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Review

Technical review of molecular markers and next-generation sequencing technology to manage plant pathogenic oomycetes

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To feed the world human population which is expected to reach 10 billion in the next three decades, agricultural sustainability is required for stable food production. However, crops always suffer from many biotic and abiotic stresses. Among them, plant pathogens often cause severe yield losses. Plant pathogenic oomycetes are one of the most destructive plant diseases, which include *Phytophthora infestans* in potato and tomato, *Phytophthora capsici* in peppers, *Phytophthora sojae* in soybean, *Phytophthora fragariae* var. *fragariae* in strawberry, *Plasmopara viticola* in grapevine, and *Pseudoperonospora cubensis* in cucurbits. Therefore, rapid, robust and sophisticated molecular technologies are required for accurate identification and characterization of the oomycetes, to manage crop diseases resistances. In addition, breeding highly disease resistant varieties is also essential for sustainable agriculture. Molecular marker technology, especially recent advanced next-generation sequencing-based methods, would provide helpful information to monitor the pathogen endemicity and to breed the resistant cultivars through a gene pyramiding strategy. In this review, there was focus on both conventional and novel genotyping techniques for oomycete characterization and resistant gene identification in crops, to discuss future outlook for successful disease management.

Key words: Oomycete plant pathogens, vegetable crops, genomics, molecular markers, next-generation sequencing technology.

INTRODUCTION

The world human population is expected to reach approximately 10 billion by 2050 (UN, 2015). To meet the challenges of poverty and the rising population, food production must be increased by at least 70% over the next three decades. Agricultural sustainability is threatened by a number of limiting factors such as water and nutrient deficiencies, infestations of insects and nematodes, and infections of plant pathogenic viruses, bacteria, fungi and oomycetes. Especially, the plant pathogens are responsible for severe yield losses in a wide range of crops throughout the world. Besides, global trade of crops among countries leads to the rapid spread

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Table 1. Characteristics of selected DNA-based molecular markers.

Marker	Dominance	Reproducibility	Precision	Speed	Relative amount of template DNA needed	Prior sequence information needed for primer design?	Restriction enzyme needed?
RFLP	Co-dominant	High	Medium	Low	High	No	Yes
RAPD	Dominant	Low	Low	High	Low	No	No
AFLP	Dominant	High	Medium	Low	High	No	Yes
SSR	Co-dominant	High	High	High	Low	Yes	No
ISSR	Dominant	Low	High	High	Low	No	No
SCAR	Co-dominant	High	High	High	Low	Yes	No
CAPS	Co-dominant	High	High	High	Low	Yes	Yes
SNP	Co-dominant	High	High	Very high	Low	Yes	No
SRAP	Dominant	High	High	High	Low	No	No
DNA sequencing	Co-dominant	High	High	Very high	Low	Yes	Yes

of plant pathogenic microorganisms and unprecedented disease outbreaks in hitherto unaffected croplands.

Molecular markers can be used for identification and taxonomic classification of species across all domains of life (Vignal et al., 2002; Singh et al., 2013). In modern plant breeding programs, a gene pyramiding strategies based on marker-assisted selection (MAS) can facilitate development of new varieties with desirable traits such as disease resistances. In addition, the molecular techniques help in discovering an array of plant disease resistance genes, which have been used for the management of several serious plant pathogens (Gururani et al., 2012). Furthermore, greater understanding of interactions between pathogens and host plants could facilitate disease outbreak forecasting and predictions of yields. In this decade, genome sequencing technology as well as the molecular marker techniques has been greatly advanced due to great advancements of next-generation sequencing (NGS) methods (Davey et al., 2011). For example, whole-genome sequencing analysis of many microorganisms including plant pathogens has contributed to understanding of pathogenicity, host preferences, secreted effector proteins and fungicide resistances of the pathogens (Grunwald et al., 2016). In this review, genomics of oomycete plant pathogens in important crops are summarized for future breeding, to overcome the disease-derived yield losses.

ADVANCEMENT OF DNA MARKER TECHNOLOGIES

Traditional morphological and biochemical markers are hampered by their reliance on particular factors, for example, developmental stages and environmental conditions. In contrast, DNA markers provide stable results independent of the factors, hence, DNA analysis is the basis of a range of techniques in basic and applied researches (Collard et al., 2005). Since the 1980s, many types of DNA markers have been developed in accordance with advancement of DNA analysis technologies, for example, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), sequence characterized amplified region (SCAR), sequence related amplified polymorphism (SRAP) and cleaved amplified polymorphic sequence (CAPS) (Table 1). The DNA markers are used to detect polymorphisms between individuals in populations, determine genetic biodiversity among genotypes, and investigate plant-pathogen interactions (Patwardhan et al., 2014).

Due to NGS technology, genome-wide SNP discovery and genotyping have been enabled with high precision and accuracy, high-throughput performance and cost-effectiveness (Yang et al., 2016). This situation has made the classical DNA markers mentioned above (Vos et al., 1995; Jarne and Lagoda, 1996) to suffer from some constraints such as time- and cost-consuming.

In Figure 1, a possible experimental approach for genotyping, SNP discovery, and gene

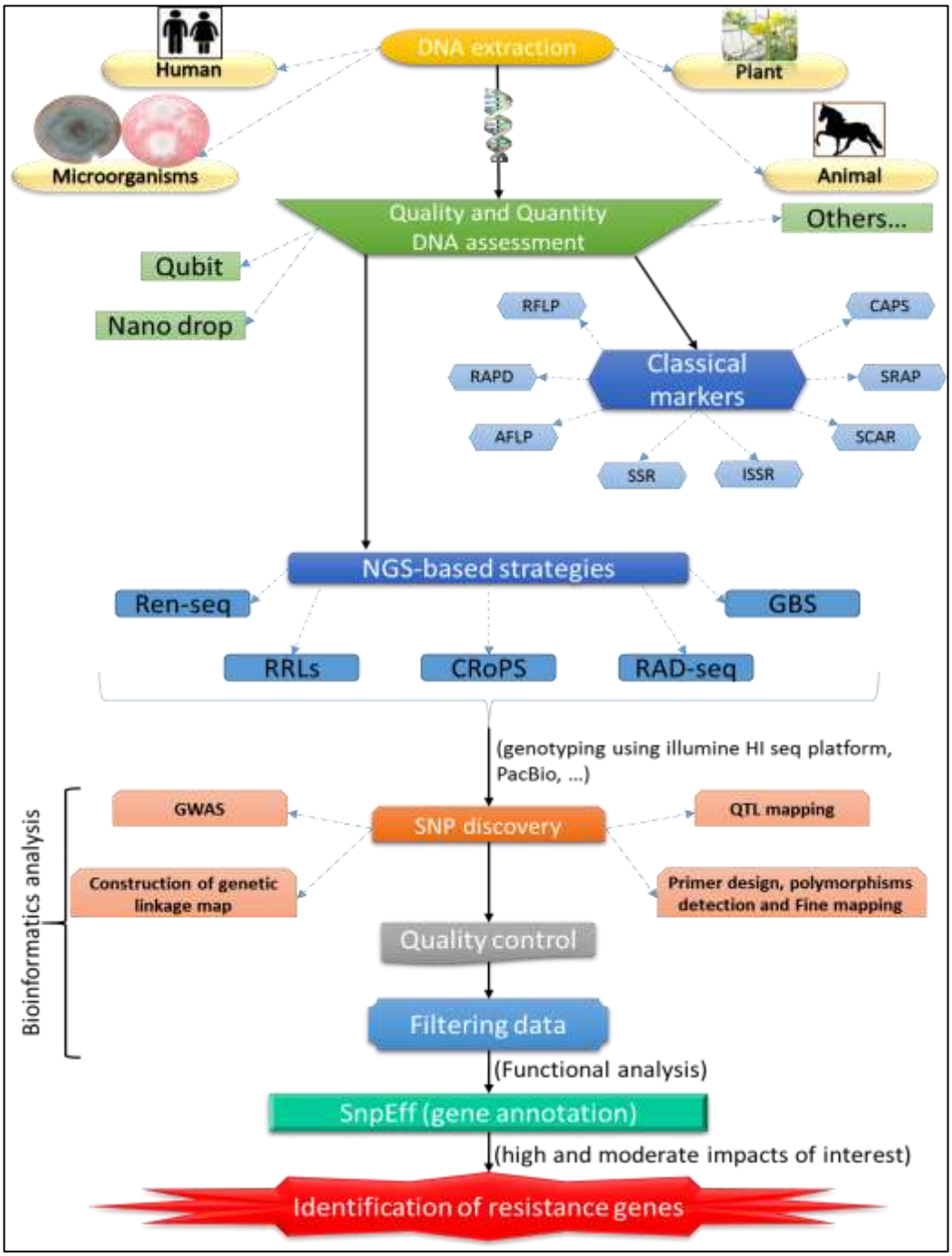

Figure 1. Workflow steps of NGS technology during identification of SNP markers and resistance loci.

identification with NGS strategies is presented. Even in non-model organisms in which genome sequences are not available (Baxter et al., 2011), NGS-based genome-wide genotyping technology has been widely applied for studies on SNP discovery, genetic variability, QTL mapping, candidate gene detection and genome-wide association study (GWAS) (Fu et al., 2014). NGS-based methods are suitable for genome-wide genotyping across large numbers of individuals, for example, reduced-representation libraries (RRLs) (Altshuler et al., 2000), complexity reduction of polymorphic sequences (CRoPS) (van Orsouw et al., 2007), restriction site-associated DNA sequencing (RAD-seq) (Baird et al., 2008) and genotyping-by-sequencing (GBS) (Elshire et al., 2011). In RRL sequencing technology, which has been originally developed in the human genome project (Altshuler et al.,

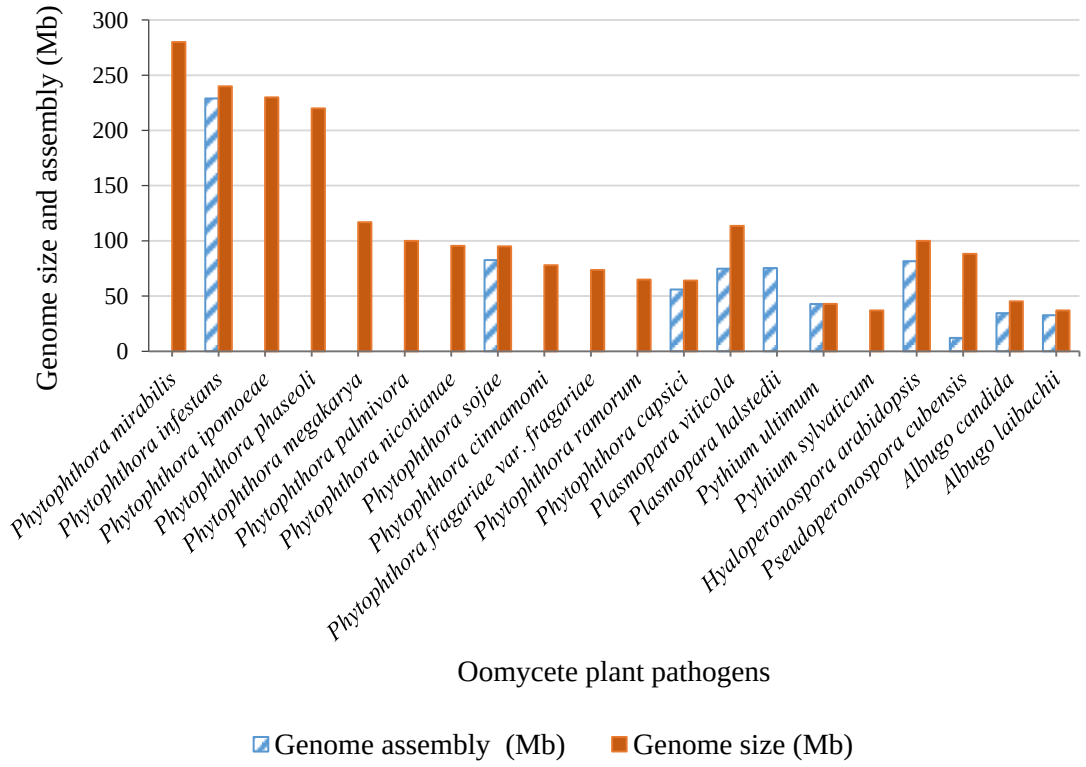

Figure 2. The genome size and assembly of selected plant pathogenic oomycetes.

2000), small genomic regions are sequenced for SNP discovery and genotyping. These techniques have been applied in other organisms including plants and animials (Van Tassell et al., 2008; Wiedmann et al., 2008; Gore et al., 2009; Hyten et al., 2010). On the other hand, CRoPS technology, another reduced-representation method, has mitigated the amounts of sequencing data to identify polymorphism in populations (van Orsouw et al., 2007). In RAD-Seq procedure, genome DNAs are cut with restriction enzymes into short DNA fragments, and sequenced to discover and genotype genome-wide SNPs (Baird et al., 2008). GBS is another approach for genotyping and developing novel molecular markers (Sonah et al., 2013; He et al., 2014). The library preparation is affordable, straightforward, rapid and precise (Elshire et al., 2011; Romay et al., 2013). Furthermore, in the fields of plant pathology and microbiology, through the NGS-based approaches, novel molecular markers associated with resistance genes were broadly identified (Devran et al., 2015). For example, resistance gene enrichment sequencing (RenSeq) is an effective genotyping technique with NGS technologies, in sequence variations of nucleotide binding-site leucine-rich repeat (NB-LRR) genes simultaneously identified (Jupe et al., 2013). Subsequently, the RenSeq approach has been widely applied to identify resistance genes in wheat and potato (Jupe et al., 2013; Steuernagel et al., 2016).

IDENTIFICATION OF KEY INTERACTIVE GENES BETWEEN PLANTS AND OOMYCETE PATHOGENS

Oomycetes are fungus-like eukaryotic microorganisms, many of which are pathogens to humans, animals and plants. Plant pathogenic oomycetes pose substantial threats to global food security. Among the oomycetes, *Phytophthora* is one of the largest genera containing almost 117 species (Martin et al., 2012), many of which cause severe disease outbreaks in horticultural, fruit, arable crops, forest trees and ornamental plants. In addition, *Plasmopara* and *Pseudoperonospora* also thrive on susceptible hosts and pose substantial risks to agriculture worldwide. To overcome the losses from the diseases, breeding new cultivars with resistance (R) genes to the pathogens is an effective strategy. However, the R-gene-derived resistances sometimes decay because of alterations of the plant pathogenic effector proteins, which suppress plant immunity system and modulate host cell functions (Hogenhout et al., 2009). Therefore, to combat the diseases completely, it there is need to understand molecular mechanisms of disease resistance as well as population dynamics of plant pathogens upon the temporal and spatial levels. The genome sequences of the several oomycete pathogens, sizes of which varied depending on the genera (Figure 2), would be useful for this purpose. In addition, methods to monitor and predict alternations of the effector genes are

Table 2. List of tomato late blight, *Phytophthora infestans* resistance genes.

S/N	Gene name	Wilde type	Accession	Chromosome	References
1	*Ph-1*	*Solanum pimpinellifolium*	West Virginia 19 and 731	7	Peirce (1971)
2	*Ph-2*	*S. pimpinellifolium*	West Virginia 700	10	Moreau et al. (1998)
3	*Ph-3*	*S. pimpinellifolium*	L3708	9	Chunwongse et al. (2002)
4	*Ph-4*	*S. habrochaites*	LA1033	2	Kole et al. (2006)
5	*Ph-5.1*	*S. pimpinellifolium*	PI270443	1	Merk et al. (2012) and Merk and Foolad (2012)
6	*Ph-5.2*	*S. pimpinellifolium*	PI270443	10	Merk et al. (2012) and Merk and Foolad (2012)

also essential. Also, genetic and genomic analyses of plant pathogens would lead to new strategies for integrated disease management of high aggressive races, which would breakdown existing resistant varieties. The current status of knowledge on important crops and the corresponding pathogens are summarized as follows.

Tomato and potato- *Phytophthora infestans*

Late blight of tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) are caused by the heterothallic oomycete pathogen, *P. infestans* (Mont.) de Bary, also known as a pathogen of the Irish potato famine in the 1840s (Aragaki and Uchida, 2001; Abad and Abad, 2003). *P. infestans* can completely destroy tomato and potato plants within a few days after infection. Unfortunately, pathogenic races of *P. infestans* have been altered in each season and growing areas (Arafa et al., 2017). Therefore, multiple resistance varieties of tomato and potato have been developed by introgression of R genes from the wild relatives. In tomato, six major and race-specific R genes have been identified from the wild relatives, which are *Ph-1*, *Ph-2*, *Ph-3*, *Ph-4*, *Ph-5.1* and *Ph-5.2* (Table 2). Among them, *Ph-3* derived from *Solanum pimpinellifolium* L3708 confers a high level resistance against a broad-spectrum of *P. infestans* genotypes. On the other hand, in potato, eleven resistance genes (*R1*–*R11*) were identified from a wild potato relative, *S. demissum*, and *R1*, *R2*, *R3*, *R4* and *R10* were broadly used in potato breeding programs (Vleeshouwers et al., 2011). Furthermore, new QTLs for *P. infestans* resistance have been reported in *S. pimpinellifolium*, *Solanum habrochaites*, and *Solanum pennellii* for tomato, and in *Solanum bulbocastanum* and *Solanum phureja* for potato. Moreover, RAD-Seq technology was used to identify SNP markers from a tomato wild relative, *S. pimpinellifolium* (Chen et al., 2014). Genetic linkage maps were constructed, and a QTL associated with late blight resistance was detected on chromosome 2. Recently, a resistance locus to an Egyptian isolate of *P. infestans* from *S. habrochaites* (Arafa et al. 2018) was also identified, in which the established analytic pipeline for ddRAD-Seq was employed (Shirasawa et al., 2016). However, most of these resistance genes might be subsequently disrupted by new pathotypes, which have been generated due to the unstableness of the *P. infestans* genomes caused by movements of transposable elements, mitotic recombinations and dispensable chromosomes (Judelson, 2002). Therefore, rapid detection methods and easy monitoring technologies of late blight would be beneficial for pathogen control to reduce yield losses of crops. For example, mitochondrial DNA (mtDNA) haplotypes detected by the RG57 RFLP marker as well as RAPD and AFLP have been used as DNA makers to examine the population diversity of *P. infestans* isolates. Subsequently, SSRs and SNPs have been also applied to investigate population structures and to monitor alterations of effector proteins of *P. infestans*. More recently, NGS-based genotyping methods including reduced representative sequencing techniques and whole-genome shotgun (WGS) approaches are used to characterize *P. infestans* isolates. A GBS approach has also been applied to detect genetic variability within four clonal lineages (US-8, US-11, US-23 and US-24) of *P. infestans*, where 3,774, 4,363, 5,070 and 4,353 SNPs were discovered, respectively (Hansen et al., 2016). These findings are considered a clear indication that the GBS method is adequately a useful method for high-resolution analysis of population structure of *P. infestans*, which would contribute to reduction in epidemiology of late blight globally. Furthermore, whole-genome resequencing strategy has also been used for six genomes of four *Phytophthora* species (*P. infestans, P. ipomoeae, P. mirabilis* and *P. phaseoli*) to detect 746,744 SNPs and to estimate genomic evolutionary rates in the genus, *Phytophthora* (Raffaele et al., 2010). This study suggested that the evolutionary level among the tested isolates

fluctuated based on copy number variations (CNVs), frequency of SNPs and the ratio of non-synonymous to synonymous substitutions. Yoshida et al. (2013) also used the genome sequencing approach to compare between ancient and modern populations of *P. infestans*, to detect 4.5 million SNPs and follow up the historical trajectory of clonal linages to comprehend the epidemiology of this destructive plant pathogen. They also presented the evolutionary process of *P. infestans* populations since the occurrence of Irish potato famine.

Pepper- *Phytophthora capsici*

P. capsici is a soil-borne plant pathogenic oomycete; this pathogen infects a wide range of host crops. *P. capsici* is considered as a major limiting factor for crop productions (Lamour and Kamoun, 2009; Roy et al., 2009; Zeng et al., 2009). Especially in pepper (*Capsicum annuum*), root, stem and foliar blights are caused by *P. capsici*. In the pepper genome, resistance loci for *P. capsici*, *Phyt-1*, *Phyt-2* and *Phyt-3* as well as *Phyto.5.2*, have been identified through a QTL mapping research. Recently, a novel resistance gene, *PhR10*, has been identified using NGS technology with Illumina HiSeq 2500 platform (Xu et al., 2016). This race specific gene can be used for breeding programs for resistant varieties to *Phytophthora* root rot with a marker-assisted selection. However, *P. capsici* readily undergoes sexual reproduction to develop new races. The new genotypes adapt to challenging environmental conditions by breakdown of pesticides. Therefore, it is important to assess *P. capsici* population dynamics and to identify new races rapidly. To date, molecular markers based on RFLP (Forster et al., 1990), mtDNA haplotypes (Martin et al., 2012), RAPDs (Yin et al., 2012), AFLPs (Hulvey et al., 2010), SSRs (del Castillo-Múnera et al., 2013) and SNPs (Gobena et al., 2012) have been available. More recently, Fulcher et al. (2014) applied the GBS approach for genotyping of *P. capsici* population to discover 368,356 SNPs. GBS has been also used to investigate population structure and genetic dynamics of *P. capsici* where 23,485 high-quality SNPs tightly linked to temporal dynamics and mating types are identified over the genome of *P. capsici* (Carlson et al., 2017).

Soybean- *Phytophthora sojae*

Root rot in soybean (*Glycine max*) is caused by *P. sojae* Kaufm. and Gerd. *P. sojae* attacks soybean plants at all developmental stages from seedling to harvest across a range of varieties (Malvick and Grunden, 2004; Kato, 2010). Soybean has at least 14 R genes, which have been used to develop *P. sojae* resistant cultivars (Burnham et al., 2003). Two R genes, *Rps8* and *Rps3*, tightly linked to each other can be used to breed new varieties, conferring durable resistance through the gene pyramiding strategy. QTL analyses have shown new resistance gene loci, *Rps1-k* (Kasuga et al., 1997; Salimath and Bhattacharyya, 1999) and *RpsYu25* (Sun et al., 2011), and novel QTLs indicating partial resistance for *Phytophthora* root and stem rot (Lee et al., 2013). The partial resistance loci have been also reported on chromosomes 3, 13 and 19 where seven QTLs were detected (Schneider et al., 2016). Therefore, pyramiding many minor genes as well as usage of R genes are efficacious ways to increase the resistance level to *P. sojae*. On the other hand, an avirulence gene, *Avr1a* in *Phytophthora* has been identified in the genome of *Ph. sojae*. This information would be useful to understand interactions between soybean and *P. sojae* (MacGregor et al., 2002). A whole genome resequencing analysis has been performed to predict effects of sequence variations on the functions of the avirulence genes, *Avr1a* and *Avr1c* (Na et al., 2014). Additionally, this study confirmed that NGS-based methods are workable techniques for breeding programs, and genetic and genomics research could be widely applied in release soybean breeding to increase crop immune system against oomycete pathogens.

Strawberry- *Phytophthora fragariae var. fragariae*

P. fragariae Hickman var. *fragariae* Wilcox & Duncan causes red core disease in *Rubus* species (Wilcox, 1989) including strawberry (*Fragaria* x *ananassa*) (Hickman, 1941), which leads to complete destruction and death of the plants. The genome sequence of *P. fragariae* is available to understand virulence, aggressiveness and evolution of this destructive pathogen (Gao et al., 2015). In strawberry, two R genes, *Rpf1* and *Rpf2*, have been reported as resistance loci (Haymes et al., 1997; Haymes et al., 2000; Gelvonauskienė et al., 2007; Mathey, 2013; Van de Weg, 1997).

Grapevine- *Plasmopara viticola*

In grapevine (*Vitis vinifera*), downy mildew disease is caused by *P. viticola* (Berk. and Curt.) Berl. and de Toni. This pathogen can infect all the green tissues of grapevine, causing substantial losses in crop productivity and quality (Gessler et al., 2011). An R gene, *Rpv3*, is responsible for the hypersensitive response against *P. viticola* in resistant grapevine genotypes (Bellin et al., 2009). Another R gene, *Rpv8*, a major QTL responsible for *P. viticola* resistance, has been identified from a grape wild relative, *Vitis amurensis* Rupr. Pyramiding of two genes, *Rpv3* and *Rpv12*+, in one line was an effective strategy to overcome downy mildew disease (Venuti et al., 2013). However, *P. viticola* exhibits extensive genetic variability (Gobbin et al., 2006), and several genotypes

3. Molecular markers used in characterization of oomycete plant pathogens.

Pathogen	Disease	Number of isolates investigated	Molecular marker	Number of polymorphic molecular markers	Number of groups obtained	Isolate origins	References
Phytophthora infestans	Late blight	326	RAPD	9	19	Canada	Punja et al. (1998)
		170	AFLPs	135	No data	Mexico	Flier et al. (2003)
		655	RFLP RG-57 probe	1	3 (8)[a]	Taiwan	Chen et al. (2009)
		32	SNP	102 by objective criteria 167 by eye	No data	Africa, Asia, Europe, North America and South America	Abbott et al. (2010)
		100	RFLP RG-57 probe	1	No data	China	Guo et al. (2010)
		104	RAPD	6	10	China, Korea and Japan	Xuanzhe and Shengjun (2010)
		200	SSR	9	169	Nordic European countries	Brurberg et al. (2011)

can be discovered from a single field (Gobbin et al., 2003). Therefore, a high-throughput genotyping is required to gain new insight into the genetic structure of *P. viticola* population (Stark-Urnau et al., 2000). For example, SNP and SSR markers have been used for the genetic variation study of *P. viticola* strains (Delmotte et al., 2011). Recently, Yin et al. (2017) employed the whole genome sequencing approach to identify pathogenicity genes and effector proteins that are associated with virulence of *P. viticola*. Moreover, NGS-based methods clarifies the origin and evolution patterns of *P. viticola*, which is completely different from *Hyaloperonospora arabidopsidis*, a pathogen for *Arabidopsis* downy mildew.

Cucurbitaceae- *Pseudoperonospora cubensis*

The family Cucurbitaceae suffers from approximately 45 diseases caused by viruses, bacteria, fungi, and oomycetes (Lebeda et al., 2006). Among them, *P. cubensis* [(Berk. and Curt.) Rost.] causes downy mildew disease. *P. cubensis* infects approximately 20 cucurbit genera, including cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima*), squash (*Cucurbita pepo*) and melon (*Cucumis melo*) (Lebeda and Urban, 2007). The GBS technique distinguishes *P. cubensis* from the relative, *Pseudoperonospora humuli* (Summers et al., 2015; Lee et al., 2016). Numbers of QTLs for *P. cubensis* resistance has been identified in cucumber (Pang et al., 2013; Yoshioka et al., 2014), while a resistance locus, *ILdm*, has been found from a wild relative of cucumber, *Cucumis hystrix* (Guo et al., 2011).

FUTURE DIRECTION OF BREEDING STRATEGIES FOR PLANT DISEASE RESISTANCE

Plant diseases are one of the main threats to global food security and sustainable agriculture. Identifying and tracking oomycete plant pathogens are critical for breeding programs for disease resistances in a range of crop species. Moreover, identification of resistance genes for crops would be required for effective integrated disease management (Table 3).

The plant disease resistances could be classified into two major categories: (i) qualitative or race-specific resistance (vertical resistance) controlled by single resistance genes (major genes or R genes), and (ii) quantitative resistance or field resistance (horizontal resistance) regulated by multiple minor genes (Poland et al., 2009). Therefore, the gene pyramiding strategy has a potential to develop varieties with durable resistance against multiple plant pathogens.

To understand molecular mechanisms of plant disease infection, responses of plants, and the interaction from both aspects of pathogens and hosts would be essential to control plant disease and maintain stability of food productions. Advancement of NGS technology enables analyzing genetic variations of pathogens and crops at whole genome level. The information would provide a beneficial knowledge in both evolutionary researches on oomycete pathogens

Table 3. Contd.

		117	mtDNA	Not available	1	Thailand	Jaimasit and Prakob (2011)
		134	SSR	15	40	China	Wu et al. (2012)
		119	SSR	12	11	United Kingdom	Stroud et al. (2015)
		24	RAPD	7	6	Illinois	Islam et al. (2005)
Phytophthora capsici	Phytophthora root rot	41	SNP	8	No data	Argentina	Gobena et al. (2012)
		51	ISSR	13	7	China	Li et al. (2012)
		98	SSR	193	2 (10)	China	Pei-Qing et al. (2013)
		400[b]	RAPD and RFLP	250	22	Australia	Whisson et al. (1995)
		99	RFLP	5	15	Australia and USA	Drenth et al. (1996)
Phytophthora sojae	Root rot	55	RAPD	23	4	Illinois, Indiana, Iowa and Minnesota	Meng et al. (1999)
		558[c]	AFLP, CAP and RAPD	16	No data	USA	MacGregor et al. (2002)
		96	RAPD	2	79	Germany	Stark-Urnau et al. (2000)
		97	SSR	4	15	Italy	Gobbin et al. (2003)
Plasmopara viticola	Downy mildew	54	AFLP and SSR	200	43	Germany, Italy, France and Switzerland	Scherer and Gisi (2006)
		93	SSR	1	234	Japan	Mochizuki et al. (2012)
		96	SSR	35	89	France, Germany and USA	Rouxel et al. (2012)
		30	AFLP	4	No data	Greece, Czech, Netherlands, and France	Sarris et al. (2009)
Pseudoperonospora cubensis	Downy mildew	262	SSR	5	5	Canada and USA	Naegele et al. (2015)
		78	ISSR and SRAP	24	No data	Turkey, Israel and the Czech Republic	Polat et al. (2014)
		No data	SNP	7	No data	South Korea	Lee et al. (2016)

[a]Numbers in parentheses indicate sub-groups and sub-genotypes; [b]F2 populations of two crosses (200 individuals for each cross) between different races of *Phytophthora sojae*; [c]F2 populations generated from two avirulent (48FPA18 and P6497) and two virulent (25MEX4 and P7064) *P. sojae* parents.

and the interactions between the pathogens and their hosts (Yin et al., 2017). Also, NGS-based genotyping techniques would confer diagnosis methods to monitor new diseases. Unambiguously, NGS technology is expected to provide useful information on adequate plant breeding programs for desirable traits such as resistance genes discovery. The plants are deeply nested with plausible future perspectives to overcome plant disease challenges in different host species. Interactive, integrative and comparative researches on plant pathology, breeding, genetics and genomics would pave way for successful disease management.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genetic structure of indigenous sheep breeds in Nigeria based on electrophoretic polymorphous systems of transferrin and haemoglobin

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The study used 100 indigenous sheep comprising 25 Balami, 25 Uda, 25 Yankassa and 25 West African Dwarf breeds reared extensively. The blood samples were taken from Vena Jugularis, processed according to standard procedure and transferrin and haemoglobin examined using cellulose acetate electrophoresis. The observed allele frequencies and genotypes (%) were tested with Hardy-Weinberg's Equilibrium (χ^2). Seven alleles Tf^A, Tf^B, Tf^C, Tf^D, Tf^E, Tf^G and Tf^P controlling 23 genotypes were observed at the transferrin locus while two haemoglobin alleles (Hb^A and Hb^B) controlling three phenotypes- Hb^{AA}, Hb^{AB} and Hb^{BB} were detected. Except for the West African Dwarf sheep, all the samples indicated genetic equilibrium revealed by significant difference between observed and expected genotypes at both loci. The observed significant difference between the frequencies of alleles and genotypes at the two studied loci in the West African Dwarf sheep can be used as a source of genetic diversity during selection for improvement. The phylogenetic analysis as viewed by the tree topology suggests that the Balami and Uda may have had the same migration route or may have been the same breed which had only just recently branched off through adaptive. Also, the West African Dwarf sheep may have been the first to branch off the path of migration and thus had more clearly defined migration route or origin.

Key words: Transferrin, haemoglobin, genetic structure, genetic diversity, Balami.

INTRODUCTION

Indigenous animal breeds in developing countries are constantly being replaced by their high-producing exotic counterparts in spite of their excellent adaptation to prevailing environmental conditions. This poses the danger of losing valuable genes for adaptation to extreme environments and disease which are of value in developing countries. Therefore, there is the need to understand the diversity of these indigenous breeds in order to develop strategies for improvement, sustainable use and conservation of the domestic animal biodiversity.

Blood protein and enzyme system have been found to exhibit heterogeneity in different farm animals (Elmaci, 2001). Polymorphic blood traits are useful in studies of relationship between populations, genetic structure of

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Table 1. Allele frequencies of transferrin (Tf) and haemoglobin (Hb) for the breeds.

Breeds	Transferrin (Tf) Alleles								Haemoglobin (Hb) Alleles		
	nTf	Tf^A	Tf^B	Tf^C	Tf^D	Tf^E	Tf^G	Tf^P	nHb	Hb^A	Hb^B
Balami	25	0.12	0.32	0.24	0.04	0.24	-	0.04	25	0.28	0.72
Uda	25	0.30	0.22	0.14	0.04	0.28	0.02	-	25	0.28	0.72
Yankassa	25	0.30	0.06	0.30	0.20	0.12	0.02	-	25	0.40	0.60
WAD	25	0.12	0.10	0.14	0.20	0.16	0.28	-	25	0.86	0.14
Total	100	0.21	0.18	0.21	0.12	0.20	0.08	0.01	100	0.455	0.545

WAD, West African Dwarf Sheep, HB, haemoglobin, TF, transferrin; A, B, C, D, E, F, G, P and S – alleles.

breeds and their evolution. Information on genetic variations of blood proteins and enzymes has also been used as an aid to parentage determination and indirect selection (Elmaci, 2001). Information on inherent genetic diversity is important in the design of breeding programmes for improvement, rational decision making on sustainable utilization and conservation of animal genetic resources. The genetically determined polymorphous systems in sheep blood and the opportunity for using them as genetic markers make it possible to conduct studies that are related to breed structure as well as changes that may have occurred in them in the process of introduction and selection (Slavov et al., 2004).

Polymorphism of blood proteins first offered the possibilities to study genetic differentiation before the advent of molecular markers. Consequently, several livestock breeds including the domestic sheep have been characterized for variations in blood proteins (Di Stasio, 1995; Mwancharo et al., 2002; Ibeagha-Awemu and Erhardt, 2004). This study therefore aims to quantify genetic diversity at the transferrin and haemoglobin loci in Balami, Uda, Yankassa and West African Dwarf (WAD) sheep breeds and to estimate the genetic distance between them.

MATERIALS AND METHODS

The four sheep populations indigenous to Nigeria, namely, Balami, Uda, Yankassa and West African Dwarf were sampled from small holder flocks and markets in Ibadan, Okene, Zaria, Iwo and Lokoja. A total of 100 blood samples comprising 25 adult healthy individual sheep of both sexes per breed were collected. Samples were collected from the jugular vein and emptied into 5 ml heparinised tubes using disposable needles and syringes and then preserved in ice boxes and transported to the Animal breeding and genetics laboratory for analysis.

The blood samples were centrifuged at 3000 rpm for 5 min. The plasma supernatant was decanted into clean plain tubes and stored until needed for transferrin analysis. The erythrocyte fraction was washed three times with physiological saline. After each centrifugation, the washing solution was removed by decanting. After the third washing, three parts distilled water was added to the erythrocyte fraction to release haemoglobin through lysis and the lysed samples were stored in a refrigerator until needed for further analysis. The cellulose acetate electrophoresis conditions were as described by RIKEN (2006) with minor modifications to suit the samples used in this study.

Red blood cells were lysed in 8 part dH_2O 0.3 μl, for haemoglobin analysis with Tris EDTA as buffer while undiluted plasma was used to run transferrin with Tris glycine as running buffer. Electrophoresis lasted for 25 to 35 min and staining was done using ponceau S while destining was with 5% acetic acid. The bands were scored visually based on their migratory pattern as described by RIKEN (2006).

Statistical analysis

Allelic variants for haemoglobin and transferrin locus were scored in order of increasing mobility, "A" being the allele with the slower of the two. Allele and genotype frequencies for each locus were computed by direct gene counting method and tested for fit to Hardy-Weinberg's Equilibrium (HWE) using χ^2 goodness-of-fit-test. The observed and expected heterozygosities were calculated according to Nei (1973). The genetic differentiation among populations and fixation indices (*Fis, Fit* and *Fst*) were calculated according to the method of Wright's (1978). The genetic distance (*D*) and genetic identity (*I*) were calculated according to Nei (1978). The Unweighted Pair Group Method of Algorithm (UPGMA; Sneath and Sokal, 1973) was used to view the tree topology of the dendrogram showing the relationship between populations. All computations were performed using Popgene (Yeh and Yong., 1999) and Tools for Population Genetic Analysis (TFPGA; Miller, 1997).

RESULTS

Gene frequencies

The two studied loci were polymorphic for all the breeds with nine allelic variants. Allele frequencies are given in Table 1. The highest number of alleles occurred at the transferrin (Tf, seven alleles) locus while Hb had two alleles. Except for the Tf which was very polymorphic, Hb alleles were present in the four breed populations studied at varying frequencies. The most frequent allele was Hb^B for Balami (0.72), Uda (0.72) and Yankassa (0.60), while Hb^A was most frequent in WAD (0.86).

Only seven alleles were found in this present study out of the twelve Transferrin alleles known in sheep breeds (Erhardt, 1986). Six alleles were detected in each of the populations at the Tf locus. The Tf^B allele was most frequent in the Balami while the Tf^A was the most frequent in the Uda population. The Tf^A and Tf^C alleles

Table 2. Genotype frequencies at the transferrin locus of the sheep populations and goodness of fit test of Hardy-Weinberg's equilibrium.

Genotype	Balami	Uda	Yankassa	WAD	Overall
AA	0	0.04	0	0.04	0.02
AB	0.04	0.08	0	0	0.03
AC	0.08	0.24	0.4	0	0.18
AD	0	0.08	0.12	0.04	0.06
AE	0.08	0.12	0.04	0.04	0.07
AG	0	0	0.04	0.08	0.03
AP	0.04	0	0	0	0.01
BB	0.12	0.04	0.04	0	0.05
BC	0.12	0.04	0	0	0.04
BD	0.04	0	0.04	0.04	0.03
BE	0.2	0.2	0	0.04	0.11
BG	0	0.04	0	0.12	0.04
CC	0	0	0	0.04	0.01
CD	0.04	0	0.12	0.08	0.06
CE	0.2	0	0.08	0	0.07
CF	0	0	0	0.12	0.03
CG	0.04	0	0	0	0.01
DD	0	0	0	0.08	0.02
DE	0	0	0.12	0.04	0.04
DF	0	0	0	0.04	0.01
EE	0	0.12	0	0.04	0.04
EF	0	0	0	0.12	0.03
FF	0	0	0	0.04	0.01
Total	1	1	1	1	1
χ^2	11.28^{ns}	12.02^{ns}	21.34^{ns}	29.61^{*}	

Ns = Not Significant (P>0.05); * = Significant (P<0.05).

Table 3. Genotype frequencies and chi squared (χ^2) test of Hardy-Weinberg's equilibrium at the haemoglobin locus.

Genotype	Balami	Uda	Yankassa	WAD	Overall
AA	0	0	0.04	0.72	0.19
AB	0.56	0.56	0.72	0.28	0.53
BB	0.44	0.44	0.24	0	0.28
Total	1	1	1	1	1
χ^2	0.66^{ns}	3.78^{ns}	3.78^{ns}	6.25^{*}	

Ns = Not Significant (P>0.05); * = Significant (P<0.05).

had equal frequencies which was the highest value in the Yankassa population while the Tf^G was the most frequent in the WAD population. Tf^G was absent in the Balami population but present in all other breeds while Tf^P was present only in the Balami and absent in all other breeds.

Genotype frequencies

The distribution of the genotypes and their frequencies are presented in Tables 2 and 3.

Transferrin

The Tf locus was the most polymorphic with twenty-three genotypes controlled by seven codominant alleles. In Balami, the most common genotypes were Tf^{BB} and Tf^{BC} with a value of 0.12; the most common genotype in the Uda population was Tf^{AC} with a frequency of 0.24 (highest genotype frequency); in the Yankassa populations, the Tf^{AD}, Tf^{CD} and Tf^{DE} were the most frequent genotypes with the same value of 0.12 while in the WAD population, the genotypes Tf^{BG}, Tf^{CG} and Tf^{EG}

Table 4. F-Statistics and gene flow based on haemoglobin and transferrin.

loci	F_{IS}	F_{IT}	F_{ST}	Nm*
Hb	-0.388	-0.069	0.230	0.836
Tf	-0.097	-0.034	0.058	4.092
Mean	-0.193	-0.047	0.123	1.791

*Nm = Gene flow estimated from F_{ST} = 0.25(1- F_{ST})/ F_{ST}. Hb = haemoglobin locus; Tf = transferrin locus.

Table 5. Nei' measure of genetic distance and genetic identity.

Population	Balami	Uda	Yankassa	WAD
Balami	****	0.966	0.891	0.527
Uda	0.034	****	0.919	0.537
Yankassa	0.115	0.085	****	0.681
WAD	0.640	0.622	0.384	****

Nei's (1972) original measure of genetic identity (above diagonal) and genetic distance (below diagonal).

were most frequent with a constant value of 0.12. Among all the breeds studied Tf^{AP}, Tf^{CC}, Tf^{CP}, Tf^{DG}, and Tf^{GG} were rare. All others were observed with varying frequencies.

Haemoglobin

Three genotypes of Hb (Hb^{AA}, Hb^{AB} and Hb^{BB}) determined by two codominant alleles were observed in Yankassa, whereas only Hb^{AB} and Hb^{BB} were observed in Balami and Uda, while the WAD had only Hb^{AA} and Hb^{AB}. The frequencies of Hb^{AB} and Hb^{BB} in Balami and Uda population were the same (0.56 and 0.44, respectively) with the frequency of Hb^{AB} being the highest. The frequency of $Hb^{AA}$was the highest (0.72) in the WAD while Hb^{AB} was 0.28. The frequency of Hb^{AA} was the lowest (0.04) in Yankassa while Hb^{AB} was the highest (0.72) and 0.24 for Hb^{BB}.

Chi square (χ^2) test of Hardy-Weinberg Equilibrium

Hardy-Weinberg equilibrium (HWE) test for single locus was conducted for the populations within the two protein loci. Results shown in Tables 2 and 3 revealed that all the breeds had no significant deviation from HWE except WAD which deviated from the HWE at both protein loci.

F-Statistics and gene flow

Population differentiation revealed by fixation indices F_{is}, F_{it} and F_{st} for each of the loci studied across four Nigerian sheep population are shown in Table 4. The global heterozygosity deficit (F_{it}) was estimated at -0.047 and the within-breed deficit in heterozygote evaluated by F_{is} was -0.388 for Hb and -0.097 for Tf with a total mean of -0.193 for both loci. Global breed differentiation evaluated by F_{st}, was estimated at 0.123. The gene flow values for each of the loci studied was 0.836 for Hb and 4.092 for Tf. The mean gene flow over all loci was 1.791.

Genetic distance and genetic identity

The distance between populations ranged from 0.034 to 0.640. The smallest genetic distance was observed between Uda and Balami populations while the farthest distance was observed between WAD and Balami populations. Results of genetic identity are presented in Table 5. It showed that the Uda and Balami populations are more genetically alike (0.966) while the Balami and WAD populations were the least genetically identical (0.527).

Dendrogram

The genetic distance estimates were used to construct dendrogram based on individual locus and the pooled distances for the four loci. When the Dendrogram of genetic distance was viewed at the haemoglobin locus, the topology differentiated two sub clusters. The sub clusters were Balami - Uda at node 1 with no distance and Yankassa with a distance of 0.0238 at node 2 (including Balami Uda and Yankassa). The WAD was totally separated from the two sub clusters with a distance of 0.5802 at node 3 (Including all four populations) (Figure 1). The tree topology of the dendrogram of genetic distance between Balami, Uda, Yankassa and WAD sheep populations at the Transferrin locus revealed two sub clusters; Balami-Uda at node 1 corresponding to a distance of 0.1258, and Yankassa at node 2 having a distance of 0.3045 (including Balami, Uda and Yankassa) while the WAD separated completely at node 3 with a distance of 0.4347 (including all four populations) (Figure 2).

The Phylogenetic tree of the genetic distances pooled for the two loci supports the genetic distance estimates where the Balami population is the most genetically distant from the WAD population. The Balami and Uda populations formed a different cluster at node 1, indicating a closer relationship between the two populations, whereas the WAD separated completely at node 3, the Uda formed a sub cluster with the Balami-Yankassa group at node 2 (Figure 3).

DISCUSSION

Haemoglobin

All of the breeds in this study were polymorphic for Hb

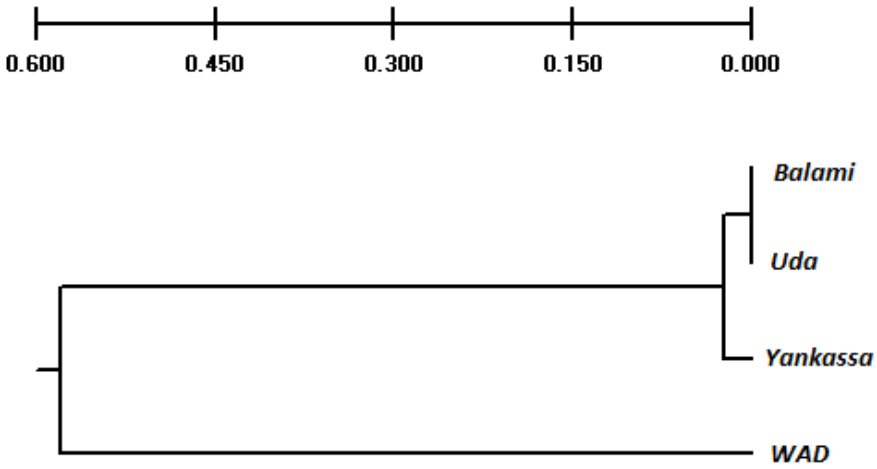

Figure 1. Dendrogram of genetic distance between four Nigerian indigenous sheep populations at the haemoglobin locus.

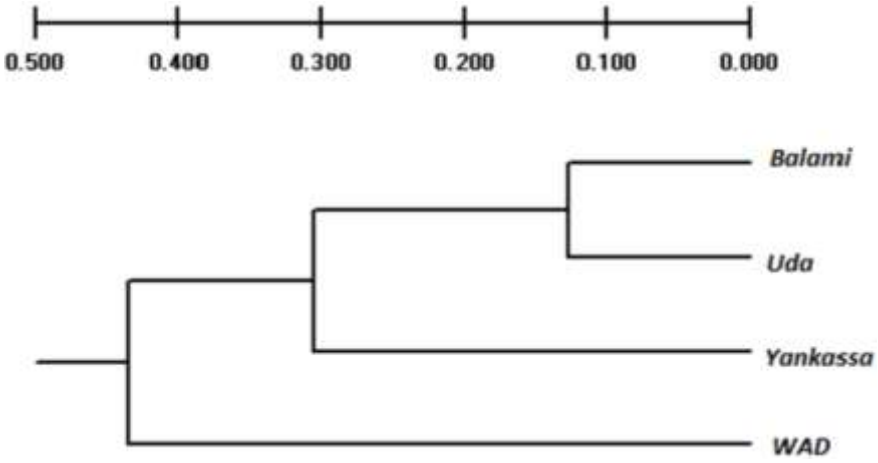

Figure 2. Dendrogram of genetic distance between four Nigerian indigenous sheep populations at transferrin locus.

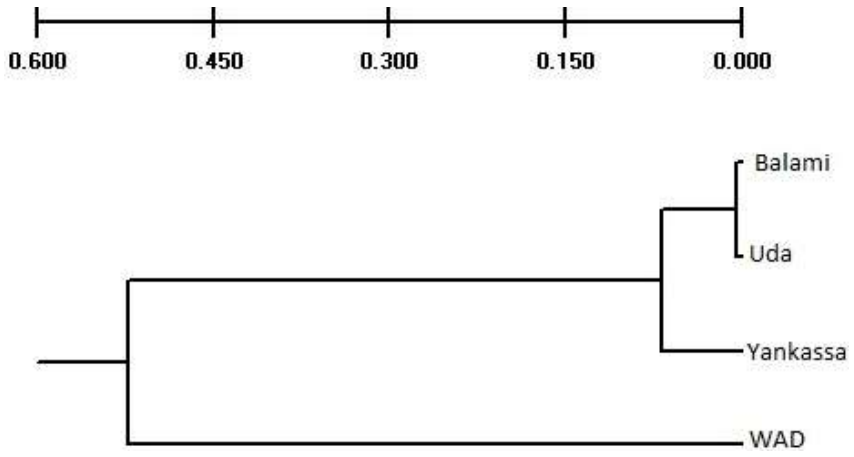

Figure 3. Dendrogram of genetic distance between four Nigerian indigenous sheep based on haemoglobin and transferrin.

with frequencies of Hb^B considerably higher than those of Hb^A in Balami, Uda and Yankassa. Similar results have been obtained by Bunch and Foote (1976), Zanotti et al.(1988), Clarke et al. (1989), Bunge et al. (1990), Mwacharo et al. (2002), Boujenane et al. (2008), and Shahrbabak et al. (2010) who reported that Hb^B is generally the most occurring allele in most sheep breeds. However, in contrast, the Hb^A was higher for the WAD population in this study; this variation may be attributed to the selective advantages of Hb in different geographical regions. The WAD being predominant in the wet humid regions may have Hb^A conferred on it for survival, while the Balami and Uda breeds may have need of Hb^B for survival in the drier savannah regions where they are found. The Yankassa however is most widely spread and had about 60.0% of its members having Hb^B while the other 40.0% were found to have Hb^A. This may have given it the advantage of survival in the regions between the extremes of the wet humid regions and the drier savannah regions. Similar results of predominance of Hb^A had been reported for other sheep populations. Buis and Tucker (1983) found that in some Dutch breeds (Friesian, Schoonebeker, Drente and Kempen), Hb^A was the more common allele compared to Hb^B. In France, Nguyen et al. (1992) also made the same observations in Rambouillet breed. Tella et al. (2000) in a study of West African Dwarf and Yankassa sheep in South West Nigeria reported that Hb^A occur at higher frequency in the two breeds with Hb^A occurring in 98.8% of the WAD population and 78.78% of the Yankassa population sampled. However, Schillhorn and Folaranmi (1978) reported that haemoglobin allele types have selective advantages in different geographical regions, while Hb^A has been shown to have advantage in sheep at higher altitudes; Hb^B occurs more commonly in lowland breeds. In Nigeria, Hb^B type has a very high frequency in sheep of the northern savannah zone, the region in which the Balami and Uda breeds are predominantly found. This predominance appears to be of adaptive significance in the arid regions to which these breeds demonstrated fitness. This is due to the decreased haematocrit values, lower blood viscosity and more availability of water associated with Hb^B blood types compared to Hb^A types. This is buttressed by the reports of Tsunoda et al. (2006) that Hb^A allele has a high affinity for oxygen and is important for survival in mountain areas at altitudes over 3000 m and Pieragostini et al. (2006) who reported that the Hb^A is more frequent in sheep living in areas above 40°C latitude.

Furthermore, Ordas (2004) reported that Hb^A has a higher affinity for molecular oxygen than Hb^B because of differences in oxygen dissociation rates. The higher availability of molecular oxygen in erythrocytes with Hb^A may be responsible for the higher incidence of parasitism. This may be due to the fact that the Hb^A erythrocytes may support parasite establishment and propagation more than those with Hb^B which have lower diffusible intra erythrocytic oxygen. Thus, Altaif and Dargie (1978) and Buvanendran et al. (1980) reported a possible correlation between haemoglobin polymorphism and genetic resistance to helminth infection in sheep and goats. The results obtained in this study demonstrates that extreme temperatures (acute cold or sultry heat), extreme relief forms (desert or mountain), precarious nutrition and breeding conditions favour the fixing of the Hb^A allele and that the temperature situated in the biological comfort zone, moderate relief forms (forest, steppe hill) or the breeding techniques or methods are correlated with a more emphasized fixing of the Hb^B allele. Thus, in the biological respect, the allele Hb^A is characterised by a great selection advantage in comparison with the allele Hb^B. In a great measure, the selective advantage of Hb^A is due to the biophysical, biochemical and physiological peculiarities of the haemoglobin molecule type A (saturation capacity with oxygen, dissociation curve of oxyhaemoglobin, erythrocyte load with haemoglobin and metabolic profile of the erythrocyte) (Raushenbach and Kamenek, 1978).

Transferrin

The seven alleles observed at the Tf locus were dispersed, in terms of their frequencies and number within each breed, and in respect of their distributions among breeds. The differences observed at the Tf alleles indicate clear genetic differentiation between the Nigerian breeds studied. The Tf^P allele is a rare allele exclusively found in the Balami breed at very low frequency and the Tf^G was found only in three of the four breeds studied (Uda, Yankassa and WAD). Manwell and Baker (1977) suggested that electrophoretic variants with low frequencies may represent, in many cases, relative recent mutations occurring after divergence of the breeds; this could be the case with the Balami breed. Akinyemi and Salako,(2012) also reported Tf^P in Balami breed and the same was also reported for SardiandBeni Ashen sheep breeds in Morocco (Boujenane et al., 2008), and it is reported to be more widely distributed in European sheep breeds (Buis and Tucker, 1983; Zanotti et al., 1990).

The gene frequencies at Tf locus were compared with those reported by other researchers to obtain information on the degree of divergence between breeds. Ashton and Ferguson (1962) reported the frequencies of alleles Tf^A, Tf^B, Tf^C, Tf^D, Tf^E and Tf^G in three different populations of Australian Merino. Stormont et al. (1968) reported the frequencies of these alleles in Mailliard Merino and Nguyen et al. (1992) published results on these alleles in Spanish Merino.

Higher frequency of Tf^A and Tf^C in Yankassa is supported by the report of Ibeagha-Awemu and Erhardt (2004) on the same breed, where Tf^C was reported to have the highest frequency in Yankassa and by the

report of Akinyemi and Salako (2012), who reported the Tf^{A} allele as the highest in the Yankassa. The presence of $Tf^{B}Tf^{D}$ and Tf^{E} alleles in this study was also reported by Ibeagha-Awemu and Erhardt (2004). The occurrence of Tf^{G} in Yankassa in this study was also reported by Akinyemi and Salako (2012) and was observed in some Moroccan sheep breeds (Boujenane et al., 2008) but was not reported by Ibeagha-Awemu and Erhardt (2004). The presence of the alleles Tf^{E}, Tf^{G} and Tf^{P} in the studied breeds were also reported by Akinyemi and Salako (2012) in similar breeds but were totally absent in a report on Kenyan breeds (Mwacharo et al., 2002). Ibeagha-Awemu and Erhardt (2004) posited that the absence of these alleles may not totally exclude their occurrence in the breeds but may have exposed the limitation of the method of starch gel electrophoresis in separating Tf variants.

Observation at Transferrin locus are generally difficult to compare with the result obtained in other studies because of the different electrophoresis media used by other researchers and subsequently different resolution power, that is, starch gel and poly acrylamide gel electrophoresis (Akinyemi and Salako, 2012). However, significant deviations of allele frequencies may occur owing to crossing and linking, inbreeding, sample error, population bottlenecks and random genetic drift. The genetic differences between the breeds are to be expected for breeds studied since they are found in separate locations throughout Nigeria, where little or no gene flow occurs.

Hardy-Weinberg equilibrium

The significant deviations from HWE ($P< 0.05$) observed for both locus within the WAD breed could be attributed to unobserved null alleles, excess of heterozygote individuals than homozygote individuals, migration, high mutation rate and artificial selection in the breeds (Aminafshar et al., 2008). Significant deviations of allele frequencies may occur owing to crossing and linking, inbreeding, sample error, population bottlenecks and random genetic drift. Ideal Hardy-Weinberg's populations do not actually occur in nature owing to various factors, which can shift the equilibrium and disrupt the stability of a population, giving rise to change in the genetic structure (Sargent et al., 1999). Deviation from HWE at protein loci have also been reported in studies such as in Southern Africa sheep (Sargent et al., 1999). Since on the overall data set, there were no significant deviations from HWE, it may be suggested that there are no biological phenomena or sampling error biases with a net effect for sufficient differences between observed and expected proportions.

Gene flow and F- statistics

F-Statistic values of F_{ST} and F_{IT} are measures of deviation from Hardy-Weinberg's proportions and total populations respectively. Positive values indicate a deficiency in heterozygotes and negative values indicate an excess of heterozygotes. F_{IS} can be interpreted as a measure of inbreeding (the measure of allelic fixation of individuals relative to the subpopulations). Thus, the negative values of F_{IT} observed at the two loci in the four breeds studied and the overall negative value of -0.047 and the negative value of F_{IS} showed the deficiency of homozygotes in the populations and that mate were less related in comparison with the average relationship of the population. This observed excess of heterozygotes could be due to non-random mating and genetic exchange between populations.

Estimated F_{ST}, which corresponds to the proportion of genetic variability accounted for by the differences among breeds, was 0.123. Thus, a large part of the total genetic diversity can be explained by the variation within breeds (0.877) and to a smaller extent by the variation among breeds. This result indicates that genetic diversity quantified by allozyme markers shows little genetic differentiation among Nigerian sheep breeds studied. The degree of differentiation observed between the Nigerian sheep breeds could be due to geographic proximity, similarities in environment and breeding practices, but most likely due to past genetic exchange among them since mean gene flow over all loci was 1.791.

Genetic distance and genetic identity

Nei (1972) standard genetic distance (D) obtained in this study (0.034-0.640) indicates the level of genetic differentiation between the breeds. Buis and Tucker (1983) reported D values of 0.181 to 0.308 between different sheep breeds and an average D value of 0.248. Different authors have reported different values of D in different sheep breeds. Ordas and Primitivo (1986) estimated the genetic distance between Spanish dairy sheep breeds and reported 0.0094-0.055 using data from 8 loci. Zanotti et al. (1990), using data from four blood groups and six protein loci, reported genetic distance ranging between 0.012 and 0.060 in five Italian sheep breeds. Mwacharo et al. (2002) obtained a closer estimate of genetic distance between Kenyan sheep breeds (0.044 - 0.169) than between Kenyan and the exotic Merino sheep (0.044 - 0.283) in a study using data on five protein-coding loci. Among six Moroccan local sheep, namely, D'man, BeniAhsen, Sardi, Timahdite, BeniGuil and Boujaad, Boujenane et al. (2008) reported a genetic distance range of 0.006 to 0.026. Distances obtained in this study between breeds were higher than those by Akinyemi and Salako (2012) who reported a range of 0.003 to 0.015. The distances obtained from this current study indicate that that the Balami and Uda which are predominantly northern breed are more closely related to each other than they are to the WAD which is a southern breed. The Yankassa however has adaptive

futures which make it the breed in-between.

Dendrogram

The phylogenetic tree constructed separated the WAD from other indigenous sheep, suggesting either early prehistoric separation of the WAD sheep or separate historical origin. The close genetic relationship between the Balami and Uda breed may be attributed to possible interbreeding between these two populations which are predominantly Northern breeds to form a homogenous population separated by administrative boundaries. Furthermore, the close genetic relationship between the breeds may also be attributed to similarity in ecological zones and production systems as well as the incidents of cross border livestock rustling contributing to the migration and movement of livestock and subsequent interbreeding between such livestock (Mwacharo et al., 2002). Based on the highest value of Nei's genetic distance (0.640), breeding programs involving the crossing of the Balami and WAD is recommended, since the crosses between breeds which are homogenous but distinctly different in their relationship would produce more hybrid vigour in the crossed progeny.

Conclusion

The populations were characterized by the presence of 7 transferrin alleles Tf^A, Tf^B, Tf^C, Tf^D, Tf^E, Tf^G and Tf^P, controlling 23 genotypes, 6 of which were homozygous and 17 heterozygous. Two haemoglobin alleles, Hb^A and Hb^B controlling 3 genotypes were found. Two of the haemoglobin genotypes were homozygous. According to the transferrin system all the breeds were in genetic equilibrium except for the WAD which had large variation in number between the observed and expected genotypes and the high χ^2 value of 29.61. According to the haemoglobin system, the WAD population was not in genetic equilibrium as revealed by the χ^2 test of Hardy Weinberg Equilibrium. All other breeds were in genetic equilibrium at the haemoglobin system. The presence of differences between the frequencies of the alleles by categories could be a source of genetic diversity.

Conflict of Interests

The authors have not declared any conflict of interests.

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Assessment of genetic diversity of Burkina Faso sweet grain sorghum using microsatellite markers

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Sweet grain sorghum [*Sorghum bicolor* (L.) Moench] is an under-harvested crop produced mainly for its sweet grains in the pasty stage. Little is known of its genetic diversity remains. This study aims to determine the level and structure of the genetic diversity of sweet grain sorghum from Burkina Faso. Thus, 93 accessions were evaluated using 15 polymorphic microsatellite markers. The analysis revealed 49 alleles in total, 6 rare alleles, an average of 3 alleles per locus, a moderate Nei diversity of 0.474, a low level of heterozygosity (0.031) in the collection and very high Wright's fixation index (Fis) of 0.934. The accessions were organized into three genetic groups: A, B and C. Groups A and B were the farthest, with an Fst and a genetic distance of 0.37 and 0.22, respectively, whereas Groups B and C were the closest, with an Fst (genetic differentiation) of 0.279 and a genetic distance of 0.142. This diversity could be exploited in Burkina Faso sweet grain sorghum breeding programs.

Key words: Burkina Faso, neglected culture, sorghum, simple sequence repeats (SSR) markers, genetic variability.

INTRODUCTION

Traditional varieties are an important source of genetic diversity whose conservation contributes to the maintenance of genetic diversity (Ahmadi et al., 1988). Knowledge of the level and structure of their genetic diversity is an important asset in defining strategies for conservation and varietal improvement (Adoukonou-Sagbadja et al., 2007).

Previous studies on sweet grain sorghum with phenotypic markers have shown an important agro-morphological variability in the collection from Burkina Faso (Sawadogo et al., 2014). However, the observation of morphological characters can lead to a biased estimation of genetic diversity because they are influenced by the environment. Indeed, phenotypic diversity may be increased but genetic diversity remains stable (Lallemand, 2004). For a better estimate of the

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Table 1. Characteristics of microsatellite markers.

S/N	Locus	Number of nucleotide repeats	Chromosome	TM (°C)
1	gpsb123	(CA) 7 (GA) 5	8	50
2	gpsb151	(CT) 12	4	50
3	xtxp10	(CT) 14	9	50
4	xcup14	(AG) 10	3	54
5	xcup53	(TTTA) 5	1	54
6	xcup07	(CCA) 8	10	54
7	xtxp320	(AAG) 20	1	54
8	xtxp15	(TC) 16	5	55
9	xtxp145	(AG) 22	6	55
10	xtxp295	(TC) 19	7	55
11	xtxp136	(GCA) 5	5	55
12	sbagb02	(AG) 35	7	55
13	sb5-206	(AC) 13 (AG) 20	9	55
14	sb6-84	(AG) 14	2	55
15	sb4-72	(AG) 16	6	55

genetic diversity of sweet grain sorghum from Burkina Faso, it is imperative to evaluate it using molecular markers.

The development of genome mapping techniques and their associated attempts to link molecular variability with phenotypic variability are likely to become increasingly acute (Pham et al., 1992). Nuclear molecular markers, in particular microsatellites, otherwise called simple sequence repeats (SSR) are very polymorphic (Doldi et al., 1997; Schug et al., 1998), with Mendelian transmission (Saghai-Maroof et al., 1994). They are codominant and abundant in the genome, easy to use and highly effective in the studying of genetic diversity (Lagercrantz et al., 1993).

The present study was conducted to get a better knowledge of the genetic diversity of the Burkina Faso sweet grain sorghum. The objectives are to: (i) determine the level and (ii) structure of the molecular genetic diversity of sweet grain sorghum from Burkina Faso.

MATERIALS AND METHODS

Plant and molecular markers

The plant materials consisted of 93 accessions of sweet grain sorghum from Burkina Faso obtained by self-fertilization in 2010. Fifteen simple sequence repeat (SSR) markers were selected based on their polymorphism revealed in previous studies on sorghum (Barro-Kondombo, 2010; Nebié, 2014), and their distribution on all the chromosomes of the sorghum genome (Table 1) were used for characterization.

Extraction of total DNA

The total genomic DNA of the 93 accessions was extracted from young freshly picked leaves of 15 days. Flinders Technology Associates (FTA) card method was used for the extraction of DNA. The method works faster from DNA extraction to DNA amplification by polymerase chain reaction (PCR). It consists of taking the fresh young leaves and crushing them on the rough side (square) of the FTA card. The samples were dried in 30 min; then disks of 1 mm diameter were punched, which were placed in Eppendorf tubes. 200 µl of FTA buffer was then added to the punched disks. The whole was incubated at a room temperature for 5 min. This operation was repeated three times by renewing the buffer. At the end, the disks were rinsed with 200 µl of TE (Tris-EDTA) buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and then dried for 5 min before transferring directly each disk in an Eppendorf tube for polymerase chain reaction (PCR).

PCR amplification of genomic DNA

DNA amplification was performed with the thermocycler Eppendorf Master Cycler Gradient in a total volume of 25 µl containing 18 µl of ultrapure water for each sample, 5 µl of premix (with 1 U of Taq (Thermus aquaticus) DNA polymerase, 250 mM of dNTPs, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$), 1 µl of each primer microsatellite F (Forward) and R (Reverse) and one disk of FTA card (0.25 to 0.5 ng/µl genomic DNA) for each sample. A marker of molecular weight of reference of 50 bp (base pairs) and a control without DNA were used. The PCR amplifications were performed in an Eppendorf Master cycler: it comprises an initial denaturation phase at 94°C for 4 min followed by 35 cycles of denaturation step at 94°C for 45 s, annealing at 50 to 55°C for 1 min and an extension at 72°C for 1 min 30 s and final extension at same temperature for 4 min.

The PCR products were separated for 1 h 30 min at 100 V on 2% agarose gel in TBE (Tris-Borate Ethidium) 0.5x and 15 µl of BET 5% (ethidium bromide), using fluorescent developer. The revelation of the amplification products was visualized on an UV transilluminator and photographed with a camera brand Canon Power Shot A620, 7.1 Mega Pixels. The size of DNA bands in base pairs was determined using the 1 kb DNA standard ladder (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Genetix 4.03 (Belkhir et al., 2002) was used to determine the

Table 2. Level of genetic diversity of each markers tested.

Locus	No d'alleles	He	Ho	PIC
gpsb123	5	0.653	0.425	0.648
gpsb151	3	0.562	0.000	0.555
sb4-72	3	0.366	0.000	0.362
sb5-206	3	0.598	0.000	0.592
sb6-84	3	0.493	0.000	0.486
sbagb02	2	0.044	0.000	0.043
xcup07	3	0.437	0.000	0.433
xcup14	4	0.485	0.011	0.48
xtxp136	2	0.498	0.00	0.493
xtxp10	3	0.533	0.000	0.528
xtxp145	5	0.668	0.000	0.661
xtxp15	3	0.579	0.000	0.573
xtxp295	2	0.087	0.000	0.086
xtxp320	5	0.653	0.013	0.646
xtxp57	3	0.484	0.000	0.478

No d'alleles: Number of alleles per locus, He: expected heterozygosity, Ho: observed heterozygosity, PIC: polymorphic information content.

observed heterozygosity (Ho). The total number of alleles (A^t), mean number of alleles per locus (A), number of rare alleles (A^r), the polymorphic information content (PIC) and the expected heterozygosity (He) were known using Fstat software V2.9.3.2 (Goudet, 2002).

The dissimilarity coefficients were used to generate an unweighted neighbor-joining tree with Jaccard's similarity coefficient and a bootstrapping value of 1,000 using the DARwin V5.0 software (Perrier et al*.,* 2006). Wright's fixation index (Fis), genetic differentiation between genetic groups based on Fst (Weir and Cockerham, 1984) and minimum distance of Nei between pairs of genetic groups were estimated using Fstat software V2.9.3.2. To verify the significance of the differences in these values, permutation (1000 to 3000 permutations) was carried out. From the Fis value, the allofecondation rate (t) was estimated using the formula Fis = (1-t) / (1 + t).

RESULTS

Genetic diversity of sweet grain sorghum

Results (Table 2) revealed that the number of alleles per locus varied from 2 for markers sbagb02, xtxp136 and xtxp295 to 5 for gpsb123, xtxp145, xtxp320 markers (Figure 1). Sbagb02 and xtxp145 markers have the extreme value of expected heterozygosity. The results indicate that only the locus: gpsb123, xtxp320 and xcup14 have observed heterozygosity values with an average of 0.03. The markers sb6-84 and xtxp145 showed extreme PIC values of 0.046 and 0.661, respectively.

A total of 49 alleles were detected in the collection with a low number of rare alleles (6), an average of 3 alleles per locus, and relatively low observed (0.031) and expected (0.474) heterozygosity. Results also show a very high value of Wright's fixation index (0.934) and a very low allofecondation rate (t) of 06.04%.

Organization of genetic diversity and description of genetic groups

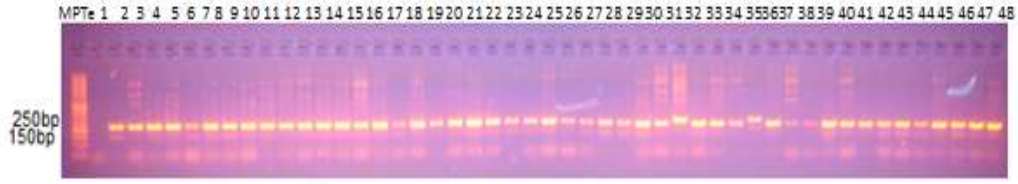
Sweet grain sorghum from Burkina Faso is divided into three genetic groups: A, B and C (Figure 2) consisting of 40, 43 and 09 accessions, respectively. The characteristics of the three genetic groups are shown in Table 3. Group A has the highest number of alleles (43) including 6 private alleles, highest expected heterozygosity (0.42), and lowest observed heterozygosity (0.0107). Group B has the highest value of rare alleles (18), observed heterozygosity (0.051), and lowest expected heterozygosity (0.30). Group C has low number of alleles (34) and mean values for other genetic diversity parameters.

Genetic distance between genetic groups

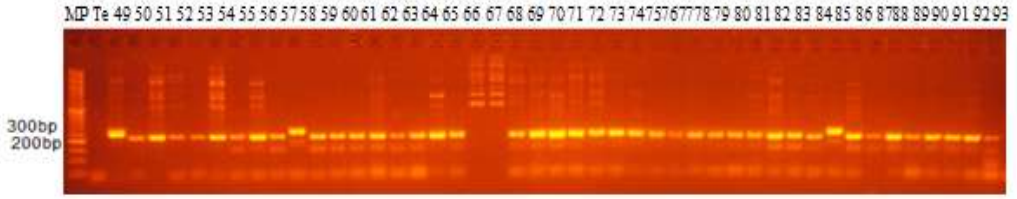
The genetic distances between the three genetic groups revealed by the minimum distance of Nei and the genetic differentiation by pair of genetic groups (Table 4) show that the three genetic groups differed very significantly from one another. Groups A and B are the most remote while groups B and C are the closest.

DISCUSSION

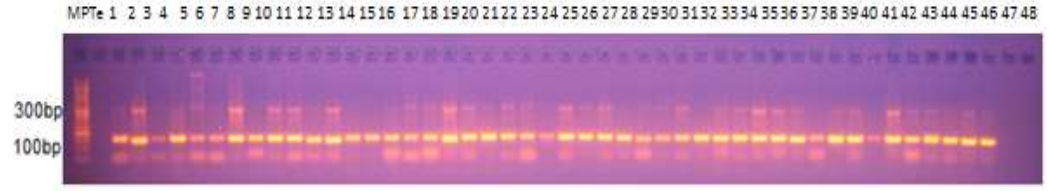
The markers xtxp145 and xtxp320 that showed 5 alleles were the most polymorphic. But, they revealed more



Marqueur xtxp145: accessions 1-48

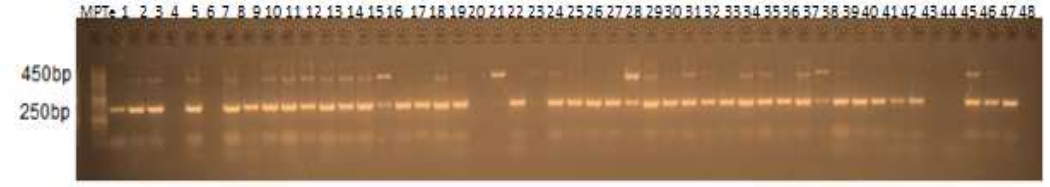


Marqueur xtxp145: accessions 49-93

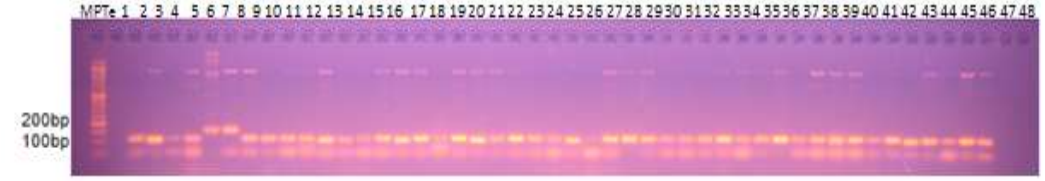


Marqueur xcup53: accessions 1-48

Marqueur gpsb151: accessions 1-48



Marqueur gpsb123: accessions 49-53



Marqueur sbag02: accessions 1-48

Figure 1. Picture of the migration profile of some polymorphic markers. MC, Molecular weight marker; Te, negative control without DNA.

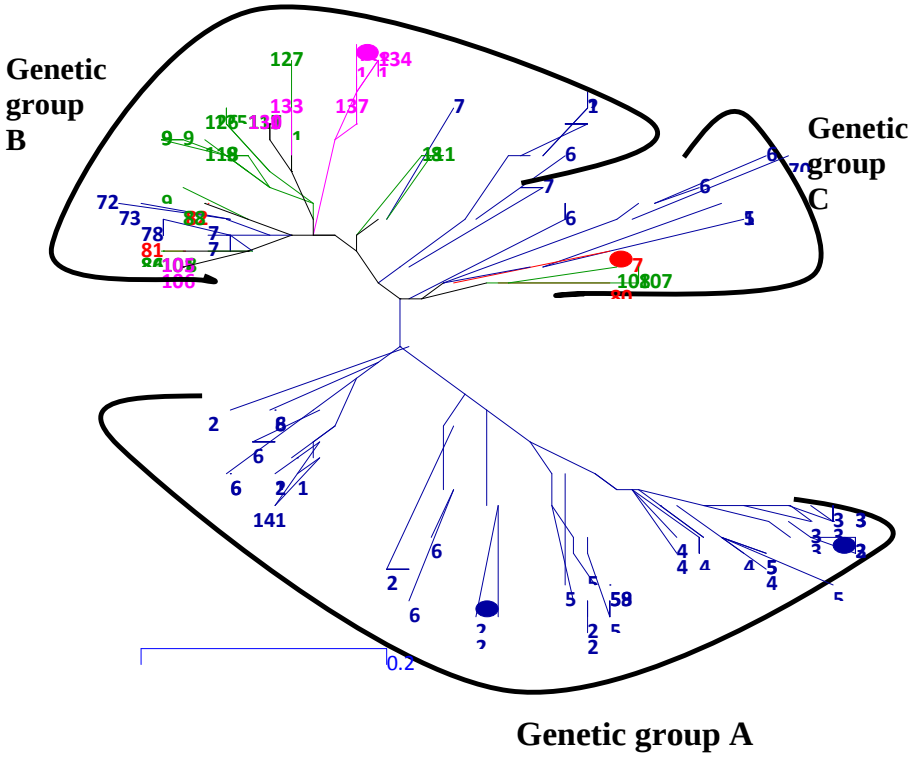


Figure 2. Radial representation of the dendrogram of the 93 accessions of sweet grain sorghum constructed from the dissimilarity matrix according to the Neighbor-Joining method.

Table 3. Characteristics of genetic groups.

Group	Genetic parameters							
	N	A^t	A^r	A^p	A	He	Ho	Fst
	92	**49**	**-**	**-**	**-**	**-**	**-**	**0.35**
A	40	43	12	6	2.87	0.42	0.01	-
B	43	39	18	3	2.6	0.30	0.05	-
C	09	34	15	0	2.27	0.38	0.02	-

N: Number of accessions, A^t: total number of alleles, A^r: number of rares alleles, A^p: number of private alleles, A: mean number of alleles per locus, He: expected heterozygosity, Ho: observed heterozygosity, Fst = genetic differentiation index.

Table 4. Genetic distance between pair of genetic groups of sweet grain sorghum.

Group	A	B	C
A	0	0.3702**	0.3093**
B	0.220	0	0.2794**
C	0.215	0.142	0

The upper diagonal is the comparison of Fst by pair of genetic group and the lower diagonal (bold) is the minimum distance of Nei. **Significant at 1%.

alleles in the sweet stalk sorghum of Burkina Faso, such as 12 for xtxp145 locus and 10 alleles for xtxp320 locus (Nebié, 2014). The PIC of SSR marker that takes into account the number of alleles detected and the relative frequencies of these alleles constitute an important estimate of the discriminating capacity of this marker (Smith et al., 2000). The relatively high value of the PIC of the SSR markers used shows that they have had a undifferentiated. These results indicate that the diversity observed was lower than those of several previous studies (Nebié, 2014; Missihoun et al., 2015; Al-Issawi, 2017). The SSR polymorphism, the level of diversity of accessions in the collection, and the sensitivity of DNA fragment separation systems could explain these strong discriminating capacity for these genetic studies. The markers xtxp145, xtxp320 and gpsb123 were therefore

the most discriminating with PIC greater than 0.6. But the maximum value of PIC per marker was lower than the value obtained by Amelework et al. (2015) and Galyuon et al. (2016): 0.88 and 0.89, respectively.

The 49 alleles detected show that the collection of sweet grain sorghum from Burkina Faso is relativelydifferences. In addition, the very low proportion of rare alleles (12%) in the collection may be related to the small sample size and the much smaller geographic origin of accessions or to a low microsatellite marker mutation rate. However, rare alleles may be of great interest if they are related only to a few particular genotypes. They can be used to identify particular genotypes or specific genome regions related to a particular type of sorghum (Agrahama and Tuinstra, 2003). Indeed, Casa et al. (2005) and Salih (2011) detected 64 and 59% of rare alleles in more extensive collections.

The collection is relatively poor in heterozygotes due to the mean observed heterozygosity rate and the very high Wright's fixation index (Fis). The Fis index was higher than the value of Barnaud et al. (2007), but similar value was obtained by Ouédraogo et al. (2017), which was 0.68 and 0.97, respectively. The observed heterozygosity rate was lower than the value of Salih (2011) but higher than the results obtained by Nebié (2014) (0.016) and Ouédraogo et al. 2017 (0.012). These results are probably related to the preferentially autogamous reproduction regime of species, the racial difference and the fact that accessions were obtained by self-pollination. The collection contains a large proportion of accessions close to the guinea whose glumes are open, which favors the allogamy whose rate can reach 24%, according to Barro-Kondombo (2010).

SSR markers are commonly used as a tool to examine the dynamics of differentiation in the population (Matsuoka et al., 2002) and for specific analyses (Liu et al., 2003; Barkley et al., 2006; Kwak and Gepts, 2009). The three genetic groups identified reveal a lower genetic diversity of sweet sorghum compared to sorghum in center and west of Burkina where Barro-Kondombo (2010) obtained 6 genetic groups. This low genetic diversity may be related to the more restricted range of these sorghum crops. The result is similar to those of Missihoun et al. (2015) who have obtained three groups with 61 accessions and 20 SSRs, while Muui et al. (2016) have obtained four groups with 20 SSRs and 44 accessions.

The study showed a weak genetic polymorphism in Burkina Faso sweet grain sorghum and a distribution of accessions into three genetic groups. The gpsb123, xtxp145, and xtxp320 markers were the most informative on the diversity of sweet grain sorghum and most discriminating. These results show that the molecular markers used were appropriate for the assessment of the genetic diversity of Burkina Faso sweet grain sorghum. A study of the phylogeny of these sorghums could allow to position them in relation to other sorghum and to complete the results of this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Biodegradation of fenthion and temphos in liquid media by *Bacillus safensis* isolated from pesticides polluted soil in the Sudan

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The objective of this study was to evaluate the capability of the bacteria *Bacillus safensis* strain FO-36b^T isolated from pesticide-polluted soil in degrading fenthion and temphos in mineral salt media (MSM). Fenthion and temphos were incubated with the isolated bacteria. Samples were drawn at 0, 3, 7, 14, and 30 days to analyze residual fenthion and temphos content with gas chromatography (GC) and high-performance liquid chromatography (HPLC), respectively. The loss of the initial pesticide concentration (400 mg/L) over time was determined and used to compute the half-lives using a biphasic model. Gas chromatography-mass spectrometry (GC-MS) was used to identify the major metabolites as well as to re-confirm the identity of starting material (fenthion). The results showed that the bacterium was still viable at the end of each incubation period. The biodegradation of fenthion and temphos followed a biphasic model. The half-lives of fenthion in the first and in the second phase were 0.29 and 3.69 days, respectively, whereas the corresponding values for temphos were 0.11 and 1.15 days. Only one metabolite "iso-fenthion" (*O*, *S*-dimethyl *O*-[3-methyl-4-(methylthio)phenyl] phosphorothioate) was detected in fenthion culture, while no metabolites were detected in temphos culture. Based on the half-lives, this bacterium was able to degrade temphos at a faster rate than fenthion.

Key words: Biodegradation, fenthion, temphos, bacteria, pesticides-contaminated soil, Sudan.

INTRODUCTION

Fenthion and temphos are organophosphorus insecticides used as larvicides in fresh and polluted waters, under urban malaria schemes (UMS). The use of the same larvicide for a long-time may, however, cause resistance in mosquito larvae (Mittal et al., 1999). Fenthion and temphos are used in Sudan to control

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larvae of malaria vectors (Bashir et al., 2012). Fenthion is available as dusts, emulsifiable concentrate, granules, liquid concentrates, spray concentrates, ultra-low volume (ULV) and wettable powder formulations (Meister, 1992). The frequent and extensive field use of temphos has caused the development of resistance in *Chironomus yoshimatsui* in the Kanda River, Tokyo (Ohno and Okamoto, 1980).

Fenthion is moderately toxic to mammals if ingested, inhaled, or absorbed through skin (Smith 1993) and highly toxic to birds. Based on its high toxicity to birds, fenthion is used in various parts of the world for weaver bird control as well as for the control of pigeons around public buildings. It has contact action and it is readily absorbed through skin. It is applied as a paste to roosting areas when utilized for such purposes (McEwen and Stephenson, 1979). Fenthion is classified by the U.S. Environmental Protection Agency (EPA) as a Restricted Use Pesticide (RUP) due to the special handling warranted by its toxicity (VanDrieshe, 1985).

Temphos is considered as a basic larvicide for immature stages of mosquito (Jamal et al., 2011). Its aerial application over aquatic sites may contaminate surface and drinking waters. The human population may be exposed to temphos via ingestion of some fish/seafood, drinking water, and dermal contact with consumer products containing this compound.

In water, temphos might be adsorbed to organic matter and slowly released to achieve steady state. Remediation of some elements pollutant using sorption process by various source materials of natural organic matter in aqueous solution was reported (Butnariu et al., 2015). Temphos adsorption to sediment steadily increased to a maximum after two days of exposure, but temphos degradation products were shown to adsorb less strongly to soils. Absorption would be expected to be less than 3% of applied dose. In mammals, elimination of mainly unchanged temphos is in the feces and urine. It might also be released to the environment through various waste streams (CASRN, 2015). US EPA concluded that there was no evidence of carcinogenicity of temphos. Temphos formulations were classified as slightly toxic end-use products (EPA toxicity class III) (US EPA, 2001).

Biodegradation is a common mechanism for fenthion and temphos degradation in the environment (HSDB, 2003). The potential use of Sudanese soil microorganisms in cleaning pesticides polluted soils in Sudan and dump sites was first argued by Abdelbagi et al. (2000, 2003).

Strains of microorganisms isolated from pesticides polluted soils in Sudan were reported to have great capability for the degradation of some pesticides such as malathion, chlorpyrifos, dimethoate, benomyl, thiram, oxyflurofen, lindane, endosulfan pendimethalin, atrazine, and azoxystrobine (Ishag et al., 2017, 2016; Elsalahi et al., 2015; Abdurruhman et al., 2015; Shaer et al., 2013; Elhussein et al., 2011; Mohamed et al., 2011; Elsaid et al., 2009; Elsaid and Abdelbagi, 2010; Osman, 2006). Their degradation capability can be enhanced by many activators such as farm manure and synthetic fertilizers (Elsaid et al., 2009). This study was initiated to evaluate the potential capability of the indigenous bacteria *Bacillus safensis* isolated from pesticides polluted soils in degrading fenthion and temphos under the condition of mineral salt media. To study the biodegradation of fenthion and temphos, the specific objectives were: (1) to characterize biodegradation rates on mineral salt media and (2) to identify bio-degradation products especially of toxicological concern.

MATERIALS AND METHODS

Chemicals and reagents

Analytical standards of the organophosphorus insecticides temphos (94.9% pure) and fenthion (95.5% pure) were obtained from the Agricultural Research Corporation, Sudan. Solvents (99.8% pure; acetone, n-hexane, ethanol, dichloromethane and other solvents) were obtained from Fischer, company, UK.

Isolation and identification of microorganisms from pesticides polluted soils

Surface soil samples were randomly collected from pesticides polluted storage soil in Hasahisa, (Gezira scheme) using a soil auger (10 cm length × 5 cm diameter). Five augers were taken and mixed thoroughly to make the composite sample (1 kg). The collected samples were placed in labeled paper bags and immediately transported to the pesticides laboratory, Crop Protection Department, Faculty of Agriculture, University of Khartoum, and then sent to the Microbiology Laboratory, Faculty of Veterinary Medicine, University of Khartoum for isolation and identification of the types of bacteria present. Isolation and identification were done according to the methods described by Cowan and Steele (1993). The identified isolate have been reconfirmed by molecular biotechnology (Ishag et al., 2016, 2017). The identified bacterial strain was subcultured on meat peptone agar for 24 h prior to their use in biodegradation study using mineral salt media (almost organic carbon free media).

Preparation of media

Meat peptone agar (MPA)

This media was prepared by adding 5 g meat, 7.5 g of peptone, 5 g NaCl, and 15 g agar to 1 L distilled water according to the methods of Tepper et al. (1993) and kept in a refrigerator at 5°C for further use.

Mineral salt medium (MSM)

MSM was prepared following the method described by Tepper et al. (1993); 1 g k_2HPO_4, 0.5 g $MgSO_4$. $7H_2O$, 0.5 g NaCl, 0.001 g $FeSO_4.7H_2O$, 0.01 g $MnSO_4.4H_2O$, and 0.05 g $CaCO_3$ were added to a conical flask (1500 mL) and then, the volume was completed to 1 L by adding distilled water. The media were autoclaved for 20 min at 121°C and then allowed to cool at room temperature and kept in a refrigerator at 5°C for further use.

Preparation of the microbial inoculums

Two hundred milliliters of MPA were taken and placed in a 250 mL conical flask and inoculated with bacteria using sterilized loops. Inoculated flask was then closed with sterilized cotton and kept in an incubator (thermostatic cabinet, Austria) at 25°C for 24 h prior to use in biodegradation experiment.

Microbial degradation of fenthion and temphos in mineral salt media

The aim of this experiment was to evaluate the capability of the isolated bacteria *B. safensis* in degrading temphos and fenthion in mineral salt media. A total of 30 clean test tubes were sterilized in an oven for 3 h at 180°C. Ten milliliters of mineral salt media (MSM) were taken from the stock flask into each test tube. One milliliter of inoculum was added to each test tube. The cultured test tubes were incubated at 25°C with 400 mg/L temphos and fenthion for 0, 3, 7, 14, and 30 days. The experimental units were arranged in a Completely Randomized Design (CRD) with two replicates. Control sets without bacterial inoculums were incubated under the same conditions. The recovery sets were immediately extracted and kept in the refrigerator for analysis by Gas Chromatograph (GC) for fenthion and High-Performance Liquid Chromatography (HPLC) for temphos.

Effect of temphos and fenthion on cultured bacteria

One milliliter of culture was taken by sterilized pipette from each test tube at the end of each period of 3, 7, 14 and 30 days and placed in a Petri dish containing sterilized meat peptone agar (MPA). The inoculated plates were then incubated at 37°C for 72 h.

Extraction of fenthion and temphos from the culture

Treated cultures were centrifuged at 800 rpm for 10 min to separate the microorganisms from the media. The supernatant was removed by careful decanting and placed in 100 ml separatory funnel and 10 ml of dichloromethane, and 10 ml saturated sodium chloride solution were added. The contents were vigorously shaken for 5 min and allowed to stand for 3 min until separation of layers. The dichloromethane layer was collected in a clean test tube and the aqueous layer was re-extracted twice with 10 ml dichloromethane. Dichloromethane fractions were recombined in a clean test tube and dried up by passing through anhydrous sodium sulfate on a filter paper. The solvent was stripped off by rotary evaporator at 70°C till dryness and the residues were reconstituted in 10 ml n-hexane and stored in the refrigerator at 5°C for Gas Chromatograph (GC) and High-Performance Liquid Chromatography (HPLC) analysis. The identity of starting materials and breakdown products were confirmed by GC-MS.

Gas chromatographic analysis

A Shimadzu GC Qp2010 system (Japan) Gas chromatograph (GC) equipped with flame ionization detector (FID) and DB-5 splitless injection fused silica capillary column of 30 m length and 0.25 mm ID was used for fenthion analysis extracts. The stationary phase (0.25 mm thickness) was 5% phenyl, methylpolysiloxane. Detector and injection temperatures were 330 and 300°C, respectively. Nitrogen was used as carrier gas at a flow rate of 4.23 ml min^{-1}. The oven temperature was programmed as follows: initial temperature was 50°C for 1 min, increased at 5°C min^{-1} until 75°C, held for 2 min, increased again at 10°C min^{-1} until 160°C, held for 6 min, increased by 5°Cmin^{-1} until 180°C and then held for 3 min, and finally increased by 3°C min^{-1} until the final temperature which was 240°C, with holding time of 10 min. Flow rates of the makeup gas (helium), hydrogen, and air were 30, 40, and 400 mL min^{-1}, respectively. Analysis of sample was done by duplicate injections of 1 µL each. Three concentrations (62.5, 125 and 250 mg/L) of the analytical standard of fenthion (95.5% pure) was injected under the same condition and response was used for the construction of the standard curve. Data was processed by GC solution software version 2.3. The limit of detection (LOD) of fenthion was 1.8 mg/L. The recovery of fenthion from the media was greater than 98%.

Gas chromatography with mass spectrometry (GC-MS) instrumentation

Three representative samples were reanalyzed using Shimadzu GC-MS Qp2010 system (Japan) with an AOC-5000 autosampler. The gas chromatograph was fitted with RSS-MS capillary column of 30 m × 0.25 mm ID, 0.25 µm film thicknesses from Restek (UK). Helium (purity ≥ 99.999%) was used as a carrier gas at a flow rate of 1.22 ml min^{-1}. The splitless injection temperature was 200°C. The oven temperature was programmed from an initial temperature of 100°C, held for 3 min, then increased to 180°C at 16°C min^{-1}, held for 6 min, and finally, increased by 16°C min^{-1} to 240°C at which it was held for 3 min. The mass spectrometer was operated with electron impact (EI) source in the scan mode. The electron energy was 70 eV, and the interface temperature was maintained at 200°C. The solvent delay was set to 2 min.

High-performance liquid chromatography analysis

A Shimadzu (Kyoto, Japan) CLASS-VP, Version 5.22 High-Performance Liquid Chromatography (HPLC) equipped with a UV/Visible detector was used for analysis of extracts of temphos. Separation was performed on a Luna C18 column. The instrument system consisted of LC-10 ADvp binary pump, DGU-14 An online degasser, SPD-M10-Avp Luna absorbance detector, Sil-10 ADvp auto-injector, CTO-10 ASvp column oven fitted with Shim- Pack VP-ODS (150 × 4.6 mm, 10 µm) column and a similar pre-column (4 × 4 mm ID). Samples were auto-injected. The detector was connected to the computer for data processing. The working condition of the HPLC was a binary gradient, with the mobile phase being acetonitrile: water (60:40), the flow rate was 1 ml min^{-1}, injection volume was 10 µL and the wavelength of the UV/Vis detector was fixed at 210 nm. Analyses of samples were done by duplicate injections of 10 µL each. Five concentrations (10, 20, 40, 80 and 100 mg/L) of the analytical standard of temphos (94.9 pure) were injected under the same condition and response was used for the construction of the standard curve. The limit of detection (LOD) of temphos was 1.58 mg/L. The recovery of temphos from the media was greater than 98%.

Statistical analysis

The data were subjected to the analysis of variance (ANOVA) and means were separated by the LSD. The probability of 0.05 or less was considered significant (SAS 2004). A biphasic model was assumed in order to calculate the loss of fenthion and temphos from the media inoculated with the bacteria. Calculations were done according to the following equation:

$$R=A_{0e}^{-\alpha t} + B_{0e}^{-\beta t} \quad (1)$$

Where, R=amount of fenthion and temphos at t days, A_0 and B_0 are the concentrations of fenthion and temphos at t=0, α and β are the

Table 1. Main concentrations (±SD) of fenthion and temphos (mg/L) following incubation with *Bacillus safensis* in mineral salt medium (MSM).

Time (days)	Fenthion (mg/L)	Temphos (mg/L)
0	400[a]±0.000	400[a]±0.000
3	372[b]±0.0039	307[b]±0.0060
7	50[c]±0.011	261[c]±0.003
14	34[c]±0.001	152[d]±0.002
30	275[d]±0.009	89[e]±0.004
LSD	21.5	12.036

Means followed with the same letter(s) in the same column are not significantly difference at p=0.05 according to LSD.

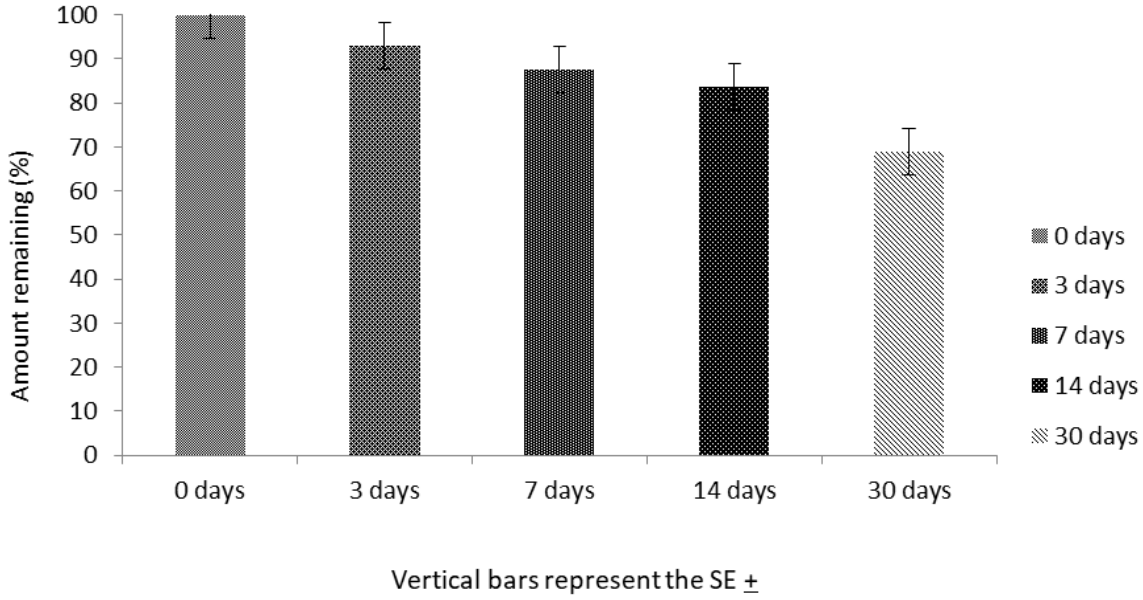

Figure 1. Amount remaining (%) of fenthion after incubation with *Bacillus safensis* in mineral salt media (MSM).

disappearance rate constants for first and second phase model, respectively. The half-life of exponential decay was calculated according to equation:

$T_{1/2}$ = (2.303log2)/rate constant (2)

RESULTS

Biodegradation of fenthion and temphos in mineral salt media (MSM)

The indigenous bacteria *B. safensis* strain FO-36b^T showed capability in degrading fenthion and temphos in mineral salt media (MSM). Data in Table 1 indicates that the concentrations of fenthion and temphos declined with the increase in the incubation periods. The concentration of fenthion (400 mg/L) was found to be 400, 372.8, 350.8, 334.6, and 275.5 mg/L after 0, 3, 7, 14, and 30 days of incubation, respectively, while the concentration of temphos (400 mg/L) was found to be 400, 307.7, 261.9, 152.4, and 89.3 mg/L following the same order. Generally, the rate of fenthion disappearance was high up to day 14 and slow thereafter while that for temphos was from day 7 onward (Table 1 and Figures 1 and 2). There were significant differences between the levels of fenthion and temphos at various time intervals. Less than 68% of the initial concentration was recorded at 30 days after the incubation of fenthion with the bacteria, whereas 22% of the initial amount was found after 30 days of incubation of temphos with the bacteria. Despite the significant drop in the starting material, only one metabolite was detected "iso-fenthion" (*O*, *S*-dimethyl *O*-[3-methyl-4-(methylthio) phenyl] phosphorothioate) in fenthion (Figures 3, 4 and 5). The recovery of the

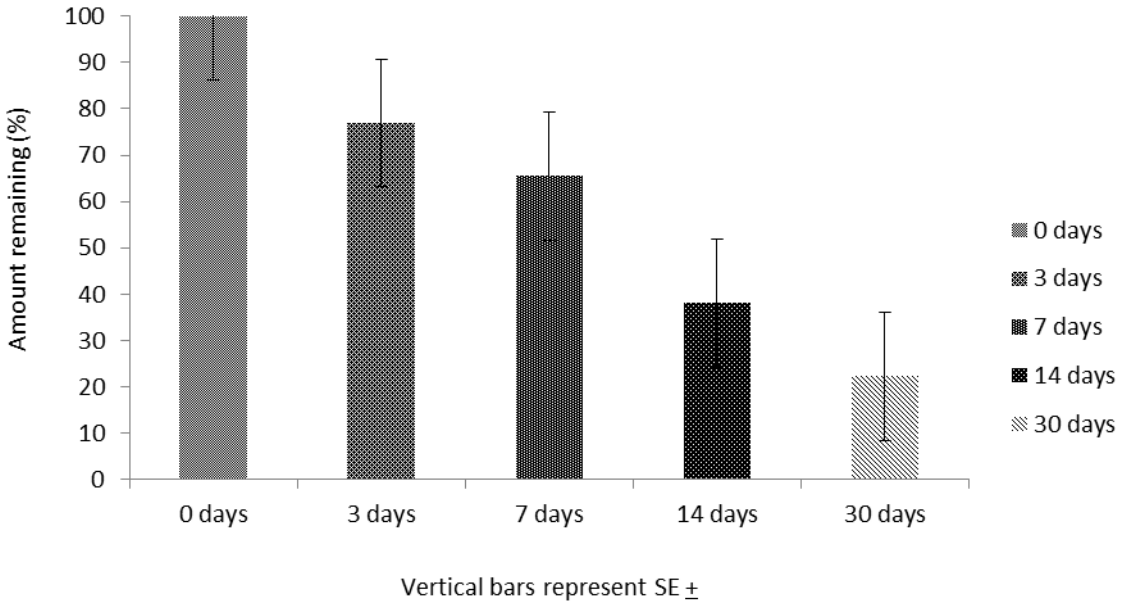

Figure 2. Amount remaining (%) of temphos after incubation with *Bacillus safensis* in mineral salt media (MSM).

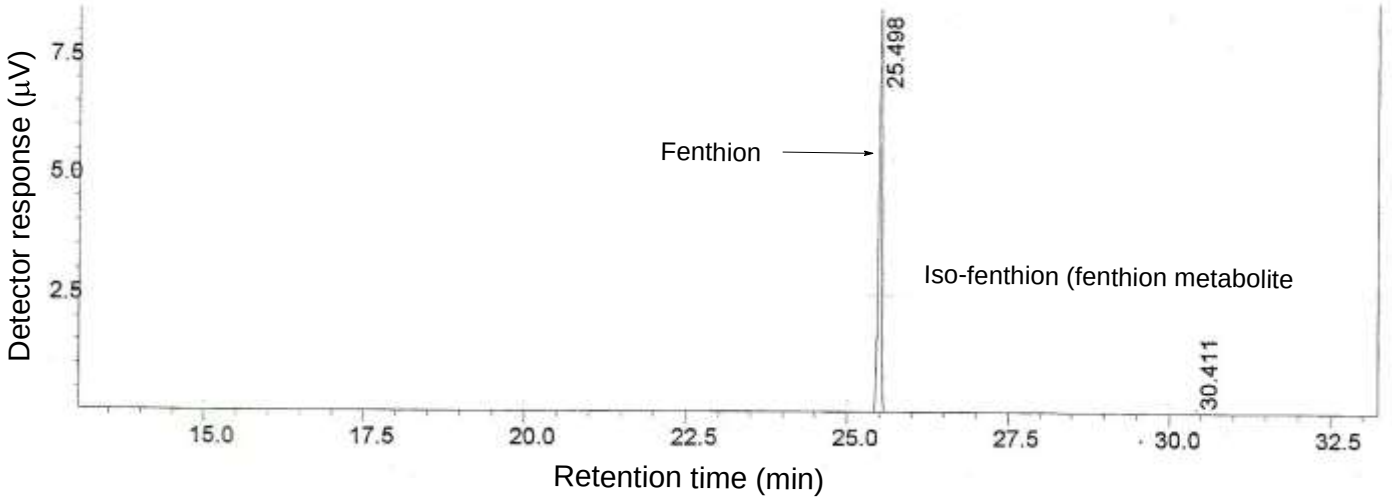

Figure 3. Typical chromatogram (Total Ion Current, TIC) of fenthion after 7 days of incubation with *Bacillus safensis* in mineral salt media (MSM).

fenthion and temphos from the media was greater than 98%. There was no change in the cultured bacteria after each incubation period. Generally, the results in Table 3 show that the degradation constant decreased with increase in the incubation period, while the mean lifetime is directly proportional to the incubation period.

Biodegradation kinetics

The data in Table 2 indicates that there was a faster rate of disappearance in the first phase than in the second. This is clearly reflected in the half-life values obtained. The half-life of fenthion and temphos in the first phase were estimated at 0.29 d and 0.11 days, respectively, while the corresponding values for the second phase were 3.69 and 1.15 days.

DISCUSSION

The results of biodegradation of fenthion and temphos by the bacteria *B. safensis* strain FO-36b^T isolated from

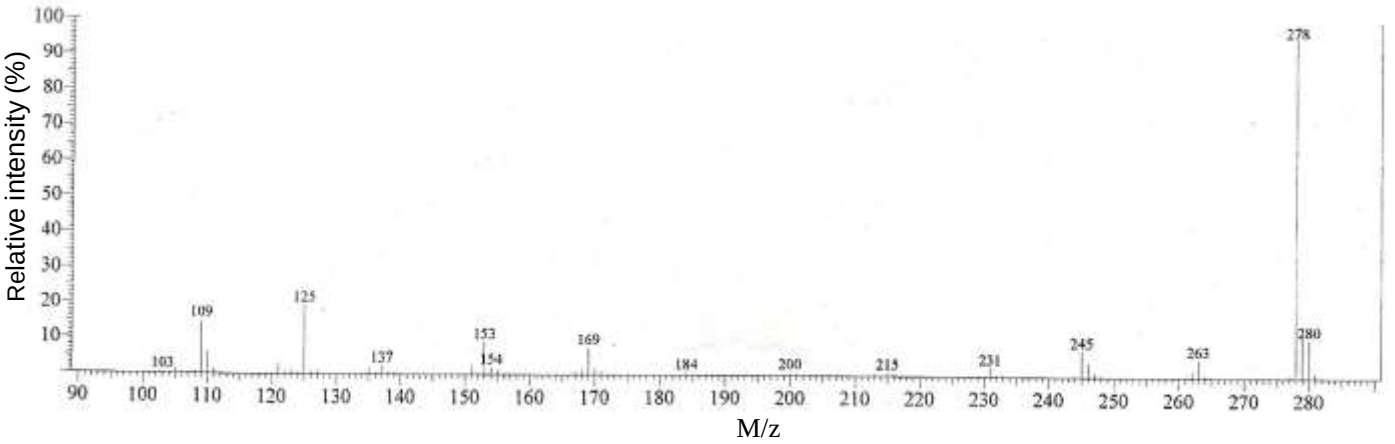

Figure 4. Mass spectrum of fenthion.

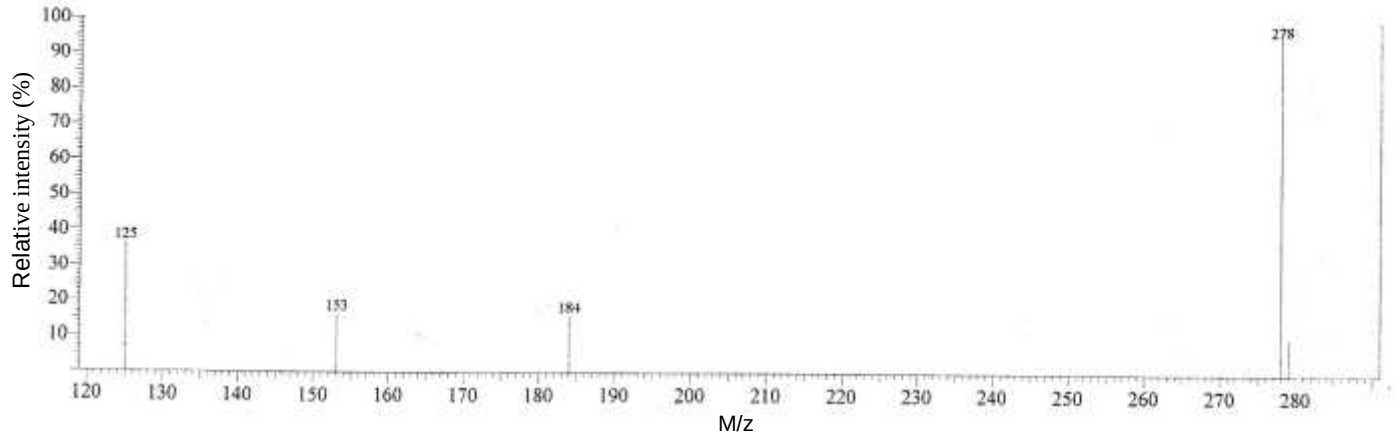

Figure 5. Mass spectrum of" iso- fenthion" *O*, *S*-dimethyl *O*-[3-methyl-4-(methylthio) phenyl] phosphorothioate.

pesticides polluted soil in Sudan was studied under mineral salt medium (MSM). Results indicate that the isolated organism is capable and efficient in degrading fenthion and temphos. The bacteria reduced the half-life of fenthion to 0.29 days in the first phase ($t_{1/2}$) and 3.69 days in the second phase ($t_{1/2}$) while for temphos it was reduced to 0.11 days in the first and 1.15 days in the second phase.

This reduction can be considered very significant compared to the reported fenthion half-lives 14 to 40 days. The degradation of temphos was followed by first-order kinetics, with a half-life of 17.2 days in the soil (CASRN, 2015). *Bacillus cereus, Bacillus mycoides,* and *Pseudomonas aerginosa* were reported as degrades of organic compounds such as petroleum products (Okerentugba and Ezeronye, 2003; Dhanarani et al., 2016) while *B. safensis* strain CFA-06 was reported to degrade aromatic compounds and petroleum aromatics (Francie et al., 2015). *B. safensis* Gram-positive and it has environmental relevance in biocatalysis and bioremediation studies (Kothari et al., 2013). Lateef et al. (2015) reported that *B. safensis* has promising biotechnological applications due to its ability to produce various industrial enzymes and industrially applicable secondary metabolites. Abiotic factors such as pH and temperature were found to have effects on biodegradability of chlorpyrifos by test microorganism (EPA, 1997). The current result agrees with those of Shaer et al. (2013) who showed that bacterial strains (*B. cereus, B. mycoides,* and *P. aerginosa*) isolated from pesticide-polluted soil are capable of degrading pendimethalin under the condition of mineral salt media. Further, this study agrees with Abdurruhman et al. (2015) who mentioned that bacteria *Psedomonas pickettii* isolated from pesticides polluted soil in the Sudan are capable and efficient in degrading pendimethalin and

Table 2. Statistical parameters of fenthion and temphos bacterial dissipation in mineral salt medium (MSM).

Statistical parameter	Fenthion	Temphos
A_0	372	333
B_0	350	261.8
α (days^{-1})	0.0236	0.0874
β (days^{-1})	0.0187	0.0604
$t_{1/2\alpha\ (days)}$	0.2925	0.0604
$t_{1/2\beta\ (days)}$	3.694	1.1457
Regression coefficient	0.559	0.8333

A_0 and B_0 are the concentration of fenthion and temphos at t = 0 and α, β are the disappearance rate constants for the first and second phase model, respectively.

Table 3. Mean lifetimes (days) and decay constants of fenthion and temphos following incubation with the *B. safensis*.

Incubation period (days)	Mean lifetime				Decay constant			
	3	7	14	30	3	7	14	30
Fenthion	41.34	52.42	77.64	80.10	0.0241	0.019	0.013	0.012
Temphos	11.34	16.40	14.50	19.96	0.088	0.061	0.069	0.050

atrazine under the condition of mineral salt media.

Temphos, when incubated in water with isolates from a sewage treatment lagoon or a farm pond, was found to slowly degrade after a 7 day lag period, forming products arising from the oxidation of the sulfide group and hydrolysis of the phosphate group (Daorai and Menzer, 1977). In soil, fenthion degradation ranges from 4 to 6 weeks and it occurs through photodegradation as well as anaerobic or non-photolytic organisms. However, soil particles strongly adsorb fenthion that makes it less susceptible to percolate with water through the soil (ATSDR, 2005). Recently, Ishag et al. (2016, 2017) reported at the first time degradation of chlorpyrifos, dimethoate, malathion, pendimethalin, and endosulfan by newly isolated bacterial strains form pesticides polluted soils. They obtained encouraging results with degradation of different pesticides and also found that the concentration of chlorpyrifos was sharply reduced in culture of *B. safensis* strain FO-36b^T than the other tested pesticides. The current study agrees with the argument of Abdelbagi et al. (2000, 2003) that indigenous soil microorganisms could be of great potential in reducing the level of contamination by pesticides in highly polluted storage soil in the Sudan. Their suggestion is in line with Elzorgani (1982) who mentioned that irrespective of a large amount of dichlorodiphenyltrichloroethane (DDT) and other pesticides applied in Gezira scheme, Sudan, yet their soil level is not high which indicate a possible and efficient degradation factors in these soils. This argument was later confirmed by Ali (2005) and Elsaid et al. (2011, 2010, 2009), who demonstrated the capability and efficiency of indigenous soil microorganisms (bacteria, actinomycetes, and fungi) in degrading endosulfan and lindane under the condition of selective mineral salt medium or soil. In addition, various types of synthetic and natural fertilizers were found to enhance the degradation rate (Elsaid et al., 2009).

Biodegradation of fenthion and temphos by *B. safensis* followed a biphasic model of initial phase of fast rate of disappearance followed by a second phase of slow disappearance. This phenomenon of biphasic disappearance rate in soil is common in many pesticides (Khaled et al., 2008; Rigas et al., 2007; Ahmed et al., 2007; Pigatello et al., 1996; Smith, 1993; Wauchope et al., 1992). The relative importance of the phase depends on the availability of the pollutants, hydrophobicity, and affinity for organic matter (Rigas et al., 2007; Pignatello and Xing, 1995).

Despite the drop in the starting material of temphos, no metabolites were detected. However, one metabolite "iso-fenthion" (*O*, *S*-dimethyl *O*-[3-methyl-4-(methylthio) phenyl] phosphorothioate) was detected in fenthion culture (Figures 3, 4 and 5). The detected fenthion metabolite (Figure 6) could be formed by rearrangement of sulfur and oxygen atom. Kouichiro and Yasuo (2006) reported that the formation of isomalathion is due to oxidation of malathion by cytochrome P-450. The absence of detectable levels of breakdown products on pesticides biodegradation studies involving bacteria and fungi was reported by many authors (Khaled et al., 2008; Ishag et al., 2016, 2017).

The bacterium was found alive after the end of each

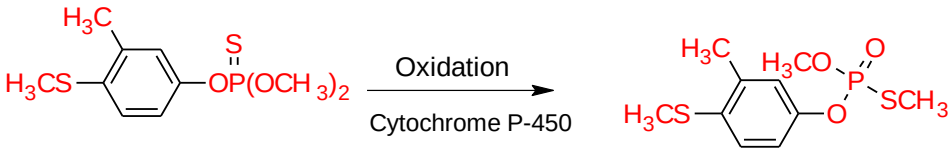

Figure 6. Degradation pathway of fenthion by *B. safensis* in MSM.

incubation period and even after the end of the whole experiment (30 days). The current results of *B. safensis* indicate its ability to live in such media.

The current results indicate that the strain of the bacteria *B. safensis* isolated from pesticides polluted soil was capable of degrading both fenthion and temphos under the conditions of mineral salt media. Based on this finding and those of previous studies (Ishag et al., 2016, 2017; Abdurruhman et al., 2015; Shaer et al., 2013; Elsaid and Abdelbagi, 2010; Elsaid et al., 2011, 2010, 2009; Osman, 2006; Ali, 2005), one can argue the significant of carrying further studies on this topic such as effects of environmental factors on soil media on the rate of degradation. Isolation and characterization of the responsible enzymes in this bacterium also deserve to be included in future work. Studies of the role of other indigenous microorganism deserve future work.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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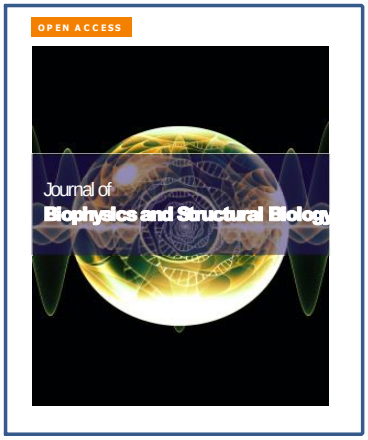

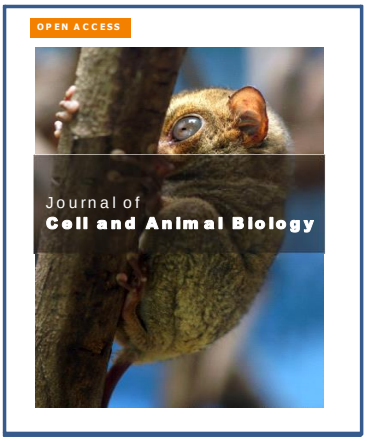

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