood collection 7 days after birth. In total, on 30 of the 55 piglets, the blood samples were collected by anterior vena cava puncture using a 21G needle and vacuum tube (vacutainer). Blood were centrifuged and serum stored in a continuous chain of cold until analysis.

The amount of IgG in samples of sows colostrum and piglets serum was assessed by a direct enzyme-linked immunosorbent assay (ELISA) using porcine IgG Kit N ° CEA544PO from EUROMEDEX® (France). The analyses were conducted at the International Centre for Research and Development of the Livestock in Subhumid zones (CIRDES) in Burkina Faso.

Statistical analysis

Two derivative data were calculated from weight: Average Daily Gain (ADG) and Relative ADG (RADG, that is, ratio of ADG to mean weight of the considered interval). Means and standard deviation for mortality rate, weight, ADG and RADG at different ages, concentrations of piglets serum IgG and sows colostrum IgG were calculated. Correlations between mortality rates, growth and immunity parameters were evaluated by using Pearson's coefficient. Binary logistic regression model was used to determine the relationships between piglets mortality from birth to 42 days as dependent variable, and both birth weight (BW) and weight gain (WG) at 24 h of life. The inclusion of sow effect in the model was not significant and thus it was not retained. Odds ratio for mortality from birth to 42 days are presented jointly with 95% confidence intervals. BW and WG at 24 h were compared between surviving and dead piglets by the Student's t-test. Associations between WG at different ages, BW and litter size were investigated using multiple regression analysis. In this case and in order to meet the conditions of the model validity (normality), the variables were transformed by the logarithm to base 10. The Effects of BW categories (BW < 500 g and BW ≥ 500 g) on the ADG, RADG at 42 days and on the concentrations of serum IgG were evaluated by analyses of variance (ANOVA) including the sow factor. The setting up of these two weight categories was based on the fact that the average BW of small West African piglets is 500 g in traditional breeding.

RESULTS

Pig farms management

The mode of animal management in the study farms was traditional. Pigs were confined in pens built of cement

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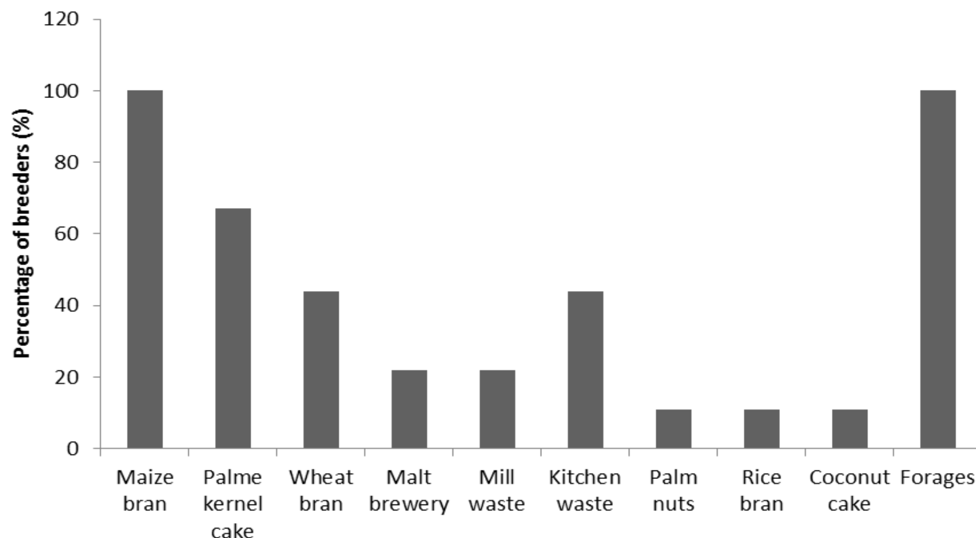

Figure 1. Feed used in local sow breed feeding in Benin.

Table 1. Reproductive performance of local sow breed in Benin.

Factor	n	Mean	SD	Min	Max
Litter size at birth	10	5.5	1.96	3	9
Number of piglets born alive	10	5.5	1.96	3	9
Litter size at 42 days	10	4.9	2.28	1	8
Mortality rate at birth (%)	10	0	0	0	0
Mortality rate 0-42days (%)	10	14.78	21.53	0	66.67

bricks walls for most (6/9), wood and bamboo for some (3/6), with individual box for farrowing. The roofs were covered with salvage sheets metals or straw and the floor was bare, cemented or made of concrete. The by-products used predominantly in feeding of sows were maize bran, palm kernel cake, kitchen wastes and wheat bran, sometimes malt brewery and mill waste. Sows were fed with a simple diet consisting of one of these by-products, according to their availability. Moreover, they were daily fed with forages in all of farms and received no veterinary care. Forages and maize bran were used by all breeders (Figure 1).

Animal survival and numeric productivity of sows

During animal follow-up, two sows died at the second week after farrowing with loss of most of their piglets the first week of life and the remainder at the onset of the second week. Lack of appetite followed by gradual refusal to eat was the main symptom observed, starting from 3rd and 5th days after farrowing in the first and the second animals respectively. No neo or perinatal mortality was observed during farrowing of the 10 sows and the mean number of piglets born alive was 5.5 ± 1.96. The average pre-weaning (0-42 days) mortality rate of piglets was 14.8 ± 21.5% and the mean number weaned at 42 days was 4.9 ± 2.28. Half of the piglet deaths occurred in the first week of life. Table 1 shows the overall reproductive performance of sows. The model of binary logistic regression (Table 2) showed that the risk of mortality was affected by the piglet BW ($P < 0.05$) and WG at 24 h ($P < 0.05$). Piglets that died before 42 days of age had lower ($P < 0.05$) average BW (338 ± 45 g versus 451.5 ± 14 g) and average WG 24 h after birth (-10 ± 22 g/day versus 44.9 ± 7.2 g/day) than surviving piglets (Table 3).

Growth of suckling piglets

Piglets average BW was 439 ± 106 g. The weight of piglets at 42 days of age was 2119 ± 720 g. Live weight increased steadily during the first two weeks of age, curbed the third weeks, to increase to a slower rate thereafter (Figure 2). This trend was the same as that observed for weekly ADG, with a higher gain obtained between 7 and 14 days (around 50 g/day), a fall between

Table 2. Mortality of piglets from local breed in Benin until 42 days of age according to BW and WG at 24 h.

Model	Coef	SE Coef	Odds ratio (95% CI)	P value
BW (P model = 0.009)				
Constant	2.75	2.02		0.172
BW	-0.01	0.00	0.99 (0.98 – 1.00)	0.025
WG (P model = 0.025)				
Constant	-1.91	0.50		0.000
WG 24h	-0.02	0.01	0.98 (0.96 – 1.00)	0.037

Table 3. Average BW and WG 24 h after birth of dead and surviving piglets from local breed in Benin.

Factors	n	Mean	SE	P value
Average BW of death piglets 0-42 days (g)	6	338	45	
Average BW of surviving piglets 0-42 days (g)	49	451.5	14	0.012
Average WG 24 h after birth of death piglets 0-42 days (g)	5	-10	22	
Average WG 24 h after birth of surviving piglets 0-42 days (g)	49	44.9	7.2	0.024

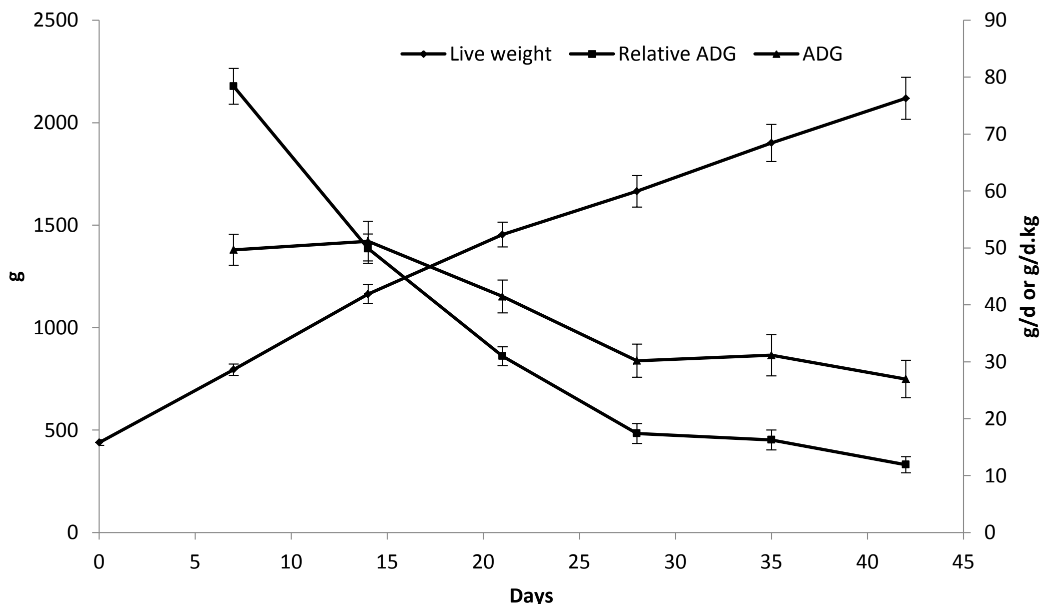

Figure 2. Evolution of live weight, ADG and relative ADG in local breed piglets in Benin.

14 and 28 days and a celling from 28 to 42 days of age reaching values just below 30 g/day the last week of measures. By contrast, RADG showed a continuous drop from 7 to 28 days and a celling from 28 to 35 days with a higher value obtained the first week (around 80 g/day.kg) and a lower between 21 and 42 days (below 20 g/day.kg). Piglets ADG until 7 days-old and until weaning at day 42 were respectively 49.7 ± 19.8 and 39.7 ± 15.7 g/day. The RADG 0-7 day and 0-42 day were respectively 78.4 ± 23 and 30 ± 4.3 g/day.kg.

Intra-litter piglets average WG at 24 h of age was 37.8 ± 30.7 g with large inter-sows variability, ranging from a minimum of -20 g and a maximum of 88 g. The average litter WG at 24 h was 215 ± 218 g. Moreover, intra-litter coefficient of variation of BW was 18.54 ± 7.55%. Likewise, growth performances are also shown in Table 4.

Table 5 shows some regression models highlighting the

Table 4. Growth performance of suckling newborn piglets from local breed in Benin.

Parameter	n	Mean	SD	Min	Max
BW (g)	55	439	106	200	675
Weight at 42 days	49	2119	720	750	3450
Litter WG at 24 h of age (g)	10	215	218		
Intra-litter average WG at 24 h of age (g)	10	37.8	30.7	-20	87.5
ADG 0-7 day (g/day)	53	49.7	19.8	7.14	85.71
ADG 0-28 day (g/day)	51	43.4	17.9	3.57	76.79
ADG 0-42 day (g/day)	49	39.7	15.7	9.52	70.23
RADG 0-7 day (g/day.kg)	53	78.4	23.0	15.0	120.6
RADG 0-28 day (g/day.kg)	51	39.5	8.43	10.2	49.5
RADG 0-42 day (g/day.kg)	49	30.0	4.31	17.3	36.2
Intra-litter average CV of BW (%)	10	18.54	7.55	6.34	27.72

Table 5. Linear models showing the relationship between weight gains at different ages, litter size and birth weight in the local piglets breed in Benin.

Model	Log_{10} (gain 0-7 day)			Log_{10} (gain 0-28 day)			Log_{10} (gain 0-42 day)		
	Coef	SE	P	Coef	SE	P	Coef	SE	P
Constant	0.41	0.76	0.597	0.53	0.84	0.530	0.08	0.63	0.901
Log_{10} (litter size)	0.47	0.20	0.025	0.27	0.23	0.250	-0.33	0.19	0.083
Log_{10} (birth weight)	0.65	0.30	0.032	0.87	0.33	0.011	1.27	0.24	0.000
R^2		0.18			0.15			0.35	
P model		0.003			0.008			0.000	

Table 6. Effect of sow and BW class on the ADG 0-42 day, RADG 0-42 day and serum IgG at 7 days in the local piglets breed in Benin.

Dependent variables	BW class		P value		SEM
	< 500 g	≥ 500 g	Sow	BW class	
ADG0-42 day (g/day)	37	46.7	0.000	0.004	2.1
RADG0-42 day (g/day.kg)	30.4	30.5	0.000	0.932	0.8
Serum IgG (mg/ml)	1.5	2.7	0.076	0.109	0.4

relationships between animal performance parameters. Positive relationships between BW and WG 0-7 day ($P < 0.05$), WG 0-28 day ($P < 0.05$) and WG 0-42 day ($P < 0.001$) were observed. Litter size was positively associated with WG 0-7 day ($P < 0.05$), but no association was observed between litter size and WG 0-28 day. The association between litter size and WG 0-42 day tended to be negative (P = 0,083). About one third of WG variation at 42 days could be explained by BW variation and litter size (R^2 = 35%, $P < 0.001$). The ADG 0-42 day was affected by the BW class (BW < 500 g and BW ≥ 500 g) ($P < 0.005$) and by the sow ($P < 0.001$) (Table 6). 72% of the variation of WG 0-42 day was explained by the sow. Piglets whose average BW was less than 500 g had lower ADG 0-42 day than piglets whose average BW was higher or equal to 500 g (33.8 ± 2.1 g/day versus 48.3 ± 2.1 g/day, $P < 0.005$). The RADG 0-42 day was not affected by the BW class (P = 0.932), but by sow ($p < 0.001$). Piglets of these two BW categories had, at 42 days of age, similar average RADG (30.4 ± 0.82 g/day.kg versus 30.5 ± 0.82 g/day.kg). Table 7 shows the significant correlations between animal performances. Both WG at 24 h and ADG 0-42 day were positively correlated with piglets BW, while ADG 1-42 day was correlated with WG 24 h.

Immune colostrum quality, IgG transfer and relationships with piglets reproductive performance

The average concentration of IgG in colostrum taken 24 h after the onset of farrowing was 21.8 ± 8.83 mg/ml, with a

Table 7. Correlations between different parameters of weight and serum IgG concentration in local breed piglets in Benin.

Parameters and correlations	r value	P value
Piglets WG 24 h (g) and BW (g)	0.40	0.002
ADG 0-42 day (g/day) and BW (g)	0.55	0.000
ADG 1-42 day (g/day) and WG 24 h (g)	0.41	0.004
Colostrum IgG concentration (mg/ml) and intra-litter average WG of piglets at 24 h (g)	0.71	0.049
Piglets serum IgG concentration (mg/ml) and piglets weight at 7 days (g)	0.37	0.049
Piglets serum IgG concentration at 7 days (mg/ml) and ADG 0-7 day (g/day)	0.50	0.008
Piglets serum IgG concentration at 7 days (mg/ml) and ADG 0-28 days (g/day)	0.37	0.059

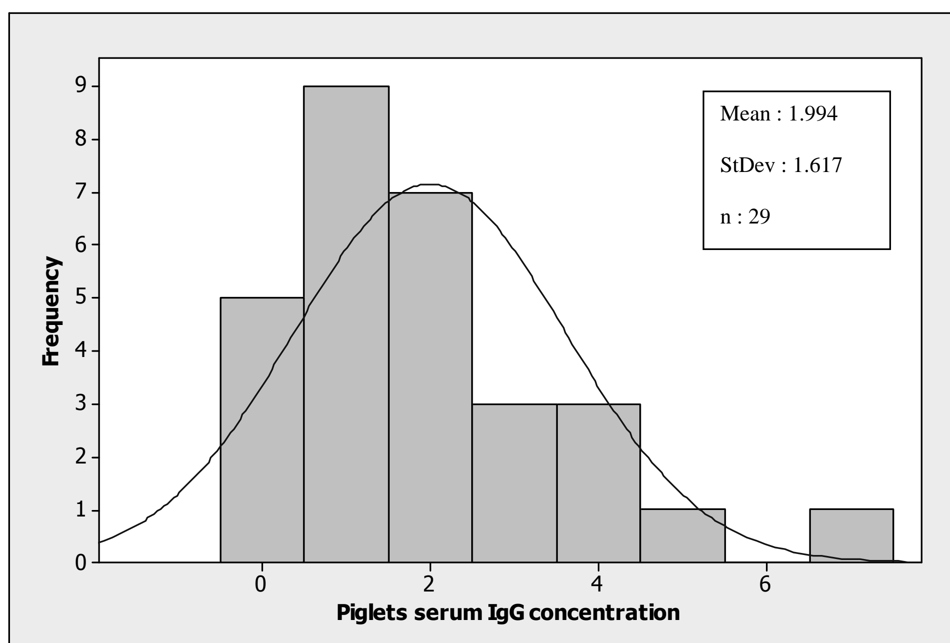

Figure 3. Distribution of serum IgG concentrations of piglets.

large variability between sows (Coefficient of Variation (CV) = 40.5%). A negative correlation was observed between intra-litter mortality rates and IgG concentration of colostrum, but it was not significant (P = 0.595). No relationship was observed between the IgG concentration in colostrum and intra-litter average ADG 0-7 day, 0-28 day and 0-42 day (with P value 0.433; 0.362 and 0.565 respectively). The correlation between IgG colostrum concentration and intra-litter average WG at 24 h of age was strong and significant (Table 7). By contrast, piglets serum IgG concentration was not correlated (P = 0.164) with average IgG colostrum concentration. The average concentration of serum IgG at 7 days of age was 2 ± 1.6 mg/ml with a very high variability between piglets of the same litter (average CV = 62.8%).

Figure 3 shows the distribution of piglets serum IgG concentration at 7 days of age. This one was not affected by BW categories (P = 0.109) with however a trend for sow (P = 0.076). Moreover, it was not correlated with BW, but was positively correlated with ADG 0-7 day, as well as with weight at 7 days of age. No significant correlation was observed between this parameter and ADG 0-28 day and 0-42 day with however a trend for ADG 0-28 day (P = 0.059).

DISCUSSION

The observed husbandry practices in this study are similar to those reported within the traditional farming system of pig in Africa by Agbokounou et al. (2016a) through driving mode (straying, confinement), housing, health, reproduction and feeding of animals. From pregnancy to lactation, sows were kept in total confinement with a sustainable or precarious housing without any hygiene measures. They were fed with one

by-product or scarcely more stemming from traditional or industrial food-processing, according to their availability. They received forages daily. The feeds used by the farmers of this study are similar to those observed within the traditional farming of local pig breed in Benin and in Africa in general (Agbokounou et al., 2016a). Forages, maize bran, palm kernel cake, wheat bran and kitchen wastes were the most ingredients used by these farmers. The values of digestible energy, crude protein (CP), Neutral Detergent Fiber (NDF) and crude cellulose reported for such by-products used in Benin and in Africa in general are respectively 3612 kcal/kg Dry Matter (DM), 197, 318 and 71 g/kg DM for maize bran, 3066 Kcal/kg DM, 146, 520 and 213 g/kg DM for palm kernel cake and 2510 Kcal/kg DM, 182, 436 and 100 g/kg DM for wheat bran (Agbokounou et al., 2016a). As for forages, CP and NDF concentrations reported are respectively between 88 and 324 g/kg DM and 279 and 688 g/kg DM (Kambashi et al., 2014). The unawareness of amounts of feed ingested by animals and use of by-products according to their availability did not allow evaluation of the satisfaction of nutritional needs of sows during their phases of pregnancy and lactation. In general, such sows receive enough fiber. The digestibility of energy should be thus limited by the high content in fiber, which could however promote intestinal health (Kambashi et al., 2014). In addition to these macronutrients, sows would have received enough minerals and vitamins because forages are characterized by high concentrations in some minerals (Ca, P, Na, Mg and Fe) and vitamins (A, D and E) (Lucas et al., 2006; Kambashi et al., 2014).

Regarding litter size, our observations are in agreement with findings of Youssao et al. (2008a) and Kiendrebeogo et al. (2012) which showed that small-sized West African local pig breed is less prolific in traditional conditions, with low litter size at birth ranging between 5 and 6 piglets. The litter size obtained in this study is lower than those reported by Agbokounou et al. (2016b) on Mukota breed of Zimbabwe (7 piglets), local pigs of Kenya (8 piglets), and of Tchad and Cameroon (7 piglets). Improving livestock farming conditions could increase this parameter. However, our values are similar to that observed on Nigerian local pigs in experimental station (between 5 and 6) (Ajayi and Akinokun, 2013).

The observed number of piglets weaned at 42 days was 5 and the average mortality rate from birth to 42 days of life was 15%. This value seems low compared to the 22% observed in traditional farms of Benin by Youssao et al. (2008a). However, the observed rate in the present experiment was obtained between birth and 42 days while the results of those authors give a mortality rate from birth to weaning. Weaning is performed at more than 8 weeks in traditional farms in Benin. At the experimental farm of the University of Abomey-Calavi in Benin, a lower rate at 12% has been obtained from birth to 42 days (Nonfon, 2005). The risk of mortality was affected by the piglets BW and WG at 24 h. For piglets with high BW and high WG 24 h after birth, the risk of mortality before 42 days was lower. Consequently, one randomly chosen piglet was around 0.99 times more likely to die than another whose BW or WG at 24 h was 1 g lower. Moreover, piglets that died between birth and 42 days of age had lower average BW and WG 24 h after birth than surviving piglets. These results confirm the results reported by Quiniou et al. (2012), which indicated that risk of piglets mortality increases when BW decreases. They are in agreement with findings of Le Dividich et al. (2004) and Devillers et al. (2011) who showed that piglets that died before weaning had lower BW, colostrum intake and WG at 24 h than piglets that survived until weaning. They also are in agreement with results obtained later by Ferrari et al. (2014) who observed that piglets from Large White × Duroc x Landrace that died before 42 days of age had lower BW, colostrum intake and serum IgG concentration 24 h after birth than surviving ones. According to Le Dividich et al. (2004), piglet WG from birth to 24 h of age is a marker of individual colostrum intake. According to this observation and in agreement with Ferrari et al. (2014) and Charneca et al. (2015), BW and colostrum intake are the major determinants of survival of local breed piglets under the sow.

The average BW obtained in the present study (439 g) is similar to those observed by Anugwa and Okwori (2008) and Youssao et al. (2008b), respectively on the small Nigerian pig in traditional breeding (500 g) and on Benin local pig in pilot farm of the Polytechnic School of Abomey-Calavi (540 g), but higher than that reported by Do Duc (2013) with Xao Va pig breed of Vietnam (200 to 250 g) characterized by small litter size (4-5 piglets). Considering this BW, the observed piglets weight and ADG at 42 days of age are slightly lower than those observed by Nonfon (2005) at the experimental farm (2660 g and 48 g/day at 42 days respectively). According to Étienne et al. (2000) and Nonfon (2005), ADG evolution would be an interesting indicator for milk production of local pig breed, and amount of milk produced by a sow is closely related to number of piglets and their average gain. Based on these results, the evolution of body weight and ADG observed in this study indicates that milk production of these sows could be more important the first two weeks with a peak between the 7th and 14th day. After a drop from the 14th day, milk production would be stable between 28 and 42 days. Piglet milk intake would attain a peak between 7 and 14 days followed by a fall from the 14th day and stability thereafter. These evolutions tend to be in agreement with that reported by Lachance (2010) on improved breeds with a peak at day 14 followed by a stability from the 22nd to the 23rd day. It is consistent with findings of Aguinaga et al. (2011), who showed that milk production by the Iberian breed (and consequently piglet milk intake) attained a peak at day 12 and leveled off thereafter. Additionally, the maximum of milk intake by improved

breed piglets is obtained between the 2nd and 4th weeks of lactation (Nonfon, 2005). However, it must be highlighted that the steady decrease in RADG from the beginning of our experiment suggests that milk production is not really sustained all over the 42 days of study.

According to Ferrari et al. (2014), BW and colostrum intake were crucial to the development of improved piglets breed from birth to day 42. This is in agreement with results obtained in this study with local breed piglets of Benin. Indeed, a positive relationship was observed between BW and WG 0-42 day or ADG 0-42 day ($P < 0.001$). Moreover, the ADG 0-42 day was to some extent affected by BW categories ($P < 0.005$) and especially by sow ($P < 0.001$). According to these observations, growth of local breed piglet from birth to weaning is dependent on environmental factors (farming practices) and factors related to the sow, such as colostrum production and genetic potential. Similarly to what was observed for colostrum production of improved breed (Le Dividich, 2006), this study showed a large variability of colostrum production between sows. This is in agreement with the observed large variability between sows of the intra-litter average WG at 24 h (-20 to 87.5 g) which would be an indicator of the colostrum yield. Regarding influence of genetic traits on growth, local piglet breed of Benin would be genetically similar to Ashanti Black pig. Indeed Darfur Oduro et al. (2009) had shown that maternal heritability of this breed was higher than direct heritability for BW and pre-weaning ADG. They showed that these parameters were influenced by the same genes and maternal additive genetic effect is an important component of variation in growth traits. Piglets of this breed with genetic capacity for high BW have better genetic capacity for growth during lactation and sows with genetic capacity to give birth to heavier piglets have a high pre-weaning growth rate in their litters (Darfour-Oduro et al., 2009). Thus it appears that BW is the most important determinant of the pre-weaning growth of local piglets breed.

This study showed the same average RADGs between piglets with BW < 500 g and BW ≥ 500 g. This finding indicates that average weight from birth to day 42 of piglets with BW < 500 g could be much lower than the one of piglets with BW ≥ 500 g because the ADG of piglets with low BW was lower than the one of animals with high BW. Moreover, the effect of the colostrum intake on growth was deduced from positive correlation between WG at 24 h and ADG obtained between birth and day 42 ($P <0.005$). In addition to these results, the observed positive correlation between BW and WG at 24 h which is a marker of colostrum intake indicates that local breed piglets with better BW has a better WG at 24 h and therefore better colostrum intake. This result is consistent with that of Ferrari et al. (2014). Furthermore, only the variation of the WG from 0 to 7 days was positively explained both by litter size and birth weight ($R^2 = 18\%$, $P <0.005$). By contrast, the relationship between the WG 0-42 days and litter size was not significant. Similar to what was observed for milk production of improved breed (Étienne et al., 2000; Ngo et al., 2012), it seems that the colostrum and milk production the first week by local sow breed increased with the litter size. This result is in contrast with findings of Foisnet et al. (2010) and Declerck et al. (2015), an indication that colostrum yield is independent on litter size. The result of the present study may be due to the fact that a high number of piglets stimulate all the teats, which would increase the number of functional ones. This higher number of functional teats coupled with the increased production of colostrum and milk from these sows the first two weeks, as indicated above, could explain this positive relationship between WG 0-7 day and litter size. The hypothesized decreased of milk production observed from the 14th day could explain the no significant relationship observed between WG 0-42 day and litter size. Finally, WGs from 0 to 42 days of suckling local breed piglets can be predicted by the equation: $log_{10}(WG\ 0\text{-}42d) = 0.079 - 0.33 log_{10}(\text{litter size}) + 1.27\ log_{10}(BW)$, but with low reliability because the predictive value was 29%. This relationship must be studied with higher sample sizes to be confirmed.

Despite weak growth performance of its piglets before weaning, the local sow breed is characterized by adequate IgG concentration of its colostrum. The observed average IgG concentration of colostrum collected at 24 h after the onset of parturition (22 mg/ml) is numerically greater than those observed with Large White x Landrace by Charneca et al. (2015) (12 mg/ml), Devillers et al. (2011) (17 mg/ml) or Decaluwé et al. (2014) (18 mg/ml). It is slightly higher than the 20 mg/ml observed by Charneca et al. (2015) on the Alentejano sow, an Iberian breed from region of Alentejo in Portugal. The combined effects of genetic and environmental (feeding practices, hygiene) factors expected in this study could explain higher colostrum IgG level in these sows. Contrary to feeding practices observed with improved breed sows, animals of this study received daily a lot of fiber through forages and by-products from traditional and industrial food-processing. According to Lucas et al. (2006) green forages are rich in β-carotene and vitamin E. Furthermore, the structural polysaccharides, including β-glucans, are the main fibers of lignocellulosic biomass (Godin et al., 2011). According to Laws et al. (2009), increased maternal intakes of β-carotene and vitamin E could increase concentration of colostrum IgG. Moreover, Leonard et al. (2010) have reported that the immune modulating properties of β-glucans could increase concentration of colostrum IgG of the sow. According to these observations, daily forages intake by local sows breed could explain their higher colostrum IgG levels. In contrast, high dietary fiber could decrease by half IgA in colostrum sampled at 24 h (Loisel et al., 2013). Feed studies taking into account sow metabolic status are

needed to confirm these results. Furthermore and similarly to what is observed for colostral IgG of improved sows breed (Le Dividich, 2006; Farmer and Quesnel, 2009), colostrum IgG concentration would be characterized by high variability between sows in local pig breed. The observed inter-sow coefficient of variation for colostrum IgG concentration was 41%. This value is close to that obtained with the Large White x Landrace sows (45%) and lower than that of Alentejano pigs (52%) for colostrum taken at 12 h after the onset of parturition (Charneca et al., 2015).

Although the high level of IgG in the colostrum from the sows, the transfer of these antibodies to piglets seems weak when compared to that observed in improved European piglets breed. The observed serum IgG level at 7 days of age (2 mg/ml) is very lower than those obtained at 7 days by Rooke et al. (2003) with Large -White x Landrace (8 mg/ml) and Yun et al. (2014) with Yorkshire x Landrace (7 mg/ml). According to Devillers et al. (2011), plasma IgG concentration of piglet is positively related to both colostrum intake and colostrum IgG concentration. Colostrum intake depends on production by sow and access to teats by newborns. Adequate transfer of IgG to piglet is the result of intake soon after birth of a sufficient amount of colostrum rich in this antibody. Furthermore, according to Charneca et al. (2015), decrease of serum IgG concentration observed from 48 h to 28 days with improved piglets breed is related to both the clearance of IgG, with a half-life of about 10 days, and the dilution effect associated with the increase of blood volume in that period. According to these considerations, when compared to improved breeds the low serum IgG level observed in this study may be related to lower colostrum production by local sows breed, lower colostrum intake, longer interval between birth and first sucking, or a more rapid clearance of IgG between 48 h and 7 days, for example, consecutively to infectious pressure or to high ability of piglets to clear IgG from the sow. According to Le Dividich et al. (2004), the litter WG at 24 h is a marker of colostrum production. The very low piglet WG at 24 h and the very low average litter WG at 24 h (215 g) observed in this study, as well as the decrease of RADG from the first week, could reinforce the thesis of low colostrum production by local sows breed. Studies need to be conducted to confirm these theories. The serum IgG concentration varied largely between piglets of the same litter and, in agreement with Cariolet et al. (2007), was not correlated with the colostrum IgG concentration. This variability could be related to the intra-litter variability of BW (CV = 19%) which is higher than the value of 15% observed with improved breeds by Quesnel et al. (2008) for litters size less or equal to 9 piglets. It also could be related to the interval between birth and first sucking and access to the teats competition. The hypothesis of intra-litter variability of BW might not be evident, because piglets' serum IgG levels at 7 days of age were not affected by BW class. Moreover they were not correlated with BW. This observation is in agreement with the results of Ferrari et al. (2014), who observed no correlation between BW and serum IgG concentrations of piglets at 24 h, 10 and 20 days. By contrast, piglet weight at 7 days of age was positively correlated with serum IgG concentration at 7 days ($P < 0.05$). Similarly, the correlation between ADG 0-7 day and serum IgG concentration at 7 days was positive ($P < 0.05$), while this relationship did not exist between this serum IgG concentration and ADGs 0-28 day and 0-42 day. The same trend was observed with the colostrum IgG concentration at 24 h and ADGs. Indeed, a strong positive correlation was observed between colostrum IgG concentration at 24 h and piglets WG at 24 h ($P < 0.05$). By contrast, no relation was observed between this colostrum IgG level and ADG (0-7 day, 0-28 day and 0-42 day). Serum IgG level appears thus important during the first stages of life in piglets from local breed in Benin.

Conclusion

In Benin, local sow breed in traditional breeding are subject to farming practices characterized by sustainable or precarious housing without any hygiene measures. Regarding feeding, they receive a significant part of nutrients by forages. Daily feeding with forages is a particularity of local pig breeding in Benin. Reproductive performance permitted by these farming practices remains weak and prolificacy, survival and growth performances of piglets under sow remains low. Birth weight and weight gain at 24 h are important indicators for survival and growth of local suckling piglets breed. Moreover, this study provides the first estimate of colostral IgG concentration and piglet serum IgG level of the local pig breed. Sows are characterized by adequate colostrum IgG concentration. However, the transfer or the retention of these antibodies at piglet level is low. Finally, feeding practices could be associated to high colostrum quality in local breed sow in Benin but the low immune transfer to piglets should be further investigated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of growth regulators on indirect organogenesis of two grapevines (*Vitis vinifera* L.) cultivars

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Establishment of efficient protocol for high-frequency of indirect regeneration of plantlets has a vital role in the analysis of genetic material in mass propagated plants. The optimal levels of growth regulators and light conditions were investigated on callus induction and organogenesis of cultured grapevine from *in vitro* tissues. Maximum calluses and shoots were produced by using medium supplemented with four different growth regulators as alone or in combinations. An observation of maximum calluses (51%) were recorded when 0.5 mg/l thidiazuron (TDZ), was combined with 0.5 mg/l indole-3-butyric acid (IBA) for Chenin *blanc* cv. Produced calluses were observed with different size and nearly similar colors. In this experiment, shoot initiation was observed in dark condition. The light condition did not induce the shoot on the same dark treatment. Different concentrations of 6-benzylaminopurine (BAP) and TDZ tested alone were not induced at any shoots from callus, but re-callusing the explant. Thus, shoot induction was observed when different concentration of BAP and TDZ were combined with auxins. The calluses produced from leaf did not produce high percentages of shoots. Further studies are needed to optimize the maximum percentage of somatic embryogenesis.

Key words: Callus, growth regulators, organogenesis, grapevine, dark.

INTRODUCTION

The organogenesis is a biotechnological tool used for obtaining mass production of mother plant with high quality of health (Bettoni et al., 2015). The explants can be grown into whole plant or produce callus. The produced callus can be utilized to regenerate plantlets or to extract or manipulate some primary and secondary metabolites (Pande and Gupta, 2013). Plant mass production can be affected by several factors such as light, temperature, plant varieties, and type of explant, components of media, sources and orientation of explants (Kumar and Raddy, 2011).

Temperature influences the various physiological processes, such as respiration and photosynthesis, which is well known and it influences plant tissue culture and micro-propagation. The most common culture temperature range has been between 20 and 27°C, but optimal temperatures vary widely, depending on genotype (Kumar and Raddy, 2011).

Most times, the optimal shoot proliferations of grapevine were reported when both hormones (cytokines

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and auxins) were combined. For instance for *Muscat of Alexandria* cv. maximum number of proliferated shoots was obtained on MS medium containing 3.0 mg/l BAP + 0.2 mg/l NAA. Similarly, the best shoot inductions were recorded at 2.0 mg/l BAP + 0.1 mg/l indole-3-acetic acid (IAA) for Canonnanon and Chenin blanc cultivars (Abido et al., 2013; Fikadu, 2011). According to Aazami (2010), different combinations of growth regulators (1.5 mgl^{-1} IBA), C (1 mgl^{-1} IBA+ 1.5 mgl^{-1} IBA) were best produced for shoots "*Soltanin*" and "*Sahebi* cultivars from meristem. Developing *in vitro* propagation of grapes was not only for the wine industry, but also due to the demand for fresh and dried fruit (Abido, 2013).

Despite the years of investigation, the application of tissue culture techniques in the grape-growing industry is still limited (Pe´ros et al., 1998). Hence, different cost effective protocols for mass propagation should be developed (Deore and Johnson, 2008). Beside the micro-propagations, an establishment of efficient protocol for high-frequency of indirect regeneration of plantlets is so much needed. Indirect organogenesis has a vital role in the analysis of genetic material in mass propagated plant (Deore and Johnson, 2008). In this finding, a protocol of producing shoots from callus was developed.

MATERIALS AND METHODS

Source and maintenance of explant

In vitro grown verities of grapevines (Chenin blanc and Canonannon*)* were obtained from Holeta Agricultural Research Center for this study. The experiments were conducted in Addis Ababa University at plant propagation and tissue culture laboratory. Sources of plant were maintained by sub culturing shoots and nodes of *in vitro* cultivated stock plant at one-month intervals on shoot induction medium (MS medium supplemented with 1 mg/l BAP + 0.1 mg/l IBA in Magenta GA-7 box vessels and sealed with Para-film). The gelling agent was 7.5 g/l agar with adjusted pH to 5.8 prior to autoclaving at 121°C for 15 min.

Preparation of media and explants culture conditions

The MS (Murashige and Skoog, 1962) nutrient medium composed of full macro, micro and vitamin A composition was used (Appendix I). Upper most leaf explant induced from the induction medium was wounded and cultured in the medium supplemented with four different growth regulators (BAP, IBA, TDZ and NAA) as alone or in combinations, to produce calluses. The cultures were incubated in a growth room at 27.5°C under different light conditions (light and darkness), where induced calluses were identified at three to four weeks intervals. Then the induced calluses were re-culturing in combinations of different growth regulators (BAP, IBA, TDZ and NAA) to produce shoots in intervals of 30 to 45 days. The length and number of induced shoots were recorded after culture at three weeks. There were 30 replicates per treatment and the experiments were repeated three times.

In vitro rooting

A 21 days sub-cultured shoots of Canonannon and Chenin blanc were rooted on full strength 20 ml MS medium supplemented with 3% (w/v) sucrose, four different concentrations of IAA (1, 2, 3 and 4 mg/l) and IBA (1, 2 and 4 mg/l). The length and number of main roots were counted and recorded after culture, at four weeks.

Acclimatization

Plantlets, having better roots and shoots systems were taken out from the culture vessels, washed under running tap water to remove the agar and sucrose. The plantlets were then transferred to 12 cm diameter plastic bag containing sterilized red soil, sand and cow dung manure at the ratio of 1:2:1, respectively. The plantlets were covered with transparent plastic bag to maintain moisture and watered within one day interval. Plastic cover was gradually removed after plantlets were successfully established in insect proof glasshouse for one month.

Experimental design and data analyses

The one-way analysis of variance (ANOVA) was used to compute the mean number and length of shoots and roots. Complete randomized design (CRD) was used. All data were analyzed at p ($\alpha<0.05$) using SPSS 16 version statistical software.

RESULTS

Effect of light conditions and growth regulators on induction of callus from leaf explant of grapevine

After culturing leaf explants in different concentrations of growth regulators, different amount of calluses were observed at dark. With similar treatments there was no record in light condition. Non wounded explants do not produce calluses both at dark and light conditions. An observation of maximum calluses (51%) were recorded when 0.5 mg/l TDZ was combined with 0.5 mg/l NAA for 'Chenin blanc' cultivar, while the highest calluses production in 'Canonannon' cultivars were obtained when 2 mg/l TDZ is combined with 0.1 mg/l NAA (Table 1).

The produced calluses had different size and nearly similar colors. Calluses produced by BAP alone or in combination with IBA were whitish while the TDZ alone or in combination of NAA were a little bit greenish white (Figure 1).

Effect of light conditions and growth regulators on induction of shoots from callus

In this experiment, shoot initiation was observed in the dark condition. The light condition did not induce shoot on the same treatment of dark. Different concentrations of BAP and TDZ alone were tested for shoot inductions from callus, which did not induce any shoots rather produced re-callus.

Shoot induction was observed when different concentration of BAP and TDZ were combined with auxins. Thus, 0.5 mg/l BAP +1 mg/l IBA and 1.5 mg/l

Table 1. Effect of light conditions and growth regulators on induction of callus from leaf explant of grapevine at 3 weeks after culturing.

Light conditions	Growth regulators (mg/l)				Canonannon	Chenin blanc
	BAP	IBA	TDZ	NAA	Callus formation (%)	Callus formation (%)
	0	0	0	0	0.0^{d}	0.0^{d}
	0.5	-	-	-	9.0^{d}	3.0^{d}
	1 .0	-	-	-	2.0^{d}	1.0^{d}
	-	-	1	-	22^{b}	9.8^{d}
	-	-	2	-	18^{c}	14^{c}
	2.5	-	-	-	26^{b}	34^{a}
	3 .0	-	-	-	13^{c}	5.0^{d}
	-	-	3	-	3.0^{d}	2.6^{d}
	-	-	-	-	3.2^{d}	3.1^{d}
	-	-	0.5	0.01	3.4^{d}	3.8^{d}
Dark	-	-	0.5	0.1	8.3^{d}	9.3^{d}
	-	-	0.5	0.5	16^{c}	51^{a}
	-	-	1	0.1	19^{c}	20^{b}
	-	-	1	0.5	8.3^{d}	9.3^{d}
	-	-	1.5	0.01	10^{c}	18^{c}
	-	-	1.5	0.5	26^{b}	9.3^{d}
	-	-	2	0.01	8.3^{d}	0.0^{d}
	-	-	2 .0	0.1	35^{a}	9.3^{d}
	1.5	0.1	-	-	32^{a}	17^{c}
	1.5	0.5	-	-	4.1^{d}	0.0^{d}
	1.5	1	-	-	32^{a}	41^{a}
	2	0.1	-	-	0.0^{d}	0.0^{d}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

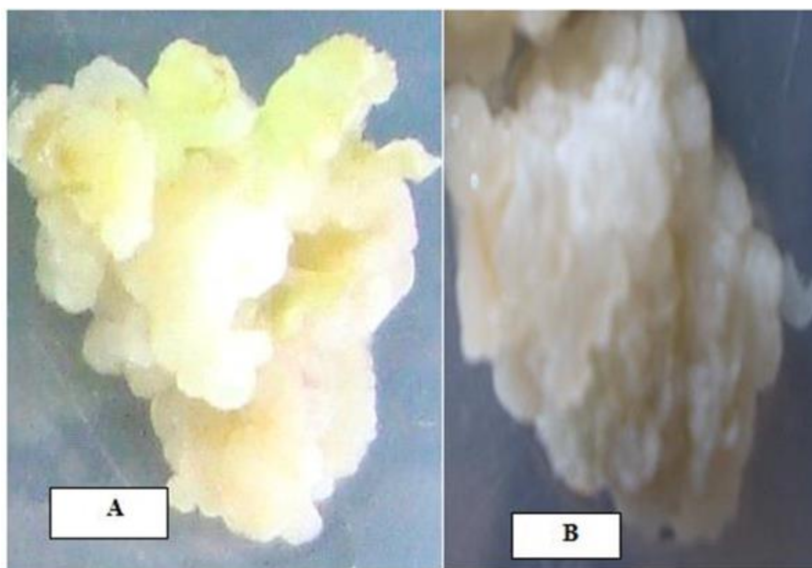

Figure 1. Induced callus from leaf explants of 'chenin blanc' cultivar (A= 0.5 mg/l TDZ +0.5 mg/l NAA; B=1.5 mg/l BAP+1 mg/lIBA) at 30 days after culture.

TZD + 0.5 mg/l NAA induced shoots for both cultivars (Canonannon and Chenin blanc), while 1.5 mg/l BAP + 1 mg/l IBA produced shoots for Chenin blanc cultivar in the dark incubation (Table 2). The induced shoots were white

Table 2. Effect of light conditions and growth regulators on induction of shoots from callus after culture at 4 weeks.

Light conditions	Growth regulators (mg/l)				Canonannon	Chenin blanc
	BAP	IBA	TDZ	NAA	Shoots induction/treatments	Shoots induction/treatments
Dark	0	0	0	0	-	-
	0.5	1			+	+
	1	1			-	-
	1.5	1			-	+
	2	0.1			+	-
	2.5	0.1			-	-
	3	0.1			-	-
			0.5	0.5	-	-
			1	0.5	-	-
			1.5	0.5	+	+
			2	0.5	-	-
			3	0.5	-	-
Light	0	0	0	0	-	-
	0.5	1			-	-
	1	1			-	-
	1.5	1			-	-
	2	0.1			-	-
	2.5	0.1			-	-
	3	0.1			-	-

+ =there was an induction of shoots; -=shows there was no shoot induction.

in color. Detail clear structures of the leaf were not observed.

Clearly visible shoots were observed at 35 days, after culture. It was gradually adapted to light incubation for further sub-culturing (Figure 2). Once the induced shoots are adapted to light, it changed to green. The general steps to obtain the green whole plantlets were summarized as the following steps (Figure 3).

Figure 2. Induced shoots of 'canonannon' cultivar from cultured callus on medium supplemented by 0.5 mg/l BAP+1 mg/l IBA at 35 days, after culture.

Effect of BAP on number and length of shoots derived from callus

The induced shoots were sub-cultured with different concentrations of BAP. Accordingly, the best mean numbers 5.5 ± 0.2 and 5.0 ± 0.1 were recorded for 0.5 mg/l BAP for canonannon and chenin blanc cultivars, respectively. Maximum length of shoots were recorded for 0.5 and 2 mg/l BAP for both cultivars (Table 3).

Effect of IBA and IAA on number and length of roots derived from callus shoot

Maximum mean numbers of roots were recorded for IAA and IBA in both cultivars. Thus, better root numbers (7.1 ± 0.5 and 6.1 ± 0.2) were produced at 4 and 2 mg/l IAA, respectively for 'canonannon' cultivar. Similarly, 6.1 ± 0.3 mean number of 'Chenin blanc roots were observed at 4 mg/l IAA. Mean length of roots were recorded at 4 mg/l IAA and 2 mg/l IBA for both cultivars (Table 4).

Even though the maximum mean number and length of

In vitro regenerated upper leaves were wounded and cultured

↓

Initiation of callus in 4 weeks (in dark)

↓

Culturing callus on shoot initiation media

↓

Initiated shoots excised from callus and cultured on multiplication media (in light)

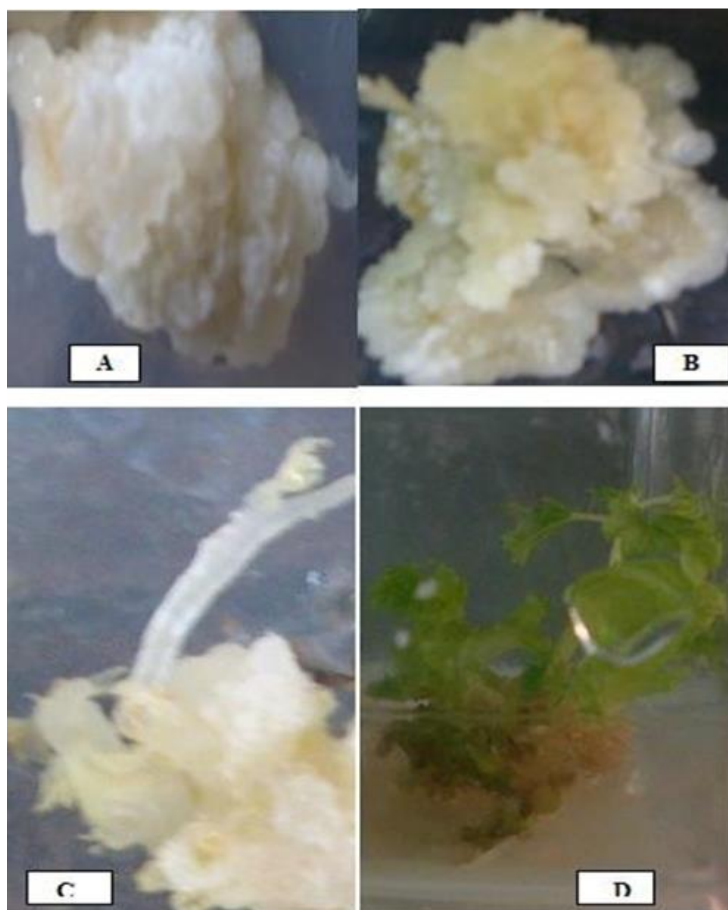

Figure 3. Shoot induction from callus (A= callus, B= Globular structure of callus on initiation, C= Induced Shoot, D= Whole plantlets).

Table 3. Effect of BAP on number and length of shoots derived from callus at 3 weeks after culture.

BAP (mg/l)	'Canonannon'		'Chenin blanc'	
	Mean number of shoots/explant	**Mean length shoots/explant**	**Mean number of shoots/explant**	**Mean length shoots/explant**
0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
0.5	5.5 ± 0.2^{a}	7.5 ± 0.1^{a}	5.0 ± 0.1^{a}	7.3 ± 0.2^{a}
1	2.6 ± 0.1^{c}	4.4 ± 0.3^{ab}	2.1 ± 0.4^{c}	4.9 ± 0.4^{ab}
1.5	3.1 ± 0.2^{b}	5.5 ± 0.2a	2.2 ± 0.3^{c}	5.2 ± 0.3^{a}
2	5.5 ± 0.1^{a}	6.8 ± 0.3^{a}	4.8 ± 0.1^{ab}	6.1 ± 0.3^{a}
2.5	2.6 ± 0.2^{c}	3.2 ± 0.3^{b}	2.2 ± 0.2^{c}	3.2 ± 0.4^{b}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

Table 4. Effect of IBA and IAA on number and length of roots derived from callus shoot at 3 weeks after culture.

Growth regulators (mg/l)		Canonannon		Chenin blanc	
IBA	IAA	Mean number of roots/explant	Mean length of roots /explant	Mean number of roots/explant	Mean length of roots /explant
00	0.0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
-	1.0	3.9 ± 0.4^{c}	5.6 ± 0.5^{ab}	2.0 ± 0.1^{d}	5.1 ± 0.5^{ab}
-	2.0	4.2 ± 0.2^{b}	6.0 ± 0.2^{a}	3.2 ± 0.2^{c}	6.3 ± 0.4^{a}
-	3.0	5.5 ± 0.1^{ab}	6.5 ± 0.2^{a}	4.8 ± 0.1^{b}	6.2 ± 0.1^{a}
-	4.0	7.1 ± 0.5^{a}	6.9 ± 1.1^{a}	6.1 ± 0.3^{a}	6.9 ± 0.8^{a}
1.0	-	4.8 ± 0.6^{b}	3.9 ± 0.3^{c}	4.5 ± 0.5^{b}	3.9 ± 0.5^{c}
2.0	-	6.1 ± 0.2a	7.5 ± 0.5^{a}	5.0 ± 0.6^{ab}	7.2 ± 0.7^{a}
3.0	-	3.4 ± 0.1^{c}	2.6 ± 0.4^{d}	3.3 ± 0.5^{c}	2.5 ± 0.3^{d}
4.0	-	3.3 ± 0.7^{c}	3.1 ± 0.2^{c}	2.5 ± 0.3^{d}	1.5 ± 0.2^{d}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

Figure 4. Induced roots of ‘Canonannon’ cultivar. A=Induced roots at 4 mg/l IAA. B= induced roots at 2 mg/l IBA.

roots were observed at medium supplemented with 4 mg/l IAA and 2 mg/l IBA, roots obtained from 2 mg/l IBA was thicker and longer than roots obtained from 4 mg/l IAA (Figure 4).

Acclimatization

The shoots with better mean length and number were survived when brought to *ex-vitro* conditions. The survival percentages were 90 and 71% for chenin blanc and canon annon cultivars, respectively.

DISCUSSION

Different concentrations of growth regulators, showed different capability of growing calluses from leaf explants. The best light condition to produce calluses were dark incubation at 27.5°C which, is confirmed with previous reports used to produce calluses from different explants of different genera (Salunkhe et al., 1997). But the same treatments incubated at light conditions did not produce callus.

For the studied varieties, non wounded leaf explants did not produce callus at both light conditions (dark and

light). An observation of maximum calluses (51%) were recorded when 0.5 mg/l TDZ is combined with 0.5 mg/l NAA for ‘Chenin blanc’ cultivar.

Meanwhile, the highest calluses production in ‘Canonannon’ cultivars, were obtained when 2 mg/l TDZ was combined with 0.1 mg/l NAA. Thus, a combinations or alone treatment of different concentrations of growth regulators are important to produce better calluses. Similar techniques were developed by Muhammad et al. (2008), while investigating effect of growth hormones on micropropagation of V*. vinifera L*. of different varieties were carried out.

The produced calluses were observed with different size and nearly similar colors but, calluses produced by BAP alone or in combination with IBA were whitish while the TDZ alone or in combination of NAA were a little bit greenish white (Figure 1). This condition indicated different concentrations of growth regulators which can produce different size of calluses while, a type of used growth regulators also contribute for the production of different color of calluses of same varieties.

Initiating shoots from leaf callus was contradicted with initiation shoots from grape tendril callus which was not successful (Salunkhe et al., 1997). Shoot initiation was found good at combinations of TDZ and BAP with auxins. Corresponding findings were reported on embryogenesis of grape from tendrils (Salunkhe et al., 1997). The induced shoots were white in color during the early development with an effect of dark when compared to light incubation, which produced green plantlets.

CONCLUSION AND RECOMMENDATION

Possible shoot inductions from callus of two cultivars of grapevine were identified in this study. Leaf callus did induce a medium rate of shoot induction but, further studies are needed to optimize the maximum percentage of somatic embryogenesis from other explants.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Appendix I. Nutrient composition and concentration of MS basal medium.

Components	Concentration (g/L)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.H_2O$	4.4
$MgS0_4.7H_2O$	1.8
KH_2P0_4	1.7
Micronutrients	
Fe-Na-EDTA	4
$ZnSo_4.7H_2O$	0.86
H_3BO_3	0.62
$MnS0_4.4H_2O$*	2.23
$MnS0_4.H_2O$*	1.69
$Cuso_4.5H_2O$	0.0025
KI	0.083
$Na_2Mo0_4.2H_2O$	0.025
$CoCl_{2.6}H2O$	0.0025
Vitamins	
Myo-inositol	0.1
Glycin (glycol)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01

*are alternative

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Full Length Research Paper

Comparative effects of plant growth promoters and earthworms (*Millsonia anomala*) on rooting of cocoa orthotropic cuttings

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Propagation of cocoa trees with high yield and disease resistance is envisioned for the renewal of the cocoa orchard. The dissemination of these elite varieties by orthotropic cuttings is strongly recommended to achieve quantitative and qualitative objectives. Some experiments reported that some *Theobroma cacao* clones produced by orthotropic cuttings do not root easily and the rate is very low, around 30 to 50% results. In this work, the impact of earthworms and chemical hormonal growth promoters on cocoa cuttings acclimatization were studied to increase the survival rate of cocoa stems during acclimatization step. The presence of earthworms identified as *Millsonia anomala* in acclimatization substrates allowed to convert more than 65% of stems into plantlets and exceeds 83% when the section of stems was a softwood part of branch. In the same profile, growth promoters as chemical hormonal solutions, regularly used in the process, are converting around 49.5% of stems into plantlets. In this study, a variability on the presence of taproots number across various parts of the branch were shown. Indeed, 69.2% of stems from hardwood, 64.3% stems from semi-hardwood and 56.4% of stems from softwood have developed at least two main roots. Indeed, this fact corroborates the capacity of these trees to able supporting the probable weight of cocoa pods and resists on wind which could appear on areas with bad weather.

Key words: Orthotropic cuttings, earthworms, somatic embryogenesis, hormones, cocoa crop.

INTRODUCTION

Cacao (*Theobroma cacao*) belongs to the genus *Theobroma* classified under the subfamily Sterculioidea of the mallow family Malvaceae. Cacao is one of 22 species of *Theobroma*. *T. cacao* L., exclusively cultivated in the inter-tropical area of the world, is a major source of income for developing countries (Alemano et al., 2007). Cocoa is the essential ingredient for chocolate and the role as the antioxidant and antiproliferative activities of *T. cacao* leaf, bark, husk, unfermented and fermented shell, pith, root, and cherelle methanolic extracts were well screened (Baharum et al., 2014; Da Silva et al., 2014).

The cocoa cropping has great economic impact in Côte

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d'Ivoire and Ghana which produced around 70% of cocoa beans around the world. These crops contribute in 10 and 8.1% of Côte d'Ivoire and Ghana gross deomestic products (GDPs), respectively. Despite its importance in the country's economy, cocoa cropping is facing different challenges in Côte d'Ivoire among which climate change, deforestation (Kouadio and Desdoigts, 2012), disease and pests pressure (Despréaux et al., 1987; Babin, 2009), aging of farmers and plantations, but also low access to inputs as fertilizers and pesticides and good quality planting materials. This results in low yields per hectare (300 to 450 kg/ha/year) with a negative impact on farmers' revenue (IITA, 2009).

Propagation of high yielding and disease resistant planting materials to farmers for renewal of the old plantations is one of the elements contributing to a sustainable cocoa cropping.

Propagation of cocoa planting material could be done by different methods (Niemenak et al., 2008). It is mainly through seeds, *in vitro* cultures such as somatic embryogenesis technics or vegetative propagation and by micropagation (Figueira et al., 1995; Lopez-Baez et al., 1993; Maxwell and Blake, 1984). However, in West African cocoa countries producers, 70% of cocoa plantlets are currently produced by seeds for plants propagation. The dissemination of these plantlets obtained by seeds is limited compared to the needs. One of the solutions could be to use orthotropic cuttings technique to address these problems. Indeed, promotion of cuttings methods particularly by orthotropic shoot growth has been achieved by the bending of mature trees or young orthotropic stems (Glicenstein et al., 1990; Bertrand and Dupois, 1992). Plants production by cuttings has an advantage in terms of capacity of production, insurance of genetic material contrary to the hybrids seeds that present a very strong heterogeneity at the level of production (N'Goran et al., 1994).

Plantlets from orthotropic shoots were obtained using branches harvested from trees bended on fields after a minimum of two months. These branches are divided into three parts to obtain stems and those planted in the polybags which contained substrates and placed under plastic tunnel during forty days.

Indeed using this system, nearly 40 to 250 plants can be produced from each somatic embryo plant as mother tree per year. The conversion of cuttings into plantlets during a large scale production is a one major challenge for the success of the process. Indeed, cultural strategies have been tested to promote the growth of orthotropic material in clonal gardens with mixed success (Miller and Gultinan, 2003).

Several methods were used to improve the success rate of cuttings conversion into plantlets by acting on acclimatization environments or by adding growth substances in the media or soil substrates and improving micro environmental conditions (Niemanak et al., 2008; Tee and Lamin, 2011). As observed in the rooting of cocoa cuttings, the application of auxin in the basal region of stem has been used worldwide to promote the adventitious rooting of woody species (Schwambach et al., 2008; Wendling et al., 2010; Hartmann et al., 2011; Hunt et al., 2011), to influence the micro-cuttings survival and then the rooting of species considered recalcitrant to rooting (Bennett et al., 2003; Schwambach et al., 2005). Beside, many environmental and genetically effects such as nursery practice and source of generative and vegetative materials in different plant species were reported by Dilaver et al. (2015) and Yazici and Babalik (2016).

At the other hand, earthworms generally are assumed to be beneficial soil animals which are mainly based on the belief that they promote plant growth (Lee, 1985; Edwards and Bohlen, 1996). However, knowledge on effects of earthworms on plant growth is very biased; most studies investigated crop plants, particularly cereals, and pastures; very little is known on plant species in more natural communities (Scheu, 2003).

Within soil organisms, earthworms are in terms of biomass and activity among the most important detritivores in terrestrial ecosystems (Edwards, 2004). Earthworms are known to impact plant growth, generally positively, via five main mechanisms: (1) an increase mineralization of soil organic matter, (2) the production of plant growth substances via the stimulation of microbial activity; (3) the control of pests and parasites; (4) the stimulation of symbionts; and (5) modifications of soil porosity and aggregation, which induces changes in water and oxygen availability to plant roots (Brown et al., 2004; Scheu, 2003).

Although, earthworms impact on plant growth through production of plant growth substances, this has never been tested on growth of cocoa cuttings. Therefore, in the current study, earthworm effects on cocoa cuttings root and shoot development were evaluated compared to classic plant growth promoters as chemical hormones. These cuttings are obtained from three types of stem: (a) hardwood (basal section of branch), (b) semi-hardwood (intermediate section), and (c) softwood (apical part of the branch).

The following hypotheses were tested: (1) Earthworms induce greater impact on root and shoot development of cocoa cuttings compared to plant growth promoters and (2) Earthworms exert greater impact on biomass production than do plant growth promoters, whatever the position of the stems on the branch (softwood, semi-hardwood and hardwood).

MATERIALS AND METHODS

Cocoa planting material

Two cocoa accessions T79-501 and PA7 from the International

Table 1. Agronomic characteristics of cocoa clones used in this study.

Selected clones	Productivity (kg/ha/year)	Weight of 100 dry beans (g)	Number of beans/pod
T79-501	2991.2	103.4	35
PA7	2255.4	103.5	35

Table 2. Different treatments done for stems acclimatization.

Treatments	Composition	Stems soaked into the hormone (g/L)
Control	Humic soil	-
*Agrinos C	Humic soil with Agrinos C (15 g/L)	-
Earthworms	Humic soil with 3-4 earthworms/bag	-
IAB 0.5%	Humic soil	1
IAB 1%	Humic soil	1
NAA 0.2%	Humic soil	1

*Agrinos C: Powder, biologically extracted micronized chitin for use in commercial outdoor agriculture. IAB: Indole-3-butyric acid; NAA: α-napthaleneacetic acid.

Cocoa Germplasm Database were used in this work as they respond highly to *in vitro* somatic embryogenesis methods (unpublished results). The agronomic characteristics of these two clones are shown in Table 1, according to information provided from Dr. Tahi (CNRA cocoa breeder).

Immature flowers buds of these cocoa trees are collected and processed to produce *in vitro* cocoa plantlets by somatic embryogenesis technique (Lopez-Baez et al., 1993; Florin et al., 2009). After five or six months of acclimatization growing period in greenhouses, these plantlets were planted on the cocoa gardens in an experimental farm to produce stems by orthotropic cuttings techniques.

Orthotropic cuttings production from mother plants

Previous plantlets produced by somatic embryogenesis technique were used as mother plants for generating orthotropic planting materials. These mother plants were arched and fixed on ground with a solid wire during four months after planting. Several orthotropic buds appeared after three weeks along the main trunk length. Three months after bending, these buds have grown and became orthotropic branches measuring around 30 to 70 cm. These branches were harvested on the base of horizontal main trunk of mother plants, and orthotropic cuttings were obtained by cutting the main trunk of these branches in three parts. For this study, each branch was divided into three portions of around 5 to 8 cm: hardwood (basal section on the branch); semi-hardwood (intermediate section); and softwood (apical part of the branch).

Growing conditions and general cultural management

Stems (2880) of each part of the branch were used as orthotropic plantlets from the two clones previously described were produced. These stems were sanitized in a solution of 2.5 g/20 L of Benomyl powder (Louis Drefus Commodities, Côte d'Ivoire), an antifungal product, during 1 to 3 min. As presented in Table 2, six different treatments were applied to acclimatize stems.

Two rooting hormones at different concentrations were prepared as 1 $g.L^{-1}$ of indole-3-butyric acid (0.5 and 1%) and 1 $g.L^{-1}$ of α-napthaleneacetic acid (0.2%).

The stems of each clone were dipped in the rooting hormone solutions during few seconds and planted in polybags which contained steppe black soil.

The other stems which were not dipped in the hormonal solutions were placed directly in the polybags which contained steppe black soil supplemented with three earthworms as *Millsonia anomala* (1 living organism/0.002 m^3 of soil) and in steppe black soil also supplemented with Agrinos C (15 g/0.001 m^3 of soil).

After transplanting, cuttings in polybags were placed under a white plastic film with 70% of shade. Closing them with plastic films has elevated tunnels' relative humidity (RH) above 80% and increased temperature around 40°C. After 30 days, plastic tunnels were opened gradually during the 10 days.

Experimental design for statistical analyses

Stems (1440) were produced per cocoa variety and the experimental design was done to establish the same experimental unit regardless of the three different positions on the branch and the six treatments. For that purpose, a split split plot with 4 replicates and 4 blocks was preferred (720 sections of stems by block 240 softwoods, 240 semi-hardwoods, and 240 hardwoods).

The following dependent variables were measured: survival rate of stems, number of plants with taproots, number of taproots, dry shoot and root dry biomasses for each treatment and taking into account the position of each stem.

Survival rate of stems was calculated on data collected after 40 days of acclimatization step under tunnels. The number of plants with taproots, shoot dry weight and root dry weight were also measured at the end of the experiment. All data were analyzed and compared with type 1 analysis of variance (ANOVA) using GLM procedure (SAS software 9.3). To determine the direction of significant effects, multiple comparison tests were used based on Least Significant Difference (LSD). General outcome of these comparisons without displaying them in detail was presented in Table 3.

Table 3. Analysis of variance F-values for ten parameters measuring plant produced by orthotropic shoot under effects of earthworms and growth promoters.

Source of variation	DF	Root dry weight (F-value)	Shoot dry weight (F-value)	Survival rate (F-value)	1 main roots (F-value)	2 main roots (F-value)	3 main roots (F-value)	4 main roots (F-value)
Bloc	3	3.86*	6.96**	9.43**	1.55	0.57	0.18	2.87*
Clone	1	0.14	5.64	3.92	0.78	0.38	1.08	0.26
Error (a)	3	-	-	-	-	-	-	-
Section	2	2.20	2.06	8.01**	11.53**	20.84**	18.43**	8.22**
Clone×section	2	13.14**	9.40**	23.40	5.70*	3.82*	1.68	9.28**
Error (b)	12	-	-	-	-	-	-	-
Treat	5	6.05**	6.06**	14.06**	2.75*	0.76	1.93	1.70
Clone×treat	5	1.52	2.00	2.06	3.38**	1.86	2.98*	0.54
Section×treat	10	1.47	1.63	1.77	1.67	1.82	1.40	1.63
Clone×section×treat	10	1.21	1.42	0.73	0.84	1.01	0.81	2.39*
Error (c)	87	-	-	-	-	-	-	-
Corrected total	140	-	-	-	-	-	-	-

RESULTS

Plant growth parameters

In this experiment, as can be seen in Table 3, treatments, section of branch and the section of branch×clone interaction were significant sources of variance for several variables. Importantly, earthworms and growth promoters (treatments) had significant effects on all of the parameters. The clone did not have a significant impact on all dependent variables measured. As shown in Table 3, this effect ranging from significant (0.01<P<0.05) for the rate of plants with one main root, to highly significant (*P*<0.01) for the survival rate, shoot dry weight and root dry weight. Furthermore, the section of stem also had a highly significant effect (*P*<0.01) on several dependent variables measured (the survival rate and number of taproots).

Finally, a significant interaction (0.01<*P*<0.05) was observed between the section of branch and the clone (section of woods×clone) for the rate of plants with 1 main root, two main roots and highly significant (*P*<0.01) for the shoot dry weight, root dry weight, rate of plants with 4 main roots.

Effects of different treatments on the conversion of stems into plantlets

Stems were immersed in concentrated solutions of growth hormones and also were buried in different substrates formulations as described in previously.

The survival rate of stems according to each treatment was determined (Figure 1). These data showed a very high variability of parameters for each treatment.

The success rate of stems converted into plantlets was 65.8% when the humic soil contained earthworms. When stems are immersed in auxin solutions or introduced on humic soil which contains Agrinos C solutions, the survival rate was 42.4 and 41.04% in only humic soil substrate as a control.

Relation between the types of roots and treatments or stems position

To evaluate the influence of each treatment on the position of each stem, the rate of its conversion into plantlet was determined and presented as shown in Figure 2. Results obtained showed that the success rate was 44.4% when stems are made by hardwood, 42.4% when it is from semi-hardwood and 58.4% when softwoods are used to produce plantlets.

The success rate on stem conversion was around 83% when softwood part of branch was acclimatized in humic soil substrate which contained earthworms.

To correlate plantlets survival rate and corresponded treatments, roots and shoots dry weights parameters were calculated. Results obtained are as shown in Figure 3. Excepted substrates of only humic soil and humic soil contained agrinos C with 7.9 and 6.8%, respectively, no difference could be observed for root dry weights between other treatments (around 11%).

The development of roots is a major asset for the future tree, not only for nutrition system, but mainly to protect the trees against the vagaries of the weather as the wind in unfavorable areas. This criterion was also determined by the enumeration of the number of taproot of each plant

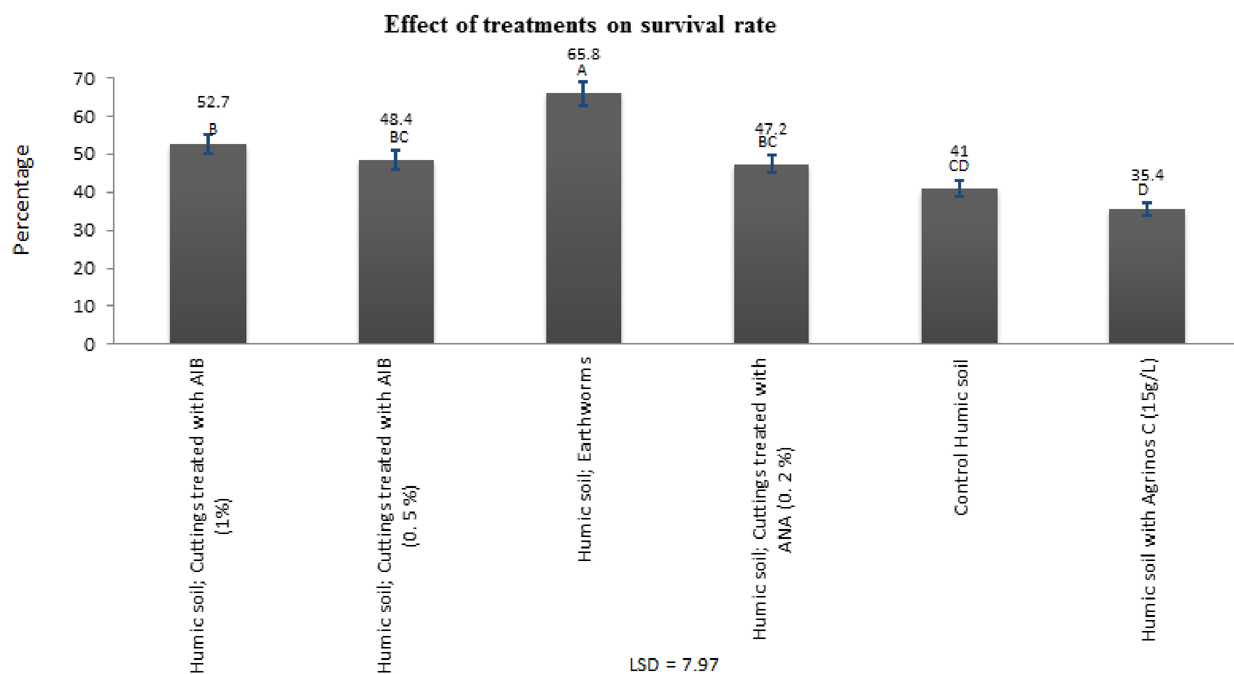

Figure 1. Stems conversion rate (%) into plantlets according to each treatment after 40 days under tunnels (AIB: IAB: indole-3-butyric acid; NAA, α-napthaleneacetic acid).

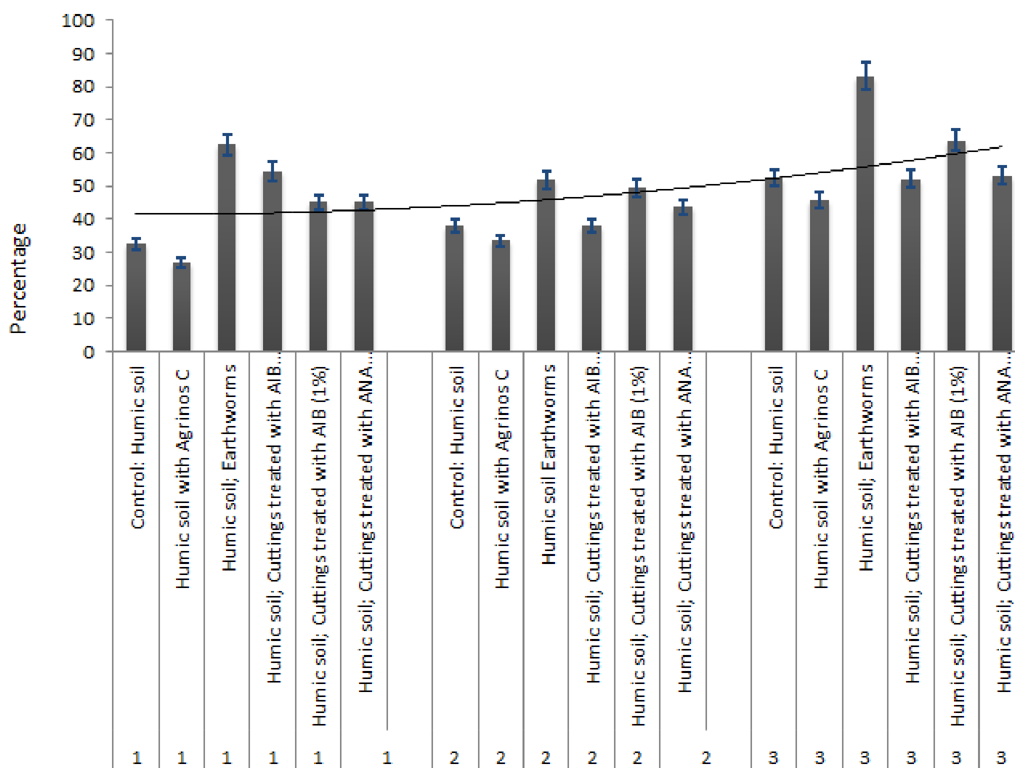

Figure 2. Stems survival rate according to each treatment and stem position on branches (AIB: IAB, indole-3-butyric acid; NAA, α-napthaleneacetic acid).

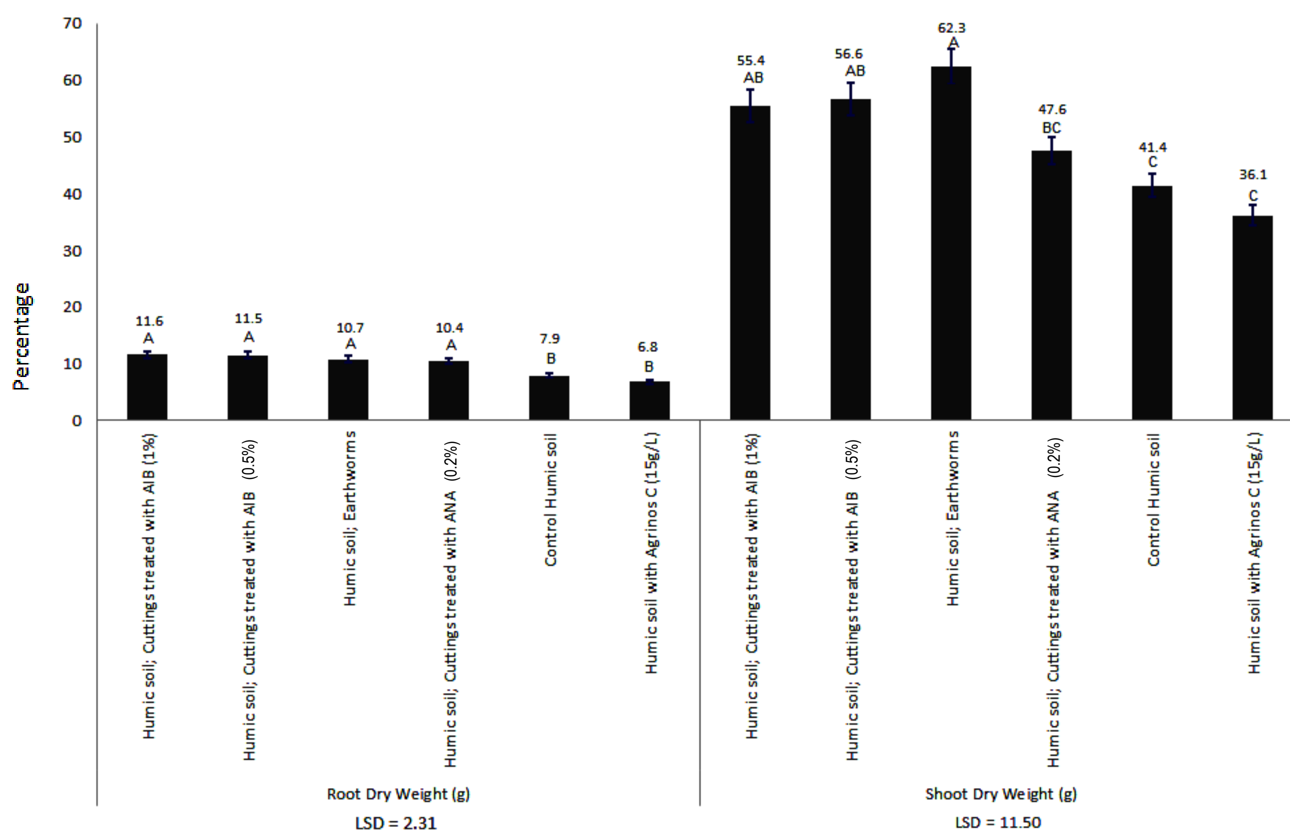

Figure 3. Determination of roots and shoots dry weights parameters according stems survival rate treatments.

according to the treatment. Figure 4 showed results obtained after 6 to 8 weeks outside acclimatization tunnels.

All plantlets obtained by orthotropic cuttings were developed one or more taproots regardless to all treatments. Notwithstanding the type of treatment, the proportion of cuttings which developed one or two taproots is ranging between 40 and 80%. No significant differences between treatments could be observed for the development of two taproots for all stems. However, the probability to develop one taproot was higher when Agrinos C (15 g/L) treatment was applied on cuttings.

These results did not explain the preferential occurrence of a precise number of roots in relation to the type of treatment. Detailed analyses of these results in function of the position of stems are shown in Table 4. One taproot was developed by 43.6% of stems obtained with softwood, 35.7% of semi-hardwood stem and then 29.7% of stems from hardwood. More than 50% of stems produced by all sections (hardwood, semi-hardwood and softwood) produced two or three taproots.

DISCUSSION

The stems from cocoa branches were better converted into plantlets when the substrate contained a humic soil with earthworms than other chemical growth hormones and also than results shown by Gehlot et al. (2014) on cuttings treated with auxins. However, all results obtained, in this study on the different rooting substrates, are higher than those obtained by Tee and Lamin (2011) on vermiculite (32.6%), perlite (23.2%) and then coconut coir fibre (21.5%).

Indeed, in the cases of auxin application, great variation was observed among the concentrations, formulations and forms of application of plant growth regulator (Wendling et al., 2000; Fogaça and Fett-Neto, 2005; Wendling and Xavier, 2005; Almeida et al., 2007; Schwambach et al., 2008) as well as other factors considered intrinsic based on the genetics (Stape et al., 2001; Bennett et al., 2003; Corrêa and Fett-Neto, 2004).

The presence of earthworms in certain rooting substrates could probably play a major role in the transformation of inorganic materials in organic matters during stems acclimatization. In their studies to investigate the effects of earthworms on N cycling processes and microbial activity, Bolhen and Edwards (1995) indicate that living organisms increased the amounts of extractable N by feeding on the microbial biomass, and increasing the turnover and mineralization of microbial

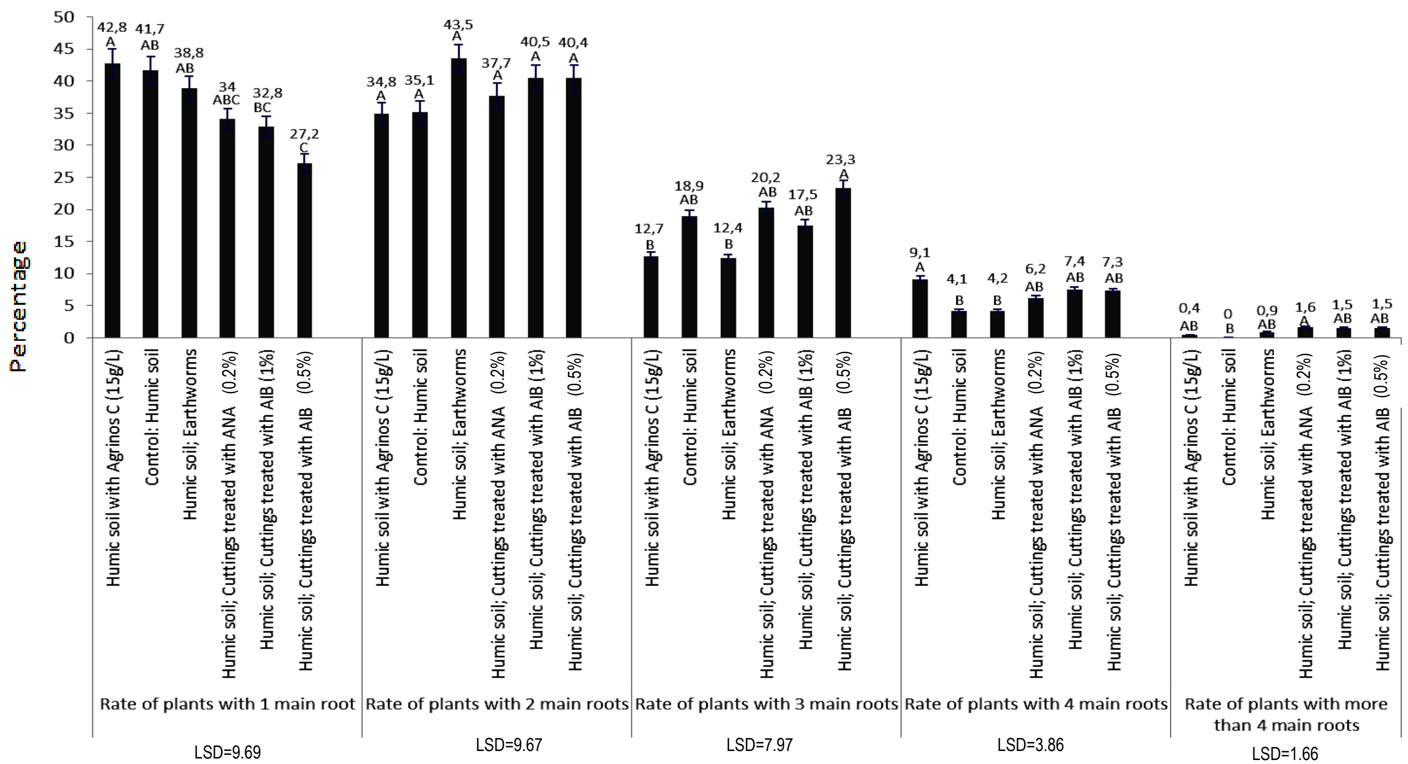

Figure 4. Effect of treatments on the number of roots×survival rate after 6 to 8 weeks stems growth after an acclimatization period of 40 days under tunnels.

Table 4. Relation between the number of taproots and the position of stem on the branch.

Type of stems section	With one root (%)	With two roots (%)	With three roots (%)	With four roots (%)	More than 4 roots (%)
Hardwood	29.67 ± 8.03	33.59 ± 7.42	25.58 ± 3.03	9.70 ± 4.22	1.44 ± 1.16
Semi-hardwood	35.71 ± 9.17	44.83 ± 7.64	13.86 ± 4.23	4.84 ± 2.6	0.74 ± 0.95
Softwood	43.6 ± 8.27	37.85 ± 7.62	13.03 ± 3.56	4.59 ± 2.46	0.91 ±1.94

tissues. Indeed, Ribeiro et al. (2008) showed that in certain cocoa genotypes, increasing levels of applied N improved growth (stem girth, dry weight of shoot and roots and shoot/root ratio), and concentration and uptake of N.

The results obtained on the relation between the position of stem and stems conversion are reported earlier. The position of stem on the branch used for producing the plant has an influence on its survival rate and showed that softwood section was easily rooted than other section.

However, the production of softwood stock plants requires a higher level of cultural management and propagation technique than those for bent semi-hardwood stock plants (Miller, 2009). Indeed, it is difficult to separate the three part of branch during cocoa trees mass propagation.

To correlate plantlets survival rate and corresponded treatments, roots and shoots dry weights parameters were determined. This showed that the root development is practically the same when earthworms were used and also chemical growth promoters too. The formation of adventitious roots is a high energy requiring process, which involves cell division, in which predetermined cells switch from their morphogenetic path to act as mother cells for the root primordia; hence, need more reserve food material for root initiation (Aeschabacher et al., 1994). No difference could be observed between IBA and NAA hormones on cocoa orthotropic cuttings rooting systems contrary to the assertion of Ghelot et al. (2014)

when these two promoters were applied to produce *Azadirachta indica* trees. However, the measurement of shoots dry weights rate showed that it is higher (62.3%) for substrates which contained earthworms than other ones. These results showed that earthworms could be involved in enriching substrates on nutrients and does not probably implicate in root systems development during the acclimatization of stems. The efficiency of earthworms tested in this study resides rather in the provision of useful nutrients for leaves development. This impact positively stems transformation into plantlets around 65.8% independent of the position of stems used. Indeed, the survival rate of stems is high when roots and shoots dry weights are high.

In this work, the presence of one or more taproots on plants obtained by orthotropic cuttings methods was observed. However, the probability of the number of taproots by type of treatment must be relativized in view of the high absolute value of the standard deviation. The presence of several main roots should be confirmed to the adult age of the tree because of these results were obtained after 6 or 8 weeks of plantlets growth.

Finally, the use of earthworms proved better compared to the tested auxins on specific concentrations. However, the direct effects of earthworms could be relativized. Indeed, Laossi et al. (2010) demonstrated that the influence of *Lumbricus terrestris* earthworm varied with the soil type and this earthworm species did not have any significant effects on *Trifolium dubium* if the soil is poor in nutrients.

Conclusion

These studies demonstrated the ability to double the survival rate of plantlets produced by orthotropic cuttings from 49 to 83%. This was achieved (1) by improving the quality of acclimatization substrate with the use of earthworms specifically *M. anomala* and (2) using preferentially a softwood part of branch to produce cocoa plantlets.

However, the advantage of the presence of earthworms on substrate media, could only be observed if the soil is rich in organic material to start with. This is not the case for poor soils present in certain part of Côte d'Ivoire's lands. The use of living organisms such as earthworms that have the ability to turn the inorganic matter into nutrients is useful to initiate rooting system for cuttings during the first 8 weeks of growth.

The understanding of earthworm mechanisms could be deepened by the fine analytical study of the soils (pH, conductivity, porosity and microflora) used as substrates to root cocoa stems.

Cocoa plants propagation in a large scale by orthotropic cuttings methods with the use of humic soil as substrate with content earthworms will be a successful pathway of increasing more the efficiency of this technique.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity in quarin clover (*T. quartinianum)* accessions of Ethiopia using ISSR markers

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Genetic diversity of *Trifolium quartinianum* accessions from Ethiopia were studied using Inter Simple Sequence Repeat (ISSR) markers. A total of 24 accessions, divided into three populations were used for the present study. DNA was extracted from a bulk of samples using a modified CTAB method. A total of 84 bands were amplified by the four di-nucleotid ISSR primers in the overall experimental materials. Genetic diversity was high at the species level (PPL = 100%, h = 0.29, I = 0.44). Comparison of population-based genetic diversity showed that Gojam population was the most diverse. Analysis of molecular variance (AMOVA) revealed high level of within-population variation with 83.13%. Unweighted pair group method with arithmetic average (UPGMA) and Principal Coordinate (PCO) analysis showed that only accessions of *T. quartinianum* from Gondar formed separate cluster. The study clearly indicates the presence of variable genotypes with their unique identity that deserve conservation attention.

Key words: Genetic diversity; Ethiopia; ISSR markers; *T. quartinianum.*

INTRODUCTION

Trifolium quartinianum A. Rich belongs to the genus *Trifolium* and section Vesicastrum (Ellison et al., 2006). It is a diploid with 2n = 16 (Badr, 1995) and self-pollinated (ILCA, 1990) species indigenous to east African highlands. It is suitable for hay and silage making to increase the quality of straw-based diets and to overcome seasonal feed shortage. Compared to other native Ethiopia clovers, it is the most productive with vigorous growth that can produce 7800 kg dry matter per ha within three months when growing conditions are favorable (Kahurananga and Asres Tsehay, 1991). It has higher seed production capacity compared to other *Trifolium* species and may adapt to a wide range of soils from heavy to clay vertisols and nitosols to loams and

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sandy loams (Akundabweni and Njuguna, 1996). It tolerates seasonal water logging. It has higher biomass production under different moisture condition (Friedericks et al., 1991). It nodulates well with most of the *Rhizobium* strains (Myton et al., 1988). This species has potential to improve natural or sown pastures in the tropical highlands of Africa (Lulseged et al., 1996). It combines well with other annual clovers and short-growing grasses. When grown in mixture with *T. quartinaianum,* grasses are known to accumulate more dry matter, crude protein and *in vitro* digestable dry matter than grass monoculture (Lulseged et al., 1997). This species can be intercropped with wheat without significant reduction of grain yield (Kahurananga, 1987). According to Zewdu (2004), *T. quartinianum* can be undersown with barley simultaneously or at first weeding without affecting the grain and straw yield of barley but significantly increasing the total fodder yield.

In recent years, a number of polymerase chain reaction (PCR)-based DNA markers has been developed to evaluate genetic variation at the intraspecific and interspecific levels (Wolfe and Liston, 1998). In *Trifolium*, primarily Random Amplified Polymorphic DNA (RAPDs) was used to evaluate genetic diversity in *Trifolium pratense* (Ulloa et al., 2003) and *Trifolium resupinatum* (Arzani and Samei, 2004). However, the Inter Simple Sequence Repeat (ISSR)-PCR method (Wolfe and Liston, 1998) using primers based on di, tri, tetra, penta nucleotide repeats without the requirement for prior knowledge of the genome sequence seems particularly suitable for germplasm comparison. For the *Trifolium* species studied, the ISSR markers were used for the first time to study genetic diversity of three South American and three Eurasiatic species (Rizza et al., 2007). ISSR markers were used to assess genetic diversity of four clover species from Europe (Dabkevičienė et al., 2011). These research findings suggest that ISSR markers systems are suitable for the analysis of genetic diversity of *Trifolium*.

Based on the above ground, ISSR markers were chosen for genetic diversity study of *T. quartinianum* collected from different geographical locations of Ethiopia. Despite the importance of this clover as livestock feed and contribution to soil fertility, so far, there were no published reports for its genetic diversity study using ISSR markers. Few genetic diversity studies based on morphological and agronomic traits were conducted. However, morphological variability often has limitations; since characters may not be observed at all stages of the plant development and traits may be affected by environment. Hence, the present study aimed to determine the genetic relationship and pattern of variation among three populations of *T. quartinianum* as well as intra population genetic diversity using inter simple sequence repeat (ISSR) markers. The study can give a base line information for efficient preservation, exploitation of the existing genetic resources and assist for germplasm management.

MATERIALS AND METHODS

Plant material

For this study, 24 accessions of *T. quartinianum* collected from three different administrative regions (AR) of Ethiopia were kindly provided by International Livestock Research Institute (ILRI) Forage Germplasm Bank, Addis Ababa, Ethiopia. Collections from each region were registered as an accession and, therefore, accessions collected from randomly selected districts of an AR were used to represent each population (Table 1). Seeds of each accession were sown in plastic pots and grown for three weeks in the greenhouse of the College of Natural Sciences, Addis Ababa University.

DNA extraction

Fresh young leaves of three individuals of an accession were bulked and ground to a fine powder by pestle and mortar in liquid nitrogen and thereafter transferred to 1.5 ml eppendorf tubes. Total genomic DNA was extracted from the fine powder following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol with slight modification established by Borsch et al. (2003). Finally, pellets were dissolved in 100 µl 1x TE solutions and kept in freezer at -20°C for subsequent use.

PCR amplification and gel electrophoresis

A total of 13 ISSR primers obtained from Genetics Laboratory, Addis Ababa university (originally bought from University of British Columbia) were screened based on previously published results in *Trifolium* and related species for their ability to generate consistently amplified band patterns (Rizza et al., 2007; Dabkevičienė et al., 2011; Arslan and Tamkoc, 2009) and to access polymorphism in two samples for each population. Four primers were selected and used for the analysis of 24 accessions based on the number of amplification products, the quality of the profiles, the level of polymorphism and the reproducibility of bands (Table 2). Each PCR of ISSR markers had a final reaction volume of 25 µl , containing 17 µl ddH_2O, 0.8 µl dNTPs (25 mM each), 2.5 µl of reaction buffer (10x taq polymerase buffer with 15 mM $MgCl_2$), 3 µl $MgCl_2$ (25 mM), 0.4 µl primer (20 pmol/µl) and 0.3 µl Taq Polymerase (1.5 unit) and 1 µl diluted template DNA. Amplifications were performed in Biometra 2003 T3 Thermo cycler programmed to run the following temperature profile: a preheating and initial denaturation for 4 min at 94°C, then 40 × 15 s denaturation at 94°C, 1 min primer annealing at 48°C, 1.30 min extension at 72°C and the final extension for 7 min at 72°C. For each primer a negative control reaction was included. Agarose gel of 1.67% concentration was prepared in 1X TBE buffer (0.86 g agarose in 50 ml 1X TBE and 2 µl ethidium bromide). PCR product (11 µl) and 1 µl 6X loading dye were loaded into the wells. The electrophoreses were done for about 3 h at constant voltage of 80 V. After electrophoresis, the gel was stained in ethidium bromide, distained with ddH_2O, visualized under UV light, photographed and documented.

Band scoring and data analysis

The ISSR band profiles were treated as dominant markers and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. Scoring was performed manually for each

Table 1. List of *T. quartinianum* accessions with their associated information used for the present study.

Acc. No	District	Zone/Adm. region	Latitude	Longitude	Altitude (m)	Lab. Code
9428	Debre Markos	Shewa	10˚08´ N	037 ˚58´ E	2430	Tq Sh49
9975	Chebo and Gurage	Shewa	08 ˚15´ N	037 ˚40´ E	1840	Tq Sh50
8321	Menagesha	Shewa	08˚48 ´N	038˚54´ E	1870	Tq Sh51
9968	Chebo and Gurage	Shewa	08 ˚24´ N	037 ˚52´ E	1870	Tq Sh52
6297	Menagesha	Shewa	09˚02´ N	038˚45 ´E	2380	Tq Sh53
14408		Shewa	-	-	-	Tq Sh54
8473	Chebo and Gurage	Shewa	08˚21´ N	037˚49´ E	1880	Tq Sh55
8464	Chebo and Gurage	Shewa	08˚17´ N	037 ˚47´ E	1880	Tq Sh56
13716		Shewa	10˚ 02´ N	038 ˚14 ´E	2010	Tq Sh57
7675	Debre Markos	Gojam	10˚14´ N	038˚06´ E	2400	Tq Goj58
2049	Debre Markos	Gojam	10˚12´ N	037˚52´E	2400	Tq Goj59
2047	Debre Markos	Gojam	10˚15´ N	037˚57´ E	2500	Tq Goj60
8521	Debre Markos	Gojam	11˚26´ N	037 ˚36´ E	2200	Tq Goj61
6277	Bahir Dar	Gojam	11˚40´ N	037˚28´ E	1900	Tq Goj62
9378	Debre Markos	Gojam	10 ˚07´ N	038˚ 09´ E	1980	Tq Goj63
8540	Debre Markos	Gojam	10˚13´ N	037˚52´ E	2450	Tq Goj64
7693	Debre Markos	Gojam	10˚14´ N	037˚52´ E	2360	Tq Goj65
8535	Debre Markos	Gojam	10 ˚42 ´N	037˚04´ E	2100	Tq Goj66
7771	Libo	Gondar	12˚03´ N	037 ˚44´ E	1840	Tq Gon67
14586		Gondar	-	-	-	Tq Gon68
13808		Gondar	-	-	1950	TqGon69
7759	Gondar	Gondar	12˚22´N	037˚17´ E	1860	TqGon70
7746	Debre Tabor	Gondar	11˚53´ N	037˚41´ E	1860	Tq Gon71
7768	Gonder	Gondar	12˚27´N	037˚31´ E	1940	Tq Gon72

(Source: ILRI). Acc. = Accession, E = East, N = North, m = meter, Adm. = Administrative, Lab. = Laboratory, TqSh = *T. quartinianum* from Shewa, TqGoj = *T. quartinianum* from Gojam, TqGon = *T. quartinianum* from Gondar.

Table 2. ISSR primers with motif, annealing temperature, amplification quality and motives screened for amplification of *T. quartinianum* accessions.

Code of primers	Primer motif	T°(℃)	Amplification quality	Motives
880	$(GGAGA)_3$	45	X	Penta-nucleotide
818	$(CA)_8G$	48	Polymorphic, Reproducible	Dinucleotide
834	$(AG)_8YT$	45	X	Dinucleotide
841	$(GA)_8YC$	48	Polymorphic, Reproducible	Dinucleotide
826	$(AC)_8C$	48	X	Dinucleotide
873	$(GACA)_4$	45	X *	Tetra- nucleotide
844	$(CT)_8RC$	48	Polymorphic, Reproducible	Dinucleotide
824	$(TC)_8G$	48	X *	Dinucleotide
816	$(CA)_8T$	48	X *	Dinucleotide
848	$(CA)_8RG$	48	Polymorphic, Reproducible	Dinucleotide
812	$(GA)_8A$	45	X	Dinucleotide
813	$(CT)_8T$	45	X *	Dinucleotide
810	$(AG)_8T$	45	X	Dinucleotide

X: Less reproducible and less polymorphic, X*: no amplification**,** T° **(**℃): annealing temperature. **Source:** Primer kit 900 (UBC 900); Y = Pyrimidines (C or T), R = purines (A or G).

primer based on presence (1) and absence (0) or as a missing observation (?) (Missing observation means those of bands which are ambiguous to say either the bands are present or absent), and each band was regarded as a locus. Only amplified bands that

Table 3. Level of genetic diversity revealed by the four ISSR primers.

Primer	NSB	NPL	PPL	h	I
818	22	22	100	0.29	0.44
844	18	18	100	0.31	0.48
841	20	20	100	0.29	0.45
848	24	24	100	0.27	0.41
Over all	84	84	100	0.29	0.44
Average	21	21			

NSB= Number of scorable band; NPL = Number of polymorphic Loci; PPL = Percent of polymorphic loci; h = gene diversity; I = Shannon's information index.

were clearly resolved were recorded, and a "0" and "1" data matrix was established. The resulting presence/absence data matrix of the ISSR phenotype was analysed using POPGENE version1.32 software (Yeh et al., 1999) to calculate the following genetic diversity parameters: percentage of polymorphic loci (PPL), gene diversity (h), and Shannon's information index (*I*). The genetic structure was investigated using Analysis of Molecular Variance (AMOVA). The AMOVA analysis was carried out using the software ARLEQUIN version 3.01 (Excoffier et al., 2006) to estimate genetic variability within and among populations without grouping. NTSYS-pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software's were used to calculate Jaccard's similarity coefficient which is calculated with the formula:-

$$S_{ij} = \frac{a}{a+b+c}$$

Where, 'a' is the total number of bands shared between individuals i and j, 'b' is the total number of bands present in individual i but not in individual j and 'c' is the total number of bands present in individual j but not in individual i.

The unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) was used in order to determine the genetic relationship among accessions and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The neighbor joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual accessions and evaluate patterns of accession clustering using Free Tree 0.9.1.50 Software (Pavlicek et al., 1999).

To further examine the patterns of variation among individual samples, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer et al., 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer et al., 2001; Statistica Soft, Inc., 2001).

RESULTS AND DISCUSSION

ISSR primers and banding patterns

An initial screening of 13 ISSR primers enabled selection for four primers, which produced satisfactory amplification profiles in *T. quartinianum* accessions. All the selected four primers have dinucleotide repeat motifs. In total, 84 very clear identifiable ISSR fragments and informative patterns were amplified using the four ISSR primers. The number of bands amplified by each primer ranged from 18 to 24 with an average of 21 bands per primer. Primer 848 produced the highest number of bands, while the lowest number of bands was amplified by primer 844 (Table 3). The size of the fragments amplified with these primers was in the range of 200 to 1000 bp. Representative gel illustrating the amplification profiles produced by the ISSR marker assay by employing primers 844 is shown in Figure 1.

Genetic diversity

Inter-simple sequence repeat (ISSR) markers have become widely used in population studies because they have been found to be highly variable, to require less investment in time, money and labor than other methods (Wolfe and Liston, 1998), and to have the ability to be inherited (Gupta et al., 1994; Tsumura et al., 1996). Several researchers used ISSR markers to study genetic diversity within and among populations of forage crops (Bolourchian et al., 2013; Jonaviclene et al., 2009; Zarrabian et al., 2013; Shirvani et al., 2013). Better reproducibility of products of ISSR bands compared to other markers such as RAPD could be due to its longer SSR-based primers with higher annealing temperatures (Huangfu et al., 2009). Moreover, as microsatellites are frequent and widely distributed throughout the genome, the ISSR targets are abundant. Compared with SSR markers, where the flanking regions of the SSR motifs have to be known in advance, ISSR amplification takes advantage of the fact that no prior sequence information is required, and the results are therefore obtained more rapidly and cost effectively (Wang et al., 2008; Yang et al., 1996; Bornet and Branchard, 2001). The present study employed ISSR markers to assess genetic diversity within and among populations of *T. quartinianum* accessions from Ethiopia. A total of 84 bands were amplified, all of which showed 100% polymorphism. All of the four primers used also showed 100% polymorphism. Over all gene diversity (h) and Shannon's information index (I) by the four ISSR primers were 0.29 and 0.44, respectively. The higher values for gene diversity (h) and

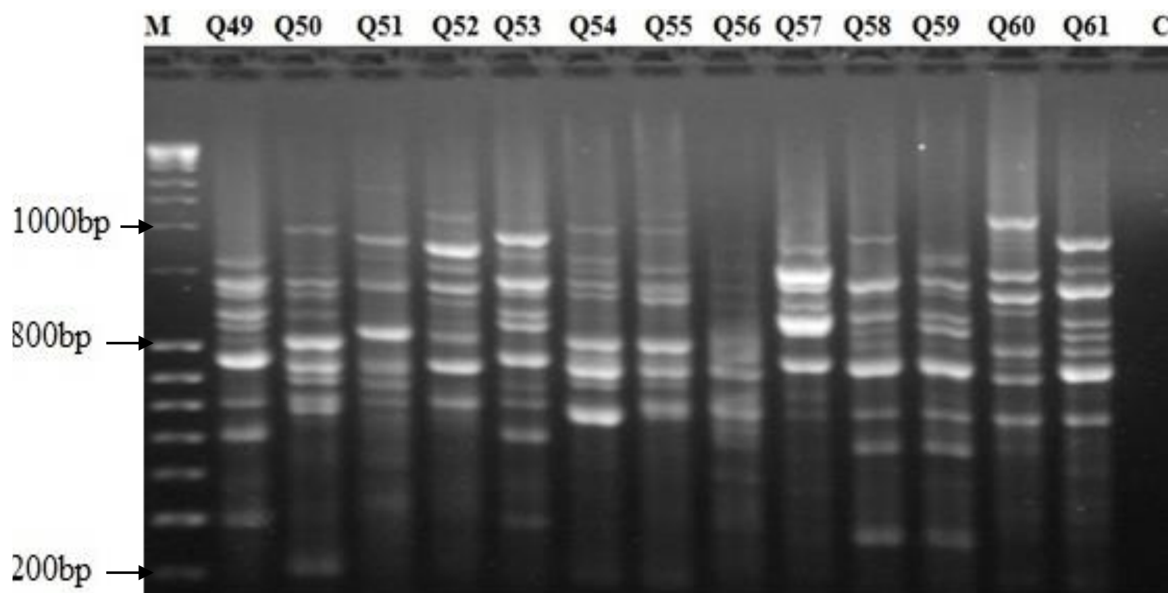

Figure 1. Banding pattern of primer 844 in *T. quartinianum* accessions. M represents a 1000 bp DNA ladder; Q stand for *T. quartinianum*, while the numbers associated with these letters represent accessions, C represents control.

Table 4. Genetic diversity within populations of *T. quartinianum*.

Population	NPL	PPL	h	I
Shewa	64	76.19	0.25	0.38
Gojam	69	82.14	0.28	0.41
Gonder	48	57.14	0.18	0.27
Average	60.3	71.8	0.24	0.35
Over all	84	100	0.29	0.44

NPL = Number of polymorphic loci; PPL = Percent of polymorphic loci; h = Gene diversity; I = Shannon's information index.

Shannon's information index (I) were in primer 844, while 848 showed the lowest indexes (Table 3).

In this study, the di-nucleotide ISSR primers 818 and 848 with CA repeat, and 844 and 841 with CT and GA motives, respectively, detected genetic diversity within and among populations. Generally primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism in plants than primers with other di-, tri- or tetra nucleotides (Reddy et al., 2002). Moreover, the choice of appropriate primer motives in ISSR fingerprint is critical to detect high polymorphism and reveal relationship within and among populations. The four ISSR primers chosen for this study amplified large number of bands, varying from 18 to 24 per primer displaying 100% polymorphism with high mean gene diversity (h = 0.29) and Shannon's information index (I = 0.44). This indicates the existence of high level of genetic diversity among the three populations of *T. quartinianum* in Ethiopia. High genetic diversity found among the population could be due to lack of selection and strict domestication under low overgrazing. Moreover, Founder effect may affect the level of genetic diversity among population.

The ISSR survey of three populations of *T. quartinianum* revealed a high level of genetic variation at the species level (PPL = 100%; h = 0.29; I = 0.44). The least polymorphic and genetically unique population was Gonder (PPL = 57.14%; h = 0.18; I = 0.27), while Gojam was the most polymorphic and diverse (PPL = 82.14%; h = 0.28; I = 0.41) (Table 4). Previous assessments of genetic diversity in 34 *T. quartinianum* accessions of Ethiopia based on eight morphologic and agronomic traits have reported that most of the accessions showed similarity in morphological characteristics (Basweti and Hanson, 2012). This finding contradicted with the present study, mainly because morphological features have a number of limitations including low polymorphism, low

Table 5. Analysis of molecular variance (AMOVA).

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	*P*-Value*
Among populations	2	69.66	2.59	16.87	P< 0.001
Within populations	23	281.49	12.80	83.13	P< 0.001
Total	25	351.16	15.40		

d.f = degree of freedom, * significance tests after 1023 permutations.

heritability, late expression, and vulnerability to environmental influences (Smith and Smith, 1992; Konarev, 2000; Muthusamy et al., 2008), which, in turn limits their utility for assessing real genetic diversity. Up to now, there are no published reports concerning ISSR method for analyzing the genetic diversity of *T. quartinianum*. However, ISSR marker was used to assess the level and pattern of genetic diversity in four *Trifolium* species represented by two varieties, one breeding sample and two wild population of *T. pratense,* four wild populations of *T. medium,* two varieties and one population of *T. resupinatum*, and two varieties and three wild populations of *T. repense*. The study showed 69.5% polymorphism in *Trifolium medium,* 68.9% in *T. resupinatum,* 76.2% in *T. pretense* and 73.6% in *Trifolium repense* (Dabkevičienė et al., 2011). Recently, ISSR marker also used for the genetic diversity study of 14 accessions of three species of *Trifolium* and 60% polymorphism in *Trifolium fragiferum*, 58.67% in *Trifolium hybridum* and 77.32% in *T. pratense* was found (Aryanegad et al., 2013). The genetic diversity investigated in the present study was higher than the ones reported by Dabkevičienė et al. (2011) and Aryanegad et al. (2013). Generally, the higher level of genetic variation found in this study may be due to the fact that geographically isolated populations in certain geographic locations could accumulate genetic differences and evolve unique genotypes as they adapt to different environment (Souframanien and Gopalakrishn, 2004).

In general, high level of genetic diversity is not expected with strictly limited distribution and a small population size. Nevertheless, ISSR markers used in this study generated higher level of polymorphism in 24 accessions of *T. quartinianum* from limited geographical location in North West Ethiopia. This shows that small populations or individuals are not always associated with a lack or low level of genetic variation (Yingjuan and Ting, 2009).

Analysis of molecular variance (AMOVA)

The AMOVA without grouping indicated that most of the total genetic variation in *T. quartinianum* populations exists within populations (83.13%), while among population variation (16.87%) was observed to be low (Table 5). High genetic variation within populations indicated that high genetic dissimilarities among the individual plants sampled from a single population. The vast majority of diversity studies across members of the Trifolieae have shown a generally high level of diversity within populations, even among other inbreeding species such as *T. subterraneum* (Pecetti and Piano, 2002; Piluzza et al., 2005). The AMOVA results obtained in the present study do not contradict with the above findings. It is a prevalent view that self-pollinated species maintain higher genetic variation among populations than within populations. Though *T. quartinianum* is self-pollinated plant, higher within populations genetic variation than among populations was obtained in the present study, contrary to this prevalent view. High genetic exchange or gene flows, which actually have a more homogenizing effect on the genetic variation among populations by the dispersal of the seeds, can likely explain higher within population genetic variation. Some seeds may be harvested as weeds together with those of crops and distributed with the crop seeds via market channels. Moreover, the pods and mature calyx of *T. quartinianum* tend to have a coarse surface and points which may attach to passing animals and be transported to other places.

Genetic relationship within and among populations

The UPGMA dendrogram of *T. quartinianum* accessions showed that most accessions of the same population formed a unique cluster, while others were distributed over the tree (Figure 2). Moreover, all accessions from Gonder population grouped in one major cluster and the genetic similarity values among accessions were relatively high. All of the accessions from each population of Gojam and Shewa were not grouped in a single major cluster but formed different clusters involving most of the accessions from the same populations and the similarity value among the accessions was relatively low. The two dimensional plot (PCO) showed that accessions collected from Gonder grouped together and appear separate from other populations, while accessions of Gojam and Shewa were not clustered based on populations of origin, rather intermixed in one group (Figure 3). Accessions of Gojam and Shewa in one group present an important genetic similarity despite of their different geographical origins.

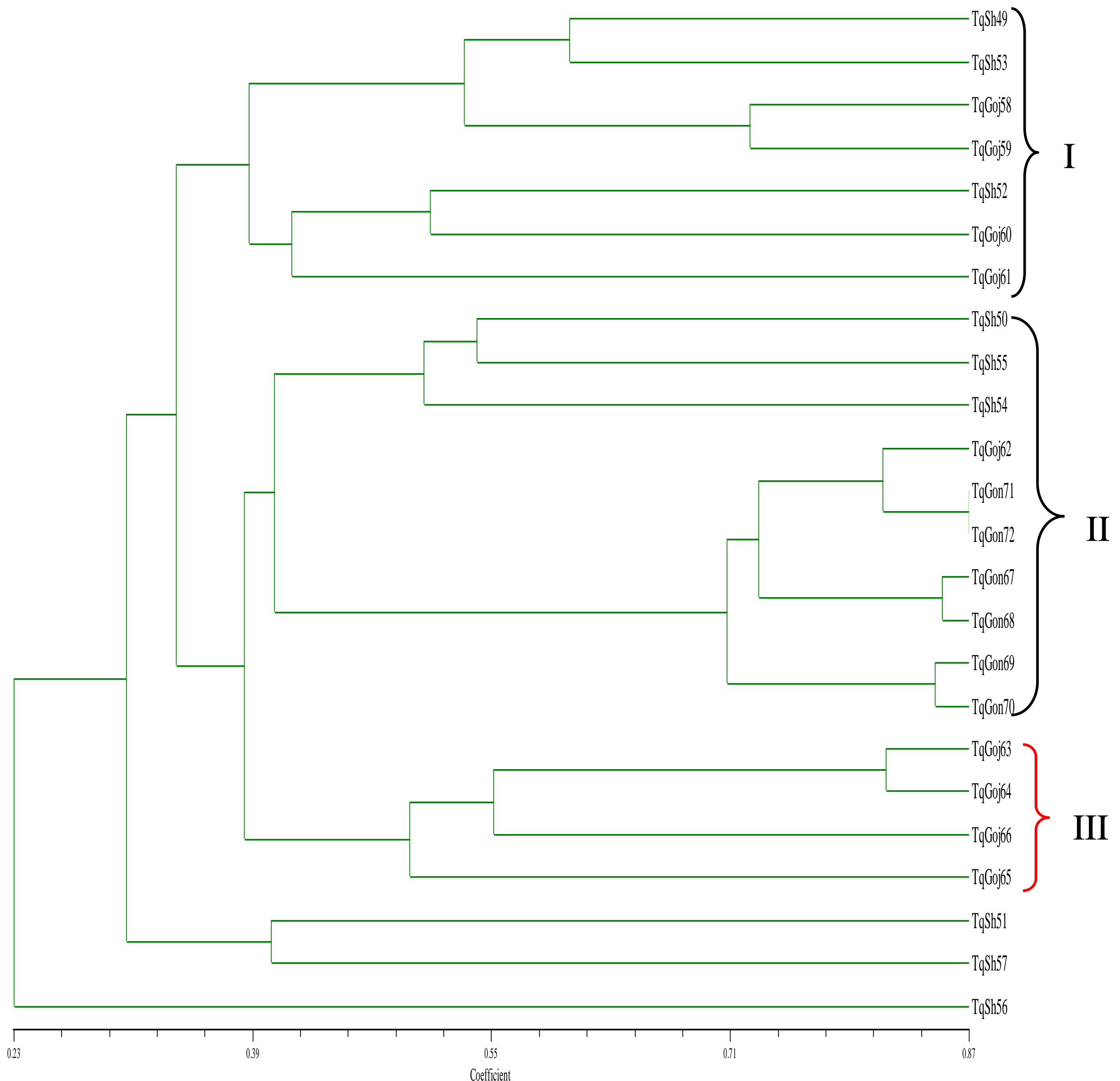

Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient among 24 *T. quartinianum* accessions using data generated with four ISSR primers.

This was further confirmed by the three dimensional plot and the pattern could be explained by long distance seed flow facilitated by human for forage cultivation for livestock feed production (Figure 4).

Conclusion

The present study is the first report on inter and intra population genetic diversity and relationships of *T. quartinianum* accessions of Ethiopia using ISSR markers. The genetic diversity data generated by four ISSR primers revealed that high genetic diversity exists at the species level.

The assessed genetic diversity level varied among populations, which could be due to different environmental conditions in which they are growing and naturally distributed. Gojam population showed relatively

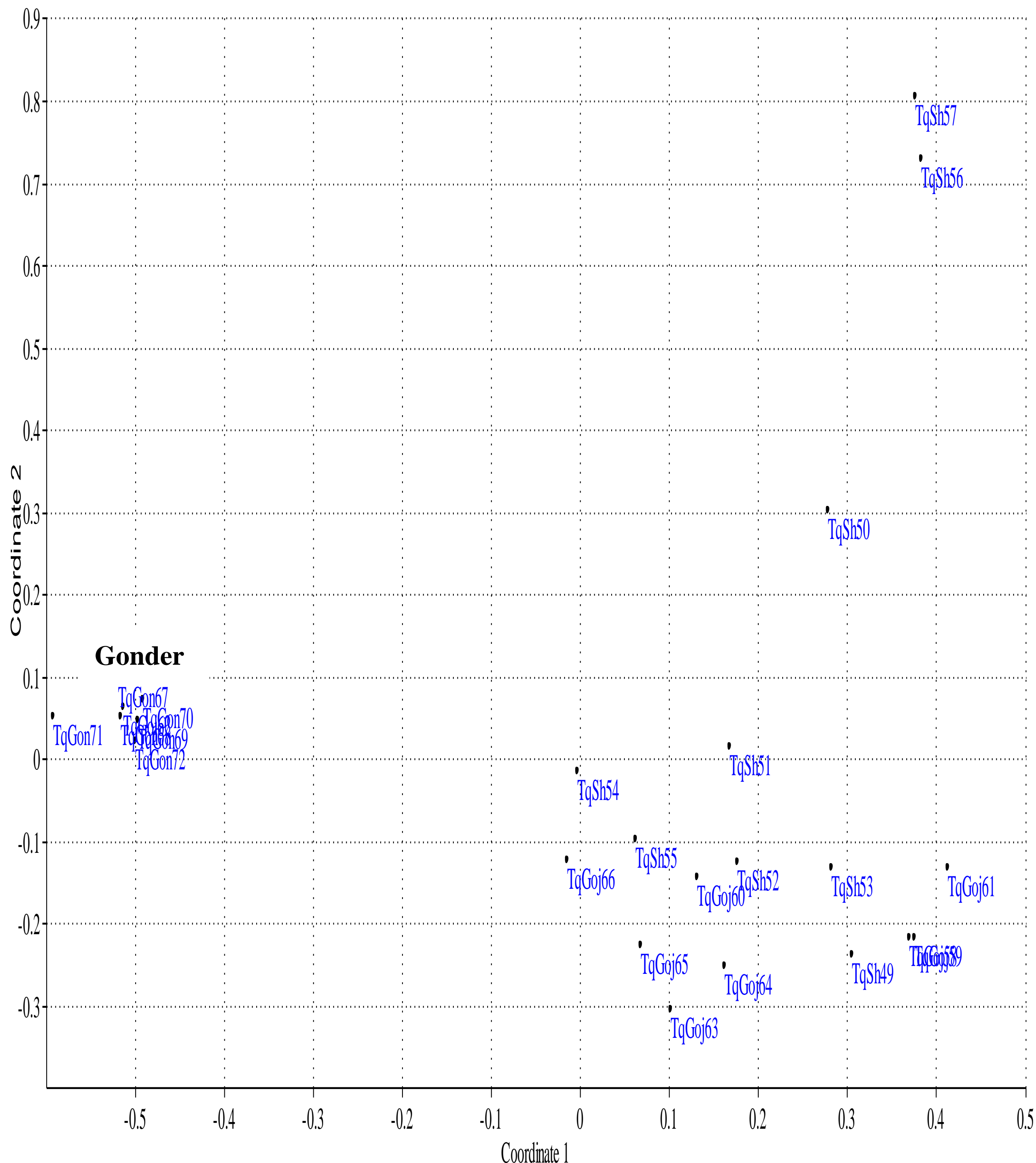

Figure 3. Two-dimensional plot obtained from principal coordinate analysis of 24 *T. quartinianum* accessions using 84 ISSR markers with Jaccard's coefficient similarity.

higher genetic diversity than Shewa and Gonder. UPGMA and PCO showed that only accessions of *T. quartinianum* from Gondar formed separate cluster. The findings of this study indicate that ISSR markers could be good tools to assess the genetic diversity and relationship at inter and intra population level of *T. quartinianum* accessions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

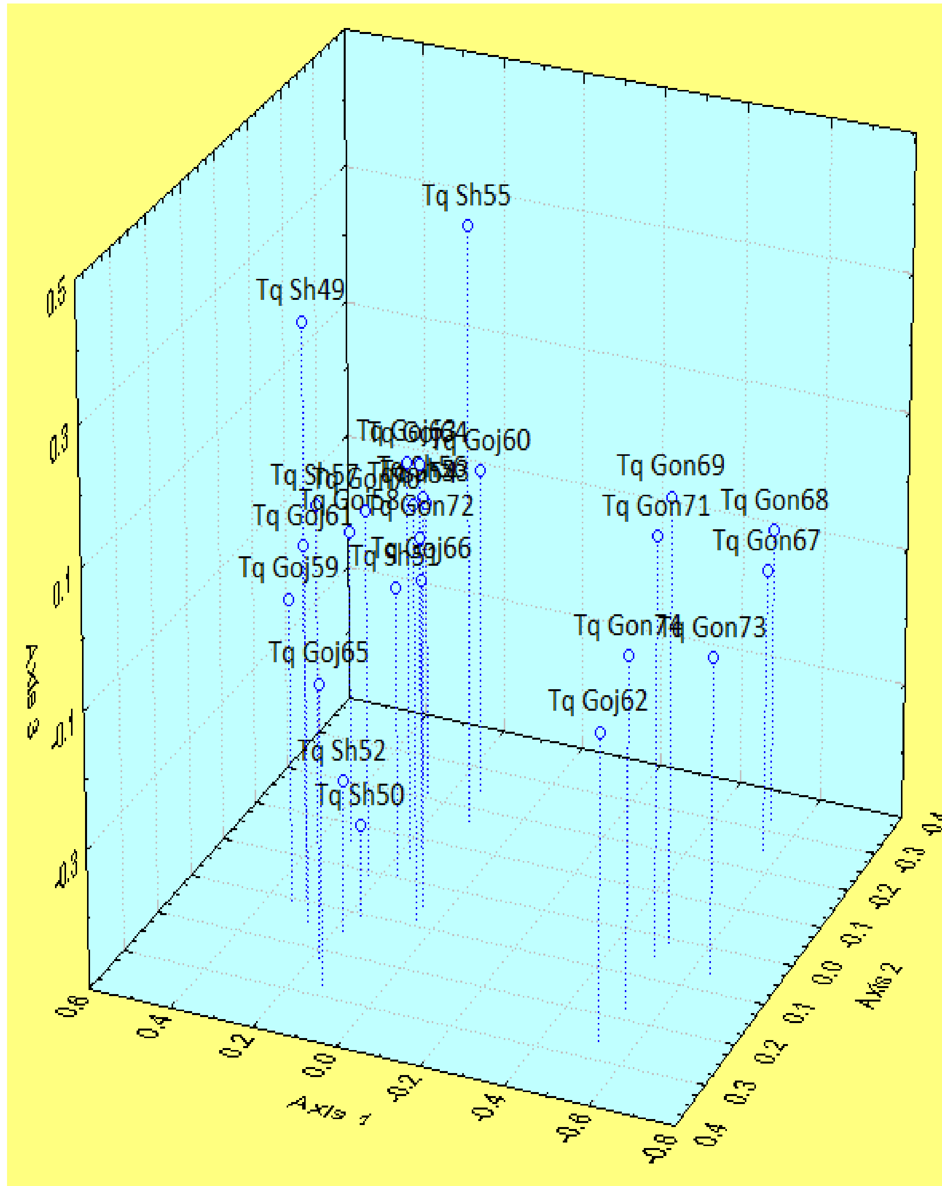

Figure 4. Three-dimensional plot obtained from principal coordinate analysis of 24 *T. quartinianum* accessions using 84 ISSR markers with Jaccard's coefficient similarity.

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Full Length Research Paper

Efficiency of sequence related amplified polymorphism (SRAP) and target region amplified polymorphism (TRAP) markers in detecting banana somaclonal variants

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Screening of micropropagated banana (*Musa* spp.) cv. Grand Naine for somaclonal variants was carried out in the open field. The total detected variants were 26 somaclones (in addition to the normal plant) and were grouped into eight groups according to their morphological and yield characteristics. Sequence Related Amplified Polymorphism (SRAP) and Target Region Amplified Polymorphism (TRAP) techniques were used to study the differences among banana cv. Grand Naine and 26 somaclonal variants of the cultivar. SRAP markers amplified 1463 fragments while 841 fragments were resulted from TRAP markers. The somaclones "double bunching from peduncle", "Giant plant" and "weak plant" somaclones were clustered with Grand Naine according to SRAP markers while "empty peduncle", "horizontal bunch" and "angled bunch" somaclones clustered with Grand Naine using TRAP markers. According to principal coordinate analysis with SRAP markers, "pale green", "black", "wavy margins", "double bunch from stem" and "vertical upward bunch" segregated from other variants; whereas, "pale green", "black" and "vertical upward bunch" segregated from other variants using TRAP marker data. Although these markers were able to distinguish some of the somaclones derived from micropropagation of Grand Naine, additional markers would be needed to identify mutations generation during tissue culture propagation of banana.

Key words: Banana, sequence related amplified polymorphism, target region amplified polymorphism, cluster analysis, principal coordinate analysis, genetic diversity.

INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops, which is grown in 128 countries especially in the developing countries of the tropical and subtropical regions. Approximately 5 million hectares are under banana cultivation with 106 Million metric tons produced worldwide (FAO, 2016). Plants propagated by *in vitro*

tissue culture techniques are known to exhibit a wide array of genetic and epigenetic variation (James et al., 2007). Variation may interfere with the use of these cultures for physical or chemical mutagenesis and/or genetic transformation. Although the causes of genetic instability are poorly understood, chromosome instability is believed to be one of the most common causes of tissue culture-induced variation (Roux et al., 2004).

Detection and analysis of genetic variation can help us to understand the molecular basis of different biological phenomenon in plants (Abdellatif and Soliman 2013) and gene expression (Eldemery et al, 2016). Sequence-Related Amplified Polymorphism (SRAP) markers have been adapted for a variety of purposes; including map construction, gene tagging and genetic diversity studies (Gulsen et al., 2006). The SRAP marker technology combines simplicity, reliability, and moderate throughput with the ability to sequence selected bands (Li and Quiros, 2001).

Target Region Amplification Polymorphism (TRAP) technique (Hu and Vick, 2003) is a rapid and efficient PCR-based methodology, which utilizes bioinformatics tools and expressed sequence tag (EST) database information to generate polymorphic markers for targeted candidate gene sequences. Thus, the TRAP technique is useful for generating markers associated with desirable agronomic traits for marker-assisted breeding. The technique has been effectively used for fingerprinting in plant species (Hu et al., 2005; Alwala et al., 2006; Liu et al., 2005). Garcia et al. (2011) used TRAP markers in 14 diploid *Musa* genotypes and produced 119 alleles. They reported that TRAP markers have been shown to discriminate A or B genome *Musa* genotypes.

The objective of this study was to genetically characterize the banana cultivar Grand Naine and 26 somaclonal variants derived from *in vitro* tissue culture of Grand Naine using SRAP and TRAP molecular markers.

MATERIALS AND METHODS

Plant material and DNA extraction

Healthy and uniform offshoots of banana cultivar 'Grand Naine' and 26 somaclonal variants of Grand Naine were selected from a farm sited in Ahmed Oraby Village, Badr City, El-Beheira Governorate, Egypt in March 2016 (Table 1). Somaclones were developed according to Hegazy et al. (2010). The somaclonal variants were categorized into eight groups based on phenotypic characteristics (Table 1 and Figure 1). Fresh cigar leaves from the 26 genotypes and Grand Naine were frozen in liquid nitrogen and transferred to the Plant Molecular Biology Laboratory, Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt where they were stored at -80°C. Total genomic DNA was isolated from 2 g of leaf tissue using the standard CTAB method as described by Saghai-Maroof et al. (1984). DNA concentrations were adjusted to 25 ng/µl and stored at -20°C.

Sequence related amplified polymorphism (SRAP)

SRAP analysis was performed using nine primer pair combinations (Table 2) specific to banana as reported by Li and Quiros (2001). PCR reactions were performed in 15 µl reaction volumes containing 7.5 µl master mix (iNtRON Biotechnology, Co.), 250 µM of each primer, and 100 ng of template DNA. PCR fragment amplification was performed for 35 cycles at 95°C for 1 min, 45°C for 1 min and 72°C for 1 min with an initial denaturation step at 95°C for 7 min and a final extension step at 72°C for 5 min.

Target region amplified polymorphism (TRAP)

Eight TRAP primer pairs combinations (Table 2) were used as described by Hu and Vick (2003). PCR amplification reactions were carried out in 15 µl reaction volumes consisting of 7.5 µl master mix (iNtRON Biotechnol. Co.), 250 µM of each primer and 100 ng of template DNA. Amplifications were conducted using a touchdown PCR program for seven cycles with annealing temperature starting from 51°C and decreasing one degree each cycle until 45°C, then a program of 30 cycles was carried out at 95°C for 1 min, 45°C for 1 min and 72°C for 1 min. An initial denaturation at 95°C for 7 min and a final extension at 72°C for 5 min were performed before holding at 10°C.

Data analysis

PCR products of both SRAP and TRAP markers were separated on 1.5% ethidium bromide-stained agarose gels using 1X TBE buffer running buffer and electrophoresed at 100 volts for 1 h. A 50 bp DNA ladder was used as indicator for DNA fragments size. Agarose gels were photographed on gel documentation system. Gels were scored as 0/1 for absence/presence of DNA fragments, respectively. The total number of band, the number of polymorphic bands and the percentage of polymorphism were calculated. The polymorphic information content (PIC) was calculated according to Anderson et al. (1993) using the following formula: $PIC_i = 1 - \Sigma p_{ij}^2$, where p_{ij} is the frequency of the *j*th allele for marker *i* summed across all alleles for the locus. Data were analyzed using cluster analysis. Similarity matrices were calculated using Jaccard coefficient (Jaccard, 1908). Dendrograms were constructed based on the UPGMA clustering method using NTSYSpc software version 2.0 (Applied Biostatistics, Setauket, New York, USA) (Rohlf, 1998). Principal coordinate analysis was carried out for the standardized decentered SRAP and TRAP data. Eigen values and Eigen vectors were calculated for the transformed interval data and the three dimensional principal coordinate analysis plot was constructed using the NTSYSpc software.

RESULTS AND DISCUSSION

SRAP and TRAP pattern

The pattern of the amplified fragments of the PCR

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Table 1. Categories of the Grand Naine banana cultivar and its detected somaclones.

S/N	Category	S/N	Sample	Remarks
1	Control	1	Normal	
2	Plant stature	1	Extra Dwarf plant	Extreme dwarfism in the whole plant
		2	Dwarf plant	Moderate dwarfism in the whole plant
		3	Giant plant	Extreme height in plant stature
		4	Weak plant	General weakness in both morphological and reproductive traits
3	Pseudostem color	1	Pale green	Pseudostem color abnormally pale green
		2	Reddish	Pseudostem color abnormally red
		3	Black pseudostem	Pseudostem color abnormally blackened
4	Peduncle length	1	Short peduncle	Peduncle is shorter than the normal one
		2	Long peduncle	Peduncle is longer than the normal one
		3	Very Long ped.	Peduncle is extremely longer than the normal one
5	Leaf orientation	1	Droopy leaf	The leaf directed downward
		2	Erected leaf	The leaf directed upward
6	Leaf shape	1	Massada	Leathery texture leaf
		2	Deformed leaf	Abnormal leaf formation and shape
		3	Narrow lamina	Lamina width shorter than the normal one
		4	Wavy margins	Unstraight leaf edges
7	Leaf color	1	Variegated leaf	Green leaves with white or yellow stripes
		2	Reddish	The red color in the leaf more than normal
		3	Bright spot	Fair spots located at the leaf upper surface
8	Bunching	1	Bunching failure	Plant fail to give bunch
		2	Double bunch from stem	The single corm gives two pseudostems
		3	Double bunch from ped.	The single peduncle divided into two parts subsequently give tow bunches
		4	Empty ped.	An empty bunch stalk without any developed fingers
9	Bunch orientation	1	Vertical upward bun.	The bunch directed upward
		2	Horizontal bun.	The bunch horizontally oriented

products of SRAP markers ranged in length from 100 to 1200 bp. All primer pair combinations produced common bands as well as polymorphic bands. In addition some specific fragments were amplified in specific somaclones. The most important fragment which was amplified at molecular weight 1000 bp in length by the primer pair "Me1+Em6" only in the pattern of the somaclone "Vertical upward bunch" could be specific for that variant. The same thing was noticed also in the pattern of the primer pair combination "Me1+Em6" for the somaclone "Short peduncle", in which a specific fragment was amplified at molecular weight 600 bp (Figure 2).

The length of the amplified fragments by the TRAP primer pairs ranged from 150 to 1000 bp and all of the primer pair combinations showed polymorphic amplified fragments. As in the SRAP pattern, some specific bands were obtained in specific somaclones. Examples of these amplified fragments are illustrated by white arrows on Figure 3. The most obvious fragments are those amplified by the primer pair combination "MUSA1+T4" at 600 bp in the pattern of "Erected leaf" and primer pair combination "MUSA2+T3" at 400 bp in the pattern of both "short peduncle" and "long peduncle" variants (Figure 3).

The above mentioned results suggest that tissue culture propagation of banana plants produce genetic variation. These variations may be genetic or epigenetic variation. Krishna et al. (2016) reported that plant tissue culture may generate genetic variability, that is,

Figure 1. Twenty-six offtypes produced from micropropagation banana cultivar Grand Naine. Giant picture is taken from Hegazy et al 2010.

somaclonal variations as a result of gene mutation or changes in epigenetic marks. Garcia et al. (2011) used TRAP markers to study genetic diversity in banana plants and they proved their efficiency in this regard. Same notes were reported by Abdellatif et al. (2012) and Krishna et al. (2015).

Table 2. SRAP and TRAP primers and their sequences used in molecular analysis.

S/N	Forward	Reverse	Amplified fragments			
			Total	Polymorphic	%Polymorphism	PIC
SRAP markers						
1	Me01-F20	Em01-R20	10	9	90	0.90
2	Me01-F20	Em03-F20	8	6	75	0.83
3	Me01-F20	Em06-R20	9	7	78	0.86
4	Me02-R20	Em01-R20	8	6	75	0.87
5	Me02-R20	Em03-F20	7	7	100	0.86
6	Me02-R20	Em06-R20	9	5	56	0.88
7	Me03-F20	Em01-R20	7	6	86	0.85
8	Me03-F20	Em03-F20	8	7	88	0.85
9	Me03-F20	Em06-R20	9	8	89	0.87
TRAP markers						
1	Musa.1-F20	T03-F20	8	8	100	0.83
2	Musa.1-F20	T04-R20	9	7	78	0.88
3	Musa.1-F20	T13-F20	3	3	100	0.65
4	Musa.1-F20	T14-R20	5	5	100	0.80
5	Musa.2-R20	T03-F20	6	3	50	0.81
6	Musa.2-R20	T04-R20	4	3	75	0.73
7	Musa.2-R20	T13-F20	5	4	80	0.79
8	Musa.2-R20	T14-R20	6	6	100	0.80

SRAP and TRAP polymorphism

For both SRAP and TRAP markers, 17 primer pairs amplified different numbers of bands through the genotypes. The primer pair “Musa1+ T13” detected only three scorable bands, all of them were polymorphic; while the primer pair “Me1+ Em1” showed amplification of ten bands with polymorphism percentage of 90%. The polymorphic information content (PIC) of the primer pairs ranged between 0.65 for the TRAP primer pair “Musa1+T13” and 0.95 for the SRAP primer pair “Me1+Em1”. Thus, it can be said that both marker types generated polymorphisms and informative amplified fragments. In general, the PIC for the SRAP primers was higher than the PIC for the TRAP markers (Table 2).

Cluster analysis

SRAP data cluster analysis generated seven clusters (Figure 4). Grand Naine clustered with “double bunch from peduncle”; whereas, the other somaclones formed the other clusters. It was observed that “horizontal bunch” and “angled bunch” which belonged to the bunch orientation category were clustered together at high rate of similarity. Also, “droopy leaf” and “erected leaf” which belonged to the leaf orientation category were clustered together at high rate of similarity. The three leaf shape genotypes “narrow leaf”, “deformed leaf” and “massada” also clustered together in one cluster (Figure 4).

TRAP data cluster analysis generated six clusters (Figure 5). “Grand Naine”, “empty peduncle”, “horizontal and angled bunch” somaclonal variants formed one cluster. It can be noticed that somaclones “droopy” and “erected leaf” showed the same trend of similarity according to SRAP and TRAP analyses. Most of the somaclonal variants clustered according to their phenotypic categories. Our results are in agreement with those of Garcia et al. (2011).

Principal coordinate analysis

From the SRAP marker data, the first three principle coordinates (PCs) accounted for 33.9% of the total variation (for example 12.5% for PC1, 11.7% for PC2 and 9.6% for PC3). The distribution somaclones is presented in Figure 6 with “Wavy margins”, “Vertical upward bunch” and “Black pseudostem” somaclones separated from the other somaclones based on PC1. “Double bunch from stem” somaclone was segregated apart from the other somaclones based on the PC2. For the PC3 coordinate, “Black pseudostem”, “Vertical upward bunch”, “pale green” and “bright spot” somaclones were separated from

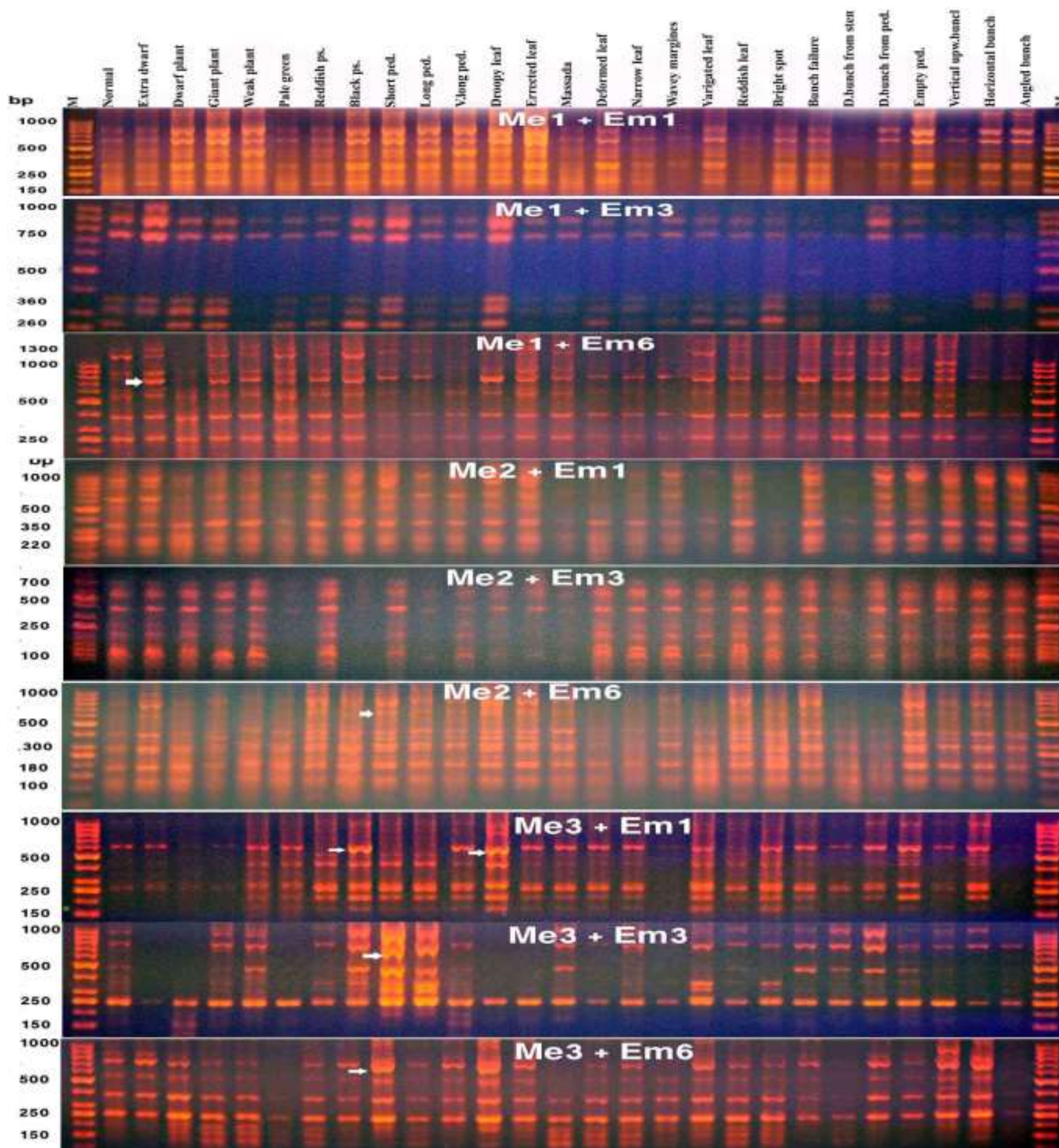

Figure 2. SRAP pattern of Grand Naine banana cultivar and its 26 somaclones. The white arrows show polymorphic bands from parent.

the other types of somaclones. “Normal plant”, “angled bunch”, “dwarf plant” and “extra dwarf” somaclones were aggregated together according to the three coordinates. These results were consistent with the data reported Abdellatif et al. (2012).

The first three principle coordinates (PCs) for the TRAP

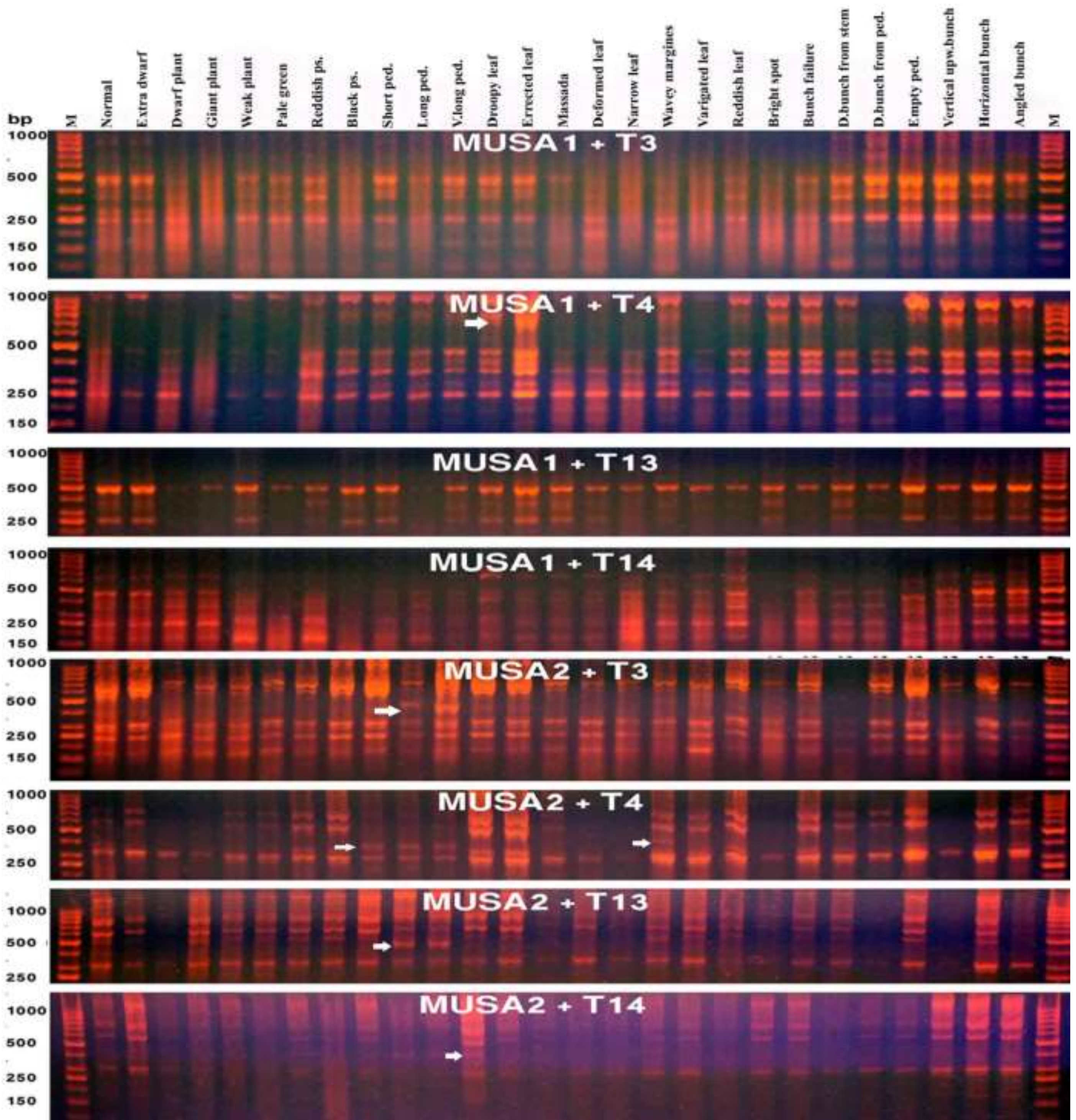

Figure 3. TRAP pattern of Grand Naine banana cultivar and its 26 somaclones. The white arrows show polymorphic bands from parent.

marker data accounted for 41.8% of the total variation (for example 16.9, 15.8 and 9.1% for PC1, PC2 and PC3, respectively). The somaclones were aggregated near from the middle of the PC1 into two groups (Figure 7). The first group included the somaclones “dwarf”, “giant”, “weak”, “reddish pseud.”, “droopy leaf”, “reddish leaf”, and “double bunch from peduncle” while the second group contained the somaclones “extra dwarf”, “short

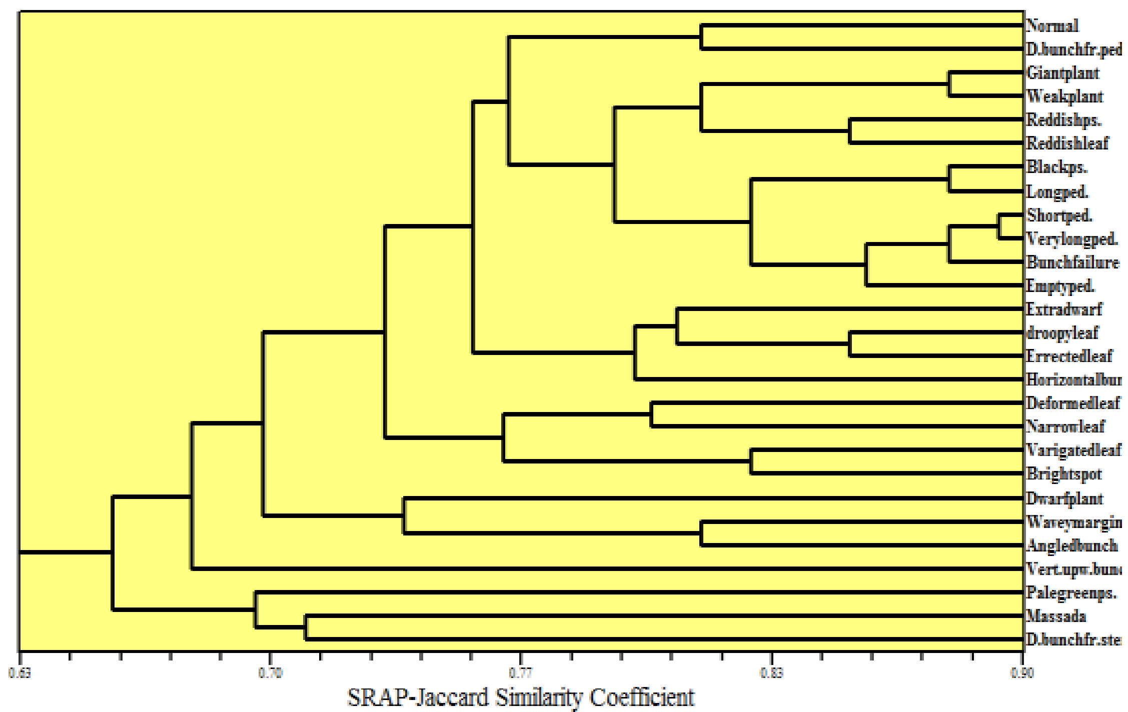

Figure 4. Cluster analysis of banana cv. Grand Naine and its 26 somaclones generated from SRAP data using Jaccard similarity coefficient and UPGMA clustering method.

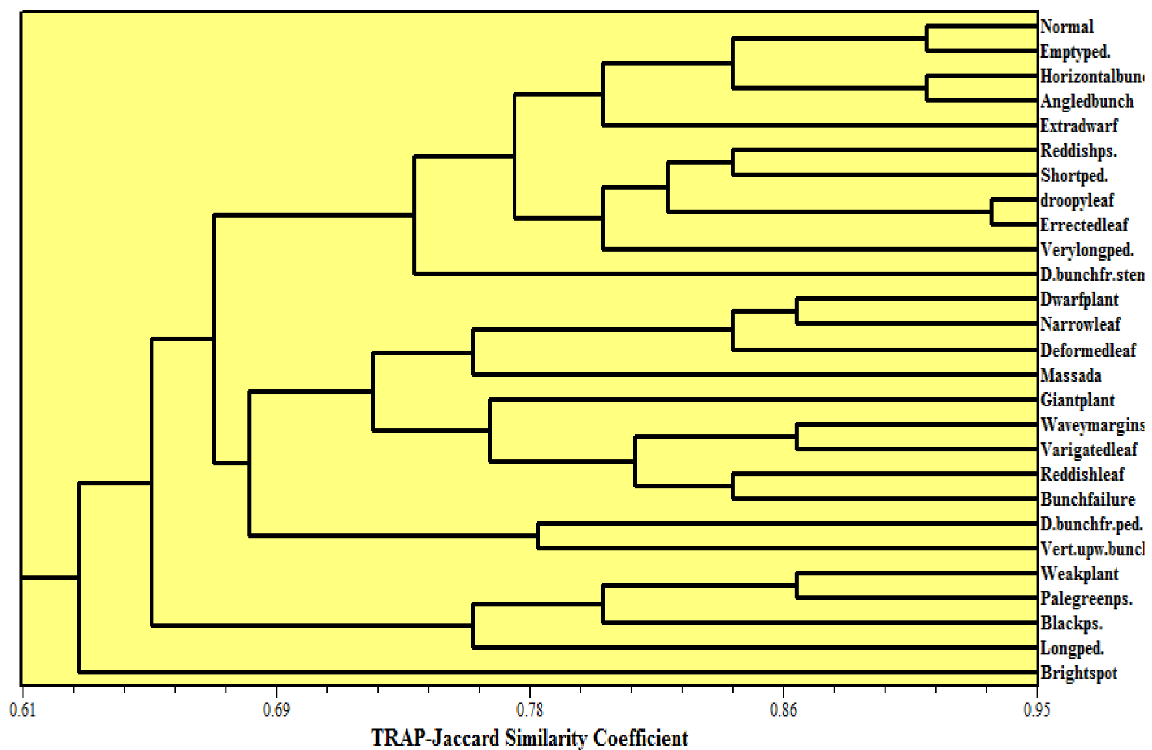

Figure 5. Cluster analysis of banana cv. Grand Naine and its 26 somaclones generated from TRAP data using Jaccard similarity coefficient and UPGMA clustering method.

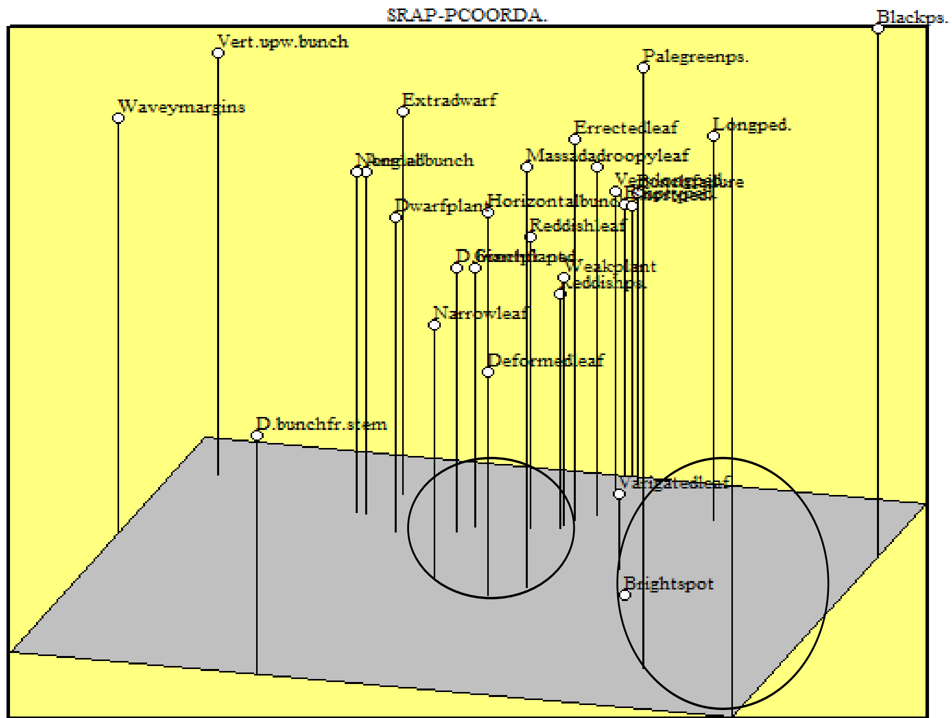

Figure 6. Principal coordinate analysis for banana cv. Grand Naine and its somaclonal variants based on SRAP analysis.

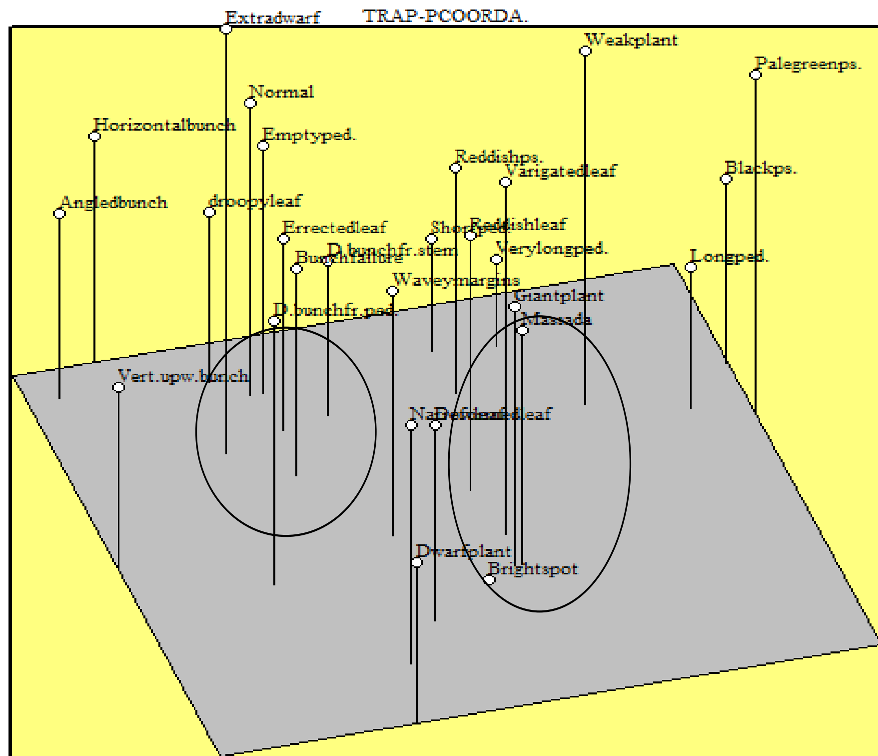

Figure 7. Principal coordinate analysis for banana cv. Grand Naine and its somaclonal variants based on TRAP analysis.

ped.", "very long ped." "Wavy margines", "bunching failure", "empty peduncle", "horizontal bunch" and "angled bunch" in addition to the "Grand Naine". The somaclones "pale green", "black" and "vertical upward bunch" were separated from other variants based on PC1. The somaclones "Dwarf plant" and "horizontal bunch" were separated from the other variants at PC2. On the other hand, the somaclones "Pale green pseudostem", "Weak plant" and "Extra dwarf" were located at the highest level and "bright spot" was located at the zero level at PC3.

The results obtained by principal coordinate analysis are similar to the results of the cluster analysis. Both analyses revealed high level of genetic variance among somaclones. Many factors are known to influence *in vitro* induced variation; however, it has been proposed that hypo/hyper methylation of DNA, which may trigger genome-wide changes, may be the underlying cause (Damasco et al., 1998). The inherent instability of the cultivar being micropropagated was another major factor influencing the production of dwarf off-types. Oh et al. (2007) reported that the molecular basis of somaclonal variation is not precisely known, but both genetic and epigenetic mechanisms have been proposed; whereas James et al. (2007) stated that the source of this variation may be derived from variation pre-existing in the mother plant or it may be induced *in vitr*o. Sales and Butardo (2014) reported that somaclonal variation is due to prolonged subculture and high 2, 4-D concentration.

Conclusion

Molecular analysis based on Sequence Related Amplified Polymorphism (SRAP) and Target Region Amplified Polymorphism (TRAP) using 12 arbitrary primers revealed genetic variability between cv. "Grand Nain" and 26 somaclones. Most of the somaclones aggregated together depending upon the different PCs, this suggests that most somaclones may reflect epigenetic changes which happened during *in vitro* culture. The somaclone "pale green" may be genetically different from the normal plant according to the principal coordinate analysis which suggests that its change is not epigenetic. The results reflect the efficiency of SRAP and TRAP markers in detecting banana somaclonal variation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity of Ethiopian emmer wheat *Triticum dicoccum* Schrank landraces using seed storage proteins markers

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Emmer wheat [*Triticum dicoccum* (Schrank)] cultivation in Ethiopia has been reducing and the genetic diversity maintained in the germplasm collection needs to be determined. The objective of this study was to assess the level of genetic diversity among populations of Ethiopian emmer wheat using seed storage protein analysis. Seeds of 85 accessions representing seven administrative regions of Ethiopia were obtained from the Ethiopian Biodiversity Institute and grown at Sinana Agricultural Research Center. Seed storage proteins were extracted from a single seed and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total genetic diversity, intra-population genetic diversity and inter-population genetic diversity were 0.33, 0.2903 and 0.0391, respectively. Low genetic differentiation (G_{ST} = 0.119) and high gene flow (N_m = 3.697) were observed among populations. The genetic distance (D) between populations ranged from 0.0424 to 0.1128. Cluster analysis revealed two main clusters and one outlier. The results provide important baseline for future germplasm conservation and improvement programs.

Key words: Cluster analysis, emmer wheat, genetic distance, genetic diversity, seed storage protein.

INTRODUCTION

Emmer wheat [*Triticum dicoccum* (Schubler)] was first domesticated in the Near East (Charmet, 2011), which was followed by subsequent hybridization and introgression from wild emmer wheat (*Triticum diccocoides*) in southern Levant (Lebanon, Syria and Israel) (Özbek et al., 2011; Civáň et al., 2013; Fuller et al., 2011; Riehl et al., 2013). It is speculated that early immigrants of Hamites brought emmer wheat to Ethiopia, some 5,000 years ago and was introduced into the Ethiopian Highlands from Egypt along the Nile River

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(Fuller et al., 2011 and Oliveira et al., 2012). Emmer wheat comprises about 7% of Ethiopia's entire wheat production (BOSTID, 1996). The major production areas in Ethiopia include Bale, Arsi, Shewa, Harerge, Wollo, Gojam and Gondar. It is known by different vernacular names such as "Aja" (in Amharic), "Hayssa" or "Matajebo" (in Afaan Oromo) and "Arras" (in Tigrigna).

Emmer wheat landraces are locally adapted to diverse ecological zones as a result of natural selection and farmers' cultivation methods. They are an important genetic resource for breeding novel genetic diversity into bread wheat and hence their genetic analysis is of importance (Oliveira et al., 2014). The diversity of emmer wheat is seriously threatened by genetic erosion due to the increase in bread wheat production. The urgent need to preserve and utilize landrace genetic resources as a safeguard against an unpredictable future is evident (Jaradat, 2011; Haile, 2012).

Genetic diversity can be estimated by different methods such as morphological traits, end-use quality traits, and molecular markers (Hailu, 2011; Oliveira et al., 2012). Seed storage proteins are suitable for diversity studies, cultivar identification and as excellent markers of the gluten content wheat (Özbek et al., 2011). According to solubility properties, seed storage proteins are classified into four classes: albumins, globulins, gliadins and glutelins. Gluten, comprising 78 to 85% of total wheat endosperm protein, is a very large complex composed mainly of polymeric and monomeric proteins known as glutenins and gliadins, respectively (Hu et al., 2012 and Konvalina et al., 2011). Glutenins confer elasticity to dough, whereas gliadins are viscous and give extensibility to dough (Riefolo et al., 2011). Due to extensive polymorphism, these proteins have been widely used for cultivar identification in hexaploid and tetraploid-wheats (Geleta and Grausgruber, 2013; Konvalina et al., 2013). Allelic variants differ in the number, mobility, and intensity of their components and can be characterized through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Colomba and Gregorini, 2011). The present study was undertaken to evaluate the genetic diversity of Ethiopian emmer wheat using total seed storage proteins markers.

MATERIALS AND METHODS

Plant sample

Eighty-five emmer wheat accessions were obtained from the Ethiopian Biodiversity Institute, Addis Ababa (Table 1). The accessions were selected evenly from the collections obtained from the main emmer wheat growing regions based on their abundances. Accessions were planted under rain fed condition in July to December of 2013 at the Sinana Agricultural Research Center (2400 m.a.s.l). Sinana Agricultural Research Center is located 463 km southeast of Addis Ababa (capital of Ethiopia). This site was selected because of its agro-ecology and its national recognition as the major emmer wheat producing regions of Ethiopia. Twenty seeds from each accession were planted in two rows with 10 cm spacing between plants and 20 cm spacing between rows. Seeds were bulked from the rows.

Table 1. Emmer wheat accessions representing seven geographical regions of Ethiopia.

Region	State/region	Number of accessions
Arsi	Oromia	15
Bale	Oromia	16
Gondar	Amhara	12
Hararghe	Oromia	11
Shewa	Oromia	11
Tigray	Tigray	9
Wollo	Amhara	9

SDS-PAGE electrophoresis

The variability of seed storage-proteins was analyzed using SDS-PAGE (Damania et al., 1983). Six seeds from each accession were randomly selected**.** Each seed was ground to a fine powder using a mortar and pestle. The grounded seed sample was transferred to 1.5 mL microcentrifuge tube and 400 µL protein extraction buffer [1.0M Tris-HCl (pH=6.8), 2.5% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol] was added. After brief vortex, the extracts were heated at 90°C for 5 min and centrifuge for 5 min at 12,000 g. The supernatant containing the dissolved seed storage proteins was transferred to a new 1.5 microcentrifuge tube and stored at 4°C. Resolving gel (10%) and stacking gel (4%) were prepared based on Laemmli protocol. The electrophoretic trays were assembled and filled with the running buffer (1.44% Glycine, 0.3% Tris-base and 0.1% SDS), the comb was removed, the wells were cleaned with running buffer and the sample (20 µl) and standard wheat (Alcatal) sample (25 µl) were loaded at the bottom of each well using micropipette. The power supply was connected at 100 V at room temperature for the time required for the tracking marker dye in extraction buffer to migrate off the gel. After electrophoresis, the gel was transferred to tray containing staining solution (125 ml distilled water, 25 ml acetic acid, 100 ml ethanol (absolute) and 0.25g coomassie blue R-250) and kept on a shaker for overnight, followed by distaining with distilled water or distaining solution (125 ml distilled water, 25 ml acetic acid, 100 ml ethanol (absolute) without commasie-blue) until the background of the gel became clear. Picture was taken by digital Panasonic photo camera (Lumix, model No. DMC-S3) (Laemmli, 1970).

Data analysis

Electrophoregrams for the accessions were scored for the presence (1) or absence (0) of each band noted. Data were entered in a binary data matrix. POPGENE version1.32 (Yeteh et al., 1999) was used to calculate genetic diversity for each population as percentage of polymorphism, gene diversity and Shannon-Weaver diversity index (H). NTSYS-pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) were used to calculate Jaccard's similarity coefficient. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare individual samples and regional populations, and generate dendrograms. The neighbor joining (NJ) method (Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 (Payne et al., 2001).

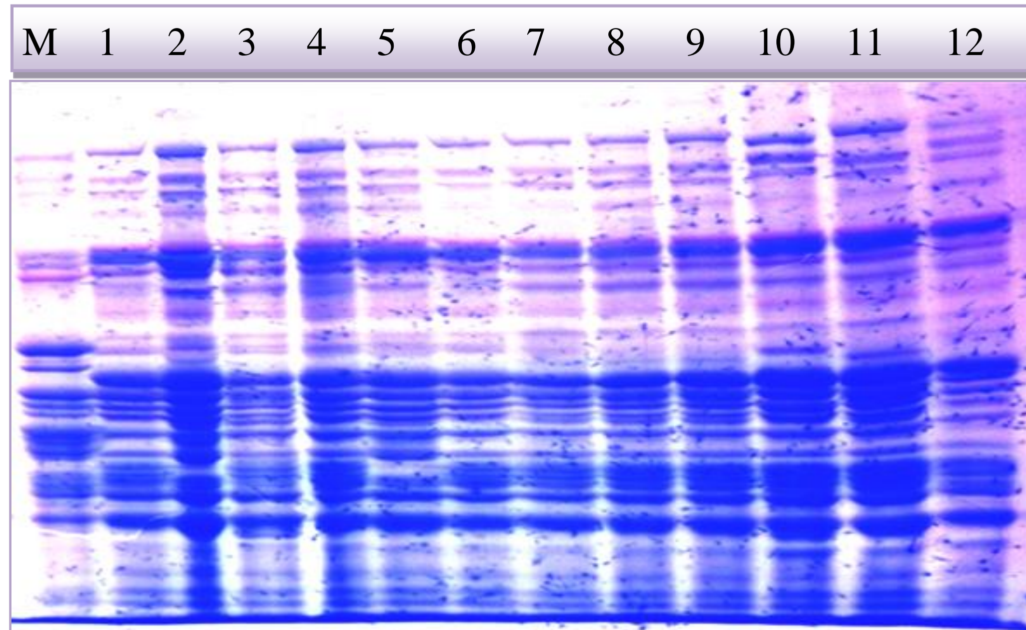

Figure 1. SDS-PAGE protein banding patterns of two *T. dicoccum* accessions. Lane M: bread wheat variety, alcatal used as a control, Lanes 1-6: Accession 238855 and Lanes 7-12: Accession 238856. Six seeds were individually analyzed for each accession.

Table 2. Percentage of polymorphic loci (PPL), Nei's mean gene diversity (H), intra-population genetic diversity (Hs), inter-population diversity (D_{ST}), gene differentiation (G_{ST}) and gene flow (N_m) for seven Ethiopian emmer wheat populations.

Population	PPL	H ± SD	Hs	D_{ST}	G_{ST}	N_m
Bale	78.79	0.301 ± 0.193				
Arsi	81.82	0.288± 0.176				
Shewa	84.85	0.318 ± 0.179				
Hararge	72.73	0.281± 0.199				
Wollo	72.73	0.308 ± 0.205				
Gondor	72.73	0.274 ± 0.192				
Tigray	69.70	0.261 ± 0.210				
Average	76.19	0.290 ± 0.240	0.290±0.240	0.039		
Total Population	90.91	0.329 ± 0.172			0.119	3.696

RESULTS

Genetic diversity analysis

In this study, SDS-PAGE of grain storage proteins was performed in order to investigate genetic diversity among different Ethiopian Emmer wheat landraces. An example of an electrophoregram showing protein banding pattern for two accessions is presented in Figure 1. A total of 33 seed storage proteins were resolved by SDS-PAGE with protein bands numbered 1, 10 and 32 in common for the accessions. However, the remaining bands showed good pattern of variation to study the diversity of this crop. Bread wheat variety Alcatal was used as standard for comparison.

The highest percentage of polymorphic loci was observed for the population from Shewa (PPL= 84.85%), while the Tigray population showed the lowest percentage of polymorphic loci (PPL= 69.70%) (Table 2). The Shewa population was also the most diverse (H =0.318).

Genetic differentiation and gene flow

The average gene diversity among populations was lower

Table 3. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Pop	Bale	Arsi	Shewa	Hararge	Wollo	Gondar	Tigray
Bale	*****	0.9549	0.9441	0.9526	0.9220	0.9415	0.9363
Arsi	0.0461	*****	0.9040	0.9208	0.8933	0.9585	0.9278
Shewa	0.0575	0.1010	*****	0.9281	0.9343	0.9199	0.9515
Hararge	0.0486	0.0825	0.0747	*****	0.9374	0.9573	0.9512
Wollo	0.0812	0.1128	0.0679	0.0646	*****	0.9253	0.9314
Gondar	0.0603	0.0424	0.0834	0.0437	0.0777	*****	0.9539
Tigray	0.0658	0.0749	0.0498	0.0500	0.0710	0.0472	*****

Table 4. Similarity matrix for the seven Ethiopian emmer wheat populations based on seed storage protein markers.

	Bale	Arsi	Shewa	Hararge	Wollo	Gondar	Tigray
Bale	…….						
Arsi	0.676	…….					
Shewa	0.699	0.643	…….				
Hararge	0.694	0.663	0.644	…….			
Wollo	0.691	0.640	0.642	0.688	…….		
Gondar	0.668	0.681	0.672	0.690	0.677	…….	
Tigray	0.679	0.644	0.678	0.701	0.683	0.705	…….

than the average gene diversity within populations. The extent of gene differentiation relative to the total population (G_{ST}) was about 0.1191. The extent of gene flow (N_m) among populations of Ethiopian emmer wheat landraces was high (Table 2).

Inter-population genetic distance and cluster analysis

Inter-population genetic distance (D) showed that the Arsi populations were the most distantly related. Genetic distance between the other pairwise combinations of populations was very low with the least genetic distance between populations from Tigray and Gondor (Table 3).

From Jaccard genetic similarity of emmer wheat landraces, comparatively the highest similarity was observed between Tigray and Gondor than other landraces combination. The lowest similarity was found in Wollo versus Arsi landraces pair. All landraces showed an average of 0.674 genetic similarities, which could mean that the landraces share an average of 67.4% of their protein band, fragments (Table 4).

UPGMA analysis of Ethiopia emmer wheat populations revealed two major clusters and one outlier (Arsi). Tigray and Gondar, which are highly, related populations (Figure 2).

DISCUSSION

Knowledge of genetic diversity within and among populations from different geographic areas is expected to have a significant impact on the conservation and utilization programs of emmer wheat germplasm. So, understanding the level and structure of the genetic diversity of a crop is a prerequisite for the conservation and efficient use of the available germplasm for plant breeding (Laido` et al., 2013).

The genetic diversity parameters: percent of polymorphic loci, gene diversity (GD), and Shannon's diversity index (I) indicated that the genetic diversity in the emmer wheats populations is indeed high. Shannon diversity index result showed that populations of emmer wheat from Shewa were more diverse. This result is also in agree with the findings of Laido` et al. (2013) in their study on 18 species of *Dicoccum* analyzed by using biochemical markers (H=0.45) and Özkan et al. (2011) on emmer wheat using HMW-gs (H=0.31). In this study, an average value of gene diversity (H=0.33) was obtained. Laido` et al. (2013) obtained exactly the same with mean genetic diversity result of the subspecies *Dicoccoides* accessions (H=0.33) collected from different parts of the world by using molecular marker and higher than that of Oliveira et al. (2014) using SNP marker (H=0.27). However, this level of gene diversity is still considered substantial for a dominantly self-pollinating and rarely out-crossed plant species such as wheat. The genetic diversity in the Ethiopian emmer landraces is most likely the result of its long cultivation history and different climatic and topographic factors which enhanced the effects of natural and artificial selection on germplasm

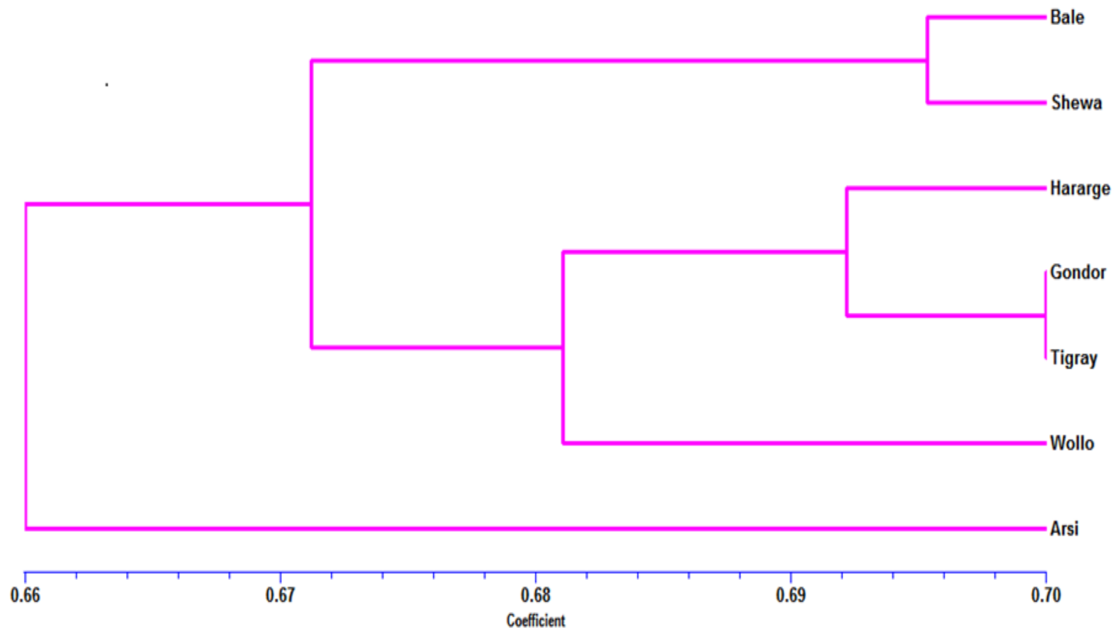

Figure 2. A dendrogram of 7 Ethiopian Emmer wheat populations derived by UPGMA from Nei's (1972) standard genetic distance based on data generated using storage protein.

diversity.

The coefficient of gene differentiation showed that the genetic variation within and among the 7 geographical regions was 88 and 12%, respectively. This result is to some extent in agreement with 73 emmer wheat accessions from Ethiopia highlands that have been recently studied with SSR markers (D=0.27) (Teklu et al., 2007). Therefore, low genetic differentiation between populations was observed due to migration or selection. The values obtained from gene flow (N_m) show the approximate number of individuals migrating from one population to the other. The highest gene flow (N_m=3.6) observed in this study may suggest that the seed exchanging system might be high among the farmers, resulting in low genetic differentiation observed among the populations (D=0.12). Considering the genetic similarity values, the results indicate that the landraces were slightly genetically different from each other. These results are comparable to mean genetic similarity coefficients reported such as 0.65 by Salunkhe et al. (2013) for emmer wheat accessions of India using SSR marker.

In general, the cluster analysis showed a considerable variation among the populations. The formation of solitary cluster might be due to intensive natural or human selection for diverse adaptive complexes.

Therefore, it is concluded that seed storage protein profiles could be useful markers in cultivar identification, registration of new varieties, pedigree analysis, and in the studies of genetic diversity currently conserved in the germplasm of landraces both in *in situ* and *ex situ* collections, thereby improving the efficiency of wheat breeding programs in cultivar development. Finally, seed storage protein analysis permits grouping of peptides patterns and of group based patterns, and is a simple, repeatable and economic procedure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Genetic diversity in Nepalese population of *Swertia chirayita* (Roxb. Ex Fleming) H. Karst based on inter-simple sequence repeats (ISSR) markers

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Swertia chirayita **is a highly valued but vulnerable medicinal plant species of Nepal. Its populations are declining in natural habitats due to over exploitation. Twenty-seven inter-simple sequence repeats (ISSR) primers were used to assess the genetic diversity and population genetic structure of 42 genotypes representing six natural populations of *S. chirayita*, from Nepal. Of the total 479 bands amplified by 27 ISSR primers, 473 (98.18%) were polymorphic, indicating very high level of genetic diversity at species level. Percentage polymorphism value for different primers ranged from 83.3 to 100% with an average of 98.18%. Polymorphism information content (PIC) value ranged from 0.88 to 0.93 with an average of 0.91. Cluster analysis performed with NTSYS pc statistical package using Jaccard's similarity coefficients generated from ISSR binary data matrix showed that, all 27 ISSR primers separated 42 individuals into two major clusters and six sub clusters at the similarity level of 0.24. The average value of Nei's genetic diversity (H) and Shannon's information index (I) equaled 0.276 and 0.423, respectively at species level. The coefficient of genetic differentiation (G_{ST}) amongst populations of *S. chirayita* was found to be high (0.548) with restricted gene flow (N_m=0.4829). Analysis of molecular variance showed that genetic diversity within populations is slightly higher (50.9%) than among populations (47.6%). The present genetic diversity assessment of *S. chirayita* populations has been of immense importance to understand the cause of its vulnerability and has furnished valuable insights for its conservation and sustainable utilization.**

Key words: Polymerase chain reaction, inter-simple sequence repeats (ISSR), genetic diversity, polymorphism, population genetics.

INTRODUCTION

Nepal is home to medicinal and aromatic plants (MAPs) diversity and over 1950 medicinal plants have been stated till date (Ghimire, 2008). Among them, 30 species of *Swertia* (Gentianaceae), including varieties are

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reported in Nepal (Press et al., 2000). *Swertia chirayita* (Roxb. ex Flem) Karst, the most valuable and dominant species in trade, is indigenous to the temperate Himalayas and is abundant in 54 districts in Nepal (Barakoti et al., 2013) throughout the altitude ranging from 600 to 5600 m (Rijal, 2010). Because of its high need in national and international markets, natural populations have been threatened and species as such has been considered vulnerable medicinal plant in Himalayan region including Nepal (Nepal, 2004; Purohit et al., 2013). Together with *S. chirayita*, eight other species of *Swertia* [*Swertia angustifolia* Buch.-Ham ex D. Don, *Swertia dilatata* C. B. Clarke, *Swertia nervosa* (G. Don) C. B. Clarke, *Swertia racemosa* (Griseb.) C.B. Clarke, *Swertia ciliata* (D. Don ex G. Don) B. L. Burtt, *Swertia multicaulis* D. Don, *Swertia alata* (Royle ex D. Don) C.B. Clarke and *Swertia tetragona* Edgew] are also traded for their medicinal properties with the name 'Chiraito' or 'Chiretta' (Barakoti, 2002). Among these nine species, *S. chirayita* is considered superior in medicinal quality and is in high demand in trade nationally and internationally (Barakoti, 2002; Joshi, 2008). Shrestha et al. (2016) have reported that *S. chirayita* is the mostly used species by the indigenous people of Sankhuwasabha district of Nepal proving the efficacy and importance in ethnomedical research. Nepal trades more than 45% of the world's total traded volume of *S. chirayita* (Barakoti, 2004). Nevertheless, Nepal uses only 1% of this volume and the rest is exported to various countries including India, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan, China, Germany, Singapore and the United States of America (Phoboo et al., 2008). Numerous studies have reported many chemical compounds in *S. chirayita* such as chiratinin, terpenoids, iridoids gentianine, amarogentin, amaroswerin, xanthones, secoiridoid, glycosides and urosilic acid (Bajpai et al., 1991; Joshi and Dhawan, 2005; Khanal et al., 2015; Kumar and Chandra, 2015; Kshirsagar et al., 2016, 2017). These compounds and their derivatives possess antihepatotoxic, antileishmanial, anticarcinogenic, antioxidant, anti-inflammatory, antidiabetic, antimalarial and antihelmithic properties (Bajpai et al., 1991; Ray et al., 1996; Saha et al., 2004; Iqbal et al., 2006; Balaraju et al., 2009; Chen et al., 2011; Nagalekshmi et al., 2011; Phoboo et al., 2013; Zhou et al., 2015; Lad and Bhatnagar, 2016). Recently, Tupe et al. (2017) have reported that *S. chirayita* showed antiglycating activity which plays crucial role in antidiabetic effects. Also, *S. chirayita* possess less inhibitory effect on the drug metabolizing isoenzymes CYP3A4 and CYP3D6 as well as inhibits the β-glucuronidase and helps in hepatoprotection (Ahmmed et al., 2016; Karak et al., 2017).

The knowledge of plant genetic diversity in their natural habitat, sustainable utilization, is important for efficient management of plant genetic resources (Mondini et al., 2009). Thus, information on genetic diversity and geographical distribution of plant species in wild conditions are essential for formulating conservation strategy (Wang et al., 2011). Analysis of the genetic diversity and population genetic structure using various molecular marker techniques is necessary to endorse the 'vulnerable' status of any species (Godt and Hamrick, 1998). Thus, molecular tools play important role in exploring genetic diversity in endangered species for the formulation conservation strategies (Kim et al., 2005).

PCR-based inter-simple sequence repeats (ISSR) markers have been widely employed for studying population genetics of various plant species, including several medicinal plants such as *Solanum tuberosum*, *Neopicrorhiza scrophulariiflora* and *Dendrobium* spp. (Bornet et al., 2002; Wang et al., 2009; Liu et al., 2011). Limited molecular studies have been carried out in *Swertia* species of the world. In a study including ISSR markers, 98.7% polymorphism was found among 19 genotypes of *Swertia* spp. (13 of *S. chirayita* and 2 each of *Swertia cordata*, *Swertia paniculata* and *Swertia purpurascenes*) collected from the temperate Himalayas of India (Joshi and Dhawan, 2007). In the investigation of endangered endemic species, *Swertia przewalskii* of the Qinghai-Tibet plateau using RAPD and ISSR analysis, Zhang et al. (2007) observed the significant genetic differentiation based on different measures including analysis of molecular variance (AMOVA) (52% for RAPD and 56% for ISSR). Misra et al. (2010) used amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for *Swertia* spp. In the study, 19 accessions (two of *S. chirayita*, three of *Swertia augustifolia*, two of *Swertia bimaculata*, five of *S. ciliata*, five of *S. cordata* and two of *S. alata*) from India were used in the study by employing 46 selected AFLP primer pairs. The species-specific markers were identified for all six *Swertia* spp. which can be used to authenticate drugs. Another study revealed polymorphism of up to 99% among various species of *Swertia* using 16 ISSR primers (Tamhankar et al., 2009). They found *Swertia lurida* to be the closest to *S. chirayita*. Another study on *Swertia tetraptera* showed high genetic diversity within species and low genetic diversity among populations (Yang et al., 2011). Also, Samaddar et al. (2015) reported on some RAPD markers that can be used for fingerprinting analysis and implementation of genetic diversity study on *Swertia* spp.

In the context of Nepal, initiation of molecular characterization of *Swertia* spp. has been reported with the phylogeny of 11 Nepalese *Swertia* spp. such as *S. augustifolia, S. chirayita, S. ciliata, S. dilatata, S. lurida, Swertia macrosperma, S. multicaulis, S. nervosa, S. paniculata, Swertia pedicellata* and *S. racemosa* (Joshi, 2008, 2011). The internal transcribed spacer (ITS) and chloroplast (*trnL-F*) regions were analyzed together with distance, parsimony and Bayesian analysis. The result indicated that ITS fragment can be used in identification as a barcoding marker for *Swertia*. The optimization of the RAPD-PCR conditions (Shrestha et al., 2011) and

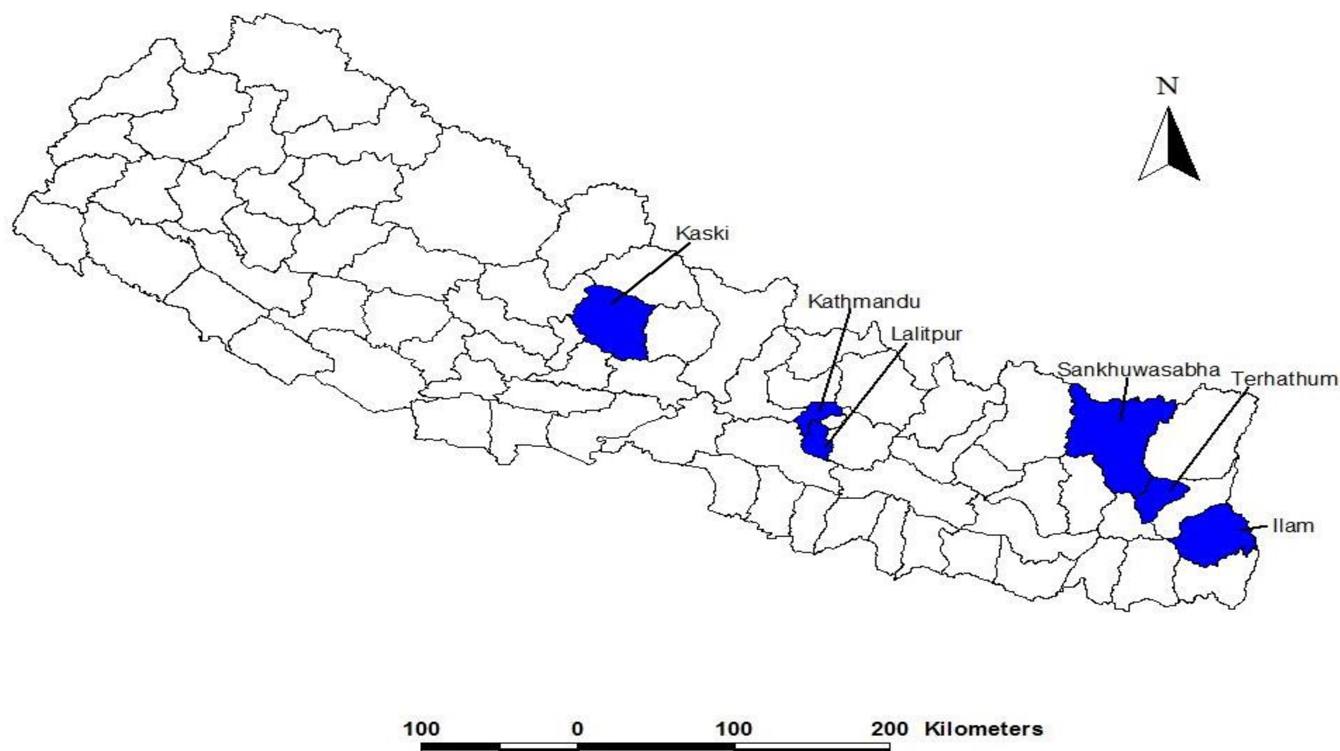

Figure 1. Geographic locations of collected *Swertia chirayita* of Nepalese populations under study.

genetic diversity of 34 accessions of *S. chirayita* including six outlier species were assessed in Nepal (Shrestha et al., 2013). Of the total 285 amplified bands scored for *S. chirayita*, 263 (92.8%) were polymorphic.

The objectives of this study were to assess the genetic diversity, unravel the genetic variation within and among populations and provide future recommendations necessary for conservation and sustainable utilization of *S. chirayita*, the highly valuable and vulnerable medicinal plant species of Nepal.

MATERIALS AND METHODS

Genetic material and genotypic data

Forty-two samples of *S. chirayita* were collected in silica gel from six different locations of Nepal: nine from Phulchowki, four from Kaski, six from Sankhuwasabha, eight from Terhathum, seven from Nagarjun, eight from Ilam along with four outlier accessions from Kaski and Sankhuwasabha districts (Figure 1, Plate 1 and Table 1). Genomic DNA was extracted by using hexadecyltrimethyl ammonium bromide (CTAB) method (Graham et al., 1994). Quantification and purity assessments of DNA were assessed by using a UV Biophotometer (EPPENDORF AG 22331, Germany). ISSR-PCR reaction parameters were optimized in 20 µl reaction volume containing 50 ng of genomic DNA, 2.4 µl of $MgCl_2$ (3.0 mM), 2.0 µl of 10X Taq polymerase reaction buffer [100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40], 1 U Taq DNA polymerase (Thermo Scientific company), 0.3 mM of dNTPs and 0.6 µM of each primer.

PCR cycling conditions described by Tamhankar et al. (2009) produced finest ISSR profiles for *S. chirayita*. The PCR program comprised of an initial denaturation step at 94°C for 2 min followed by 45 cycles of 95°C for 30 s, 51°C for 45 s and 72°C for 2 min and final extension of 72°C for 5 min. Amplification of DNA was performed using BIOER Xp thermal cycler [BIOER Technology Co. LTD, Taiwan, China]. The PCR amplified ISSR fragments were assessed by gel electrophoresis using 1.5% agarose (Promega Co.) in 1X TAE stained with ethidium bromide (10 mg/ml solution, Promega Co.) buffer at 50 V (4.2 V/cm) in EMBI TEC (Santiago, CA) gel tank for 2 h 30 min. The gel documentation was done using Gel Doc system (IN GENIUS, Syngene Bioimaging, UK). 100 ISSR primers (UBC primer, University of British Columbia, Oligonucleotide Synthesis Laboratory, Vancouver, British Columbia, Canada) were employed to screen against DNA of *S. chirayita* from Nagarjun. Twenty-seven UBC primers (Table 2) that engendered reproducible and scorable bands were selected for the ISSR profiling. Both polymorphic and monomorphic bands were scored as “1” for presence and “0” for absence whereas, the failure in amplification was scored as “9”, as an indicator of missing data (Jaccard, 1908). The size of the ISSR-PCR products was determined using Gene rulerTM100 bp plus DNA ladder (Thermo Scientific Company) (Plate 1).

Genetic diversity and clustering analysis

The reproducible bands scored across all samples were included in the analysis. The binary data matrix was investigated using MS-Excel 2007 for the assessment of total number of bands (TNB), number of polymorphic bands (NPB), percent polymorphism (PP), polymorphic information content (PIC), band informativeness (I_B), and resolving power (R_P) for each primer. These are calculated by,

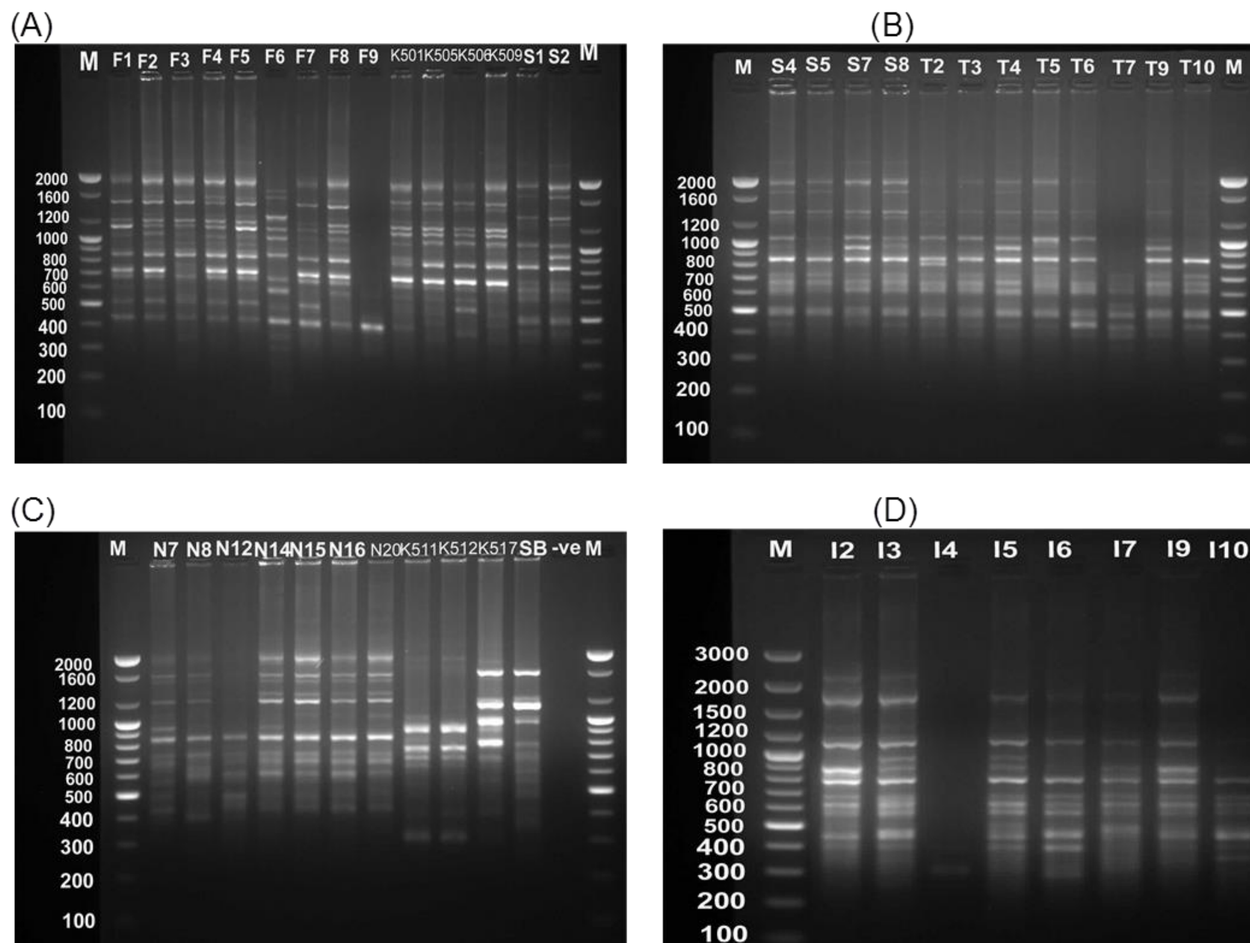

Plate 1. ISSR profile generated by primer UBC 842 for *Swertia chirayita* populations. Lanes marked with M are 100plus DNA ladder. (A) Lanes F1-F9 from Phulchowki, K501-K509 from Kaski and S1-S2 from Sankhuwasabha; (B) S4-S8 from Sankhuwasabha and T2-T10 from Terhathum; (C) N7-N20 from Nagrajun and K511-K517, SB as outliers (*Swertia sp.*); (D) I2-I10 from Ilam.

PP = NPB/TNB engendered by each primer; PIC = 1- $\Sigma(P_{ij})^2$, where P_{ij} denotes the frequency of the ith pattern revealed by the jth primer added throughout patterns revealed by the primers (Botstein et al., 1980); Rp = ΣI_B, where I_B is the band informativeness with IB = 1-[2×(0.5-P)], where P denotes the proportion of accessions containing the band (Prevost and Wilkinson, 1999). The similarity indices were computed using a similarity for qualitative data (SIMQUAL) computer algorithm via NTSYS-PC (Numerical Taxonomy and Multivariate System, version 2.21i, Exeter software; Setauket, New York, USA).

The program POPGENE 1.31 was used to estimate intra and inter-population genetic variation. The parameters used were Nei's gene diversity index (H), Shannon's information index (I), the observed number of alleles (Na) and the effective number of alleles (Ne). Nei's gene diversity statistics along with total genetic diversity (H_T), genetic diversity within populations (H_S), and the extent of genetic differentiation among populations (Gst) was estimated to study the genetic construction (Nei, 1978). Gst was calculated using formula as $(H_T - H_S)/H_T$. The gene flow among populations (Nm) was calculated using formula $(1 - G_{st})/4G_{st}$ by Slatkin and Barton (1989). GenAlEx ver 6.5 was used for analysis of molecular variance (AMOVA) and Mantel test done to estimate the genetic variation among and within populations (Peakall and Smouse, 2012). AMOVA was tested by nonparametric randomization tests using 999 permutations in variation attribute. 3D plot of the distribution of all *S. chirayita* accessions was constructed with the analysis of Eigen vector for the illustration of variation as compared to the dendrogram using Jaccard's similarity matrix using NTSYS-PC.

Principal coordinates analysis (PCA) was performed through Nei's genetic distance (Nei, 1978) matrix of populations using GenAlEx software (Peakall and Smouse, 2012). Finally, F_{st} was calculated employing Tools for Population Genetic Analysis (TFPGA) through 1000 permutations (Miller, 1997).

RESULTS

ISSR analysis

The total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (PP), amplicon size range, PIC, I_B and R_P values of the 27 ISSR primers used to generate ISSR profiles of *S. chirayita* accessions are presented in Table 2. The 27 selected primers

Table 1. Sample details of *S. chirayita* and other outlier species used in the present study (locality, number of samples, altitude, plant accession codes, latitude/longitude of the plant samples collected; C= Central, W= Western, E= Eastern).

District/Locality	Sample size	Altitude (m)	Plant accession codes	Latitude/Longitude
C. Nepal, Lalitpur, Phulchowki, Godavari	9	2100-2150	F1, F2, F3, F4, F5, F6, F7, F8, F9	27.34°N, 85.23°E
W. Nepal, Kaski, Sikles	4	2000-2500	K501, K505, K506, K509	28.21°N, 84.60°E
W. Nepal, Kaski, Sikles	3	2000-2500	K511, K512, K517	28.20°N, 84.59°E
C. Nepal, Kathmandu, Nagarjun, Jamacho,	7	2050-2100	N7, N8, N12, N14, N15, N16, N20	-
E. Nepal, Sankhuwasabha, Lamapokhari-Manlabre	6	2600	S1, S2, S4, S5, S7, S8	27.37°N, 87.78°E
E. Nepal, Sankhuwasabha, Lamapokhari-Manlabre	1	2600	SB	27.37°N, 87.80°E
E. Nepal, Terhathum, Tirkhimti-Gupha Pokhari	8	1500-2800	T2, T3, T4, T5, T6, T7, T9, T10	27.11°N, 87.30°E
E. Nepal, Ilam, Maipokhari	8	-	I2, I3, I4, I5, I6, I7, I9, I10	26.90°N, 87.92°E

Total: 46 samples. Outliers (K511 and K512: *Swertia dilatata* C. B. Clarke and K517 and SB: *Swertia pedicellata* Banerji).

Table 2. Primer sequences, total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (PP), amplicon size range, PIC, I_B and R_P values of the 27 ISSR primers used to generate ISSR profiles of *S. chirayita* populations.

Primers	Primer sequence	TNB	NPB	PP (%)	Amplicon size (bp)	PIC	I_B	R_P
UBC811	GAG AGA GAG AGA GAG AC	17	16	94.12	350-2000	0.914	0.45	8.62
UBC842	GAG AGA GAG AGA GAG AYG	22	22	100	300-2200	0.931	0.58	12.86
UBC 810	GAG AGA GAG AGA GAG AT	19	19	100	250-2000	0.911	0.57	12.00
UBC 820	GTG TGT GTG TGT GTG TC	11	11	100	600-2000	0.859	0.46	5.52
UBC 830	TGT GTG TGT GTG TGT GG	8	8	100	500-2000	0.805	0.43	3.43
UBC812	GAG AGA GAG AGA GAG AA	12	10	83.33	350-2000	0.825	0.31	5.62
UBC 817	CAC ACA CAC ACA CAC AA	15	15	100	400-2100	0.906	0.59	9.43
UBC 809	AGA GAG AGA GAG AGA GG	20	18	90	250-2000	0.927	0.51	11.67
UBC 807	AGAGAGAG AGA GAG AGA GT	16	16	100	400-2100	0.912	0.63	11.3
UBC836	AGA GAG AGA GAG AGA GYA	24	24	100	250-2200	0.938	0.54	13.62
UBC 848	CAC ACA CAC ACA CAC ARG	21	21	100	300-2200	0.916	0.43	9.8
UBC 850	GTG TGT GTG TGT GTG TYC	22	22	100	350-2100	0.937	0.49	11.43
UBC 818	CAC ACA CAC ACA CAC AG	18	18	100	300-2200	0.886	0.36	6.48
UBC 840	GAG AGA GAG AGA GAG AYT	16	16	100	500-3000	0.914	0.51	9.62
UBC 841	GAG AGA GAG AGA GAG AYC	22	22	100	300-2800	0.93	0.58	14.95
UBC 857	ACA CAC ACA CAC ACA CYG	18	17	94.44	350-2600	0.915	0.56	12.29
UBC825	ACACACACACACACACT	18	18	100	300-2500	0.924	0.67	12.76
UBC 834	AGA GAG AGA GAG AGA GYT	16	16	100	400-2200	0.904	0.60	10.33
UBC 864	ATGATGATGATGATGATG	14	14	100	500-2500	0.895	0.70	10.52
UBC 900	ACTTCCCCACAGGTTAACACA	21	21	100	400-2500	0.931	0.46	9.71

Table 2. Contd.

UBC 824	TCTCTCTCTCTCTCTCG	18	18	100	450-2500	0.920	0.33	5.86
UBC 886	VDVCTCTCTCTCTCTCT	16	16	88.89	200-2100	0.889	0.60	10.81
UBC 889	DBDACACACACACACAC	19	19	100	150-1600	0.933	0.72	13.67
UBC 890	VHVGTGTGTGTGTGTGT	18	18	100	200-2200	0.923	0.76	14.52
UBC 895	AGAGTTGGTAGCTCTTGATC	17	17	100	200-3000	0.921	0.80	13.62
UBC 873	GACAGACAGACAGACA	21	21	100	400-2200	0.924	0.60	12.62
UBC 880	GGAGAGGAGAGGAGA	20	20	100	250-1800	0.902	0.44	9.76
Total		479	473	98.18	Average	0.91	0.54	10.47

R: GA (Purine); Y: TC (Pyrimidine); V: GCA (All but T); H: ACT (All but G); D: GAT (All but C); B: GTC (All but A).

Table 3. Genetic variability within population of *S. chirayita* as shown by POPGENE using ISSR-PCR primer data.

Population of *S. chirayita*	Sample size	NPB	PPB (%)	Na	Ne	H	I
Phulchowki, Lalitpur	9	173	72.90	1.7362	1.474	0.2672	0.3947
Kaski	4	109	51.42	1.507	1.2961	0.1749	0.2643
Sankhuwasabha	6	219	78.21	1.7821	1.4597	0.2706	0.4073
Terhathum	8	181	77.35	1.7702	1.4419	0.2589	0.3900
Nagarjun, Kathmandu	7	143	63.9	1.6356	1.3726	0.2192	0.3296
Illam	8	165	77.47	1.771	1.489	0.2801	0.4159
Average	-	-	70.20	-	-	0.2451	0.3669
Species level	42	473	98.18	1.9865	1.4514	0.276633	0.423056

generated altogether, 479 unambiguous and reproducible bands, of which 473 (98.18%) were polymorphic, the sizes ranged from 150 to 3000 bp. The numbers of bands varied from 8 to 24, with an average of 18 bands per primer. Polymorphism information content (PIC) score of primers under study ranged from 0.805 (UBC830) to 0.938 (UBC836) with an average of 0.91. The average band informativeness (I_B) of the 27 primers was 0.54 and it ranged from 0.31 (UBC 812) to 0.80 (UBC 895). Whereas, the resolving power (R_P) ranged from 3.43 for primer UBC 830 to 14.95 for primer UBC 841 with an average of 10.47.

Genetic diversity within populations

The percentage of polymorphic loci (PPB) ranged from 51.42 to 78.21%, with an average of 70.20% in individual populations (Table 3). Nei's gene diversities (H) varied from 0.174 to 0.270, with an average of 0.245, and Shannon's indices (I) ranged from 0.264 to 0.407, with an average of 0.366. In this study, the high genetic diversity was found in Sankhuwasabha populations (H and I values of 0.270 and 0.407, respectively), while low genetic diversity was found in Kaski populations (H and I values of 0.174 and 0.264, respectively). The genetic diversity of populations from high to low ranked as follows: Sankhuwasabha > Ilam > Terhathum > Phulchowki > Nagarjun > Kaski. It was also evidenced from the number (Na) and effective number of alleles (Ne) (Table 3). At species level, the H and I values equaled 0.276 and 0.423, respectively, and the Na and Ne values equaled 1.986 and 1.451, respectively.

Genetic construction of populations

The considerable level of genetic differentiation was observed among various populations of *S. chirayita* studied. The total gene diversity (H_T) and gene diversity within populations (H_S) were 0.278 and 0.120, respectively. The coefficient of genetic differentiation (G_{st}) amongst inter-populations of *S. chirayita* 0.548 indicated 54.8% variation in inter-populations and 45.2% variation within the populations (Table 4). AMOVA showed 48.0% genetic variation in inter-populations (Table 5); which strongly supports the result shown in genetic differentiation of *S. chirayita* as it was affected more in inter-population units. The gene flow was estimated to be 0.482. The correlation between genetic distance and geographical distance (r) value was found to be 0.418 ($p<0.001$), which indicated no significant correlation between the two matrices, based on genetic and geographical distances. F_{ST} value was found to be 0.6529.

Table 4. Genetic differentiation and diversity within and between the populations of *S. chirayita.*

Primers code	H_T	H_S	G_{ST}	N_m
UBC811	0.2719	0.1065	0.6084	0.3218
UBC842	0.2807	0.1432	0.4900	0.5204
UBC 810	0.2664	0.1113	0.5381	0.4292
UBC 820	0.2399	0.1154	0.5191	0.4632
UBC 830	0.2633	0.0732	0.7220	0.1926
UBC812	0.1613	0.1153	0.2850	1.2545
UBC 817	0.3370	0.0865	0.7433	0.1727
UBC 809	0.3033	0.0569	0.8123	0.1155
UBC 807	0.3331	0.1274	0.6176	0.3096
UBC836	0.2821	0.1432	0.4923	0.5156
UBC 848	0.254	0.0998	0.6072	0.3235
UBC 850	0.2667	0.1718	0.3557	0.9055
UBC 818	0.1671	0.0988	0.4088	0.7230
UBC 840	0.2986	0.1484	0.5032	0.4937
UBC 841	0.2874	0.1453	0.4942	0.5117
UBC 857	0.3117	0.1091	0.6500	0.2692
UBC825	0.3483	0.0674	0.8066	0.1199
UBC 834	0.2935	0.1174	0.6001	0.3331
UBC 864	0.3174	0.1941	0.3884	0.7872
UBC 900	0.2470	0.1444	0.4154	0.7036
UBC 824	0.1746	0.1253	0.2825	1.2701
UBC 886	0.2445	0.1022	0.5820	0.3590
UBC 889	0.3877	0.1340	0.6542	0.2643
UBC 890	0.3584	0.0986	0.7249	0.1897
UBC 895	0.3319	0.1517	0.5431	0.4207
UBC 873	0.2742	0.1428	0.4790	0.5439
UBC 880	0.2176	0.1116	0.4873	0.5260
Mean	0.2785	0.1200	0.5485	0.4829
Standard deviation	0.0561	0.0311	0.1432	0.2953

Table 5. Analysis of molecular variance (AMOVA) for 42 individuals in six populations from three regions [degree of freedom (d.f.), sum of squares (SS), mean square (MS), estimated variance, percent (%) and its associated significance (n=999 permutations)].

Source of variation	d.f.	SS	MS	Estimated variance	Total variance (%)	P-value
Among populations	5	1904.740	380.948	47.658	48	<0.01
Within populations	36	1835.117	50.975	50.975	52	<0.01
Total	41	3739.857	-	98.633	100	-

Cluster analysis based on the ISSR genotyping profiles

The results from Mantel test (Matrix comparison) using NTSYS-PC (Version 2.21i) showed that the correlation between Jaccard and Dice similarity matrices was the highest and significant (0.99252) (Table 6 and Plate 1). Clustering based on unweighted pair group method of arithmetic averages (UPGMA) for Jaccard coefficient was observed to give a high cophenetic correlation value of 0.96875 and comparatively lowest cophenetic correlation value of 0.94904 was observed for UPGMA clustering using simple matching coefficient. Because of their highest correlation value and comparison of standard chart of goodness of fit, Jaccard's coefficient of similarity with UPGMA clustering method was the best for studying relationship among *S. chirayita* accessions. In the study, Jaccard similarity with UPGMA yielded the highest correlation coefficient value but the difference between Jaccard, Dice and simple matching coefficients was not

Table 6. Correlation coefficients from Mantel test (2 way) of original matrices.

Correlation parameter	Simple matching	Jaccard	Dice
Simple Matching	****	-	-
Jaccard	0.92823	****	-
Dice	0.90743	0.99252	****

Table 7. Correlation coefficient value (r) obtained from cophenetic values of similarity matrices (simple matching, Dice and Jaccard's coefficient) and clusters computed by UPGMA module using MXCOMP (matrix comparisons) option of NTSYS.

Clustering module of similarity	Simple matching	Dice	Jaccard
UPGMA	0.94904	0.95197	0.96875

Table 8. Consensus fork index (CI_C) among the UPGMA based phenograms produced by similarity coefficients among *S. chirayita* accessions by ISSR marker.

Correlation parameter	Jaccard	Dice	Simple matching
Jaccard	****	0.9805	0.7500
Dice	-	**-**	0.7500
Simple Matching	-	-	****

so far (0.96875, 0.95197 and 0.94904 respectively). From this test, the three coefficients were in the decreasing order as J > D > SM (Table 7). Consensus indices (CI) were calculated for each combination of coefficient and UPGMA clustering for the evaluation of trees constructed from UPGMA clustering by genetic similarity coefficients. Highest Consensus fork index (CI_c = 0.9805) was found for Jaccard and Dice coefficients. The CI_c values for J and SM and D and SM were lower (CI_c = 0.7500) (Table 8).

Based on the Jaccard's similarity matrix (01-0.86) clarified the genetic relationship of *S. chirayita* accessions from geographically diverse population. Individuals (42) were found separated into two major clusters (Clusters I and II) and six sub clusters A, B, C, D, E and F at the similarity level of 0.24 (Figure 2). The sub clusters A and B were separated at the similarity coefficient level of 0.27. Likewise, the clusters C and D were separated from A and B at the similarity coefficient of 0.25. The sub-cluster A contained accessions from Phulchowki and Kaski. All accessions (except F9) from Phulchowki were clustered at the similarity coefficient level of 0.65, while F9 accession was clustered at the similarity coefficient level of 0.425. Likewise, all the individuals from Kaski were clustered as the similarity coefficient level of 0.68. The sub cluster B contained accessions from Nagarjun and were clustered at the coefficient level of 0.62. The sub cluster C contained accessions from Sankhuwasabha (S1 and S2), whereas sub cluster D contained accessions from both Sankhuwasabha (S4, S5, S6, S7 and S8) and Terhathum. The accessions from Sankhuwasabha, S1 and S2 were separated from rest of the individuals at the similarity coefficient level of 0.35, while the rest of individuals were clustered at the level of 0.675. The accessions T7 and T6 were separated from the rest of the individuals of Terhathum at similarity coefficient level of 0.52 and 0.50, respectively. The sub clusters E and F contained accessions from Ilam. The individuals of Ilam were clustered at the similarity coefficient level of 0.49 except the accession I4 at 0.345. The PCoA analysis was carried out based on the Euclidean matrix. In the plot, first (percentage of variance = 18.33%) and second (percentage of variance = 16.90%) axis with a cumulative variance of 35.23% was seen. The PCoA plot supports the result of dendrogram by clustering the individuals according to their geographical locations (Figure 3). It showed the congruence with 3D-plot of the distribution of all *S. chirayita* accessions (Figure 4).

DISCUSSION

The genetic polymorphism observed in the present study for *S. chirayita* was very high (that is, 98.18%) as compared to the result from 13 Indian *S. chirayita* genotypes from temperate Himalaya (42.5%) using ISSR marker (Joshi and Dhawan, 2007). When over 50% of the total genetic variation existed within populations, six populations of species should retain 95% of their genetic

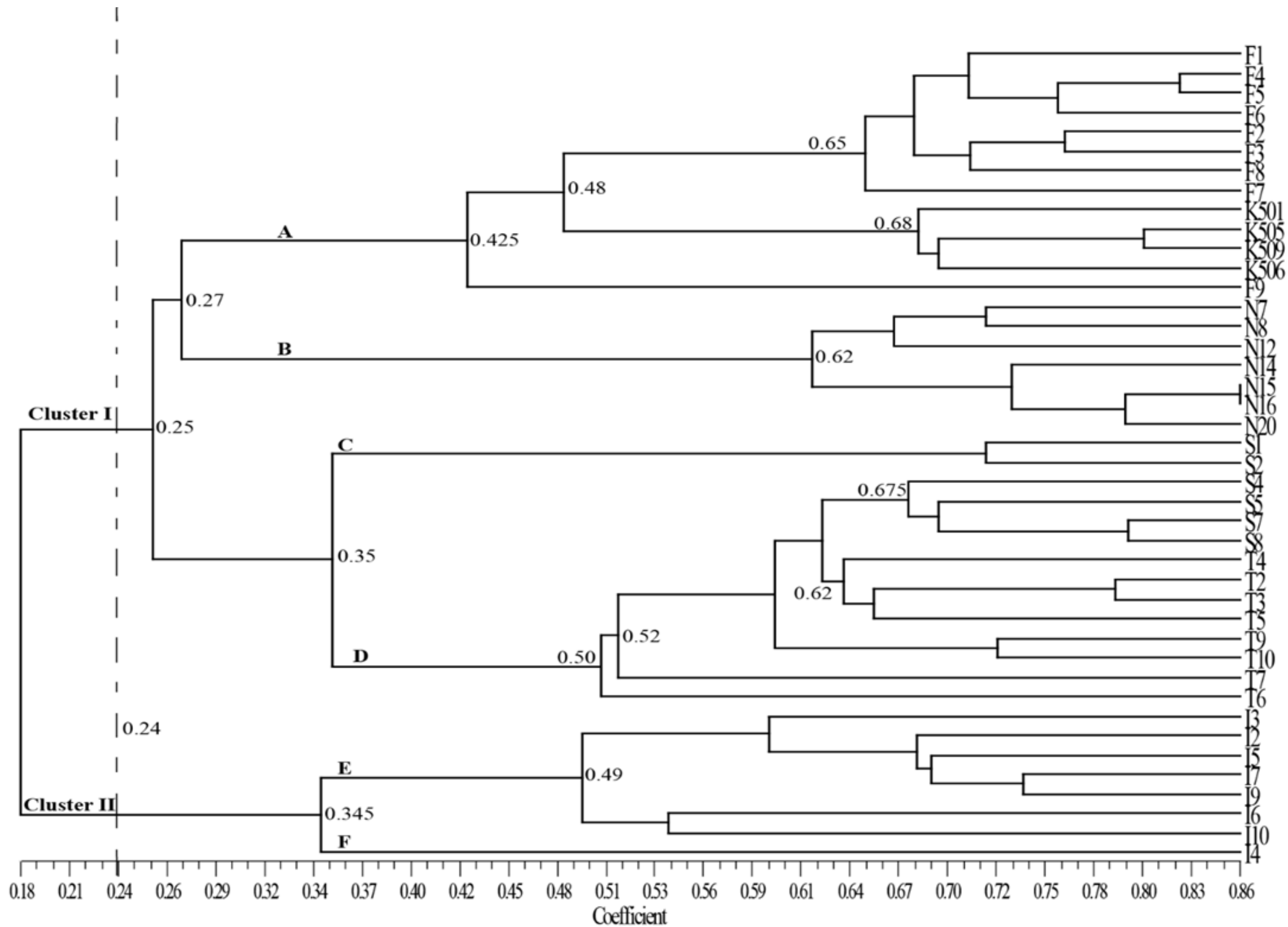

Figure 2. Dendrogram generated for 27 polymorphic ISSR-PCR primers data of 42 *Swertia chirayita* accessions using Jaccard's similarity coefficient by UPGMA method of Cluster analysis. The clusters are labeled as A, B, C, D.

diversity (Hamrick and Murawski, 1991). The investigation showed substantial genetic differentiation among *S. chirayita* populations. However, RAPD based analysis of 34 accessions of *S. chirayita* (used in this present research) revealed 92.28% polymorphism (Shrestha et al., 2013). *S. tetraptera*, an endemic species of Qinghai-Tibetan Plateau revealed 98.9% polymorphism using ISSR fingerprinting (Yang et al., 2011) and *S. przewalskii* from the same region showed 56% polymorphism using ISSR marker (Zhang et al., 2007). Genetic polymorphism shown with ISSR analysis for *Neopicrorhiza scrophulariiflora* also showed high level of polymorphism of 100% (Liu et al., 2011), likewise, 100% for *Dendrobium* studies (Wang et al., 2009). The high level of both polymorphism and reproducibility is observed using ISSR-PCR technique because of the use of longer primers which increases stringency in annealing temperatures than in RAPD-PCR technique (Kojima et al., 1998).

The present study revealed Jaccard's similarity coefficient values ranging from 0.1 to 0.86. This values suggest the presence of high genetic diversity within *S. chirayita* species of six populations. Similar result was observed among the *S. chirayita* species collected from temperate Himalaya of India as Jaccard's coefficient was observed in the range of 0.68 to 0.97 (Joshi and Dhawan, 2007). Although, there was a different cluster pattern in PCoA, the Mantel test revealed no significant correlation between genetic distance and geographical distance ($r = 0.418$; $p < 0.001$). This might be due to clustering of accessions from Phulchowki and Kaski together, which are approximately 200 km apart. The accessions from Ilam were grouped together in a separate cluster.

For the formulation of conservation strategies, understanding of the diversity of threatened species is quintessential (Kareem et al., 2012). The species should possess enough genetic variability as it plays important role in adaption in the changing environment (Schaal et al., 1991). The polymorphism study can be shown in terms of the Nei's genetic diversity (H), Shannon's information index (I), total heterozygosity (H_T), average heterozygosity (H_S), coefficient of population differentiation

Axis	1	2	3
Percentage	18.33	16.90	12.97
Cumulative Percentage	18.33	35.23	48.20

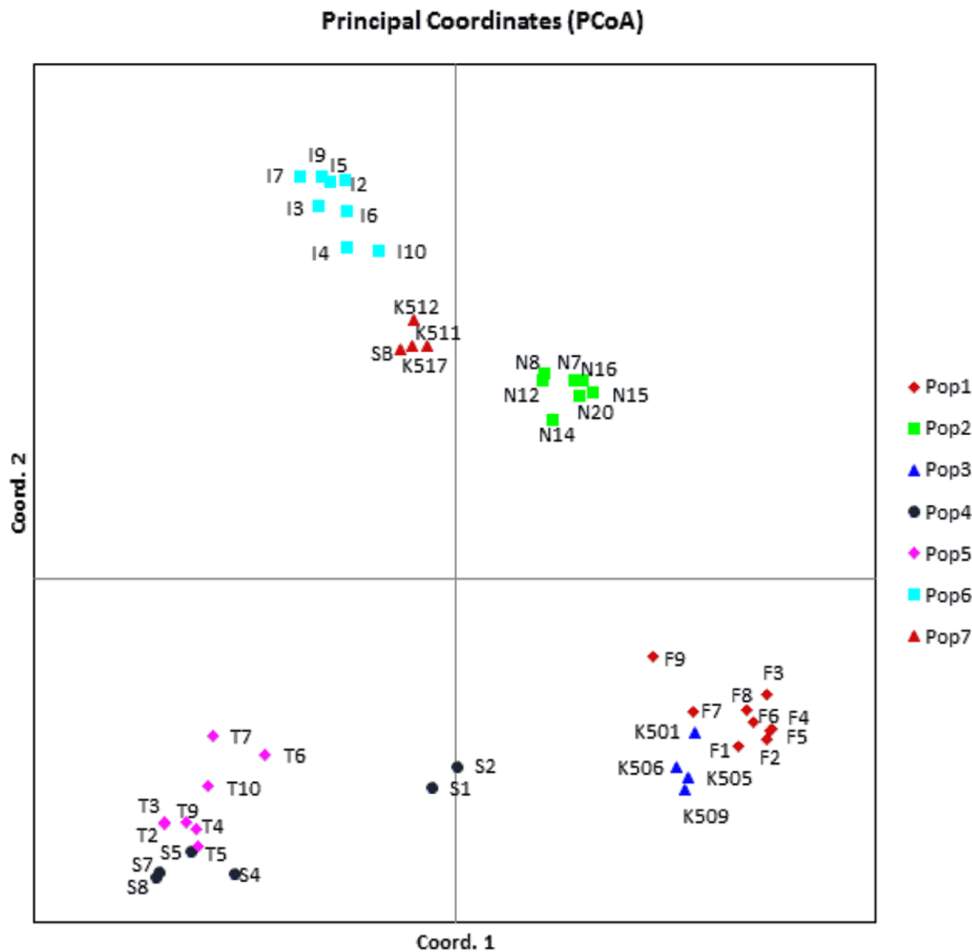

Figure 3. Two-dimensional scatter plot of PCoA axis generated for 42 *Swertia chirayita* accessions by 27 ISSR-PCR primers through Principal Co-ordinate Analysis.

(G_{ST}) and gene flow (N_m) (Zhao et al., 2006). The value of gene differentiation (G_{ST}) ranging < 0.05, 0.05 to 0.15 and > 0.15 is grouped as low, medium and high population differentiation, respectively (Nei, 1978). Also, the value of gene flow (Nm) < 1 which denotes less than one migrant per generation into a population is the threshold value at which the differentiation occurs in population in a significant amount (Slatkin and Barton, 1989). When the condition arises where N_m is found to be less than one, it is suggested that the diversity maintained in the population is prone to genetic drift (Wright, 1949). High G_{ST} value (0.5485) and the low N_m value (0.4829) were observed in the present study and showed rapid genetic differentiation among the six populations of *S. chirayita*. The reason behind the high level of population differentiation can be the geographic separation of the populations (Hogbin and Peakall, 1999). The genetic variation in *S. chirayita* could be due to genetic drift within the population. Also, effect of the gene flow among inter-populations of *S. chirayita* is not significant. The genetic heterogeneity showing index, the Shannon's index was observed to be the highest (0.416) for accession from Sankhuwasabha along with high Nei's gene diversity index (0.280), whereas the lowest Shannon's index (0.0.264) was found for Kaski accession with low Nei's gene diversity index (0.175) and PPB (51.42%). Study of species level diversity showed high level of genetic differentiation with 98.18 of PPB and high Shannon's index of 0.423, which shows the high polymorphism in the *S. chirayita* populations.

The data on the genetic structure of *S. chirayita* obtained in the present study suggest that the differentiation coefficients (G_{ST}=0.5485 and F_{ST}=0.6529) are higher than the average coefficients of outcrossing

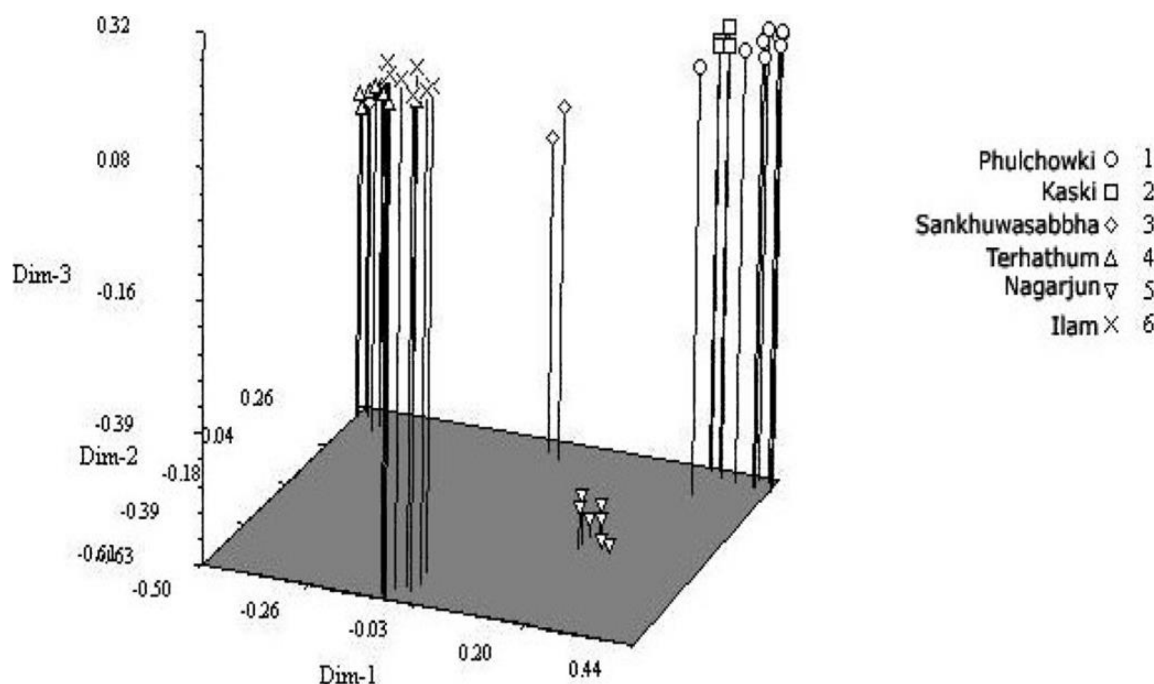

Figure 4. Three-dimensional plot constructed using NTSYS-PC ver2.2i to assess the dispersion of *S. chirayita* accessions.

species (G_{ST}=0.22 and F_{ST}=0.27) and similar to the average coefficients of inbreeding species (G_{ST}=0.59 and F_{ST}=0.65) (Nybom, 2004). High genetic differentiation in this species may suggest that the individual populations have been reproductively isolated and there is little current gene flow between them. The result is in agreement with Soumendra et al. (2009) in which *S. chirayita* has demonstrated the self-pollination in natural pollination of formation of seeds. However, it contrasts with the aspect of high genetic diversity observed among the *S. chirayita* individuals of different populations that indicate the existence of significant cross-pollination among the individuals in the population. The natural environment of the plants from Gentian family shows the probability of out crossing by 16 to 20% (Dudash, 1990). The pollinators such as bees and insects are responsible for cross pollination as they collect nectar from nectar glands (Khoshoo and Tandon, 1963). The morphological reason behind the self-pollination can be the structure pattern of androecium and gynoecium (Kulkarni et al., 2005). In *S. chirayita*, the distance between the anther sac and stigma is less which creates the condition for self-pollination (Proctor et al., 1996). Also, the various studies revealed that *S. chirayita* is mostly cross pollinated with potential of self-pollination (Shah et al., 2011; Raina et al., 2013).

Conclusion

S. chirayita is considered a highly valued medicinal plant of Nepal. In the present investigation, microsatellite based ISSR technique was employed for the assessment of existing genetic diversity among six *S. chirayita* populations from eastern, central and western regions of Nepal. The conservation of *S. chirayita* populations *in situ* to preserve its genetic diversity is suggested. The findings provide insights into important genetic information for formulating and effecting conservation strategies and cultivation of *S. chirayita*. Easily identifiable and confusion with the other *Swertia* spp. twinned with capacity of possessing chemical compounds from the early stage are the main reasons for premature harvesting of *S. chirayita*. This has resulted into the major problem as seeds could not be dispersed in the natural environment. *In vitro* tissue culture technique to be followed in order to reduce harvesting pressure on wild populations of *S. chirayita* is suggested. Additionally, to implement effective conservation strategies of *S. chirayita*, it is crucial to understand species pollination, breeding system associated with the genetic structure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analysis of similarities within different date palm cultivars of Al-Ahsa oasis in Saudi Arabia

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Date palm (*Phoenix dactylifera* L.) is widely cultivated in Saudi Arabia, with annual production estimated to be above one million tons. Of the 450 cultivars that are commonly used, 70 cultivars are found in the Eastern province and 20 cultivars are well known and common in the Al-Ahsa oasis. In this study, thirty seven date palm samples representing nine cultivars collected from six locations in the Al-Ahsa oasis, Saudi Arabia. Total proteins were extracted and subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results revealed several differences within the cultivars. Overall polymorphism ranged between 12 and 76%, with the highest being 76% within Helali cultivar, and the least being 12% within OmRohaim cultivar. These results support the conclusion that some palms are grown from seeds resembling a known cultivar, which are later distributed as the known cultivar. These results revealed differences in the degree of similarity between the cultivars from 0.059 to 0.579, estimated according to Jaccard`s similarity method based on the presence and absence of protein bands. Khosab and Shehel cultivars were closely related to each other with 0.579 degrees of similarity, both separated by phylogenetic dendrogram in mini-cluster, which have been confirmed by the 1st principal components. The mini-cluster was related to OmRohaim and Khonaizi cultivars within a larger cluster. Shishi, Garrah and Helali were related cultivars, with degrees of similarities ranging between 0.250 and 0.455, and were separated in one cluster by phylogenetic dendrogram. The close relationship between the cultivars may indicate that they originated from the same ancestor. Further molecular and biochemical studies are needed to enrich our knowledge about the relationships among date palm cultivars.

Key words: Components, dendrogram, patterns, principal, protein.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a dioecious species belonging to the Arecaceae family that includes 225 genera and 2600 species (Corner, 1966). It is cultivated in the Middle East and North Africa (Hamza et al., 2011), where it is considered a valuable fruit crop (Racchi et al., 2014). The annual global production of dates is estimated to be about 7.4 million tons, while Saudi Arabia accounts for more than one million tons (FAOSTAT, 2013). In

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Table 1. Cultivars and names of locations with their corresponding abbreviations.

Abbreviation	Khs	Shi	Shl	Hel	Hat	Hat	OmR	Khn	Grr
Cultivar	Khosab	Shishi	Shehil	Helali	Hatemi	Hatemi	OmRuhaim	Khonaizi	Garrah
Abbreviation	**OY**	**HF**	**KL**	**MN**	**SH**	**SB**			
Location	Al Oyoon	Al Hafof	Al Kelabiah	Al Menaizlah	Ash Shgaig	Ass Sabatt			

Saudi Arabia, about 450 cultivars are cultivated with more than 70 cultivars being grown for centuries in the eastern province (Asif et al., 1982), and ~ 20 cultivars are well known and common in the Al-Ahsa oasis (Al-Baker, 1972), which is the largest date palm oasis of Saudi Arabia (Al-Abdolhadi et al*.,* 2012), with an estimated three millions date palms (Al-Abbad et al*.,* 2011). In general, date palms produce inferior fruits when propagated by seeds, therefore offshoots are preferred for conventional propagation, as offshoots produce fruit identical to the mother tree (Abass, 2013; Kenna and Mansfield, 1997).

Utilizing DNA markers is most accurate than protein patterns in studying similarities between cultivars, but still protein profiles characterized by high stability and reproducibility, which produces reliable results to verify the differences within and between species and cultivars (Dakhil et al., 2013; Rouholamin and Saei, 2016; Alege et al., 2014; George et al., 2013), electrophoretic analysis of protein bands has been used as an effective tool in recognizing the origin of cultivated plants (Ladizinsky and Hymowitz, 1979). It is a fast and inexpensive tool (Smykal et al., 2008; Rouholamin and Saei, 2016). Electrophoretic protein analysis has been used traditionally in higher plants to study genetics, *P. dactylifera* L. (Dakhil et al., 2013), *Punica granatum* L (Rouholamin and Saei, 2016), taxonomy, *Trifolium* L. (George et al., 2013), physiology, *P. dactylifera* L. (Al-Helal, 1994) and for the study of phylogenetic relationships, *P. dactylifera* L. (Attaha et al., 2013), as well as for the genotoxicity of different chemicals on date palm callus (Abass et al., 2017). Phylogenetic electrophoresis has been used to study the genetic relationships between and within date palm cultivars (Attaha et al., 2013), and in identifying the cultivars (Stegemann et al*.,* 1987), and other plants (Barta et al., 2003). Date palm leaflets have been used extensively as a source of proteins in cultivar identification and phylogenetic studies (Ahmed and Al Qaradhawi, 2009; Attaha et al., 2013), therefore, thirty seven leaflet samples representing nine different cultivars, namely, Khosab, Shishi, Shehel, Helali, Hatemi, Khalas, OmRohaim, Khonaizy and Garrah, were collected from six locations in the Al-Ahsa oasis, namely, Al Oyoon (OY), Al Hafof (HF), Al Kelabiah (KL), Al Menaizlah (MN), Ash shgaig (SH) and Ass Sabatt (SB) (Table 1 and Figure 1). The purpose of the present study was to verify that the palms of each cultivar are identical and to analyze the phylogenetic relationships between the cultivars using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Young green leaflets were collected from date palm trees of age 30 years and above. The samples were snap frozen immediately in liquid nitrogen, and then stored at -20°C.

Protein extraction

The samples were cut into small pieces, and ground in a mortar with liquid nitrogen. Total proteins were extracted according to Al-Helal (1994) with some modifications, homogenizing 200 mg of ground leaflet powder in 1.2 ml extraction buffer [0. 5 M Tris/HCl (pH 6.8) + Glycerol 10% (v/v) + PVP 4% (w/v)] using a Bullet Blender Homogenizer. The extracts were incubated overnight at 4°C, vortexed, boiled at 95°C for 5 min, and centrifuged at 12500 rpm for 10 min, after that loaded in 20 µl.

SDS-PAGE Electrophoresis

Discontinuous vertical electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) with some modifications. 12.5% resolving gels were prepared with 6.67 ml acrylamide-bis acrylamide (30/2.6%) + 4 ml 1.5 M tris/HCl buffer (pH 8.8) + 5.05 ml distilled water (DW) + 0.16 ml 10% SDS + freshly prepared 0.12 ml 10% amm. persulfate $(NH_4)_2S_2O_8$ + 0.008 ml TEMED and stacking gels were prepared with 0.95 ml acrylamide-bis acrylamide (30%\2.6%) + 1.25 ml 0.5 M tris/HCl buffer pH 6.8 + 2.72 ml DW + 0.05 ml 10% SDS + freshly prepared 0.025 ml 10% ammonium persulfate + 0.005 ml TEMED. The running buffer was prepared from 3.0 g Tris + 14.1 g glycine + 1 g SDS, dissolved in 1 L DW, the pH adjusted by hydrochloric acid to 8.3. Electrophoresis was carried out at 100 to 150 V using Bio-Rad Broka 0.75 mm mini electrophoresis system and Bio-Rad PowerPac Basic.

Staining and destaining

The gels were washed with 100 ml of gel fixing solution (Ethanol: Acetic Acid: Distilled Water, 5:1:4%, respectively GFS) for 1 h with agitation at room temperature. The gels were then covered with 100 ml of gel washing solution (Methanol: Acetic Acid: Distilled Water, 5:1:4%, respectively GWS) overnight with agitation at room temperature. The gels were incubated with 100 ml Coomassie brilliant blue R-250 stain, prepared by dissolving 0.4 g of Coomassie Brilliant Blue R-250 in 200 ml methanol (40%) and 200 ml acetic acid for 3 to 4 h with agitation at room temperature The

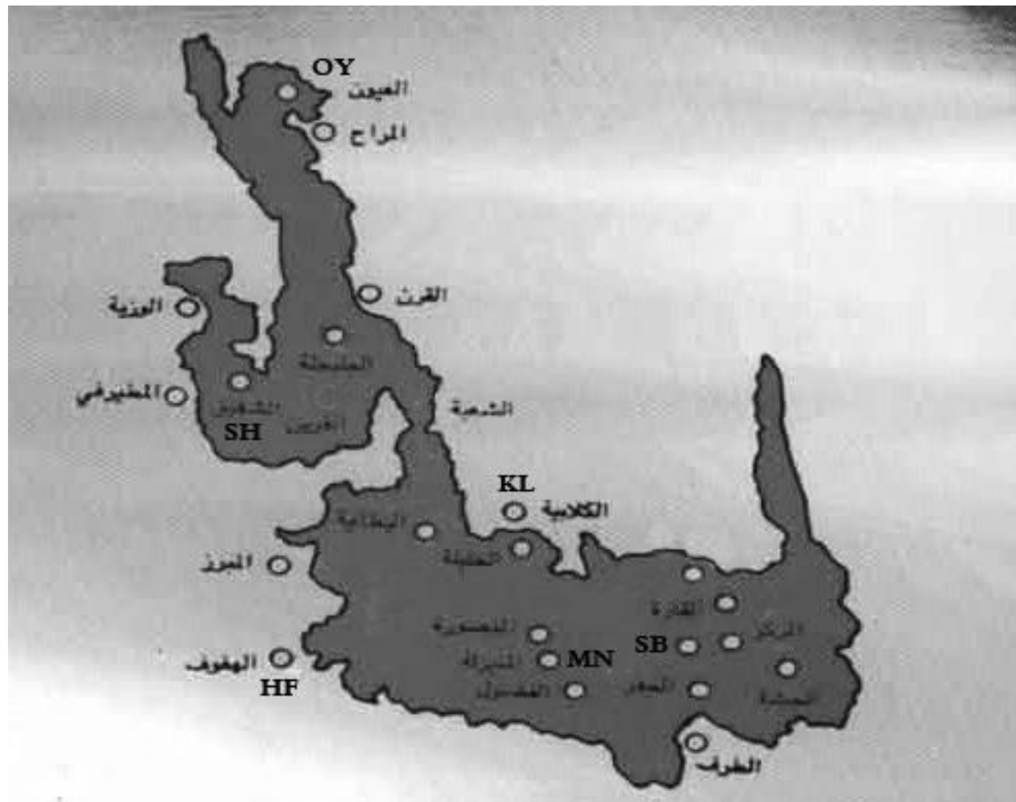

Figure 1. Map of the Al-Ahsa oasis and the locations of samples, mentioned by abbreviations.

gels were then rinsed with several changes of 100 ml gel washing solution.

Band scoring and analysis

The electrophoresis was carried out three times and the gel with best resolution of protein bands was analyzed using a Gel Analyzer 2010a densitometer software and was scored according to the presence (I) or absence (O) of protein bands (Gel Analyzer, 2010). Molecular weights of the protein bands were calculated, with reference to the standard protein marker, starting at the top with 250 KDa band followed by 150, 100, 75, 50, 37, 25, 20, 15 and 10 KDa band, using a Gel Analyzer 2010a densitometer software. The results obtained from protein patterns were analyzed statistically. Jaccard's similarity was determined based on the presence (I) or absence (O) of protein bands (Ahmed and Al Qaradhawi, 2009), hierarchical clustering was performed, and principal components analysis was conducted using IBM SPSS Statistics for Windows software (IBM Corp., 2010).

RESULTS

Total protein extracts from date palm cultivars were subjected to SDS-PAGE analysis. Electrophoretic protein bands varied in intensity, with some bands showing very low resolution. Upon analyzing the protein patterns (Figures 2 and 3) with densitometer software, low resolution protein bands with raw volumes below 10 were excluded.

The number of protein bands ranged between 15 in Hatemi cultivar (Table 2) to 25 in Helali cultivar (Table 3).

The percentages of polymorphism observed was higher 76% (Table 4) in Hel cultivar for 25 electrophoretic bands representing six locations because the electrophoretic bands varied between 12 bands in OY and SB locations to 20 bands in KL location (Table 3).

High polymorphism ratio 70% (Table 4) in Khl cultivar for 20 electrophoretic bands representing six locations because the electrophoretic bands varied between 11 bands in SH location and 16 bands in KL location (Table 5); high polymorphism ratio 68% (Table 4) in Shi cultivar for 22 electrophoretic bands representing five locations because the electrophoretic bands varied between 12 bands in KL and MN locations and 16 bands in HF (Table 6); low polymorphism ratio 44% (Table 4) in Grr cultivar for 16 electrophoretic bands representing five locations because the electrophoretic bands varied between 11 bands in SB location and 13 bands in HF location (Table 7); low polymorphism ratio 40% (Table 4) in Hat cultivar for 15 electrophoretic bands representing three locations because the electrophoretic bands varied between 11 bands in MN and SH locations and 14 bands in OY location (Table 2); low polymorphism ratio 38% (Table 4) in Shl cultivar for 24 electrophoretic bands representing four locations because the electrophoretic bands varied

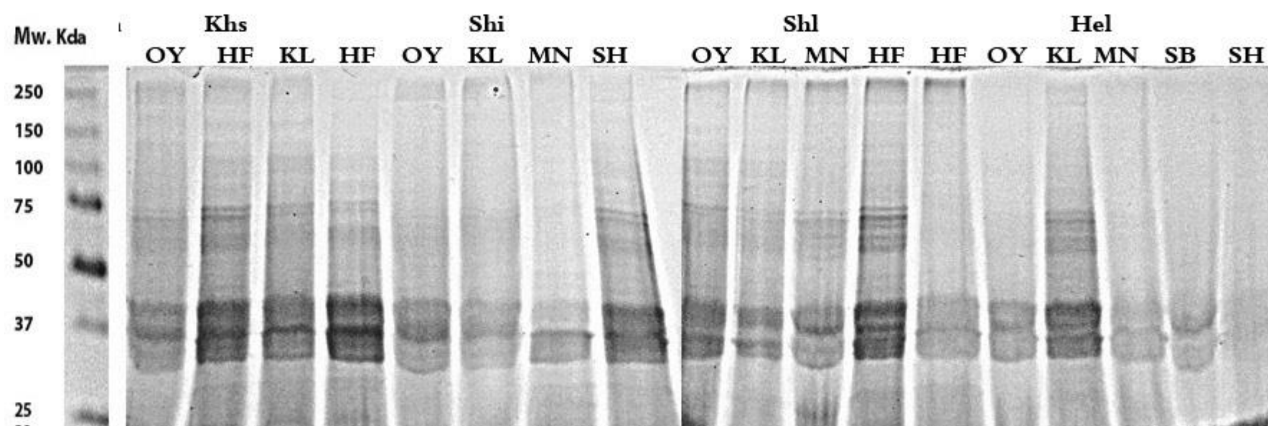

Figure 2. Electrophoretic protein patterns of the different cultivars from the different locations, with protein molecular weight standard marker.

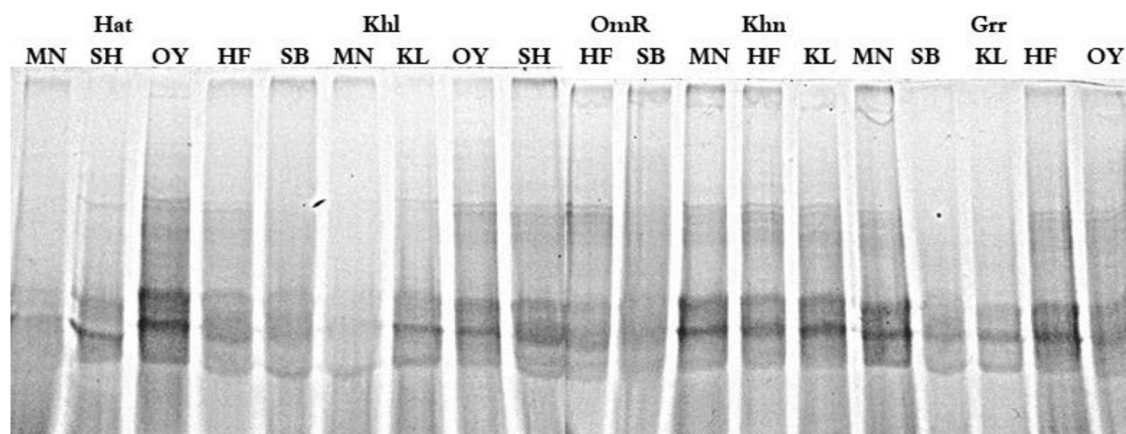

Figure 3. Electrophoretic protein patterns of the different cultivars from the different locations.

between 16 bands in OY location and 22 bands in HF location (Table 8); low polymorphism ratio 37% (Table 4) in Khn cultivar for 19 electrophoretic bands representing three locations because the electrophoretic bands varied between 14 bands in MN location and 18 bands in HF location (Table 9); low polymorphism ratio 25% (Table 4) in Khs cultivar for 20 electrophoretic bands representing three locations because the electrophoretic bands varied between 17 bands in KL location and 19 bands in HF location (Table 10); and very low polymorphism ratio 12% (Table 4) in OmR cultivar for 17 electrophoretic bands representing two locations because the electrophoretic bands varied between 15 bands in SB location to 17 bands in HF location (Table 11).

Phylogenetic analysis of protein patterns according to nearest neighbor single linkage hierarchical clustering dendrogram based on Jaccard's similarity, regarding the presence and absence of all bands for all the cultivars locations, showed that the different cultivar palms of the different locations were grouped within clusters on the phylogenetic tree (Figure 4).

The similarity between protein profiles according to Jaccard's similarity method, ranged between 0.059 and 0.579 degrees of similarity (Table 12).

The highest similarity value 0.579 was observed between Khs and Shl cultivars, and the least similarity value 0.059 was observed between Hat and Grr cultivars (Table 12). Phylogenetic analysis of protein patterns according to nearest neighbor single linkage hierarchical clustering dendrogram based on Jaccard's similarity, regarding the presence and absence of common bands for all of the cultivar locations (Figure 5) showed two clusters, in addition to two cultivars Khl and Hat, which were separated out singly.

The first cluster was composed of a sub-cluster, in addition to one line, the line represented by Khn cultivar. The sub-cluster was composed of a mini-cluster for two closely related cultivars, Khs and Shl, in addition to OmR

Table 2. The protein bands of Hatemi cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	MN[a]	SH[b]	OY[c]
193	O	O	I
142	O	O	I
128	I	I	I
99	I	I	I
91	I	I	I
77	I	I	I
67	I	I	I
44	I	O	I
41	I	I	I
38	I	I	I
37	O	I	O
35	I	O	I
32	O	I	I
30	I	I	I
26	I	I	I
Total band =15	11	11	14

a: Menaizlah; b: Ash Shgaig; c: Al Oyoon.

Table 3. The protein bands of Helali cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	HF[a]	OY[b]	KL[c]	MN[d]	SB[e]	SH[f]
180	I	O	I	O	O	O
170	I	O	I	O	O	O
154	I	O	I	O	O	O
128	I	O	O	I	I	I
116	I	I	I	I	I	I
112	I	O	I	I	I	I
105	I	O	O	O	O	I
99	O	O	I	O	I	I
95	I	O	O	O	I	O
91	O	I	O	I	I	O
86	O	I	I	O	O	O
82	O	O	I	I	O	O
77	O	O	I	I	O	O
71	O	I	I	I	O	I
67	O	I	I	O	O	O
62	I	I	I	I	I	I
60	O	I	O	O	O	I
53	O	O	I	I	O	O
50	I	O	I	O	O	O
44	I	I	I	I	I	I
38	I	I	I	I	I	I
35	I	I	I	I	I	I
32	I	I	I	I	I	I
28	O	I	I	I	I	I
26	I	O	I	O	O	O
Total band=25	15	12	20	14	12	13

a: Al Hafof; b: Al Oyoon; c: Al Kelabiah; d: Menaizlah; e: Ass Sabatt; f: Ash Shgaig.

Table 4. The polymorphism ratios of each cultivar for the different locations.

Cultivar	Khs	Shi	Shl	Hel	Hat	Khl	OmR	Khn	Grr
No. of samples	3	5	4	6	3	6	2	3	5
Total Bands	20	22	24	25	15	20	17	19	16
Monomorphic	15	7	15	6	9	6	15	12	9
Polymorphic	5	15	9	19	6	14	2	7	7
Polymorphism (%)	25	68	38	76	40	70	12	37	44

Table 5. The protein bands of Khalas cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	HF[a]	SB[b]	MN[c]	KL[d]	OY[e]	SH[f]
164	O	I	O	I	I	O
142	O	I	I	O	I	O
128	O	O	O	I	I	O
112	O	O	I	I	I	O
99	O	I	O	I	O	I
95	I	I	I	I	I	I
86	I	I	I	I	I	I
82	I	O	I	I	O	I
77	I	I	O	O	I	I
71	I	I	O	O	I	O
60	I	I	I	I	O	I
53	O	I	O	I	O	O
44	I	I	I	I	I	I
43	O	O	O	I	O	O
41	I	I	I	I	I	I
38	I	I	O	I	I	O
32	I	I	I	I	I	I
30	I	I	I	I	I	I
26	I	O	I	O	I	I
24	O	I	I	I	O	O
Total band=20	12	15	12	16	14	11
-	a:Al Hafof	b: Ass Sabatt	c: Menaizlah	d: Al Kelabiah	e: Al Oyoon	f: Ash Shgaig

a: Al Hafof; b: Ass Sabatt; c: Menaizlah; d: Al Kelabiah; e: Al Oyoon; f: Ash Shgaig.

cultivar, which was separated out individually. The second cluster composed of Shi, Grr and Hel cultivars.

Principal component analysis (PCA) revealed six components while two components were targeted, according to the variance results summarized in Table 14, because the first component represented 42.580% of the total variations, the second component represented 41.621% of the total variations, hence the cumulative value for the targeted components represented 84.201 % of the total variations. The results of Kaiser-Mayer-Olkin (KMO) and Bartlett's test for adequacy and sphericity were 0.761 and 0.025, respectively (Table 13).

However, the first component was characterized by three variables; 134, 122 and 82 KDa bands, while the second component was characterized by three variables; 180, 86 and 50 KDa bands (Table 15) because the component variables were mostly positioned close to each other, on the 2-dimensional scatter gram (Figure 6).

DISCUSSION

It is known locally that some palms of the same cultivar differ in the quality of their fruits, for example, Khalas cultivar palms at the Ash-Shgaig and Al Hafof locations, produce higher quality dates, which command higher prices in comparison with Khalas dates produced in the other locations, additionally, some Shishi cultivar dates were known locally as the surrounding dates, since the surrounding part at the base of the date, continue at the

Table 6. The protein bands of Shishi cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	HF[a]	OY[b]	KL[c]	MN[d]	SH[e]
170	O	I	O	O	O
134	O	I	I	O	I
128	I	O	I	O	O
105	I	O	O	I	O
95	I	I	O	O	I
91	I	O	O	O	I
86	I	O	O	O	I
82	O	I	I	O	O
77	I	I	I	I	I
71	I	I	I	I	I
67	O	I	O	O	I
60	I	I	I	O	O
53	I	I	I	I	I
50	O	O	I	I	I
44	I	I	I	I	I
41	O	O	O	I	O
38	I	I	I	I	I
35	I	I	I	I	I
32	I	I	I	I	I
28	I	O	O	I	O
26	I	I	O	O	O
25	I	O	O	I	I
Total band=22	16	14	12	12	14

a: Al Hafof; b: Al Oyoon; c: Al Kelabiah; d: Menaizlah; e: Ash Shgaig.

Table 7. The protein bands of Garrah cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	MN[a]	SB[b]	KL[c]	HF[d]	OY[e]
180	I	O	O	O	O
112	O	O	O	I	I
86	I	O	I	I	I
82	I	I	I	I	I
74	O	O	O	I	I
71	I	I	I	I	I
60	I	I	I	I	I
53	I	I	I	I	I
44	I	I	I	I	I
41	O	I	O	O	O
38	I	I	I	I	I
35	I	I	I	I	O
32	I	I	I	I	I
28	I	I	I	I	I
25	O	O	I	O	O
24	I	I	I	I	I
Total band=16	12	11	12	13	12

a: Menaizlah; b: Ass Sabatt; c: Al Kelabiah; d: Al Hafof; e: Al Oyoon.

Table 8. The protein bands of Shehil cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	OY[a]	KL[b]	MN[c]	HF[d]
180	O	O	I	I
170	O	O	I	I
154	I	I	I	I
134	I	I	I	I
128	O	O	O	I
122	I	I	I	I
112	I	I	I	I
99	I	I	I	I
86	I	I	I	I
82	I	I	I	I
77	O	O	I	I
71	I	I	I	I
67	I	I	I	I
62	O	O	I	O
60	O	I	I	O
53	O	I	O	I
50	I	I	I	I
44	I	I	I	I
38	I	I	I	I
35	I	I	I	I
34	O	O	O	I
32	I	I	I	I
28	I	O	O	I
24	I	I	I	I
Total band=24	16	17	20	22

a: Al Oyoon; b: Al Kelabiah; c: Menaizlah; d:Al Hafof.

bisser stage, not fully matured (tamer stage), which command higher prices in comparison with the regular Shishi dates, as well as, some Hilali cultivar dates were known locally as scratchy dates, since the texture was scratched, which command higher prices in comparison with the regular Hilali dates. These differences are in harmony with the results of the present study, since the polymorphism within Helali, Khalas and Shishi were 76, 70 and 68%, respectively (Table 4), even though the diversity of protein bands between varieties and within species were generally low (Hastuti et al., 2009; Khalifah et al., 2012). Due to the high polymorphism, 76% within Helali cultivar palms (Table 4), Hel at KL location and Hel at HF location were separated out of the Hel cluster (Figure 4), as well as the Hel at MN location and Hel at OY location were separated out of close related Hel at SB and SH locations, within the cluster of Hel cultivar, it could be concluded that the original Hilali cultivar is within this cluster, while it could assumed the Hel at KL location and Hel at HF location were seedling palms. The differences within Helali cultivar were reported regarding

Table 9. The protein bands of Khonaizi cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	MN[a]	HF[b]	KL[c]
180	I	I	I
170	I	I	O
134	O	I	O
128	I	I	O
105	I	I	I
91	I	I	I
86	I	I	I
82	O	I	O
77	I	I	I
71	I	I	I
67	O	I	I
60	I	I	I
53	I	I	I
50	I	I	I
44	O	O	I
38	I	I	I
35	O	I	I
32	I	I	I
28	I	I	I
Total band=19	14	18	15

a: Menaizlah; b: Al Hafof; c: Al Kelabiah.

Table 10. The protein bands of Khosab cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	OY[a]	HF[b]	KL[c]
180	I	I	I
164	I	O	O
154	O	I	I
134	I	I	I
122	I	I	I
112	I	I	I
99	O	I	I
86	I	I	I
82	I	I	I
71	I	I	O
67	I	I	I
60	I	I	O
53	I	I	I
50	I	I	I
44	I	I	I
38	I	I	I
35	I	I	I
32	I	I	I
30	I	I	I
26	I	I	I
Total band=20	18	19	17

a: Al Oyoon; b: Al Hafof; c: Al Kelabiah.

Table 11. The protein bands of OmRuhaim cultivar in the different locations represented by (I) present and (O) absent.

Mwt.	HF[a]	SB[b]
180	I	I
170	I	O
154	I	I
134	I	I
91	I	I
86	I	I
82	I	I
77	I	I
71	I	I
53	I	I
50	I	I
44	I	I
38	I	I
35	I	I
30	I	I
28	I	O
24	I	I
Total band=17	17	15

a: Al Hafof; b: Ass Sabatt.

of the ripening and season of harvesting (Al-Qurashi, 2010, Awad, 2006). Due to the high polymorphism 68% within Shishi cultivar palms (Table 4), Shi at OY location and Shi at KL location were separated as close related within a sub-cluster (Figure 4), where as Shi at HF location and Shi at SH location were included within the first sub-cluster with Khn cultivar palms, while Shi cultivar at MN location was separated out singly. It could be concluded that the close related palms, Shi at OY location and Shi at KL location representing the original Shishi cultivar, whether the regular or the surrounding Shishi, while it could assumed that the rest of Shishi palms were seedlings. The results of Shishi cultivar were in agreement with the results of previous study which revealed genetic differences between palms of the same cultivar grown in different locations (Al-Issa, 2006).

Due to the high polymorphism 70% within Khalas cultivar palms (Table 4), Khl at SH location and Khl at HF location were separated as close related within a Khl cluster (Figure 4), whereas the rest of Khl palms each represented by one line within the Khalas cluster, it could be concluded that the close related palms, Khl at SH location and Khl at HF location representing the original Khalas cultivar, which agree with what is known locally that Ash-Shgaig and Al Hafof locations, produce higher quality of Khalas dates, while in a previous study, different locations were not included in present study of date palms were proposed as representative for the original Khalas cultivar; Al-Oyouni, Al Omran, Al Taraf

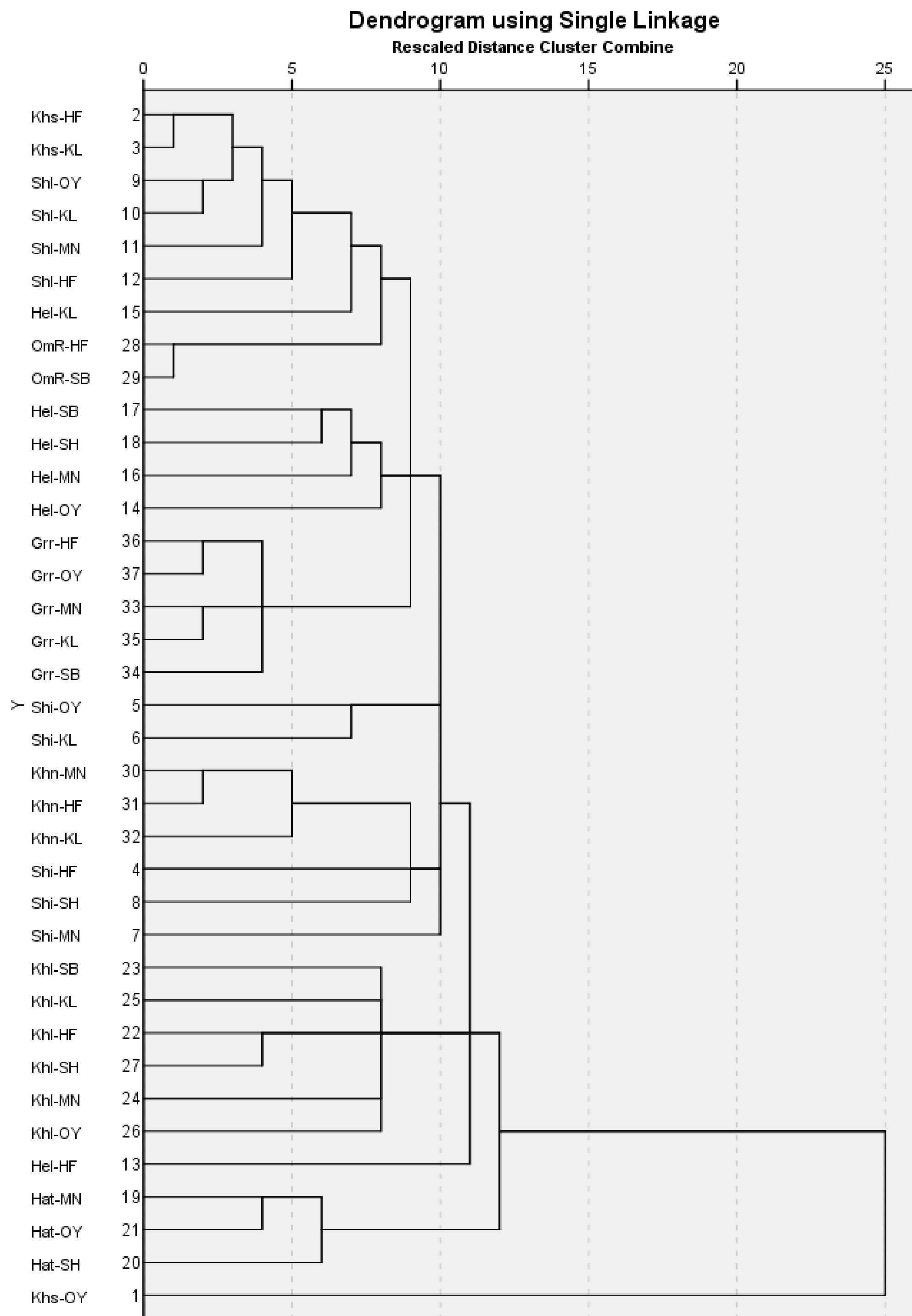

Figure 4. Hierarchical clustering dendrogram based on Jaccard's similarity showing genetic relationships between all the cultivars locations.

and Ashoa'bah (Al-Issa, 2013). It could be interpreted that the Khalas cultivar is an elite cultivar, and in high demand for its fruits and offshoots, therefore, some farmers treated seedlings produced from seeds as the original cultivar if they produced fruits similar to the original cultivar. This resulted in different lines of Khalas cultivar, which differed genetically, and in the quality of their dates, being sold as the Khalas cultivar. Representation of the Khalas cultivar was 15 to 20% of all Al-Ahsa palms in 1972 (Al-Baker, 1972), which later

Table 12. Data matrix of Jaccard`s Similarity analysis between Cultivars based on common bands.

Cultivars	1:Khs	2:Shi	3:Shl	4:Hel	5:Hat	6:Khl	7:OmR	8:Khn	9:Grr
1:Khs	1								
2:Shi	0.294	1							
3:Shl	0.579	0.294	1						
4:Hel	0.235	0.444	0.235	1					
5:Hat	0.200	0.143	0.143	0.071	1				
6:Khl	0.235	0.182	0.167	0.200	0.154	1			
7:OmR	0.286	0.357	0.227	0.125	0.167	0.125	1		
8:Khn	0.500	0.375	0.500	0.167	0.200	0.167	0.421	1	
9:Grr	0.263	0.455	0.333	0.250	0.059	0.154	0.400	0.333	1

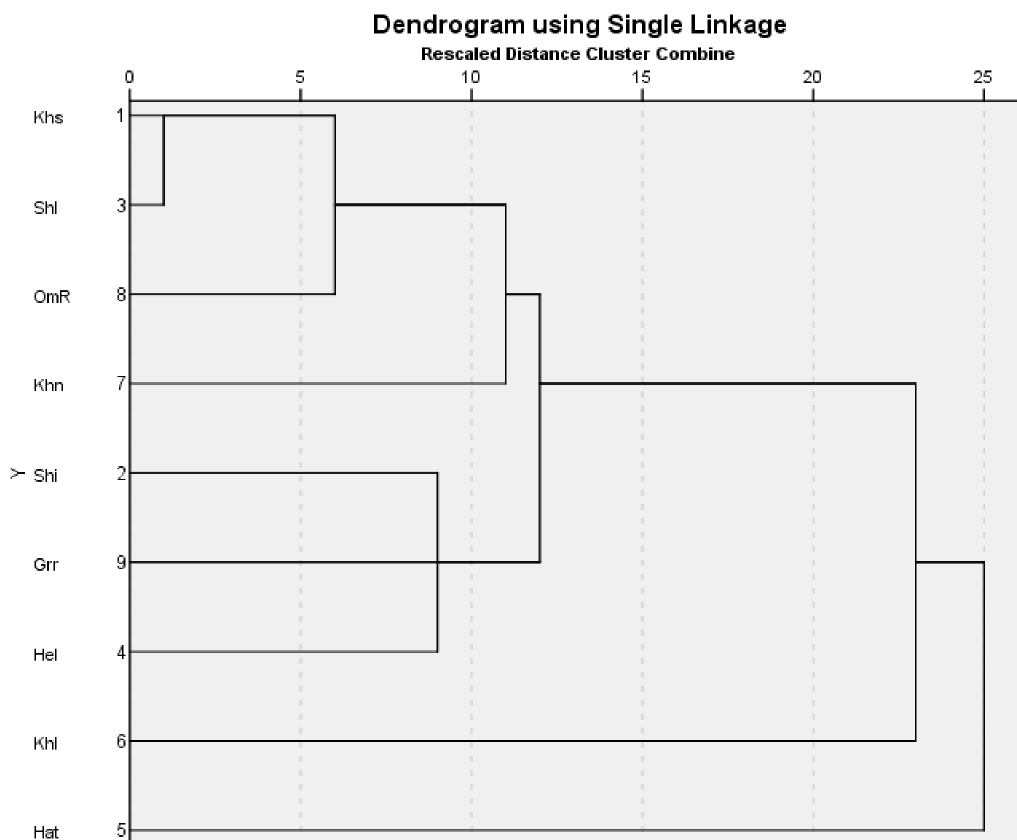

Figure 5. Hierarchical clustering dendrogram based on Jaccard's similarity showing genetic relationships between the cultivars.

Table 13. The KMO and Bartlett's tests for adequacy and sphericity.

Parameter		Value
Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		0.761
Bartlett's Test of Sphericity	Approx. Chi-square	27.498
	df	15
	Sig.	0.025

Table 14. Eigenvalues of 6 components, % of variance, % of cumulative variation for each component, first three components targeted.

Component	Total variance explained								
	Initial Eigenvalues			Extraction sums of squared loadings			Rotation sums of squared loadings		
	Total	% of Variance	Cumulative (%)	Total	% of Variance	Cumulative (%)	Total	% of Variance	Cumulative (%)
1	3.972	66.202	66.202	3.972	66.202	66.202	2.555	42.580	42.580
2	1.080	17.999	84.201	1.080	17.999	84.201	2.497	41.621	84.201
3	0.504	8.395	92.596	-	-	-	-	-	-
4	0.252	4.193	96.789	-	-	-	-	-	-
5	0.114	1.894	98.683	-	-	-	-	-	-
6	0.079	1.317	100.000	-	-	-	-	-	-

Table 15. First two components represented by the variables and their loadings.

Rotated component matrix[a]	Component	
	1	2
Kda_180	0.071	0.930
Kda_134	0.834	0.470
Kda_122	0.875	0.173
Kda_86	0.329	0814
Kda_82	0.856	0.182
Kda_50	0.498	0.829

Extraction Method: Principal Component Analysis. [a]Rotation converged in 3 iterations. Rotation Method: Varimax with Kaiser Normalization.

became more than 50% of the oasis area (Al-Abbad et al., 2011). In general these results indicate that some palms were grown from seeds, and produced fruits similar to the fruits of the original cultivar, which were then sold and distributed as the original cultivar (Devanand and Chao, 2003). The present study revealed differences between the cultivars, based on common electrophoretic bands, and these data passed the KMO and Bartlett tests for adequacy and sphericity, with 0.761 and 0.025, respectively, meaning that they are valid for principal component analysis (Andy, 2005). Khs and Shl cultivars were closely related among the studied cultivars with the degree of similarity being 0.579 (Table 12), separated by phylogenetic dendrogram in a mini-cluster (Figure 5), as well as confirmed by the first principal component with three variables 134, 122 and 82 KDa bands (Table 15), with high loading 42.580% (Table 14), which agree with Elmeer et al. (2011), who additionally revealed that the previous mini-cluster of Khs and Shl cultivars is related to OmR cultivar with degrees of similarities 0.286 and 0.227, respectively (Table 12) because they were separated by phylogenetic dendrogram in a sub-cluster (Figure 5), which is confirmed for Khs cultivar by the second principal component with three variables 180, 86 and 50 KDa bands (Table 15), with high loading 41.621% (Table 14), because the component variables collected together on the 2-dimensional scatter gram (Figure 6) agree with Al-Issa (2015). Previous studies reported that clusters constructed by phylogenetic dendrograms can be confirmed using principal component analysis (Ahmed and Al Qaradhawi,

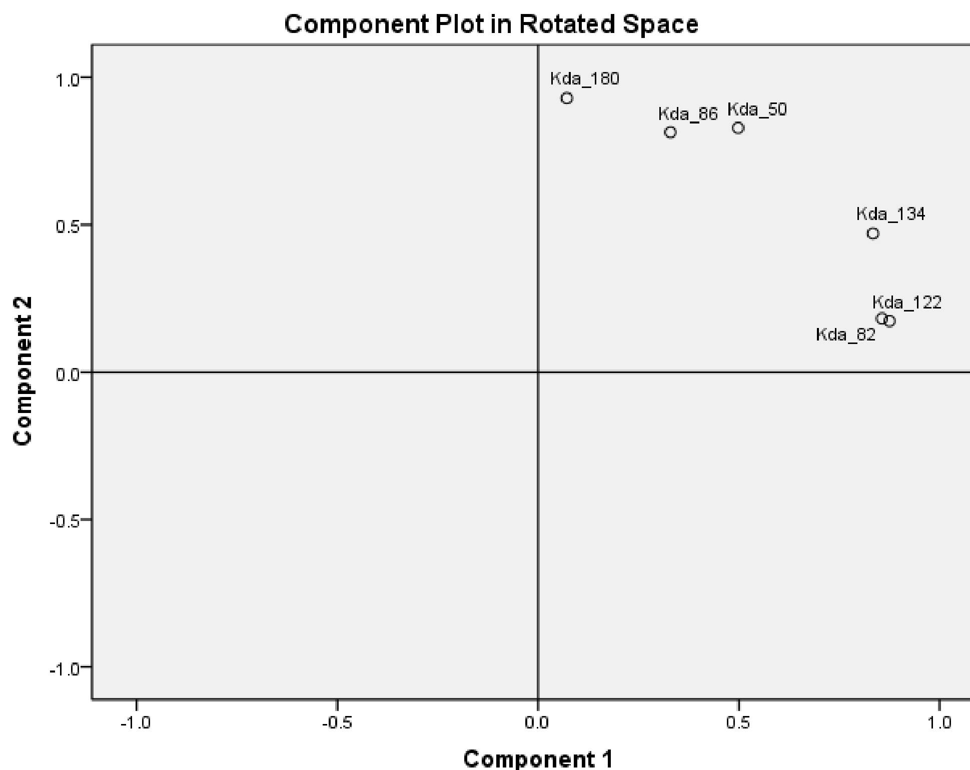

Figure 6. Scatter diagram of electrophoretic bands according to the first two components.

2009; Attaha et al., 2013). The results revealed that the Khs, Shl and OmR cultivars were close related to Khn cultivar, with degrees of similarities ranging between 0.375 and 0.500 (Table 12) because they were separated in one cluster by phylogenetic dendrogram (Figure 5), which agree with Elmeer et al. (2011), but disagree with Al-Issa (2015). The results revealed that the Shi, Grr and Hel were related cultivars, with degrees of similarities ranging between 0.250 and 0.455 (Table 12) because they were separated in one cluster by phylogenetic dendrogram (Figure 5) which agree with Elmeer et al. (2011) but disagree with Al-Issa (2015). Khl cultivar was separated out individually, followed by Hat cultivar (Figure 5), which agrees with Ahmed and Al Qaradhawi (2009). It could be concluded that Khs, Shl, OmR and Khn cultivars, share a common ancestor, such results have been reported previously (Ahmed and Al Qaradhawi, 2009; Elmeer et al., 2011). Based on gene expression the results revealed an allelic polymorphism, according to the common bands; the 128 KDa band was specific for Hat cultivar, the 116 and 62 KDa bands were specific for Hel cultivar, the 95 KDa band was specific for Khl cultivar, and the 105 KDa band was specific for Khn cultivar. These bands may be suitable as genetic markers for cultivar identification, even though environmental factors have been reported to affect protein profiles (Hanna et al., 2003; Kong-mgern et al., 2005). The 193 KDa band in the Hat cultivar at OY location, 37 KDa band in the Hat cultivar at SH location, 43 KDa band in the Khl cultivar at KL location and 34 KDa band in the Shl cultivar at HF location, which appeared unique among all studied cultivars, might indicate that the cultivar palm is a mutant, and useful for breeding programs, as has been reported previously (Al-Helal, 1992). It may be concluded that these results provide helpful basic information for breeding programs to improve the fruit characteristics of date palms. However, more biochemical and molecular studies are needed to confirm the genetic relationships within and between date palm cultivars and to identify the cultivars of the Al-Ahsa oasis and surrounding areas. Differences observed in the quality of dates from some cultivars may be due to genetic differences. Hence, comprehensive studies are recommended to ascertain the true-to-type characteristics of each cultivar.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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