# DELINEATING MIXED POPULATIONS OF *ROTYLENCHULUS RENIFORMIS* AND *MELOIDOGYNE INCOGNITA* WITH FAME ANALYSIS

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## Abstract

Previous studies have indicated that mixed populations of plant-parasitic nematode species can be present in agricultural fields. Since we have previously demonstrated that *Meloidogyne incognita* and *Rotylenchulus reniformis* have distinct fatty acid profiles as determined using FAME analysis, it is hypothesized that it will be possible to use this system to differentiate among mixed-population samples of these two species. Samples containing five separate ratios of *M. incognita* to *R. reniformis* (100-0, 75-25, 50-50, 25-75, 0-100) were prepared at both 500 and 5000 total individuals per sample. Ten replicates of each ratio were analyzed using GC FAME analysis. Gradual shifts in fatty acid profiles from one species to another were observed throughout the ratios. Most of the fatty acids observed during the analyses were significant to differentiation among the various ratios. Statistical analysis with SAS (SAS Institute, Inc) indicated that all ratios are significantly different in samples containing 5000 total individuals ($P \leq 0.0028$). In samples containing 500 individuals, only when comparing the 50-50 ratio to the 25-75 ratio are the ratios not significantly different ($P=0.3956$); all other comparisons are significantly different ($P \leq 0.0075$). Additional analysis with the Sherlock Analysis Software (MIDI Systems, Inc) indicates that samples containing mixed populations can be completely differentiated from single-species samples. Accurate identification with this software is possible at frequencies of 93.3%, 61.5%, and 100% for the ratios 25-75, 50-50, and 75-25, respectively. It may be possible to use the developed ratio profiles to identify mixed-species samples in diagnostic laboratories.

## Introduction

The plant-parasitic nematode species *Meloidogyne incognita*, the Southern root-knot nematode, and *Rotylenchulus reniformis*, the reniform nematode, are present in many agricultural areas and can cause significant crop damage; *M. incognita* caused a loss of 489,000 bales of cotton in 2007 alone (Blasingame *et al*. 2008). Quick and accurate identification of these nematodes in need to indicate any management practices that should be enacted to prevent significant losses to agriculturists. To complicate matters, these nematodes can be found in fields in mixed populations. Gazaway and McLean (2003) found that 61% of the fields that were surveyed in Alabama contained more than one species of plant-parasitic nematode, with 56% of those multi-species fields infected by two species. Knowing if a mixed population is present can have a significant impact on what crop should be planted in agriculture.

Our previous research has indicated that *M. incognita* and *R. reniformis* can be differentiated from each other using statistical analysis (Sekora *et al*., 2008b). We have also been able to determine that these nematodes can

be accurately differentiated in samples containing greater than 250 total individuals (Sekora *et al*. 2008). As a result of these analyses, we hypothesize that it will be possible to differentiate among mixed-population samples of *M. incognita* and *R. reniformis* using the FAME analysis procedure that we have adapted in our previous studies.

## Materials and Methods

Samples were prepared using *M. incognita* and *R. reniformis* that had been grown on cotton under greenhouse conditions for sixty days. Vermiform life stages were extracted by combined gravity-screening and sucrose centrifugation while eggs and infective females were extracted from roots by agitation in 0.6% NaOCl. Both extractions for each species were combined and enumerated to determine life stage ratios. Five distinct ratios were prepared at two concentrations of total individuals (Table 1).

Table 1. Ratio percentages of samples at two concentrations of individuals

| Individuals | Ratio % Mi-RR |
|---|---|
| 500 | 100-0 |
| | 75-25 |
| | 50-50 |
| | 25-75 |
| | 0-100 |
| 5000 | 100-0 |
| | 75-25 |
| | 50-50 |
| | 25-75 |
| | 0-100 |

To prepare ratios at a given concentration, the desired number of nematodes of each species was placed into a microcentrifuge and mixed thoroughly. For example, a 75-25 ratio of 5000 individuals required mixing 3750 *M. incognita* individuals and 1250 *R. reniformis* individuals. Ten replications of each ratio were prepared for a total of 100 samples. Samples were extracted and analyzed using the adapted FAME procedure (Sekora *et al*., 2008).

Canonical discriminant analysis and comparison matrices were used to determine the statistical significance of each ratio at a given concentration. Canonical analysis was performed using the STEPDISC and CANDISC procedures of SAS (SAS Institute, Inc.). Comparison and similarity matrices were generated using the Sherlock Analysis Software (MIDI Systems, Inc.) by preparing a library of fatty acid profiles for *M. incognita, R. reniformis*, and each of the three intermediate ratios across the two concentrations of individuals and any previous samples used to generate profiles.

## Results and Discussion

### 5000 Individuals

All ten replicates were averaged to produce mean profiles for each ratio. As the ratios progress from 100% *M. incognita* to 100% *R. reniformis*, gradual changes in the expression of each fatty acid can be observed. For instance, when a sample contains 100% *M. incognita*, the fatty acid 18:1 ω7c has a mean concentration of 31.54%; this percentage increases as the ratio of *R. reniformis* increases to a value of 53.57% in samples containing 100% *R. reniformis*. Both 16:1 ω5c and 18:1 ω5c are found in *R. reniformis* and not *M. incognita* using our system. There is no expression of these fatty acids in *M. incognita* samples, but as the ratio of *R. reniformis* increases, these fatty

acids are found with increasing abundance until they reach 1.46% and 1.67%, respectively, in samples containing 100% *R. reniformis* (Figure 1).

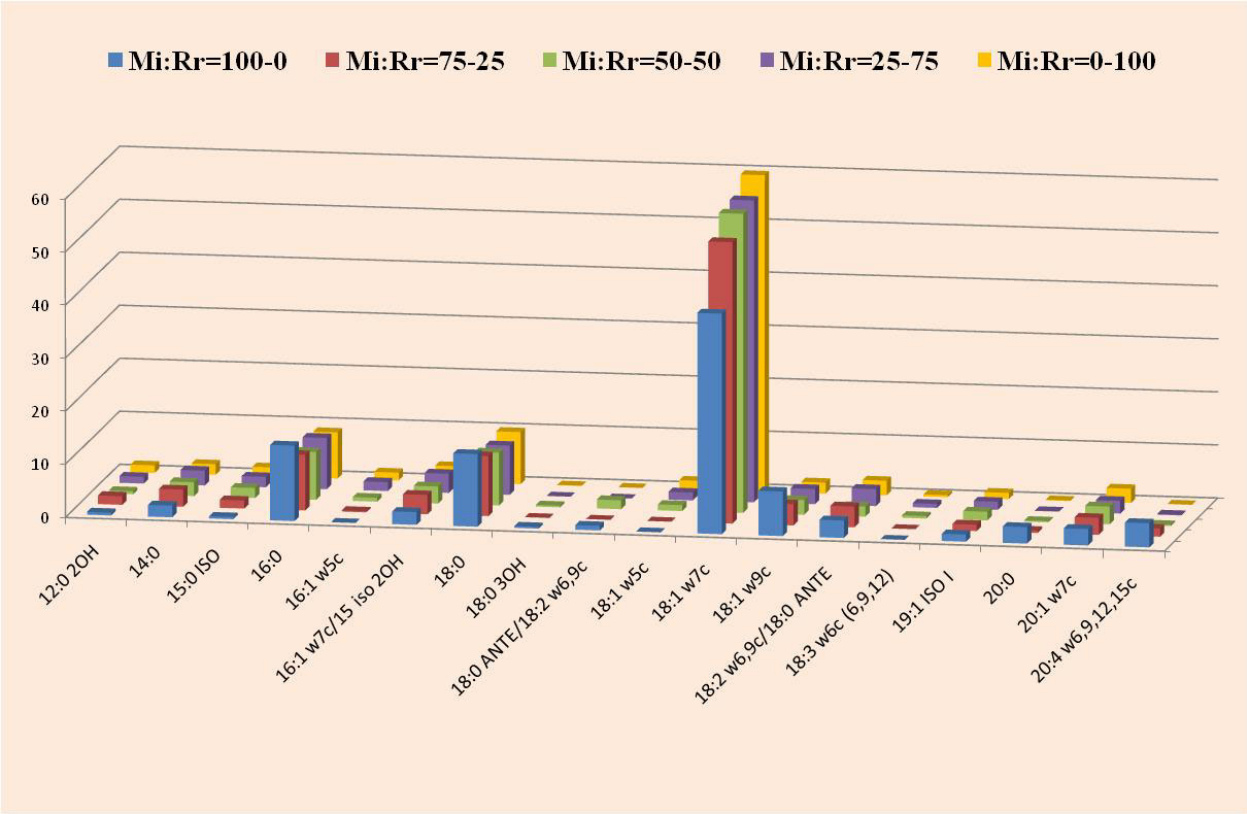


Figure 1. Mean fatty acid profiles of varying ratios of *Meloidogyne incognita* and *Rotylenchulus reniformis* with a total of 5000 individuals per sample.

Canonical analysis of these samples indicates that each ratio is distinctly different from the others, with all comparisons significant ($P \leq 0.0028$). The greatest difference among samples is among 100% *M. incognita* samples and all other samples (96% canonical difference); the minimum Mahalanobis distance ($D^2$) lies between the 100-0 ratio and the 75-25 ratio ($D^2=393$) (Figure 2).

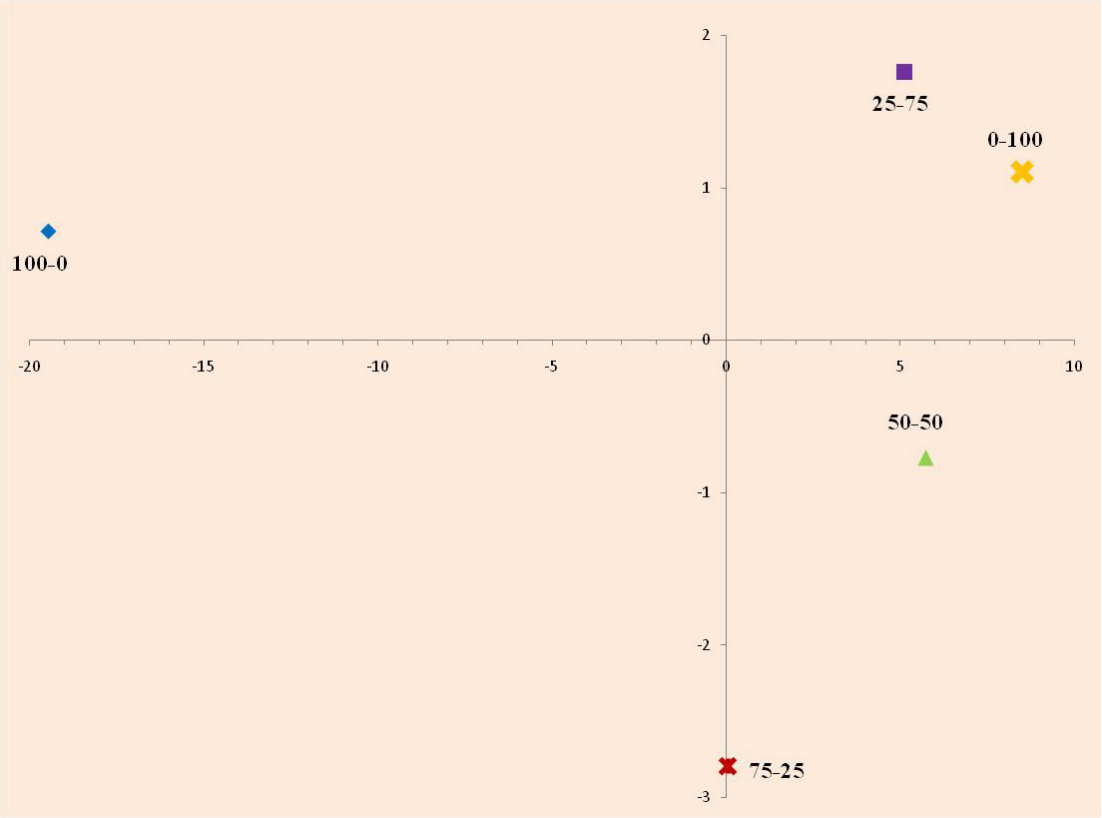


Figure 2. Canonical distribution of *Meloidogyne incognita* to *Rotylenchulus reniformis* ratios at concentrations of 5000 total individuals. The x-axis describes the first canonical axis (96% DIFFERENCES) while the y-axis describes the remaining differences along the second canonical axis.

The closest relationship lies between the 50-50 ratio and 25-75 ratio comparison. While this comparison is significant ($P \leq 0.0028$), this is the only comparison with a p-value greater than 0.0004. Of the 18 fatty acids observed among samples, fifteen are significant to discrimination among ratios, with four of the fatty acids nearly perfectly aligned along the first canonical axis (Canonical value>0.99).

**500 Individuals**

Mean profiles for each ratio were produced in the same way as those for the samples containing 5000 individuals. Similar changes in the expression of fatty acids was observed as the ratios progressed from 100% *M. incognita* to 100% *R. reniformis*, though the changes were less gradual than those in the samples of 5000. For these samples, the 18:1 ω7c fatty acid had a mean percentage of 43.11% in 100% *M. incognita*, which increased to a mean percentage of 49.85% in 100% *R. reniformis* samples. The percentage of 18:1 ω7c was decreased in 100% *R. reniformis* samples due to the increased expression of the 18:0 ANTE/18:2 ω6,9c peak; the peak had a mean percentage of 13.9% compared to 0% in the samples of 5000 individuals (Figure 3).

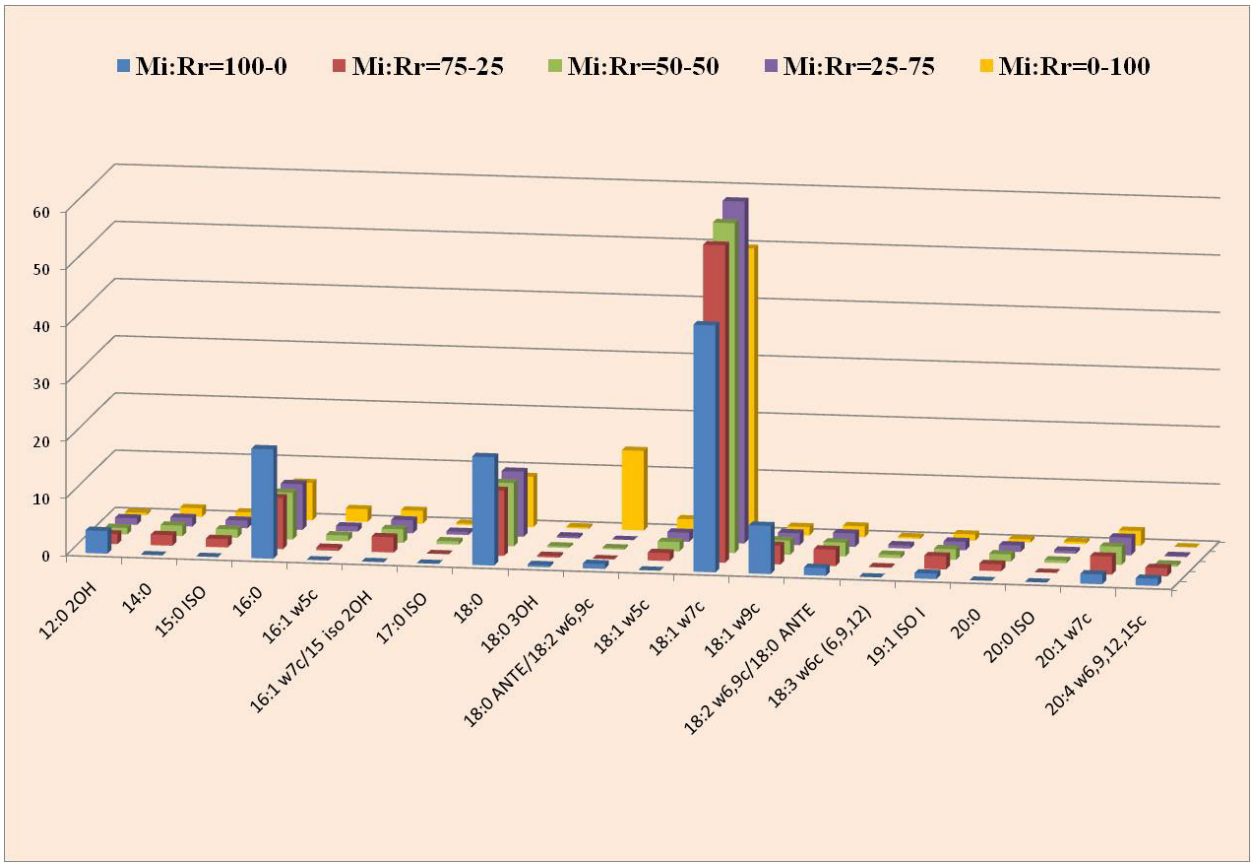


Figure 3. Mean fatty acid profiles of *Meloidogyne incognita* to *Rotylenchulus reniformis* ratios containing 500 total individuals.

The canonical relationships of the ratios were distributed in much the same way as those in the 5000 individual samples, though the canonical graph is rotated 180 degrees. For these comparisons, 97% of the total differences were described by the first canonical axis and were between the 100% *M. incognita* samples and the remaining ratios. All comparisons were significant ($P \leq 0.0075$) except the 50-50 ratio to 25-75 ratio comparison; these comparisons were similar ($P=0.3956$) at this concentration of individuals. For these samples 14 of the 20 observed fatty acids were significant to allow differentiation of the two genera. The two fatty acids 16:0 and 18:1 ω9c were nearly perfectly correlated along the first canonical axis (canonical value >99.6) (Figure 4).

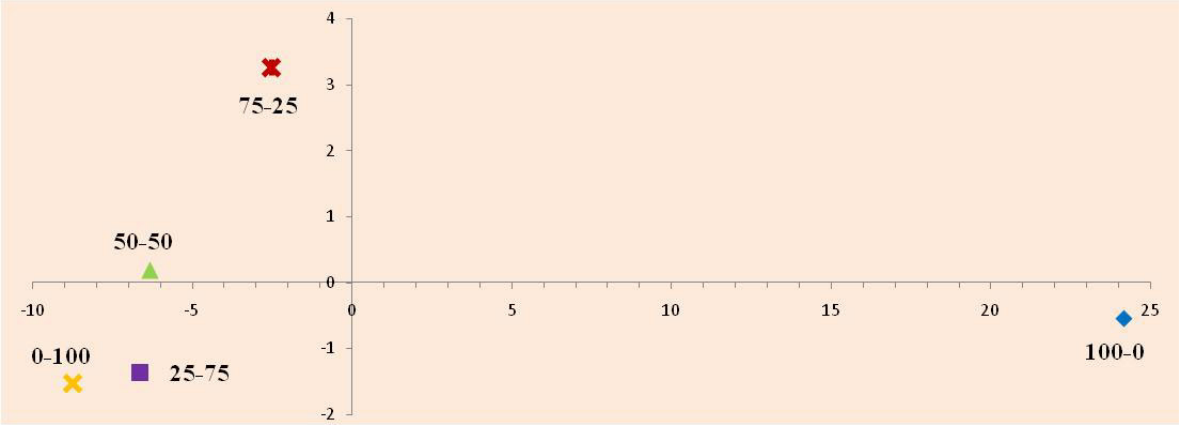


Figure 4. Canonical distribution of *Meloidogyne incognita* to *Rotylenchulus reniformis* ratios at concentrations of 500 total individuals. The x-axis describes the first canonical axis (96% DIFFERENCES) while the y-axis describes the remaining differences along the second canonical axis.

**Sherlock Identification**

The comparison matrix generated by the Sherlock software indicated that each of the ratios could be identified correctly 100% of the time from pure samples of either *M. incognita* or *R. reniformis*. The similarity among the ratios and pure cultures was also low, with only 1.1% and 4.9% similarity of the ratios to the pure cultures of *M. incognita* and *R. reniformis*, respectively. The comparison matrix within the three mixed-population ratios indicated that both the 25-75 and the 75-25 ratios could be accurately identified more than 93% of the time. However, the 50-50 ratio can only be accurately identified by the software 61.3 % of the time; 30.8 % of the time it is identified as the 25-75 ratio (Table 2).

Table 2. Comparison matrix for mixed-population ratios of *Meloidogyne incognita* and *Rotylenchulus reniformis*. Values listed are percentage of accurate identification with the Sherlock Analysis Software.

| | 25-75 Ratio | 50-50 Ratio | 75-25 Ratio |
|---|---|---|---|
| 25-75 Ratio | 93.3 | 30.8 | . |
| 50-50 Ratio | 6.7 | 61.5 | . |
| 75-25 Ratio | . | 7.7 | 100.0 |

**Conclusions**

These analyses indicate that mixed-populations of *M. incognita* and *R. reniformis* can be differentiated from each other and identified 95% of the time. The statistical analysis can differentiate 98% of the total comparisons across both the 500 and 5000 total individual samples. Only when trying to differentiate between the 50-50 Ratio and 25-75 ratio at 500 total individuals, is demarcation not accurate at the $P \leq 0.05$ level.

The statistical analysis strengthens the results from the Sherlock library identification. Mixed-population samples can be accurately identified from samples containing only *M. incognita* or *R. reniformis*. Within those ratios, the 75-25 ratio can always be identified by the software, while the 25-75 ratio can be correctly identified 93% of the time. The software can only indentify 61.5% of the 50-50 ratios correctly, assigning 30.8% of those samples to the statistically similar 25-75 ratio.

Although not all of the ratios can be correctly identified consistently, the software can correctly indicate the presence of a mixed-population of *M. incognita* or *R. reniformis* over a single population of either species. This

could be advantageous to diagnostics labs that will have to recommend management practices to their clients. Knowing if a mixed population is present can have a significant impact on what crop should be planted in agriculture.

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