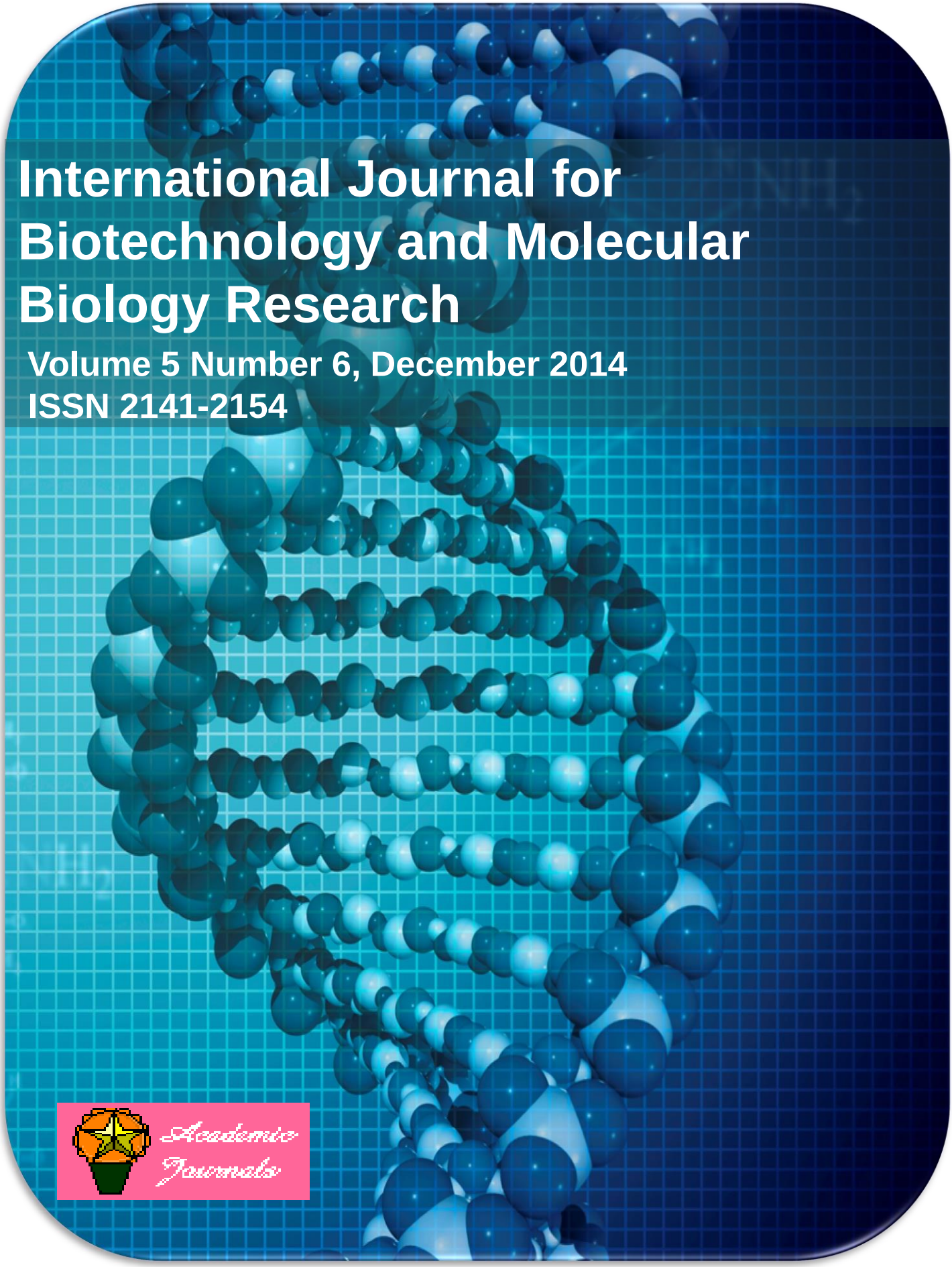

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Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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

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

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Full Length Research Paper

Molecular identification of *Lactobacillus plantarum* isolated from fermenting cereals

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The identification of a microbial isolate to genus level only amounts to a partial characterization of the isolate, but this can tell us a lot about that organism. Knowing the species allows the laboratory access to the body of knowledge that exists on that species. Identification schemes using phenotypic characteristics such as colony and cell morphology, Gram reaction and other staining characteristics, nutritional and physiological requirements for growth and metabolic characteristics have been developed and improved over many decades to a point where laboratories are able to identify isolates to species level using simple conventional methods. This phenotypic method however have some limitations apart from being laborious and time consuming, some organisms may however be misidentified either at genus or species level. This work aims at looking directly at the genome of lactic acid bacteria (LAB) and from this identifies some species using its genotypic and phenotypic characteristics. These bacteria species were identified by sequencing specific sections of ribosomal DNA - the 16S rRNA gene, after amplification by PCR, and then comparing the results to sequences stored on a related database. The results from both conventional and molecular methods were then compared. Twenty (20) *Lactobacillus plantarum* were isolated from spontaneously fermented cereals made into *"Ogi"* and identified using classical methods. They were further characterized using molecular methods by polymerase chain reaction (PCR) amplification of 16S rDNA genes to confirm their identities. The genotypic characterization however showed that 85% of the organisms identified using conventional method as *L. plantarum* correlated, while 15% did not correlate; 2 were identified as *Lactobacillus pentosus* and one unidentified *Lactobacillus* sp. The method is a rapid and reliable way of producing a large number of copies of a specific DNA sequence for the identification of LAB. This method is however, able to solve the problem of poor identification that is usually associated with the identification of this fastidious organism that is regularly used as probiotics, starter culture and bio-preservatives in fermented foods that are consumed and in biotechnology because they are generally regarded as safe.

Key words: Molecular methods, conventional, *Lactobacillus plantarum* identification, fermented foods, species and genera level, rapid, reliable.

INTRODUCTION

Microorganisms have been isolated from different sources especially from different food samples and grown in pure cultures over the centuries. A major aspect of microbiology and the work of food microbiologists and various microbiology laboratories is the ability to identify and characterize various isolates so that they can be

differentiated from one another. Different schemes that can be used to describe the characteristics and properties of microbial isolates are essential in every branch of microbiology. These schemes have been undergoing different forms of development and refinement over the years. The various methods are not static; but have been improved from time to time and proper identification is very essential when it has to do with foods that are consumed (Lucke, 2000; Olaoye and Onilude, 2009). The advent of molecular biology in the 1980s contributed a set of powerful new tools that have helped microbiologists to detect the smallest variations within microbial species and even within individual strains (Olaoye and Onilude, 2009). This is because different organisms have different genetic combination.

In fact, the technology has progressed far beyond the level needed by most routine laboratories, where identifying the species of any isolate is likely to be sufficient. Distinguishing between different strains of the same species (typing) is more likely to be of value in a research laboratory. Nevertheless, methods and equipment designed to help with both species identification and typing are commercially available for a range of applications (Lucke, 2000).

There are different molecular characterization techniques namely genotyping, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), ribotyping, repetitive sequence-based PCR (rep-PCR) and the use of 16S rDNA genes which relies on the relative stability of the 16S and 23S rRNA genes coding for ribosomal-RNA and so on (Ogier et al., 2002; Gomes et al., 2008; Paula et al., 2012).

Molecular characterization of microorganisms however has some distinct advantages over the known conventional methods. The molecular method of identification and characterization of microorganisms have been preferred over the classical ones which make use of the biochemical reactions and proteolytic activities of the organisms (Morgan et al., 2009). The classical and conventional method of identification is slow, laborious, time consuming and may not be 100% specific and accurate. It is also problematic and subjective due to ambiguous biochemical or physiological traits.

Bulut et al. (2005) reported that identification of lactic acid bacteria (LAB) by phenotypic methods such as sugar fermentation may be uncertain and complicated owing to the increase in species that vary with few characters. The commercially available system based on this technology is a valuable complementary tool to other routine identification technologies. However, identification based on the 16S rRNA gene is by no means infallible as the sequence stretch analysed is a reduced section of the full genome and the variability of this marker is low.

The development of molecular typing methods has offered the possibility of accelerating a great deal of bacterial identification which avoid so many biases that are related to the classical methods. The polymerase chain reaction (PCR) has however provided a method to detect DNA sequences with high speed and sensitivity. This technique is emerging as a new tool in identifying and selecting bacteria with specific and desirable functions (Bulut et al., 2005). A combination of different approaches in the identification of different organisms offer a solution to the use of the conventional method that makes use of the ability of LAB to produce acid from carbohydrate and other metabolic activities only (Morgan et al., 2009).

According to Merien et al. (2013), the nucleotide base sequences of *Lactobacillus* spp. 16S ribosomal DNA also provides accurate basis for phylogenetic identification of organisms that are slow growing, fastidious and are therefore poorly identified by conventional methods. These small ribosomal units exist universally among bacteria and include regions with species-specific variability which makes it possible to identify bacteria to species level.

The use of *Lactobacillus* sp. as probiotics in man has been found to enhance their immunity and increase their ability to fight and survive against food related pathogens. Also, nursing mothers prefer natural products with fewer artificial preservatives in foods that are used for weaning infants with natural fortification or supplements. They have also been found to be consumed in fermented foods that contain them for their health benefits (Adeyemo and Onilude, 2013).

Lactobacillus plantarum particularly has also been implicated in the reduction of raffinose- family of oligosaccharide content of soybeans used in the formulation of a weaning food blend by their ability to hydrolyse the raffinose to simple sugars and hence improve the weaning food (Adeyemo and Onilude, 2014). Fermentation with cultures containing LAB is able to produce healthy, safe, high quality and nutritious beneficial food products such as fermented milk, meat, vegetables, grains, cereals, legumes, meat, beverages, etc. These organisms produce lactic acid which has a way of preserving such fermented foods and also improve the flavour, texture and nutritional compounds of such foods through the metabolic activities of LAB during fermentation. Also, the metabolism and physiology of LAB is used in different biotechnological processes in industries to formulate LAB starters with useful metabolic activities and capabilities so as to ensure a wide range of quality fermented products with consistent characteristics (Adeyemo and Onilude, 2013).

Being used as probiotics and starter culture in many

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food industries and in fermentation technology, a prompt and rapid identification of *L. plantarum* is of utmost importance so as not to confuse this very important organism with other organisms of the same genus or species that are closely related. As a result of this, there is need for accurate identification of this organism, the importance of which cannot be over emphasized.

MATERIALS AND METHODS

Sample collection

Local varieties (LV) of sorghum (*Sorghum bicolor*) were obtained from a market and typed varieties (TV) from Institute of Agricultural Research & Training, Ibadan, Nigeria. They were all processed to o*gi* in the laboratory using the traditional method of Banigo and Muller (1972). *Ogi* was also obtained from traditional sellers within Ibadan (CO) and used for comparative studies. The samples were collected in clean polythene bags and transported to the laboratory.

Isolation of lactic acid bacteria

One gram each of the samples listed above was subjected to ten-fold serial dilutions using the method of Harrigan and MacCance (1976). Isolation of organisms was done with the pour plate method using molten MRS agar. After solidification, they were incubated anaerobically in an anaerobic jar at 30°C for 48-72 h. Pure cultures were selected and stored on slant overlaid with sterile glycerol.

Identification of the isolates

Morphological and macroscopic characteristics

For proper identification of the isolates, the cultural, morphological, biochemical and physiological characterization including microscopic and macroscopic examinations of the various isolates were carried out according to Sneath et al. (2009). Gram positive and catalase negative organisms were subjected to further biochemical tests.

Biochemical characteristics

Isolates were identified phenotypically on the basis of the following biochemical test after Gram's staining, catalase, oxidase, methyl red test, Voges Proskaeur, nitrate reduction, starch, casein and gelatin hydrolysis, growth at different pH and temperature and NaCl ranges and the ability to produce CO_2 from glucose and production of acid from carbohydrates such as fructose, lactose, maltose, galactose, arabinose, mannose, xylose, dulcitol, inositol, mannitol, raffinose, trehalose, rhamnose, etc.(Sneath et al., 2009).

Genetic characterization of isolates

Extraction of genomic DNA of LAB isolates

DNA extraction from the LAB isolates was carried out using a modified GES (5M guanidine thiocyanate (Fisher scientific, England), 0.1 N EDTA (Sigma, England) and 0.5% N-lauroyl - sarcosine sodium salt (Sigma, England) (w/v) DNA extraction method (Pitcher et al., 1989). Aliquots of 1.5 ml of overnight cultures grown in appropriate broth were centrifuged (Biofuge, Heraeus, Germany) in Eppendorf tubes at 13,000 g for 1 min. Pellets obtained were washed in 1 ml of ice cold lysis buffer (25 mM Tris-HCl (Sigma, England), 10 mM EDTA, 50 mM sucrose (BOH GPR 303997J), pH 8). The pellets were re-suspended in 100 µl of lysis buffer in addition to 50 $mgml^{-1}$ lysozyme (Sigma, England.) and incubated at 37°C for 30 min. 0.5 ml of the GES solution were added and mixed thoroughly. This was incubated at room temperature for 15 min. The lysate was then placed on ice for 2 min and 0.25 ml of 7.5 M ammonium acetate (Fisher scientific, England). Cooled ice was also added, vortexed and incubated on ice for 10 min. Aliquots (0.5 ml) of 24:1 chloroform : isoamyalcohol (Sigma, England) were added, vortexed and centrifuged for 10 min at 13,000 g. Aliquots of 800 ml of the upper phase were removed quantitively and placed in a clean Eppendorf tube. Cold isopropanol (Fisher scientific, England) was added and mixed for 1 min. This was then centrifuged at 13,000 g for 5 min and the supernatant removed from the pellet. The pellet was washed three times in 500 µl of 70% ethanol and dried at 37°C for 15 min. Aliquots (50 µl) of TE buffer were added and 5 µl of the DNA were checked on 1% agarose (Biogene, Kimbolton, UK) gels in 200 ml 1X TAE buffer and the DNA samples were then stored at -20°C for future use.

Polymerase chain reaction (PCR) amplification of 16S rDNA gene

The method of Bulut et al. (2005) was used. Amplification of 16S rDNA gene - ITS region, was performed by using the following primer pairs. Forward (16S ITS For), 5/- AGAGTTTGATCCTGGCCTCAG-3/ and reverse (16S - ITS Rev), 5/ - CAAGGCATCCACCGT - 3/, 16S rDNA V3, forward 5/ - CCTAGGGGAGGCAGCAG - 3/ and 16S rDNA V3, reverse, 5/ - ARRACCGCGCTGCTGC-3/. The forward 5'-CCTACGGGAGGCAGCAG-3' and reverse, 5'-ATTACCGCGGCTGCTGG-3', primers used occupied positions 341-358 and 518-534, respectively of the V3 region in the 16S ribosomal DNA *of Escherichia coli*. The primers specify about 200 bp of the PCR products (as could be seen on the gel after electrophoresis).

The V3 primer pair was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates.

Each of the polymerase chain reactions (PCR) was performed in a 50 µl reaction volume containing 50 µg genomic DNA as the template. 10 µl of 0.2 mM deoxynucleoside triphospates, dNTPs (Promega UI20A - UI23A, Madison, WI, USA), 10 µl of 2.5 mM $MgCl_2$, 10 pmol each (0.1 µl volume) of the DNA primer in PCR buffer (Promega, UK), and 10 µl of 1.25 units Taq DNA polymerase (Promega, UK) and 18.9 µl distilled water. Amplification conditions were as follows: an initial denaturation step of 5 min at 94°C, 40 amplification cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C. Reactions were terminated with a final extension step for 10 min at 72°C. PCR amplification was performed in a Thermocycler (Techne- Progene, Cambridge, UK).

Gel electrophoresis of 16S rDNA PCR Products

Electrophoresis of the amplified 16s rDNA PCR products were performed on the Bio-Rad contour - clamped homogenous electric field (CHEF) DRII electrophoresis cell. This was done through 1.5% (w/v) agarose gel (Biogene, Germany) in 0.5 X TAE buffer at 84 V for 1.5-2 h. This was prepared by boiling 1.5 g of agarose powder in 100 ml of 0.5X TAE buffer. A 100 bp ladder (Promega, U.K) and 1 Kb DNA ladder (Promega, U.K) were used as molecular size markers.

Sequencing and analysis Of 16S rDNA gene

Purification of PCR 16S rDNA gene

75 µl of the PCR 16S rDNA amplified products (obtained above) were resolved in 1% agarose gels with the conditions earlier described. PCR products were resolved by gel electrophoresis, using an agarose gel (1.5%; Biogene) that was stained with of 0.5 µg/ml ethidium bromide, in 1xTAE buffer at 84 V for 1.5 - 2 h.

The DNA bands were then visualised using a UV transilluminator (Amersham Pharmacia Biotech, UK) with 313 nm emission and pictures were taken using Fuji Film Imaging system FT1-500 (Amersham Pharmacia Biotech, UK).

The resulting bands in agarose gel were carefully excised with sterile scalpels and then purified the Wizard PCR preps DNA purification kit (Promega, USA). The purified DNA was kept at 4°C until used.

Drying of the purified 16S rDNA genes

To a 50 µl of the purified DNA, 0.1 µl of sodium acetate buffer (3M, pH 5.0) and 2.0 µl of 100% ethanol were added. This was then incubated at -20°C for 1 h. It was brought out and left to stand at room temperature for 5 min, and then centrifuged at 13,000 g at 4°C for 45 min. The liquid was removed, leaving only the DNA in the Eppendorf tubes. The DNA was dried in an incubator at 37°C for 30 min.

Sequencing of 16S rDNA gene

The dry DNA samples (obtained using V3 primers) were sequenced using a computer analytical sequencer (MGW - Biotech, Germany) with the V3 and V5 primer Rev, acting as the basis according to manufacturer's instructions. The generated nucleotide sequences were subjected to analysis. Sequencing of the purified 16S rDNA DNA products was performed using the sequencing unit of the University of Nottingham; a 373 DNA sequence (Perkin-Elmer Applied Biosystems) was used with the Taq Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). The full identities of the isolates were then obtained by subjecting the nucleotide sequences to searches in the Gene Bank (http://www.ncbi.nlm.nih.gov/blast/) with the Blast search program.

Analysis of the 16S rDNA gene sequence

The generated sequences of the 16s rDNA genes were subjected to alignment in the databases at the BLAST, Basic Local Alignment and Search Tool, Website: http://www.ncbi.nih.gov/blast/.igi. The isolates were then identified based on the result of the analysis.

RESULTS

Table 1 shows the result of the conventional method of identification of LAB, the carbohydrate utilization pattern and biochemical characteristics of the isolates. All the 20 isolates were identified as *L. plantarum*. The result obtained agrees with the characterization pattern of other authors (Sneath et al., 2009).

Table 2 shows the comparison between the phenotypic method and the genotypic method using the 16S rDNA gene sequence of the 20 isolates that were initially identified as *L. plantarum.* The topmost sequences producing significant alignments when the nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (http://www.ncbi.nlm.nih.gov/blast/Blast.igi) for *L. plantarum* isolates.

Altogether, seventeen *L. plantarum* isolates that have been identified before showed a significant alignment in the gene database. The result of the PCR sequencing correlated in 17 out of 20 isolates while there was no correlation in 3 out of 20. The names and accession numbers of these seventeen isolates have significant alignments with the *L. plantarum*. All the seventeen topmost species was shown to produce significant alignment with the marker and have expected value (E value) of between 1e - 73 and 5e - 7 and maximum identification (Max identity) of between 95 and 100%. They were all *L. plantarum*. Three out of the twenty isolates did not have significant alignment with the others. They were identified as *L. pentosus* and one unidentified *Lactobacillus* sp*.* There was significant difference in the molecular method and the conventional methods. The result however did not correlate but a divergent view was presented which shows a difference in their gene sequence.

Table 3 shows the qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification.

The 16S rDNA of the 17 species after amplification with primers was found to belong to the *L. plantarum* group as they were identified as *L. plantarum* by partial gene sequencing. The 16S rDNA genes of the other of the three organisms were not shown because a different gene sequence was presented.

Figure 1 shows the *L. plantarum* strain 16S ribosomal RNA gene, the partial sequence alignment of 16S rDNA after amplification of the gene by PCR in the gene bank data base. Molecular characterisation of the isolates was done by extracting the DNA gene sequence using universal primers and when compared, it was identified as *L. plantarum* with alignment.

Figure 2 shows the nucleotide sizes in base pairs (bp) of the plasmids of the selected seventeen *L. plantarum* isolate that were used for further work after their identities have been confirmed by 16S rDNA. This base sequence provides significant information on the 16S rDNA gene sequence of the *L. plantarum.* The nucleotide base sequence of the 16S rDNA has provided a basis for phylogenetic identification and analysis.

DISCUSSION

Accurate and definitive microorganism identification is essential for a wide variety of application including biotechnological, industrial, biomedical, pharmaceutical and environmental studies. The 16S rDNA sequence based analysis is a central method to understand not only

Table 1. Physiological and biochemical characteristics of isolates.

Isolate code	Gram reaction	Cell morphology	Catalase	Oxidase	Casein hydrolysis	Gel HND	M.R	V.P	H_2SP	Growth at 15°C	45°C	pH at 3.9	pH at 9.2	pH at 5	4% NaCl	CIT.UTI	Glucose	Xylose	Rhamnnose	Triammonium citrate	Raffinose	Sucrose	Lactose	Maltose	Galactose	Fructose	Arabinnose	Mannose	Dulcitol	Mannitol	Inositol	Motility	Indole	NH_3Arg	Nitrate red	Probable identity
1	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
2	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
3	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
4	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
5	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
6	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
7	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
8	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L .plantarum*
9	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L .plantarum*
10	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
11	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
12	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
13	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
14	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
15	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
16	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
17	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
18	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
19	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*
20	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	*L. plantarum*

R = Rod; + = A positive reaction; = A negative reaction; D = A delayed reaction; W = A weakly positive reaction; M.R = methyl red test, V.P = Voges Proskaeur

Table 2. Comparison of phenotypic and genotypic methods of identification of *L. plantarum*.

Isolate code	Conventional identity	Closest relative (using 16srDNA gene sequencing)	Identity	Gene bank accession no
L. plantarum CO1	*L. plantarum*	*L. plantarum*	95%	GQ180906.1
L. plantarum CO2	*L. plantarum*	*L. plantarum*	95%	GQ166663.1
L. plantarum CO3	*L. plantarum*	*L. plantarum*	95%	GQ166662.1
L. plantarum CO4	*L. plantarum*	*L. plantarum*	95%	GQ166661.1
L. plantarum CO5	*L. plantarum*	*L. pentosus*	91%	GQ180915.1
L. plantarum CO6	*L. plantarum*	*L. plantarum*	95%	GQ180902.1
L. plantarum LV1	*L. plantarum*	*L. plantarum*	95%	FJ861114.1
L. plantarum LV2	*L. plantarum*	*L. plantarum*	95%	FJ861113.1
L. plantarum LV3	*L. plantarum*	*L. plantarum*	95%	FJ861112.1
L. plantarum LV4	*L. plantarum*	*L. plantarum*	95%	FJ861111.1
L. plantarum LV5	*L. plantarum*	*L. plantarum*	95%	FJ851111.1
L. plantarum LV6	*L. plantarum*	*L. pentosus*	91%	FJ851122.1
L. plantarum LV7	*L. plantarum*	*L. plantarum*	95%	FJ851116.1
L. plantarum LV8	*L. plantarum*	*L. plantarum*	95%	FJ851113.1
L. plantarum TV1	*L. plantarum*	*L. plantarum*	95%	FJ84495.1
L. plantarum TV2	*L. plantarum*	*L. plantarum*	95%	FJ844955.1
L. plantarum TV3	*L. plantarum*	*L. plantarum*	95%	FJ844949.1
L. plantarum TV4	*L. plantarum*	*L. plantarum*	95%	FJ844954.1
L. plantarum TV5	*L. plantarum*	*Lactobacillus* sp.	90%	FJ843956.1
L. plantarum TV6	*L. plantarum*	*L. plantarum*	95%	FJ844953.1

Table 3. Qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification.

S/N	Sample ID	16S rDNA			
		Conc. (ug/L)	A260nm	A260/280	0.0260/230
5	H101	18.34	0.367	1.82	0.02

the microbial diversity within and across the group but also to identify new strains. Bacterial species have at least one copy of the 16S rDNA gene containing highly conserved regions together with hyper variable regions, which is used for identification of new strains. However, a considerable variation can occur between species in both the length and the sequence of 16S rDNA ITS region, therefore this region is useful in characterization of bacterial species (Mohammed et al., 2011). The 16S rDNA gene is very useful because the genome of all bacteria contains this conserved gene and any small variability in this region is unique and specific to each species. This characteristic is usually harnessed in their identification (Mohania et al., 2008).

Considering the conventional method for identifying LAB isolates, the objective of this study was to compare the phenotypic method and the 16SrDNA sequencing which is a species-specific PCR reaction for the proper identification of the twenty *Lactobacillus* sp. The genus level was however the same for all the isolates, they were further characterized using PCR reactions to perform complete identification. The results obtained with 95% reliability and higher were considered; those lower than this were not considered because their gene sequences were identified as different organisms. Considering that species-specific PCR reactions target specific genes of genera and species, the molecular method was considered reliable. Molecular bacteria identification is based on the full length of 16S rDNA gene sequence by several studies have shown that the initial few base pair sequence provides sufficient discrimination between strains because this region shows a high genetic diversity.

Of the 20 isolates used in this work, three presented divergent results as compared to 16S rDNA sequencing and species-specific PCR reaction. This confirmed the result of 17 out of 20 isolates tested (17/20), that is, 85% and divergent result were obtained in 3 out of 20 (15%) isolates that were screened (3/20). Out of these, 2 were identified as *L. pentosus* while the last was a

|GQ180905.1|*Lactobacillus plantarum* strain TJ2 16S ribosomal RNA gene, partial sequence, Length=182, Score = 283 bits (153), Expect = 1e-73,

```
Identities = 153/153 (100%), Gaps = 0/153 (0%) Strand=Plus/Plus

Query
GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT

Query
AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query        CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA
             ||||||||||||||||||||||||||||||||
Sbjct        CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA
```

Figure 1. Alignment of 16S rDNA nucleotide sequences of *L. plantarum* against *L. plantarum* strain LpT2 (accession no GQ166663.1) and *L. plantarum* strain LpT1 (accession no GQ166662.1) in the gene bank data base.

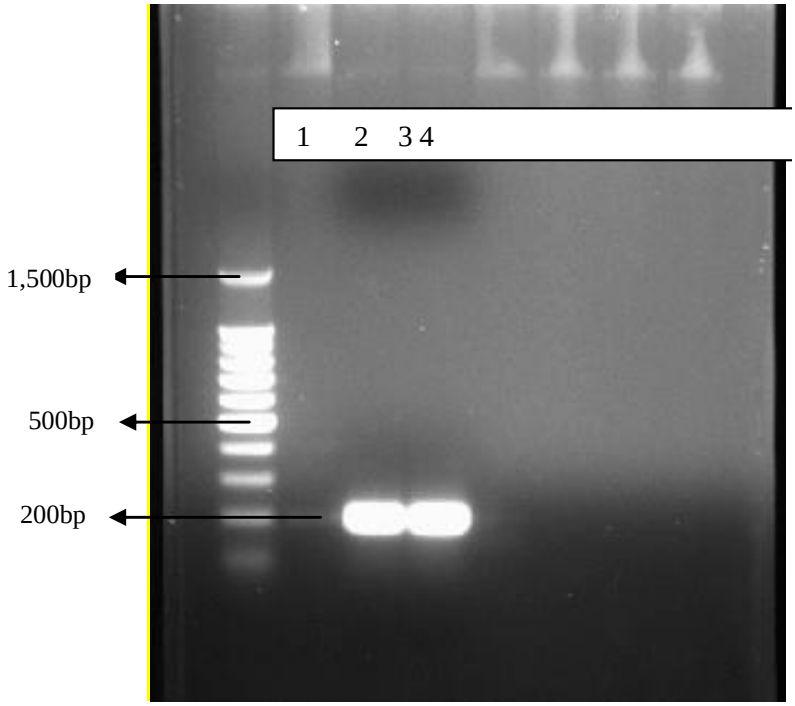

Figure 2. The nucleotide sizes of the plasmids of the selected nine *L. plantarum* isolate.

Lactobacillus sp. that could not be identified. This result agrees with the report of Marroki et al. (2011) who reported a similar view stating that *L. plantarum* and *L. pentosus* have very similar 16S rDNA sequences that only differ by 2 base pair. Other authors also reported that both organisms belong to the same phylogenetic group and they can only be differentiated when analysis of 16S-23S larger spacer is done (Ennahar et al., 2003). This may also be the same reason for the other *Lactobacillus* sp. that was not identified by this method presenting a result that did not correlate with those obtained earlier by phenotypic method. However, the result of the conventional method cannot be discarded completely but it can be regarded as giving a clue or presumptive result which can then be confirmed by molecular method.

Differences between genotypic and phenotypic tests have been identified previously not just for LAB but also for many other bacteria (Gomes et al., 2008; Paula et al., 2012). They also noted that this tool is useful for identifying microorganisms at sub species level which cannot easily be identified by other common technique. Phenotypic method may also have poor reproducibility as a result of changes that occur during the growth and metabolism of different organisms. This also agrees with the report of Mohania et al. (2008) who reported that bacterial isolates do not express their genes at the same time or they may lose some characteristics such as plasmids during culturing. This may however be responsible for the inconsistencies that are usually identified in sugar fermentation patterns and other biochemical tests that rely on physiological characteristics of different organisms for identification.

Gill et al. (2006) also stressed another importance of this molecular method being a desirable advantage of 16S rDNA over the conventional one. Apart from being rapid, the sequence could also be performed not only on bacterial culture but also on the sample so as to study the diversity of the organisms without culturing. The efficacy and efficiency of this method was clearly demonstrated in this work by differentiating strains belonging to the same species and it has been clearly identified by various authors such as Gill et al. (2006) and Morgan et al. (2009) because the results are not subjective.

The molecular method used in this work further confirmed the real identities of *L. plantarum* that were used for further work in the fermentation pattern for the formulation of a weaning food blend as earlier reported by Adeyemo and Onilude (2013). The real identities of the organisms are usually revealed by molecular methods and the results can be reproduced at any time and in different places without environmental variations. Based on the result of this study, the 16S rDNA sequencing method is specific for the gene of target and broader strategies that can characterize lactic acid bacteria without prior knowledge of genetic targets, this is however a desirable characteristics of this method, it is thus recommended for proper identification of organisms to be used in fermented foods as starter culture or bio-preservative.

The result obtained in this work agrees with the result obtained by Parker et al. (2001). They opined that several PCR methods have subsequently been developed to overcome difficulties experienced with phenotypic methods. The method described in this work allows the amplification of specific PCR products. This enables direct sequencing of unknown regions without the need for DNA cloning but makes use of analysis of microbial genetic elements. Shittu et al. (2006) also noted the accuracy of the molecular diagnostic method in the ability to rapidly identify microorganisms isolated from clinical samples from genus level to species level using automated systems. Reduction of analysis time and reproducibility would be advantageous, especially for organisms that are fastidious, slow-growing and of medical and industrial importance.

The result obtained also solves the problem of misidentification. This agrees with the work of Woo et al. (2008) who reported that some LAB species are closely related to *Lactobacillus* sp. The importance of accurate identification need to be emphasized in LAB obtained from fermented foods that are used as probiotics or starter cultures. This is because some LAB are also involved in clinical infections such as *Leuconostoc* sp., *Pediococcus* sp. *and Enterococcus* sp. These organisms are of medical importance and should not be misidentified with other *Lactobacillus* sp. The use of 16S rDNA will lower the risk of inaccurate or poor identification of these pathogens that are also similar to other *Lactobacillus* sp.

However, in industrial microbiology for example, there are various importance of rapid methods of identification of microorganisms. First, it is of paramount importance to food/industrial microbiologists for screening and identify-cation of organisms that are of great industrial and biotechnological purposes. Rapid detection and identify-cation of microorganisms also allows for continuous monitoring of microbial growth in relation to various metabolites that are produced by them especially in pharmaceutical industries such as enzymes, vaccines, antibiotics, organic acids etc. Also, the ease of producing a large number of copies of a specific DNA sequence can be applied in the industry for the production of many important products from microorganisms using some specific genes from them.

Finally, the advantage of genotyping is that it is an accurate method for the identification of *L. plantarum* in that the genome is stable; the genetic composition of the organism is independent of cultural conditions and method of isolation; it can easily be subjected to auto-mation and the results can be analysed statistically with ease. LAB are referred to as “probiotics’’ and it belongs to the group of organisms that are generally regarded as safe (GRAS). Its prompt and quick identification is a

useful tool in distinguishing between these probiotics and other opportunistic pathogens that may also be present as contaminant in fermented foods.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Molecular characterization of resistance to Russian wheat aphid *(Diuraphis noxia* Kurdjumov) in bread wheat (*Triticum aestivum* L.) line KRWA9

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The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) causes extensive economic damage to wheat (*Triticum aestivum* L.) in most wheat growing regions of the world. Control of RWA using systemic insecticides is expensive and pollutes the environment therefore the most effective method of RWA control is the development of RWA resistant cultivars. This study was initiated to determine inheritance of RWA resistance in a wheat resistance source KRWA9, and identify the chromosome location of the resistance gene. Inheritance was studied in parent materials, F_1 populations, F_2 populations and $F_{2:3}$ families of a cross between resistant line KRWA9 and a susceptible variety NjoroBW2. Seedlings were infested with RWA then scored for damage on a visual scale of 1 to 9 after 21 days of infestation. The segregation data from NjoroBW2 × KRWA9 population depicted monogenic dominant inheritance of the resistance gene with phenotypic ratios of 3:1 in F_2 populations and 1:2:1 in $F_{2:3}$ families. Bulk segregant analysis approach was used for the mapping of resistance. Nine simple sequence repeat (SSR) primers were tested between parental lines and bulks, and only chromosome 7DS SSR marker *Xgwm111* produced clear polymorphism between the parental lines and the resistant and susceptible bulks. Detailed analysis of this marker with the full population revealed very close linkage to resistance with a coefficient of determination (R^2) value of 85%. This marker provides good opportunities for the marker-assisted breeding towards improving Russian wheat aphid resistance.

Key words: Russian wheat aphid, resistance, susceptibility, simple sequence repeat (*SSR*) markers.

INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), a pest of wheat and barley, is indigenous to southern Russia, Iran, Afghanistan and countries bordering the Mediterranean Sea (Hewitt et al., 1984). The pest has spread widely and is now found in all the continents except Australia (Ennahli et al., 2009), and

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causes economic damage to wheat in many parts of the world. In Ethiopia, Miller and Haile (1988) reported 68% yield loss in wheat. In South Africa, 21−92% yield losses were reported (Du Toit and Walters, 1984). In Kenya, it can cause losses of up to 90% in wheat (Malinga, 2007) and sometimes up to 100% due to prolonged drought conditions. RWA attacks the plant by infesting the young growing tip, deep in the leaf whorls where it feeds from the phloem of longitudinal veins. Symptoms of RWA attack appear as chlorotic spots that coalesce to form white, yellow or purple streaks running parallel to the mid rib of leaves (Botha and Matsiliza, 2006). In young plants, heavy infestation leads to prostate tillers while adult plants show trapped ears within the flag leaf looking like a fish hook. Severe infestation may lead to head sterility and death of host plant.

Insecticide use and particularly contact foliar applications are ineffective because of the feeding nature of the aphid. The aphid feeds within the rolled leaf whorl so cannot be easily reached by contact foliar sprays. This necessitates the use of more expensive systemic insecticides which apart from being harmful to the environment promote development of resistant biotypes and destroys biological agents. RWA resistant cultivars have been observed to have a yield advantage as compared to susceptible cultivars (Tolmay et al., 2000) and resistant cultivars have low cost as seed is usually the least expensive component in the production system besides being environment friendly. Host plant resistance is therefore, the most desirable alternative that could form part of an integrated pest management programme (IPM).

The first RWA resistant cultivar, TugelaDn (containing resistance gene *Dn1*), was released in South Africa in 1992 (Van Niekerk, 2001). A new biotype-designated RWASA2 was identified in 2005 virulent to *Dn1, Dn2, Dn3* and *Dn9* (Jankielsohn, 2011). Most of the RWA resistant cultivars available for commercial production in South Africa (Tolmay et al., 2007) were overcome by RWASA2. Similarly, resistant cultivar Halt (containing *Dn4*) was released in the United States in 1994 (Quick et al., 1996), but a new biotype, USARWA2 with virulence to resistance genes *Dn4* and *Dny* was reported in 2004 (Haley et al., 2004), also overcoming the majority of commercially available resistant cultivars. Although RWA resistance expression is known to be influenced by genetic background (Randolph et al., 2005; Tolmay and Van Deventer, 2005), it is nonetheless assumed to function on a gene-for-gene basis in terms of the resistance/biotype interaction (Ricciardi et al., 2010). Recently a third biotype, RWASA3 virulent to *Dn1, Dn2, Dn3, Dn4* and *Dn9* was reported in South Africa by Jankielsohn (2011). Notably, neither *Dn4* nor *Dny* had been deployed against RWA in South Africa. In Kenya, two biotypes with genetic differences have been discovered in the major wheat growing areas, that is, Njoro and Timau (Malinga et al., 2007a). Amplified fragment length polymorphism markers used to detect genetic differences showed that the Njoro biotype may contain more virulent populations as compared to Timau biotype (Malinga et al., 2007a). This was the first confirmatory report on biotypes in Kenya and it raised great challenges to resistance breeding programs for Russian wheat aphid.

Breeding for RWA resistant cultivars requires a reliable method of selecting plants containing a resistant gene. While phenotype based selection method is straight-forward, it has several limitations like the environmental influence on symptoms of damage expression. It is therefore highly desirable to employ a screening technique that is based on molecular markers linked to the resistance genes. Aside from overcoming the problems associated with phenotypic screening, marker-assisted selection (MAS) would enable gene pyramiding which is the combination of two or more resistance genes efficiently. This will expedite the process of breeding for multiple and durable resistance.

Most of the known wheat genes conferring resistance to RWA, have been mapped using microsatellite markers. Nine of these resistance genes are located on the D genome of wheat and one on the 1RS/1BL translocation (McIntosh et al., 2003). A study by Liu et al. (2001) revealed that the locus for wheat microsatellite GWM111 (*Xgwm111*), located on wheat chromosome 7DS (short arm), is tightly linked to RWA resistance genes *Dn1, Dn2* and *Dn5*, as well as *Dnx* in wheat resistance source PI 220127. The segregation data indicated that RWA resistance in PI 220127 is also conferred by a single dominant resistance gene (*Dnx*) (Liu et al., 2001). These results by Liu et al. (2001) confirmed that *Dn1, Dn2* and *Dn5* are tightly linked to each other, and this provided new information about their location, being 7DS, near the centromere, instead of as previously reported on 7DL. According to Miller et al. (2001), the marker *Xgwm437* is closely linked to *Dn2* at 2.8cM. *Xgwm106* and *Xgwm337* flanked *Dn4* on chromosome 1DS at 7.4 and 12.9 cM, respectively (Liu et al., 2002). Nkongolo et al. (1991a) reported RWA resistance gene *dn3* in *Triticum tauschii. Dn5* is located on wheat chromosome 7DS rather than 7DL and microsatellite marker *Xgwm635* shows close linkage to the gene (Liu et al., 2001). The markers *Xgwm44* and *Xgwm111* are linked to *Dn6* near the centromere on chromosome 7DS at 14.6 and 3.0 cM, respectively (Liu et al., 2002). This was the first report of the chromosome location of *Dn6*, which is either allelic or tightly linked to *Dn1, Dn2, Dn5* and *Dnx*. *Xgwm635* (near the distal end of 7DS) clearly marked the location of a previously suggested resistance gene in PI 294994, which was designated as *Dn8* (Liu et al., 2001). *Xgwm642*, in a defense gene-rich region of chromosome 1DL, marked another new gene *Dn9* from PI 294994 (Liu et al., 2001). A third new gene *Dny* from the Chinese wheat Lin-Yuan207 was localized on chromosome 1DL between *Xgwm111* and *Xgwm337* (Liu et al., 2001). A

study was carried out with PCR markers for Russian Wheat Aphid Resistance Gene *Dn7* on Chromosome 1RS/1BL and two markers which amplified rye-specific fragments proved to be useful for MAS. *Xrems1303* amplified a 320-bp band only in cultivars with high-level resistance to USA biotype 2 and was effective for MAS of *Dn7*. *Xib267* was found to be linked to the susceptible locus and amplified a fragment specific for rye Petkus 1RS (Lapitan et al., 2007).

Most of the Kenyan commercial wheat varieties are susceptible to RWA (Kiplagat, 2005) and since breeding of RWA resistant cultivars is further complicated due to presence of RWA biotypes, rapid breeding for and deployment of additional wheat cultivars resistant to RWA is urgently needed to reduce further losses from RWA outbreaks. This study was carried out to determine the inheritance and chromosome location of RWA resistance gene in the wheat source KRWA9.

MATERIALS AND METHODS

Plant materials and population development

Seeds were obtained from the Kenya Agricultural Research Institute, Njoro and planted in the crossing block in a row spacing of 30 cm. Crossing was carried out between resistant line 'KRWA9' and susceptible commercial variety 'Njoro BW2' to obtain F_1 progeny. The F_1 progeny was planted the following season and selfed to obtain F_2 seeds. $F_{2:3}$ families were obtained by planting seeds harvested from individual F_2 plants. Plants grew under normal rainfall regime with occasional irrigation supplement.

Phenotyping

The parents, F_1 plants, 100 F_2 plants and $F_{2:3}$ families were screened for RWA resistance under greenhouse conditions. Parents, F_1 and F_2 seedlings were grown in 20-cm-diameter pots containing *sterilized forest soil and sand at a ratio of 3:1* mixed with 5 g Di-ammonium phosphate (18-46-0) fertilizer. Each pot contained two to four seedlings. Fifteen (15) seeds from each $F_{2:3}$ family were planted on evaluation flats (1.5 × 1.0 × 0.75 m) containing *sterilized forest soil and sand at a ratio of 3:1* mixed with 75 g Di-ammonium phosphate (18-46-0) fertilizer. Due to poor germination, screening data was collected from ten plants of each $F_{2:3}$ family. The plants were watered regularly to ensure that they did not suffer moisture stress.

The virulent RWA colony that had earlier been biotyped by Malinga et al. (2007b) was collected from symptomatic bread wheat in the screenhouse and multiplied in preparation for infestation. The aphid colony was established on 'Kenya Kwale', a wheat variety that is highly susceptible to RWA and maintained in the greenhouse with temperatures 25:18°C, photoperiod (LD 12:12) and relative humidity varying between 60-80%. The pots and evaluation flats were caged with a 60 cm high wire cage and covered with a polyester screen mesh (68 meshes per square cm) to prevent aphids from getting in or escaping. Five adult aphids (3 - 5 instar stage) were placed on the whorls of seedlings at the two leaf stage using a camel hair brush. Five aphids were used for each plant to ensure maximum infestation pressure was achieved. RWA infestation was rated at twenty one days after infestation and scoring done according to a modified 1 - 9 visual scale (Malinga, 2007). Plants showing damage scale of 1 - 5 were grouped as resistant and 6 - 9 susceptible.

Statistical analysis

The data of RWA reaction for individual F_2 plants was tested against an expected phenotypic segregation ratio of 3:1 using the Chi square (χ^2) goodness of fit test, to confirm the mode of inheritance at probability level of P = 0.05. The data on RWA reaction for individual $F_{2:3}$ families was tested against an expected phenotypic segregation ratio of 1:2:1 using the Chi square (χ^2) test to also confirm the mode of inheritance at probability level of P = 0.05. The segregation of $F_{2:3}$ families was expected to confirm the segregation ratios observed in F_2 populations and aid in the classification of F_2 lines for the bulk segregant analysis.

Genotyping using microsatellite markers

DNA was isolated from parents and 100 F_2 plants following the protocol by Dellaporta and Woods (1983) with some modifications. Approximately 500 mg of leaf tissue was ground with liquid nitrogen before adding and mixing with 500 µl of extraction buffer (0.1 M Tris-HCl pH 8.0, 0.05 M ethylenediaminetetraacetic acid (EDTA), 0.5 M NaCl, 1% polyvinylpyrolidone, 1.6% sodium dodecyl sulphate (SDS). This was followed by the addition of 50 µl of 20% SDS, and after mixing by inversion the tubes were incubated for 15 min at 65°C. The samples were removed from incubator and 250 ml of potassium acetate (-20°C) followed by incubation in freezer for 10 min at -20°C. The samples were then centrifuged at 13,000 rpm for 5 min and 500 µl of isopropanol (at -20°C) was added to the supernatant in new tubes. The mixture was incubated for 10 min at -20°C followed by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded, DNA pellet washed with 500 µl of 70% ethanol (at -20°C) followed by air-drying. The DNA pellet was resuspended in 100 ul of 10:1 TE (10 mM Tris:1 mM EDTA) buffer. The samples were RNase treated by adding 2.25 µL of 10 mg ml^{-1} RNase and incubating for 30 min at 65°C followed by storing at -20°C till further use.

DNA was quantified spectrophotometrically and quality checked by 1% agarose gel electrophoresis, against lambda DNA of known quantity. Presence of DNA was confirmed by visualizing the bands on the gel under a UV transilluminator (Alpha Innotech, Taiwan). Comparison of the concentration of DNA was done against known standards of 100, 125, 250 500 and 1000 ng/µl lambda DNA to determine quantity. DNA was diluted to a working stock of 30 ng/µl for PCR reactions.

Bulk segregant analysis (BSA) with microsatellite markers was used to identify DNA markers associated with RWA resistance. Nine primers for *Xgwm* microsatellites were used in this study. These microsatellite markers have been mapped in wheat chromosome 7D. They included *Xgwm30*, *Xgwm44*, *Xgwm46*, *Xgwm56*, *Xgwm111*, *Xgwm297, Xgwm333, Xgwm437 and Xgwm644* (Roder et al., 1998). BSA was done using DNA from KRWA9, NjoroBW2, resistant homozygous plants, resistant heterozygous (segregating) plants, homozygous susceptible plants and control resistance sources PI 137739 (*Dn1*), PI 262660 (*Dn2*), USA9 (*Dn7*) and PI 294994 (*Dn5*, *Dn8* and *Dn9*). DNA solution was bulked into their respective resistant and susceptible bulks. The resistant bulk consisted of equal amounts of DNA 10 µl from eight homozygous resistant plants. The susceptible bulk contained DNA from eight susceptible plants. The third bulk contained DNA from segregating plants. There were two more bulks with equal amounts of DNA 10 µl from each parent NjoroBW2 and KRWA9. All PCR reactions were performed in 13 µl reaction volumes containing 1.25 µl of 10X PCR buffer, 8.5 µl of ddH_2O, 0.5 µl of 10 mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 0.25 µl of 10 mM each of forward and reverse primer and 0.05 µl of Invitrogen Taq DNA polymerase recombinant

Table 1. Chi-square values for seedling reaction to Russian wheat aphid in KRWA9, NjoroBW2, F_1, F_2 and $F_{2:3}$ populations of KRWA9 × NjoroBW2 cross.

Parents and crosses parents	Pop	Total	R	S	Observed R:S	Expected R:S	χ^2	P-value
KRWA9	P1	44	44	0	44:0	44:0	-	-
NjoroBW2	P2	45	0	45	0:45	0:45	-	-
Crosses								
KRWA9 × NjoroBW2	F_1	24	24	0	24:0	24:0 (1:0)	0.00	1.00
	F_2	100	77	23	77:23	75:25 (3:1)	0.21	0.644
KRWA9 × NjoroBW2	**Pop**	**Total**	**R:Seg:S**		**Observed R:Seg :S**	**Expected R:Seg:S**	**χ^2**	**P-value**
	$F_{2:3}$	100	28:49:23		28:49:23	25:50:25 (1:2:1)	0.53	0.767

R = Resistance, S = Susceptible, Pop = Population, χ^2 = Chi-square, Seg = Segregating, Significance at P = 0.05 level (df = 1, CV = 3.841 and df = 2, CV = 5.991).

(5 U/µl) and 1.5 µl template DNA. PCR amplifications were carried out on PCR machine (Applied Biosystems 2720 Thermal Cycler, Singapore). The microsatellite products were resolved on 2.0% agarose gels in TAE buffer. The bands were visualized under a UV transilluminator (Alpha Innotech, Taiwan). The electrophoresis products were captured on a camera and transferred to a computer.

Once a specific polymorphism between resistant and susceptible bulks had been identified by BSA screening, individual co-segregation analysis, based on the associations between marker genotype and RWA reaction phenotype, was carried out on the total F_2 segregating population to determine the genetic linkage between a RWA resistance gene and a marker.

Microsatellite marker, *Xgwm111* (linked to RWA resistance) and weighted at 210 bp was used to confirm the presence of RWA resistance gene in NjoroBW2 × KRWA9 F_2 population. The PCR profile was a follows: an initial denaturing step at 94°C for 3 min followed by 45 cycles at 94°C for 1 min, annealing for primer *Xgwm111* at 55°C for 1 min.

This was followed by primer elongation at 72°C for 2 min and final 10 min primer extension at 72°C. The simple sequence repeat (***SSR***) products were resolved on 2.0% agarose gels in TAE buffer and bands visualized under a UV transilluminator (Alpha Innotech, Taiwan). The electrophoresis products were captured on a camera and transferred to a computer.

Marker analysis

Informative bands were scored as present (+) or absent (-) and since SSRs are co-dominant markers, it was expected that alleles from both parents would be observed in some samples. Single marker analysis was done using the JoinMap software (Stam and Van Ooijen 1995) to detect QTL associated with *Xgwm111.* Linear regression was done to obtain coefficient of determination (R^2) that explains the phenotypic variation arising from QTL linked to a marker. Chi-square goodness-of-fit test was carried out to test conformity to Mendelian segregation patterns. The Chi square (χ^2) value and segregation ratios from gel data were later compared against Chi square (χ^2) value and phenotypic segregation ratios resulting from RWA reactions of individual F_2 populations and $F_{2:3}$ families.

RESULTS

Inheritance analysis

The resistant parent KRWA9 showed resistance reactions having minimal levels of chlorosis and rolling, with damage scores of 1 - 3. This indicated high levels of resistance in the resistant parent. The susceptible parent NjoroBW2 showed a susceptible reaction with damage scores of 7 - 9. Most NjoroBW2 seedlings had severe leaf chlorosis, streaking and rolling leading to death after 21 days of infestation. The F_1 population of cross NjoroBW2 × KRWA9 showed resistance reaction with damage scores of 1 - 4. The resistance reaction of F_1 population was not significantly different from the reaction of KRWA9 indicating that the resistance gene in KRWA9 is dominant. The χ^2 statistics for NjoroBW2 × KRWA9 F_1 population was significant at $P<0.05$ with a fit in ratio of 1:0 (Table 1). In NjoroBW2 × KRWA9 F_2 generation, the hybrids segregated and were classified into their respective phenotypic classes. The F_2 population showed both susceptible and resistant reactions with damage scores of 1 - 9. The χ^2 statistics was significant at $P<0.05$ with a fit in ratio of 3:1 (Table 1). The $F_{2:3}$ progenies were classified as homozygous resistant and heterozygous resistant (segregating) based on the seedling reactions to RWA. The $F_{2:3}$ homozygous resistant progenies showed damage scores of 1 - 5, indicating resistance. Heterozygous resistant progenies showed damage scores of 1 - 9 indicating both resistance and susceptible reactions. The χ^2 statistics for $F_{2:3}$ population of NjoroBW2 × KRWA9 was significant at $P<0.05$ (Table 1) with a fit in ratio of 1:2:1. These results confirmed the model of 3:1 at F_2 populations with a fit of 1:2:1 at $F_{2:3}$ families for monohybrid inheritance.

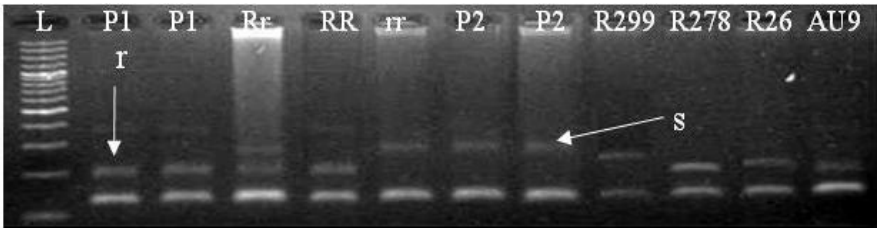

Figure 1. DNA bands amplified from F_2 DNA bulks using primer pair *Xgwm111* and electrophoresed in a 2% agarose gel. L = 100kb ladder, P1 = resistant parent bulk, P2 = susceptible parent bulk, RR = homozygous F_2 plant bulk, Rr = heterozygous F_2 plant bulk, rr = susceptible F_2 plant bulk, AU9 = resistance source having gene *Dn7*, R299 = (PI 294994) resistance source having genes *Dn5, Dn8, Dn9*, R26 = (PI 137739) resistance source having gene *Dn1*, R278 = (PI 262660) resistance source having gene *Dn2*, r = resistance band, s = susceptible band.

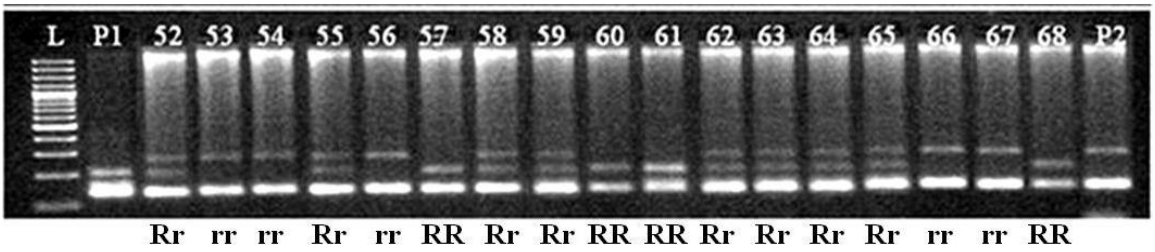

Figure 2. DNA bands amplified from F_2 progeny of NjoroBW2 × KRWA9 using primer pair *Xgwm111* and electrophoresed in a 2% agarose gel. P1 = resistant parent, P2 = susceptible parent, RR = Homozygous resistant, Rr = Homozygous susceptible, rr = Homozygous susceptible, L = 100 bp ladder.

Genotypic analysis

Nine primers (*Xgwm30, Xgwm44, Xgwm46, Xgwm56, Xgwm111, Xgwm297, Xgwm333, Xgwm437* and *Xgwm644*) were screened for polymorphism and only chromosome 7DS primer *Xgwm111* produced a distinguishing polymorphism. Primer *Xgwm111* produced a band that clearly and consistently differentiated the parents, resistant and susceptible bulks (Figure 1). A band was produced on control resistance source PI 137739 which was similar to the one on resistance source KRWA9. The band was approximately 210 bp and was subsequently tested on F_2 population individuals. Other bands were produced on resistance sources PI 262660 (*Dn2)*, PI 294994 (*Dn5, Dn8* and *Dn9*) and AUS9 (*Dn7*). Figure 1 shows the banding patterns for KRWA9, NjoroBW2, homozygous resistant plants, heterozygous resistant plants, homozygous susceptible plants and control resistance sources “R299”, “R278”, “R26” and “AU9”. KRWA9 showed two distinctive bands; one was 210 bp while the other was 160 bp. The susceptible parent NjoroBW2 showed two distinctive bands; one was 280 bp while the other was 160 bp (Figure 1). It was observed that both parents had a common 160 bp band. The 210 bp band was present in the resistant parent but absent in the susceptible parent. This band was designated as the band of interest. The inclusion of different resistant sources helped to accurately identify the DNA markers for gene of interest. The primer *Xgwm111* also produced a 210 bp band that clearly and consistently differentiated the parents, resistant, heterozygous and susceptible plants in the F_2 population (Figure 2). Based on the banding patterns observed in the F_2 population, 28 plants were homozygous resistant, 49 heterozygous and 23 homozygous susceptible (Table 3). This ratio did not differ from the expected 1:2:1 segregation ratio ($\chi^2 = 5.991$, df = 2, $P \leq 0.05$).

Linkage analysis

The F_2 population of NjoroBW2 × KRWA9 cross showed a wide range of segregation for response to infestation by RWA. The frequency distribution of RWA feeding damage on the F_2 population was somewhat bimodal, indicating the presence of one major resistance gene in KRWA9 (Figure 3). Simple regression analysis identified marker *Xgwm111 to be* highly significantly associated with resistance in KRWA9. The marker had an LOD score of 40.1 and high R^2 value of 85% indicating that it is a very significant marker for the resistance in KRWA9 (Table 2). Genetic data for *Xgwm111* marker showed a

Table 2. Statistical indicators for SSR marker *Xgwm111.*

Marker	LOD*	R^{2}**	P***	Source of resistance
Xgwm111	40.1	85%	0.000	KRWA9

* = p ≤ 0.1, ** = p ≤ 0.05, *** = p ≤ 0.01

Table 3. Summary of primer *Xgwm111* F_2 gel data.

Genotype	Observed values	Expected values	Chi square (χ^2)	P value
A	28	25	0.53	0.767
B	23	25		
H	49	50		
Total	100	100		

A = Homozygous resistant, B = homozygous susceptible, H = heterozygous (Significance at P = 0.05 level, df = 2, CV = 5.991).

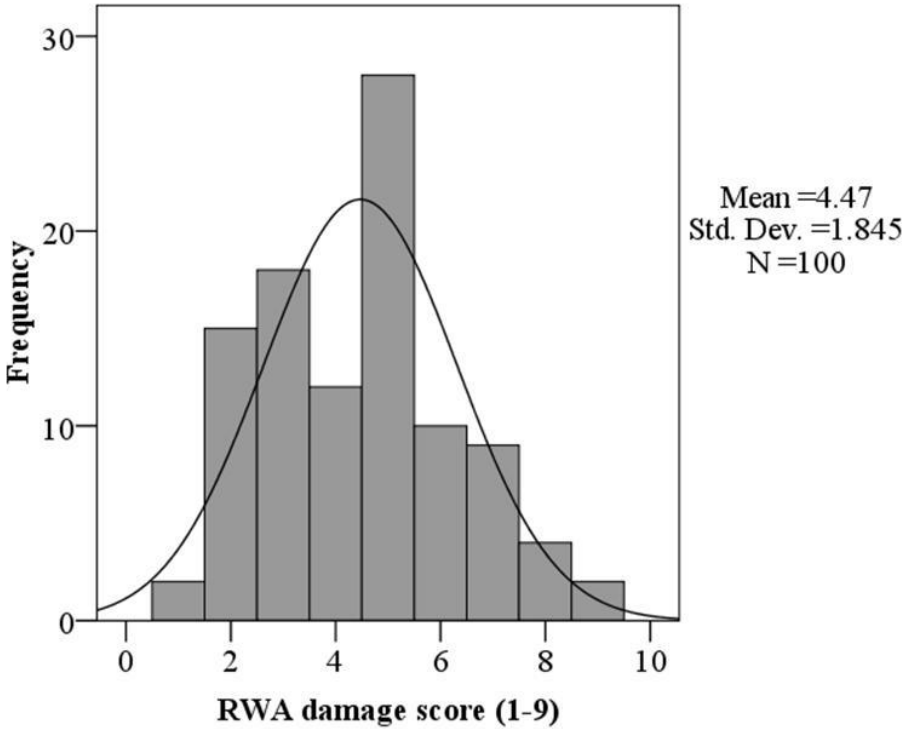

Figure 3. RWA damage distribution in F_2 population.

complete co-segregation with the disease data in the mapping population indicating a very tight linkage to the RWA resistance gene in KRWA9.

DISCUSSION

KRWA9 was selected for this study because visual observations of RWA feeding damage on it suggested that this source of resistance has high level resistance (Pathak et al., 2007; Malinga et al., 2008). This resistance could be transferred to NjoroBW2 a popular commercial wheat variety which is susceptible to RWA. The F_1 seedlings of the cross between NjoroBW2 and KRWA9 were all resistant indicating the resistance in KRWA9 is dominant. The segregation observed in the F_2 population and the $F_{2:3}$ families further confirmed the dominance of resistance in KRWA9. Most RWA resistant genotypes have single dominant genes located on chromosome 1D and 7D (Du toit, 1987; Nkongolo et al.,

1991b; Saidi and Quick, 1996; Liu et al., 2001; Liu, 2001). Resistance sources reported to have single dominant genes include PI137739 (Dn1), PI262660 (Dn2), PI372129 (Dn4) and PI243781 (Dn6) (Du Toit, 1989; Nkongolo et al., 1991b; Saidi and Quick, 1994). The dominant nature of RWA resistance gene could be easily identified in the segregating populations. However, the major problem with single gene inheritance is that insect can develop biotypes very fast if the resistant cultivar is grown on a large scale. Colorado State University has developed several commercially available RWA resistant varieties of winter wheat such as Halt, Prairie Red, Prowers 99 and Yuma (Thomas et al., 2002). All these varieties have the *Dn4* resistance gene derived from PI 372129 (Turcikum 57). It was later reported that RWA resistant cultivars with the *Dn4* gene were susceptible to a new biotype designated as "Biotype 2" (Haley et al., 2004). This led to sourcing of more resistant materials. Gene *Dn7* that was previously transferred from rye to wheat background via a 1 RS/1BL translocation had been reported to be resistant biotype 1 and 2 and depicts high levels of resistance as compared to other Dn genes (Collins et al., 2005; Turanli et al., 2012). However, part of the rye chromosome containing *Dn7* has detrimental genes resulting to poor bread making quality (Graybosch et al., 1990). Breeding for resistance with *Dn7* gene is no longer a desirable strategy and identification of diverse sources of resistance would be a highly desirable to keep ahead of biotype development in RWA. Pyramiding two or more resistance genes in a single cultivar will also increase the longevity of resistance.

The marker *Xgwm111* has previously been found to be linked to genes *Dn1*, *Dn2* and *Dn5* in resistance sources PI 137739, PI 262660 and PI 294994, respectively (Liu et al., 2005). In their study, the marker *Xgwm111* produced band sizes 210 bp in PI 137739 for gene *Dn1*, 200 bp in PI 262660 for gene *Dn2* and 200 bp in PI294994 for gene *Dn5* (Liu et al., 2005). The results are in agreement with Liu et al. (2001, 2002), who reported that *Xgwm111* amplifies functional fragments from DNA of RWA-resistant wheat sources with expected sizes of 200 to 225 bp that are associated with RWA resistance.

In the F_2 population, marker *Xgwm111* followed the expected Mendelian segregation ratio of 3:1 or 1:2:1 (Table 3). These findings are consistent with Pathak et al. (2007) on a single dominant gene controlling resistance in KRWA9. The marker also completely co-segregated with the disease data and it is believed that the resistance gene in KRWA9 must be tightly linked to the marker. This offers a good opportunity for breeders to use this marker to select for resistance to RWA.

Conclusion

The usage of host plant resistance at the low cost is environmentally safe and is an ideal method to control the Russian wheat aphid. KRWA9 is a good source of resistance to RWA biotypes in Kenya and marker *Xgwm111* could be used for marker assisted selection of resistance associated with this line. Similarity exists between KRWA9 and PI 137739, therefore there is a need to screen more markers in order to find more polymorphic markers in this region of chromosome 7DS. Most RWA resistance sources are monogenic and the challenge is that insects can develop biotypes very fast which could overcome the resistant cultivars. Identification of many sources of RWA resistance would be highly desirable to keep ahead of biotype development in the RWA by way of deploying multiple resistance genes to new breeding lines.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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