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Molecular marker variability for southern root-knot nematode resistance in sweetpotato

Mwamburi Mcharo[1,*], Don R. LaBonte[1], Chris Clark[2], Mary Hoy[2] & James H. Oard[3]
[1]*Department of Horticulture, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, U.S.A.;* [2]*Department of Plant Pathology and Crop Physiology, Louisiana State University. Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, U.S.A.;* [3]*Department of Agronomy, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, USA (*author for correspondence: e-mail: mwamburin@yahoo.com)*

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Summary

Amplified fragment length polymorphism (AFLP) marker profiles for individuals in two F1 populations of sweetpotato [*Ipomoea batatas* (L.) Lam] were used in association studies to identify AFLP markers suitable for identification of plants possessing a resistant reaction to southern root-knot nematode race 3 [*Meloidogyne incognita* (Kofoid and White) Chitwood]. Population one consisted of 48 half-sib genotypes developed at the Louisiana State University (LSU) AgCenter. The second population consisted of 54 full-sibs developed by the East African and International Potato Center (CIP) sweetpotato breeding programs. Results for plant nematode resistance indicate a bimodal distribution among the genotypes for the LSU population and a normal distribution for the CIP population. Using analysis of molecular variance (AMOVA) at $P < 0.001$ and two multivariate analysis techniques i.e logistic regression and discriminant analysis, 5 and 4 AFLP markers that had a strong and significant association with respect to the resistance trait were selected for the LSU and CIP populations, respectively. A comparative analysis of the power of discriminant analysis models for southern root-knot nematode resistance class prediction achieved 88.78% (LSU) and 88.04% (CIP) classification efficiencies.

Introduction

Among the more than fifty described species of plant parasistic nematodes, root-knot nematodes (*Meloidogyne* spp.) are the leading cause of crop loss (Roberts, 1995). Agriculturally important species include the southern root-knot nematode, *M. incognita*, which is a major pest of sweetpotatoes. Other species that have been known to affect sweetpotato production are *M. arenaria* and *M. javanica* (Giamalva et al., 1963). Jones et al. (1986) reported success in finding resistance to southern root-knot nematode using mass selection techniques. The advantage of mass selection is the possibility of exploiting a wide gene base. Using mass selection, Jones et al. (1991) released two sweetpotato populations designated as I/13 and J/8 to provide a wide genetic base for use with introductions and exotic materials in order to develop enhanced germplasm.

Southern root-knot nematode research is complicated by the existence of different races. Lawrence (1984) showed that for some southern root-knot populations, there was greater reproduction of nematodes on the resistant 'Jewel' and 'Jasper' than on the susceptible 'Centennial'. Cervantes-Flores et al. (2002) also found differences in reaction (none, low, medium and high infection levels) of different *Meloidogyne* populations to different genotypes. Ukoskit et al. (1997) concluded that since more than one race is capable of infecting sweetpotatoes, the nematode population being tested must be identified in order to specify the type of resistance from a given source. The challenge

for breeders has so far been to design effective quantitative gene identification protocols through classical breeding methods like mass selection. Previous studies have shown that identification of suitable genes is an important prerequisite for the success of a breeding program aiming to develop sweetpotatoes with resistance to root-knot nematode (Cervantes-Flores et al., 2002). Molecular marker techniques, as suggested by Barker and Koenning (1998), would therefore be an important consideration in combining markers for parasitism (virulence) within different nematode populations and host-resistance genes for faster breeding advances.

The AFLP marker system combined with discriminant analysis has been shown to identify important markers that would otherwise be difficult to identify. Discriminant analysis applications in molecular marker selection have been extensively discussed by Capdevielle et al. (2000) and Aluko (2003) who associated microsatellites and agronomic traits in rice. Fahima et al. (2002) who investigated microsatellite polymorphism in wheat also used discriminant analysis in their characterization. In sweetpotatoes, Mcharo et al. (2004) used discriminant analysis to select useful AFLP markers that identified variability in dry matter in a USDA sweetpotato collection. Logistic regression has been used by Thurston et al. (2002) to select AFLP markers associated with the semen freezability trait in boars (*Sus scrofa* L.).

In this study we evaluated the efficiency of using two multivariate analysis methods i.e. discriminant and logistic regression analysis to identify AFLP markers that are associated with southern root-knot nematode resistance in two sweetpotato populations. We also compared the similarity or differences among groups of selected markers between the populations.

Materials and methods

Planting material

Two sweetpotato [*Ipomoea batatas* (L.) Lam] F_1 populations were used for southern root-knot nematode race 3 (*Meloidogyne incognita*) resistance expression. Population one consisted of 48 half-sib genotypes developed at the Louisiana State University AgCenter. Maternal clones used to obtain the open pollinated F_1 half-sibs were 'Beauregard', 'Excel', 'L94-96', 'L89-110', 'L86-33' and 'L96-117'. Approximately ten progeny were randomly selected from each parent. The second population consisted of 55 full-sibs developed by the National Agricultural Research Organization, Kampala Uganda, and International Potato Center, Lima Peru, sweetpotato breeding program. The crosses from which the second population was derived were 'Beauregard × Wagabolige', 'Kyukei No. 63 × Jonathan W218', 'Jonathan W154 × Wagabolige', 'CN1732-4 × Jonathan W218', 'Tanzania × Wagabolige' and open pollinated 'Tanzania'. Approximately eleven progeny were randomly selected from each parent. 'Beauregard' was included as a susceptible control and 'L94-96' was used as a resistant control.

A southern root-knot nematode population was increased on Bell pepper (*Capsicum annum* L.) cv 'Yolo Wonder' in a greenhouse. Nematode eggs were then extracted from the roots of the 2-month-old seedlings with 0.6% sodium hypochlorite for 4 min. The sodium hypochlorite with the eggs was poured through an 80-mesh sieve to remove root and leaf debris then onto a 500-mesh sieve to collect the eggs. The eggs were then washed under running water and suspended in water in standard volumes containing 5000 eggs. Fresh sweetpotato cuttings were planted in 4 inch clay pots in a sterilized 1 sand: 1 soil mixture (v/v). Each of the cuttings was inoculated with 5000 eggs 4 days after planting for population 1 and at planting for population 2.

The experiment was laid out as a randomized complete block design with three replicates in a greenhouse and watered as necessary. Observations were made 8 weeks after planting for population 1 and 6 weeks after planting for population 2. Rating for resistance or susceptibility was on the scale of number of egg masses per plant as follows: 0 (0–5); 1 (1–3); 2 (4–10); 3 (11–30); 4 (31–100); 5 (>100), with 0 being the most resistant and 5 the most susceptible. Mean egg mass rating for each genotype was then determined from the three replicates.

Nematode eggs were extracted from the fresh vigorously growing sweetpotato plants by dipping the combined roots of the three replications in 0.6% sodium hypochlorite for 10 min. As above, the resulting suspension was poured through an 80-mesh sieve and then onto a 500-mesh sieve to collect the eggs. The 500-mesh sieve was backwashed into a beaker, the volume adjusted to 20 ml and diluted as necessary to count the total number of eggs among the three plants that represented each genotype. Mean number of eggs per plant, henceforth referred to as mean number of eggs per genotype were computed for each genotype. Egg

mass rating was computed as a validation criterion on the number of eggs counted.

DNA extraction

DNA extraction was previously described by Mcharo et al. (2004). Young leaves were harvested and stored at −40 °C until needed. Total DNA was isolated from 100 mg of fresh leaf tissue using the Genelute plant genome kit (Sigma-Aldrich Inc., St. Louis, Mo).

AFLP analysis

The DNA samples were amplified in a three-step process using a GeneAmp PCR system 9600 thermocycler (Perkin Elmer, Fulerton, CA). Reagents for AFLP were obtained from Invitrogen™ (AFLP starter primer kit, Cat No. 10483-014) and LI-COR Inc. (Lincoln, NE, Cat. No. 420032). Genomic DNA (120 ng/μl) was digested using an EcoRI/MseI restriction enzyme mix at 37 °C for 3 h. The enzymes were then inactivated by incubating the mix at 70 °C for 10 min. Double stranded adaptors were then ligated to the restricted DNA fragments resulting in template DNA which was used for pre-amplification. Diluted template DNA (1.5 μl) was added onto 10 μl pre-amp primer mix, 1.25 units Taq DNA polymerase (Invitrogen™) and 1.25 μl RedTaq™ PCR reaction buffer 10 × with $MgCl_2$ (Sigma-Aldrich™) to make a 13 μl reaction volume. The pre-amplification conditions were 20 cycles each of 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s and a final hold at 4 °C.

The reaction volume for selective amplification consisted of 3.0 μl pre-amplified diluted DNA, 0.4 μl of EcoRI (fluorescently labeled) primer (AAG), 4.4 μl of MseI (unlabelled) primer, 2.0 μl RedTaq™ PCR reaction buffer 10× with $MgCl_2$ (Sigma-Aldrich™), 1.35 μl $MgCl_2$ (where 1.35 μl were insufficient, the volume was raised to 1.4 μl), 1 unit Taq DNA polymerase (Invitrogen™) and 6.38 μl of double distilled or AFLP grade water. Four primer pairs as identified by Fajardo (2000) were used for selective amplification (CAG, CTA, CTG, CTT). Blue stop solution (3.0 μl) (LI-COR, Lincoln, NE) was added onto each amplified DNA sample. The amplified DNA was then denatured at 95 °C for 3 min and thereafter covered in aluminium foil and placed in a freezer at −20 °C for 10 min to prevent annealing of complementary fragments, before loading onto a 25 cm acrylamide gel. PCR amplification fragments were separated by 6.5% acrylamide gel electrophoresis using a LI-COR Global IR^2 sequencer (LI-COR, Lincoln, NE) for 3 h. The AFLP fragments were automatically detected and recorded during electrophoresis using the LI-COR SAGA^{MX} v 3.1.0 software. Data were collected and presence (=1) or absence (=0) of bands scored using LI-COR SAGA^{MX} v 3.1.0 software. The markers were named starting with the three letters coding for the primer followed by the molecular weight of the marker in base pairs.

Statistical analysis

Two nematode reproductive measures were used to assign genotypes to either the resistant or susceptible class. The two measurements in the present study were mean number of eggs per genotype and mean egg mass rating The mean egg number per genotype was log transformed and the frequency distributions plotted to illustrate resistant and susceptible patterns of progeny within each population. The egg mass ratings was assessed to enable correlation with the mean number of eggs. This correlation provides confimatory data that our class assignments are appropriate (Jones & Dukes, 1980).

Discriminant analysis previously described by Mcharo et al. (2004) was used to select informative molecular markers that are associated with root-knot nematode resistance in the two populations. The efficiency of the classification model constructed using the selected markers was then tested by cross-validation using the leave-one out method as described by Mcharo et al. (2004). Classification error rates derived from cross-validation provided a measure of model efficiency. Logistic regression (Hosmer & Lemeshow, 1989) was also used to model molecular markers as variables associated with the resistance trait. Significance level to include a marker for both discriminant analysis and logistic regression was set at $P = 0.03$ for the LSU population. Values for the CIP population were set at $P = 0.05$ (discriminant analysis) and $P = 0.03$ (logistic regression). These P values ensured we selected an optimum set of markers without compromising on model efficiency due to too few markers or over-fitting due to too many markers selected. A higher P value was used for discriminant analysis on the CIP population to include markers that increased the efficiency of the model developed. Logistic regression and discriminant analysis were done using SAS® (1999, 2001). AMOVA on the selected AFLP markers was used to test genotypic variability between the resistant and susceptible groups (Excoiffer et al., 1992).

Results and discussion

Response to inoculation

A correlation analysis of mean egg mass rating with mean eggs per genotype showed positive significant ($P < 0.0001$) associations in both CIP ($r = 0.654$) and LSU ($r = 0.674$) populations. Results obtained from plotting the logarithm of mean number of eggs (Figures 1 and 2) suggest that resistance to root-knot nematode may be qualitatively as well as quantitatively controlled. The LSU population showed a bimodal response to inoculation indicating that a major gene may be controlling the resistance trait. We found no obvious trend that resistant parents, e.g., 'Excel', 'L94-96', 'L89-110', 'L86-33' and 'L96-117' produced progeny with greater propensity for resistant reactions. Ukoskit et al. (1997) also obtained a bimodal response among the genotypes they worked on. The cross from which they obtained their F_1 genotypes involved 'Vardaman' and 'Regal' as the parents. Material used in the United States breeding programs has narrow genetic base (D.R. LaBonte, personal communication) and this may partially explain similar qualitative responses between our population and the population used by Ukoskit et al. (1997). In contrast, genotypes from the CIP population exhibited a quantitative response suggesting that a few major genes may be involved in inheritance of nematode resistance. We also noted that the progeny from various CIP parents did not exhibit any differences in resistance. These results agree with those of Cervantes-Flores et al. (2002) who suggested that under some conditions resistance to root-knot nematode may be quantitative. These workers also found that Porto Rico and Pelican Processor had different reactions to the *M. incognita* populations, regardless of the host race. These responses suggest that different genes could be involved in the resistance of sweetpotato to root-knot nematodes.

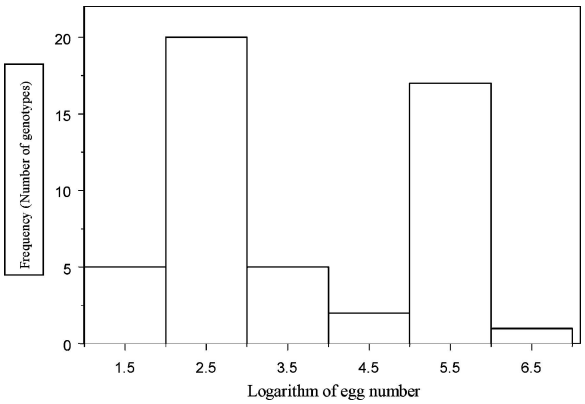

Figure 1. Frequency of log total root-knot nematode eggs for the LSU sweetpotato genotypes.

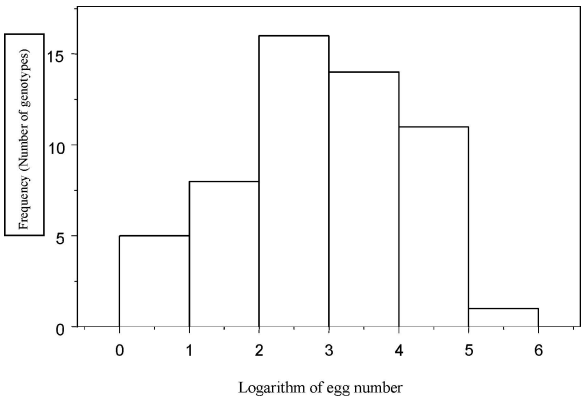

Figure 2. Frequency of log total root-knot nematode eggs for the CIP sweetpotato genotypes.

Early work on sweetpotato resistance to the root-knot nematode includes that of Giamalva et al. (1963), Davide and Struble (1966) and Struble et al. (1966). Resistance to root-knot nematode has previously been explained to be either qualitatively or quantitatively controlled by these and other investigators. In their study on inheritance to resistance, Struble et al. (1966) suggested a multigenic control on inheritance of resistance. They found varying degrees of resistance among the tested progenies from different varieties expressing differing levels of resistance. They also noted differential reactions of the same variety to different populations of *M. incognita*, but no inheritance was determined. Lawrence and Clark (1986) also noted that different populations of *M. incognita* varied in their virulence on sweetpotato with some of the populations capable of overcoming resistance previously exhibited by cultivars in the study. Lawrence et al. (1986) concurred with Struble et al. (1966) when they concluded that the benefits of a resistant cultivar may be affected by the nematode population specifically infecting a given field. Resistance can be assessed in sweetpotato, but it must be in reference to the chosen southern root-knot nematode population. This reinforces the value in identifying molecular markers associated with resistance to various southern root-knot nematode populations as means of circumventing multiple population testing.

Molecular marker variation

From our statistical analysis, we did not obtain informative markers that were common to both populations at the set significance levels (Tables 1 and 2). This may partly be explained by the fact that the two populations exhibit different modes of resistance gene inheritance. This may suggest that there is need for a more extensive study involving different populations with different trait distribution patterns to define an array of markers that may be universally applicable with a certain level of confidence. Due to observed differential interactions by other workers (Lawrence, 1984; Cervantes-Flores et al., 2002) an array of markers may also be needed to account for differing genes for resistance to different populations of *M. incognita.* Lack of universal applicability of markers is the same challenge that is faced by traditional QTL mapping studies. The quest for marker assisted selection in breeding for resistance to root-knot nematode is a result of complications associated with tedious field observation experiments. For example in a heritability study of resistance to two Meloidogyne species, *M. incognita* and *M. javanica*, Jones and Dukes (1980) found high heritability estimates (0.57 to 0.78) for reactions of sweetpotato parental lines to both species. Although they concluded that development of resistant cultivars is possible, these workers also postulated that determining resistance levels with a high degree of confidence would require experimental analysis using egg mass, gall indices and root necrosis concurrently. This is because a variety may exhibit resistance based on one evaluation while exhibiting susceptibility using another method. Results from our study show that mean egg mass rating and mean number of eggs were positively and significantly correlated. It is thus expected that marker assisted selection will obviate the need for screening with multiple populations of the pathogen and the tedious phenotypic tests to select progeny carrying the desired allele (Bent & Yu, 1999).

Table 1. STEPDISC marker selection for AFLP DNA markers associated with resistance to southern root-knot nematode in two sweetpotato populations

Population	Marker[a]	Partial R-square[b]	Wilks' lambda[c]	Pr < lambda
LSU	ctg218	0.133	0.867	0.0107
	ctg227	0.110	0.771	0.0029
	cta098	0.111	0.685	0.0008
	ctg232	0.164	0.573	<0.0001
	cag267	0.185	0.467	<0.0001
	cag259	0.190	0.378	<0.0001
	ctt113	0.112	0.336	<0.0001
CIP	ctg228	0.247	0.753	0.0001
	ctt126	0.239	0.573	<0.0001
	cag118	0.149	0.488	<0.0001
	cag108	0.081	0.448	<0.0001
	cta148	0.104	0.401	<0.0001

[a]Markers are named starting with the three letters coding for the primer followed by the molecular weight of the marker in base pairs.
[b]Partial *R*-square is the marginal variability accounted for by a variable when all others are already included in the model.
[c]Wilks' lambda is the likelihood ratio measure of a marker's contribution to the discriminatory power of the model.

Table 2. Logistic regression selection for AFLP DNA markers associated with resistance to southern root-knot nematode in two sweetpotato populations

Population	Marker selected[a]	χ^2 score[b]	Pr> χ^2
LSU	ctg218	6.400	0.0114
	ctg227	5.534	0.0186
	cag198	5.596	0.0180
	cta098	5.127	0.0236
	cta183	8.008	0.0047
	cag267	13.792	0.0002
	cag232	8.000	0.0047
CIP	ctg228	13.335	0.0003
	cag118	12.015	0.0005
	Cta148	4.999	0.0253
	cag108	6.790	0.0092
	cta081	6.300	0.0121
	cta237	9.473	0.0021
	Cta172	19.001	<0.0001

[a]Markers are named starting with the three letters coding for the primer followed by the molecular weight of the marker in base pairs.
[b]χ^2 score is the largest significant score for maker not in model to be included in the model.

LSU genotypes

An important aspect of marker assisted selection is the selection of as few informative markers as possible without losing out on the prediction efficiency. Out of a total of 229 polymorphic markers that were generated, five (ctg218, ctg227, cta098, cag267 and ctg232) were selected by both discriminant analysis and logistic regression as having a significant effect on resistance variation. AMOVA found significant differences

($P < 0.001$) between resistant and susceptible groups using the five markers for the LSU population. Markers ctg218 and ctg227 were consistently selected by the two statistical procedures as being strongly associated with the resistance trait. We suggest that future studies focus on the two markers strongly associated with resistance in an effort towards identifying markers that would be useful for breeding against root-knot nematode. Using the seven significant markers selected by discriminant analysis (Table 1) we achieved a prediction efficiency of 88.78% with 6 out of 48 genotypes misclassified by the model. Two of the 6 were susceptible according to the nematode count but were classified as resistant using molecular markers. The other four were phenotypically classified as resistant with three of them being of intermediate resistance but all four were classified as susceptible by the molecular marker method. Further model expansion resulted in a 97.22% correct classification rate using 14 markers and 100% using 18 markers (cag279, cag267, cag259, cag108, cta213, cta155, cta144, cta098, ctg284, ctg232, ctg227, ctg218, ctg188, ctg146, ctg088, ctg079, ctt113 and ctt081). Consequently a gain of only 12% in a selection program using an extra 12 markers may not be very cost effective.

CIP genotypes

Compared to the LSU population there were four common markers (ctg228, cag118, cag108 and cta148) selected by both logistic regression and discriminant analysis out of a total of 220 polymorphic markers generated. AMOVA found significant differences ($P < 0.001$) between resistant and susceptible groups in the CIP population using the four markers. Consequently it is recommended that further research involve these four markers from the CIP population. A group prediction model created using the five significant markers selected by discriminant analysis (Table 1) achieved 88.04% prediction efficiency with 4 out of 55 clones misclassified. Out of the 4 genotypes, 3 were previously classified as being highly susceptible with means 13, 867; 27,467 and 42,667 eggs while the fourth had intermediate resistance with a mean of 192 eggs. Further investigations revealed that gains from additional markers in the model were minimal with 15 markers giving 93.86% correct classification and 16 markers (cag268, cag217, cag213, cag195, cag118, cag116, cag108, cta309, cta211, cta148, cta124, cta081, cta071, ctg228, ctg110 and ctt126) resulting in 100% correct classification.

Investigations by Ukoskit et al. (1997) found a low level of linkage (0.2421) between the identified marker and the resistance gene. These workers further recommended that it is important to find more molecular markers associated with the resistance trait to increase the efficiency of screening seedlings. The multiple markers identified for both populations in our study show that such markers exist. The high levels of classification efficiency provide further proof that there are significant gains to be achieved in using multiple markers for progeny selection. Use of only the top ranked marker (ctg218) in discriminant analysis for the LSU population resulted in a lower cross-validated classification efficiency to 67.78% while use of only the top ranked marker for the CIP population reduced the classification efficiency to 73.69%. We therefore recommend that future investigations for nematode resistance in sweetpotatoes involve use of multiple markers whether the genotype frequency distribution suggests qualitative or quantitative inheritance.

In their review, Bent and Yu (1999) showed that in disease resistance investigations, molecular markers have been primarily used to select for single genes that have a clear, major and dependable effect on phenotype. Such selections ensure that the phenotype of interest will most likely be advanced through breeding lines. Breeding efficiency may be further improved by determining the type of linkage because if a resistance locus is linked in repulsion to other desirable loci, marker-based selection can greatly reduce the time and space needed to generate the desired allelic combinations. Marker assisted selection will likely play an important role in evaluating nematode susceptibility and will help to close the debate on the mode of resistance inheritance; quantitative, qualitative or a mixture of the two. In potatoes the gene H_1, which confers a high level of resistance to the golden nematode (a cyst nematode) has been bred into several potato cultivars (Brodie, 1999). Mapping of the H_1 gene has led to the development of a molecular marker to screen segregating populations for resistance to the golden nematode. According to Brodie (1999) limited success has been realized in control of root-knot nematodes in potatoes although sources of resistance have been identified. It is possible that the golden nematode is an introduced species that has had little time to evolve in the US. Compared to the root-knot nematode there is a possibility that the golden nematode represents a much more genetically homogeneous pathogen (Clark, personal communication).

Models based on large samples are expected to be more reliable. Our samples were about 50 clones in

each group and hence may not have provided sufficiently large numbers for efficient marker selection. This may partly explain why one single marker with a large effect could not be found for the LSU population that has a bimodal distribution as in the case of Ukoskit et al. (1997). Large samples are expected to have a wider range of markers from which to choose and more clones which provide greater variability within the disease tolerance response variable. However our results suggest that there is utility in using small sample sizes not only for development of protocols but also in studying important traits that may affect only a small sample of the population. According to Cruz-Castillo et al. (1994) the combination of too many variables and a small sample size may cause unreliable estimates. They further suggest that discriminant analysis may not provide meaningful results where there are too many markers selected even with large samples. However according to them a reliable approach would be to use a sample size that is about 10 times the number of markers selected per phenotypic group. In addition samples sizes smaller than the corresponding number of markers should be avoided.

There is need to use well defined biological classes that have clear physical interpretation for modeling. Such classes will show clear differences in sweetpotato susceptibility to root-knot-nematode infection. The advantage of such clear classification is that the models that are constructed are likely to have low error rates during classification. A discriminant analysis model constructed with such populations is also likely to classify an unknown genotype with greater precision because the nearest neighbor distance used for grouping is less ambiguous compared to using populations with fuzzy descriptive boundaries. The study reported by Mcharo et al. (2004) used a larger population of 68 genotypes from extreme low and high ends of a population tested for root dry matter content. Consequently the phenotypic classes were clear cut unlike the present nematode study that used smaller populations with boundaries that did not clearly separate resistant from susceptible genotypes.

A fundamental difference between multivariate analysis and other marker selection techniques like bulked segregant analysis or QTL analysis is that while the latter two seek markers that may be linked to the gene of interest, multivariate analysis selects an array of markers that can be used to predict a clone of unknown resistance status into a predefined resistance group. As indicated in Table 1, each of the selected markers is associated with a certain weight as represented by Wilk's lambda and the presence or absence of a marker in the genotype will therefore contribute towards its resistance group classification. In the complicated sweetpotato genome where mapping is very resource consuming, multivariate analysis therefore seems to present a better approach for trait-linked marker selection especially in quantitative traits where the probability of finding a marker strongly associated with the trait is low. Our results indicate that whether studies on resistance to root-knot nematode resistance involve segregating populations (Ukoskit et al., 1997) or non-segregating populations, the mystery of mode of resistance inheritance still needs to be unraveled, regardless of the marker generation technique used.

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