The 4th National Plant Diagnostic Network meeting. March 8-12, 2016. Washington, DC.

How To Get Your Lab Involved In Testing For Export Or Import

Weimin Ye, Ph.D.

Nematologist

NCDA&CS Agronomic Division, Dr. Colleen M. Hudak-Wise, Director
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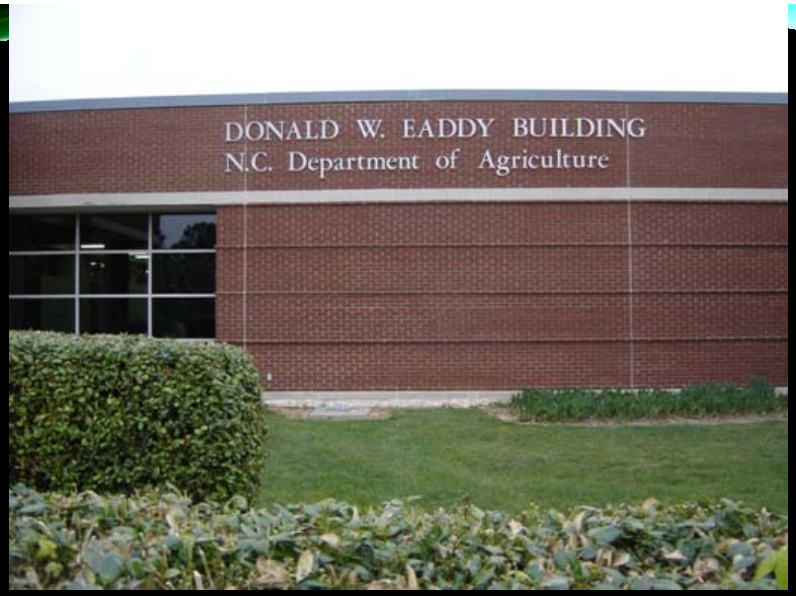
Phone: (919) 733-2655; FAX: (919) 733-2837
Email: Weimin.Ye@ncagr.gov


DONALD W. EADDY BUILDING
N.C. Department of Agriculture

Workload In FY2014 and FY2015

	FY2014	FY2015
Soil Testing	328,216	306,111
Nematode Assay	42,383	40,172
Plant/Waste/Solution/Media	26,679	30,681
Total	**397,278**	**376,964**

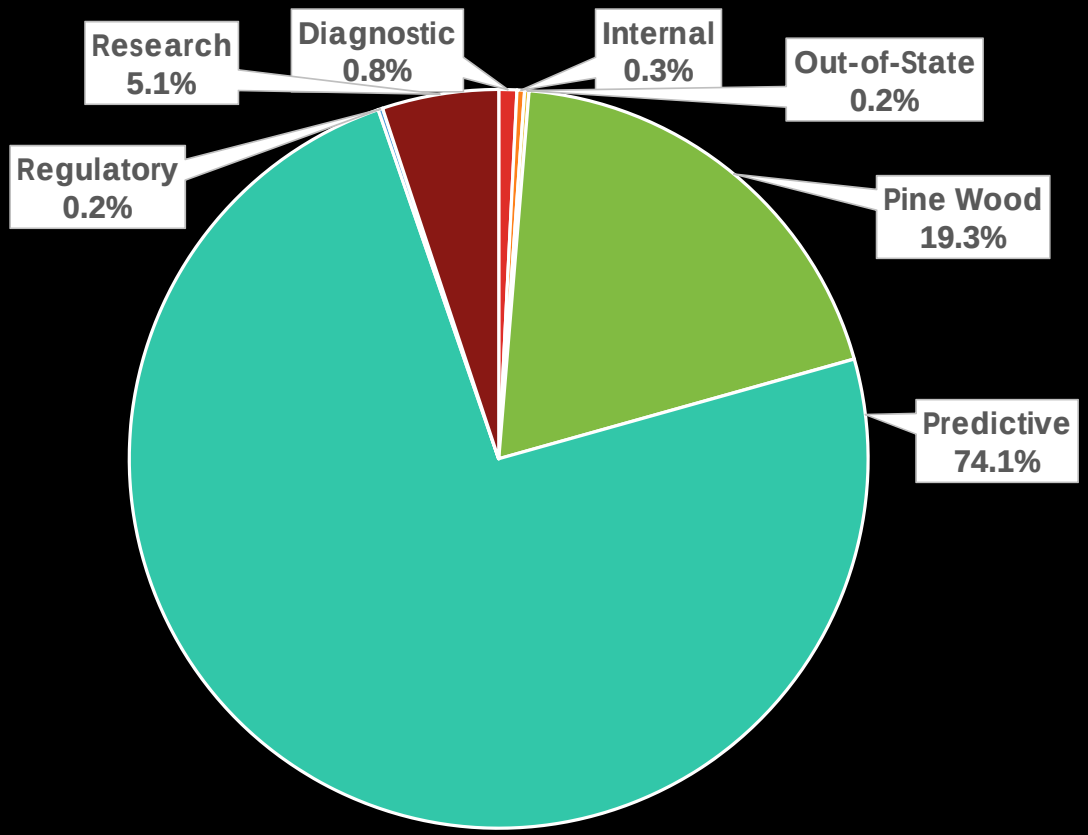
Sample Classification and Fees

- Predictive: Determine nematode threat to next crop: $3/sample
- Diagnostic: Determine nematode problem: $3/sample
- Regulatory: $6/sample
- Research: $10/sample
- Pine wood: $10/sample
- Out-of-State: $10/sample
- Internal: free
  - PDIC samples from NCSU
  - Regional agronomists

Sample Loads In FY2015

Total samples: 40,172

Diagnostic	316	0.8%
Internal	135	0.3%
Out-of-State	76	0.2%
Pine Wood	7767	19.3%
Predictive	29752	74.1%
Regulatory	80	0.2%
Research	2046	5.1%
Total samples	40172	



Serial number

Start with 1, and sequentially number the samples listed on your sample information form. Doing so will help the lab line up the samples easily and reduce turn-around time.

Lab number
For lab use only

PLEASE PRINT PLAINLY

5 characters)

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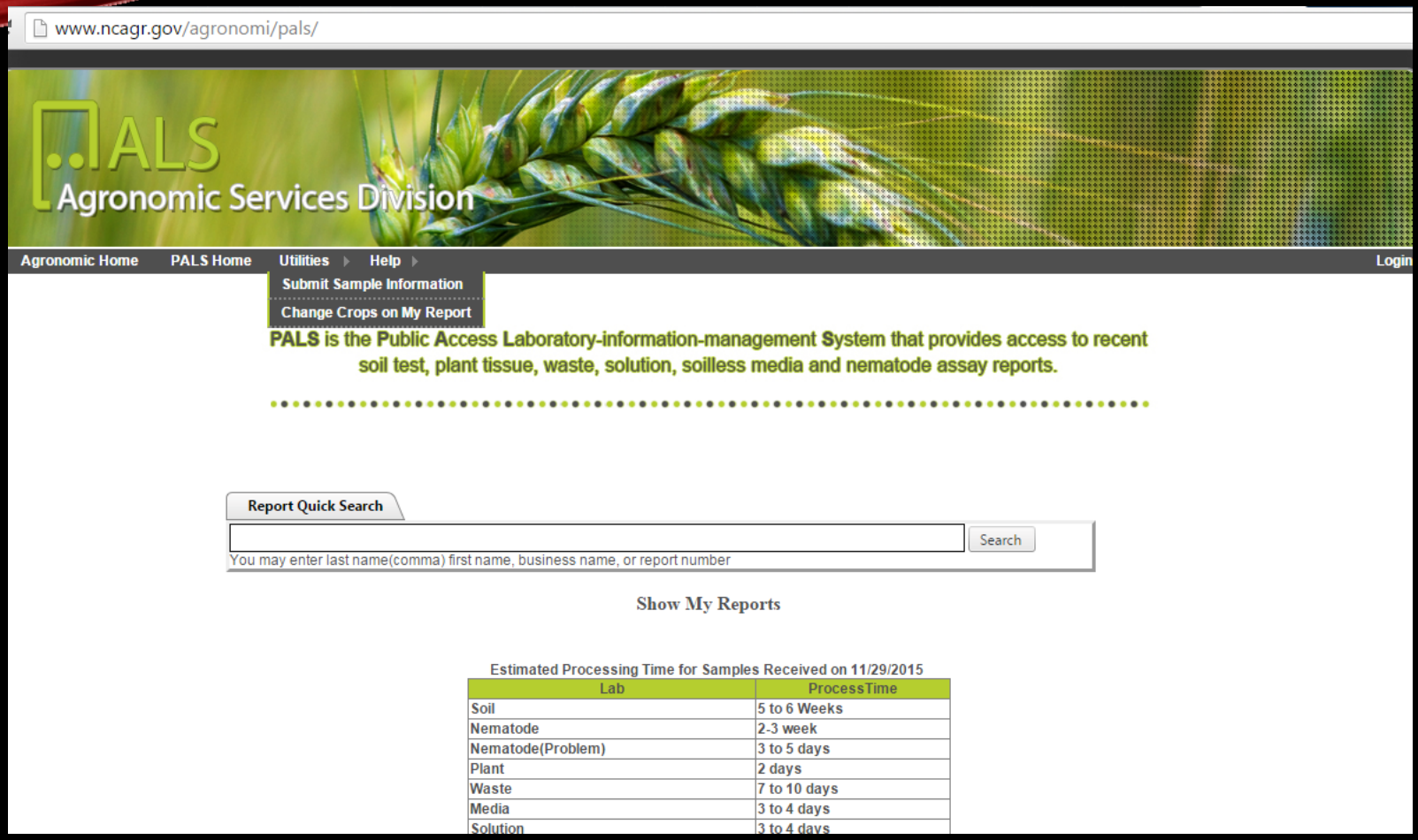
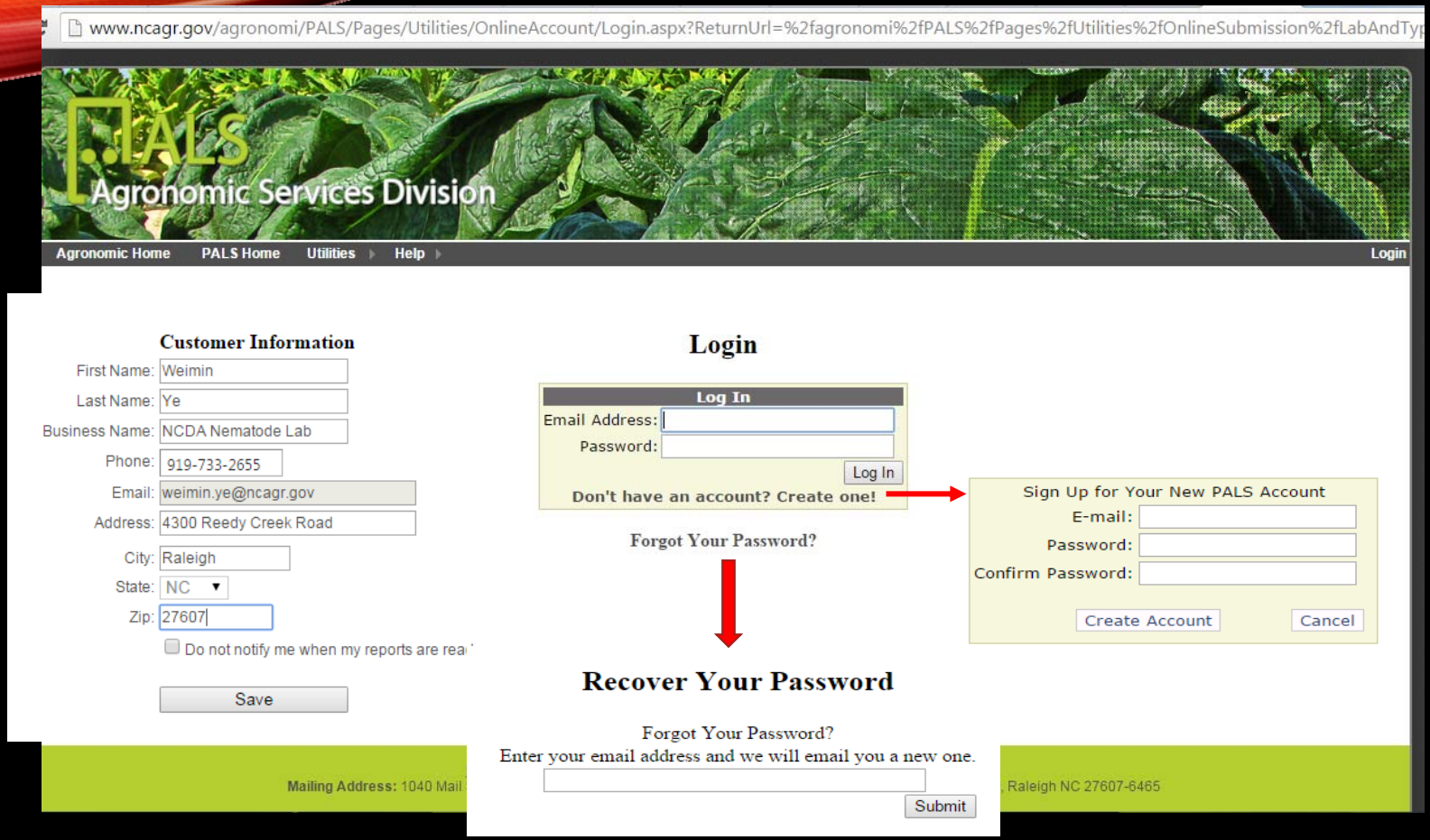
n the package,
mple number.

PROCEDURES FOR TAKING SOIL SAMPLES FOR NEMATODE ASSAYS

Carefully follow these instructions for collecting samples for nematode assay. Good results depend on good samples.

Nematode Sample On-line Submission and Prepayment with Credit Card




www.ncagr.gov/agronomi/PALS/Pages/Utilities/OnlineAccount/Login.aspx?ReturnUrl=%2fagronomi%2fPALS%2fPages%2fUtilities%2fOnlineSubmission%2fLabAndTyp
PALS
Agronomic Services Division
Agronomic Home
PALS Home
Utilities
Help
Login
Customer Information
First Name: Weimin
Last Name: Ye
Business Name: NCDA Nematode Lab
Phone: 919-733-2655
Email: weimin.ye@ncagr.gov
Address: 4300 Reedy Creek Road
City: Raleigh
State: NC
Zip: 27607
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Sign Up for Your New PALS Account
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Password:
Confirm Password:
Create Account
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Recover Your Password
Forgot Your Password?
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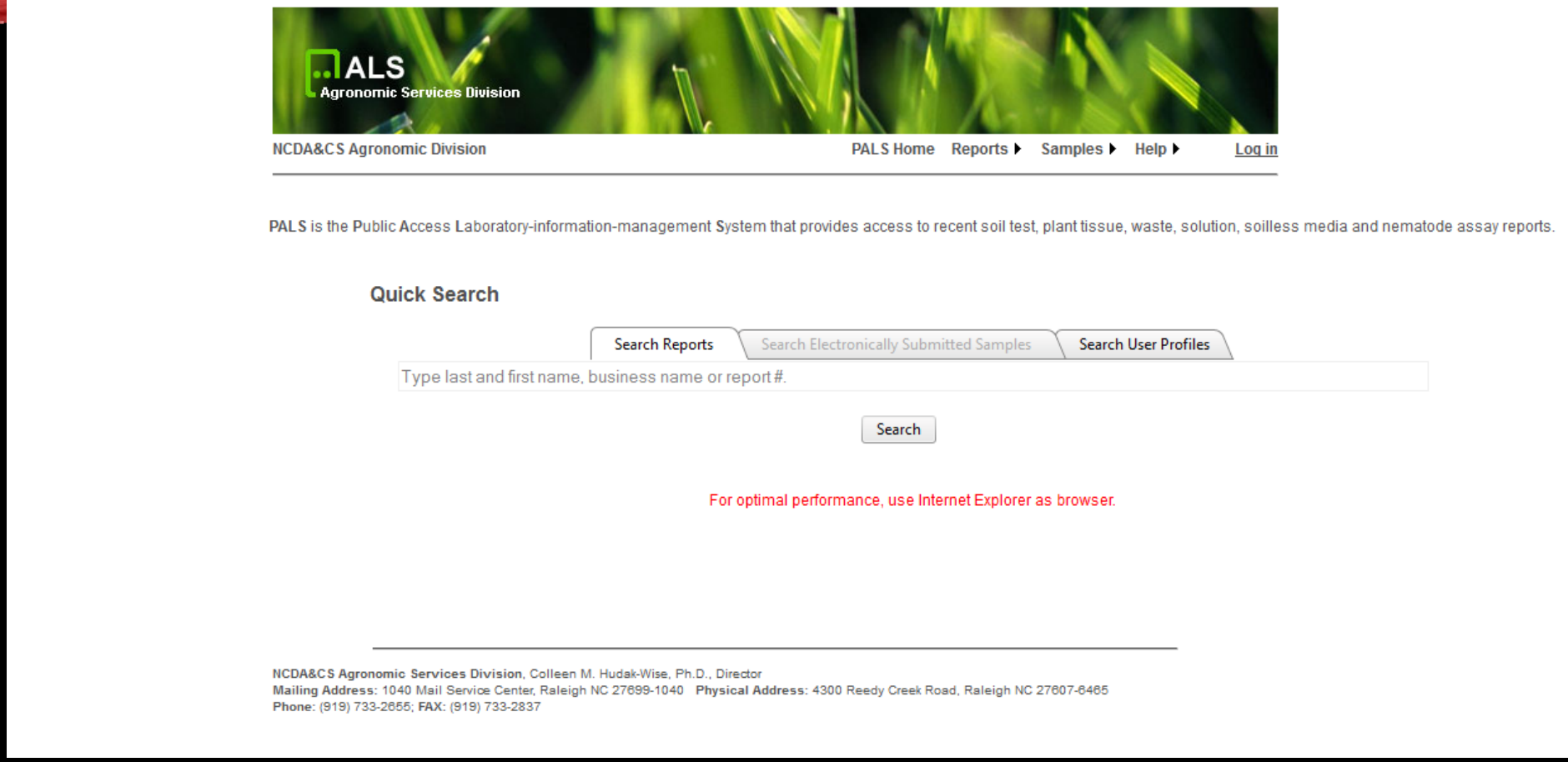
Access to Reports



Report Search Result for Pierce, Danny

Customer: Danny Pierce
674 Lassiter Rd.
Princeton

Client Advisor Agronomist

From: 2011 To: 2013

Download CSV
Download XML

2832 report(s) found.

Fiscal Year	Client	Report	Lab	Report Type	Status	Status Date	Farm ID	Number of Samples	PDF File	Spreadsheet	Sel... All
2013	Kornegay, Danny	N001253	Nemato...	Research	Released	2012/11/06	PA 2	1	New Report !	Download Data	
2013	Kornegay, Danny	N001252	Nemato...	Research	Released	2012/11/06	BIZ 2	1	New Report !	Download Data	
2013	Wellons, Mark A	N001251	Nemato...	Research	Released	2012/11/06	MUM	1	New Report !	Download Data	
2013	Howell, Frank	N001181	Nemato...	Predictive	Released	2012/11/09	NEIL	4	New Report !	Download Data	
2013	Howell, Danny	N001180	Nemato...	Predictive	Released	2012/11/09	BILLY	1	New Report !	Download Data	
2013	Kornegay, Danny	N001179	Nemato...	Predictive	Released	2012/11/09	WO... 6	5	New Report !	Download Data	
2013	Kornegay, Danny	N001178	Nemato...	Predictive	Released	2012/11/09	WHI 1	9	New Report !	Download Data	

NCDA&CS Agronomic Division Phone: (919)733-2655 Website: www.ncagr.gov/agronomi/ Report No. FY13-N000442

Predictive

Nematode Report

Client: Barnes Farming Corp
7840 Old Bailey Hwy
Spring Hope, NC 27882

Advisor:

County: Wilson

Farm: MR-1

Sampled: 09/15/2012 Received: 09/19/2012 Completed: 09/25/2012

Links to Helpful Information

Sample Information

Results and Recommendations:
For each species listed, column S = # nematodes per 500 cc soil and column H = hazard index for crop.

Total # Samples on Report: 42

Sample ID	Lab ID	Next Crop / Alternative Crop	Action Code(s)	Nema Notes	Dagger		Ring		RootKnot		Spiral		StubbyRoot		Stunt					
					S	H	S	H	S	H	S	H	S	H	S	H	S	H	S	H
01	N001973	sweetpotato	B						80	5-40			20		200					
		tobacco	A	3-2					80	5-15			20		200					
02	N001974	sweetpotato	B						40	5-40					120					
		tobacco	A	3-2					40	5-15					120					
03	N001975	sweetpotato	C						410	55-85					160					
		tobacco	C or D	3-3					410	10-45					160					
04	N001976	sweetpotato	B						50	5-40			10		160					
		tobacco	A	3-2					50	5-15			10		160					
05	N001977	sweetpotato	C						680	55-85					160					
		tobacco	C or D	3-3					680	10-45					160					
06	N001978	sweetpotato	C						1120	30-100					120					
		tobacco	C and D	3-4					1120	40-75					120					

North Carolina Tobacco Trust Fund Commission

Reprogramming of the laboratory-information-management system that makes this report possible is being funded through a grant from the North Carolina Tobacco Trust Fund Commission.

Thank you for using agronomic services to manage nutrients and safeguard environmental quality.
- Steve Troxler, Commissioner of Agriculture.

Lab Work Flow



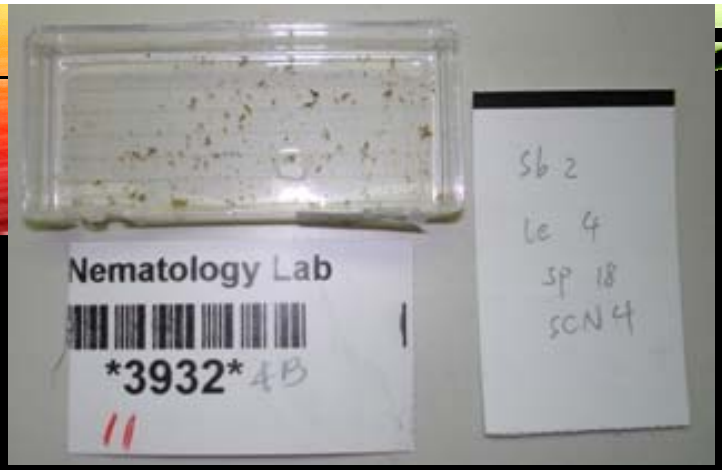


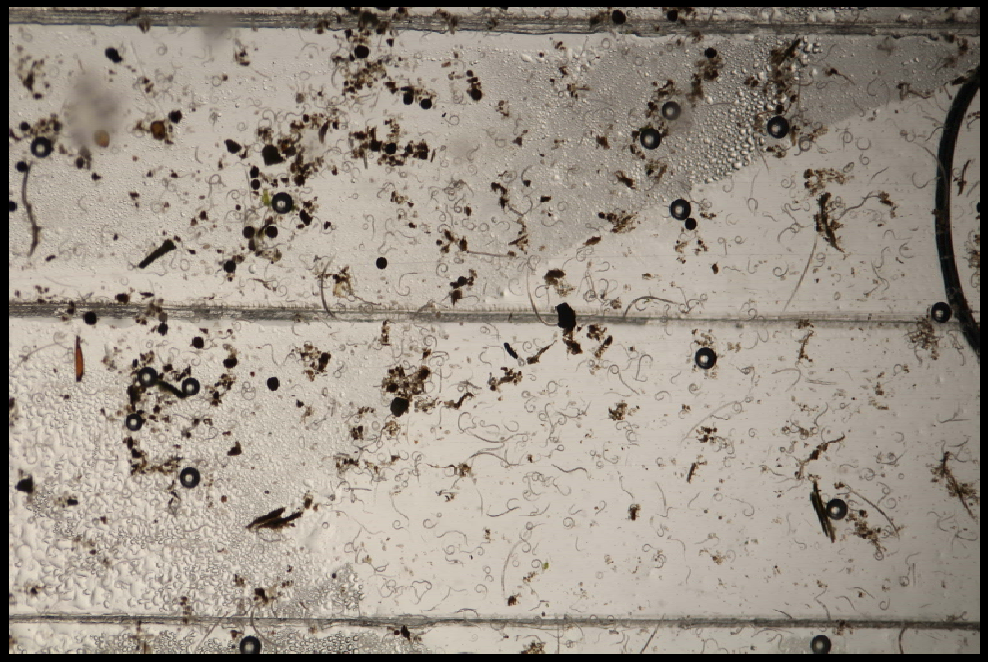


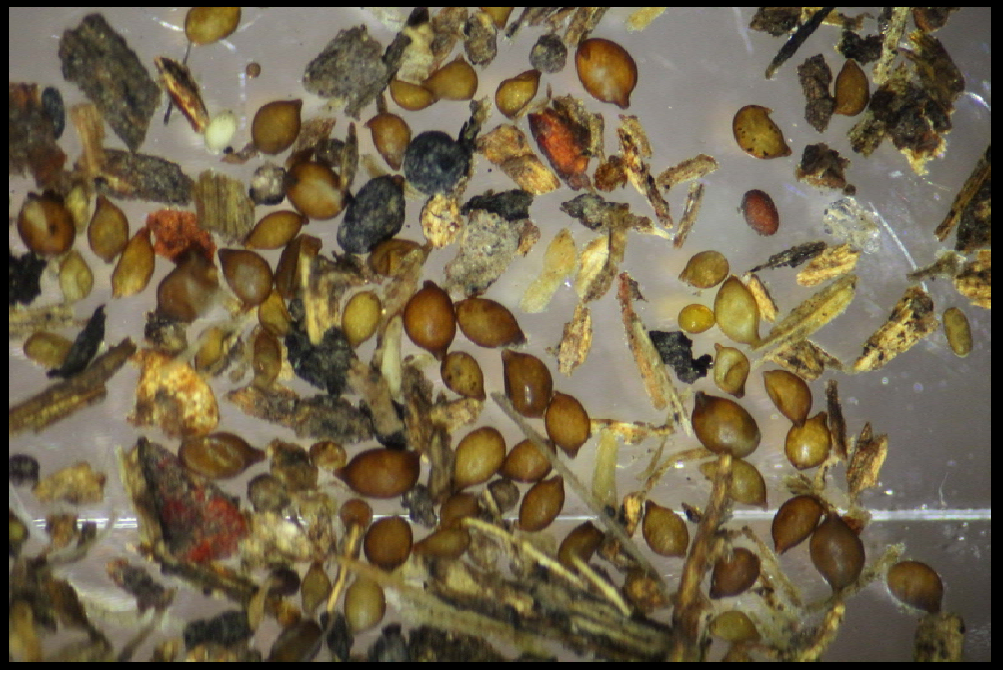



plus 3 temps

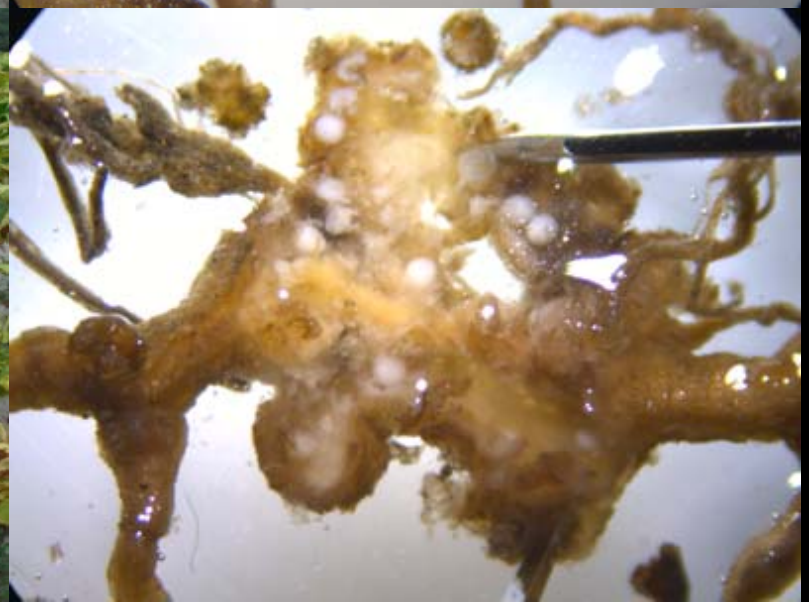
13 in total


Nematology Lab
3932
Sb 2
Le 4
SP 18
SCN 4





Root-knot Nematodes

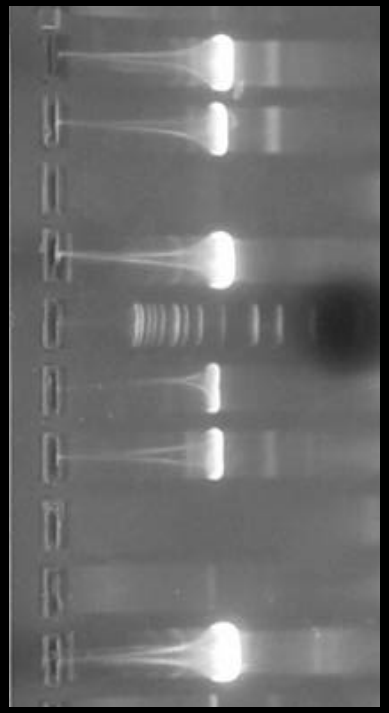


Molecular Diagnosis

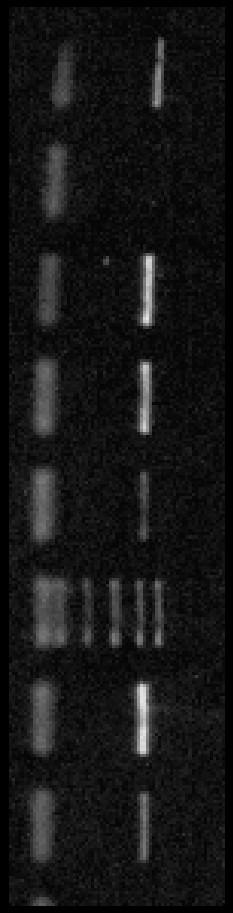
- PCR and DNA sequencing
- PCR by species-specific primers
- Real-time PCR

Nematode PCR of LSU D2/D3, SSU, ITS and mtCOI

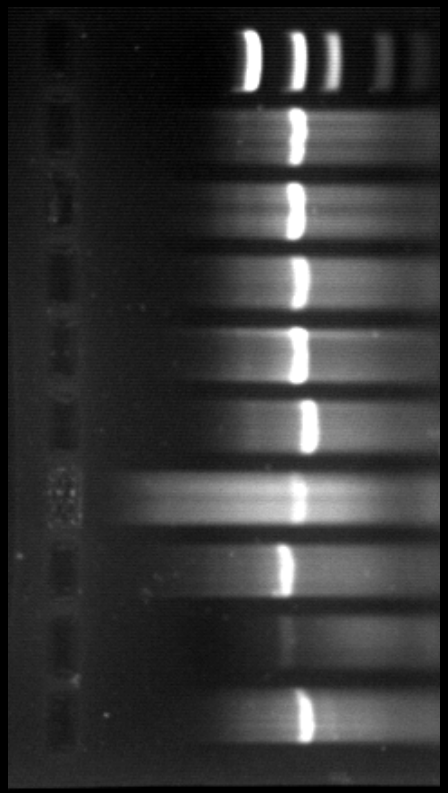
18S, 1750bp



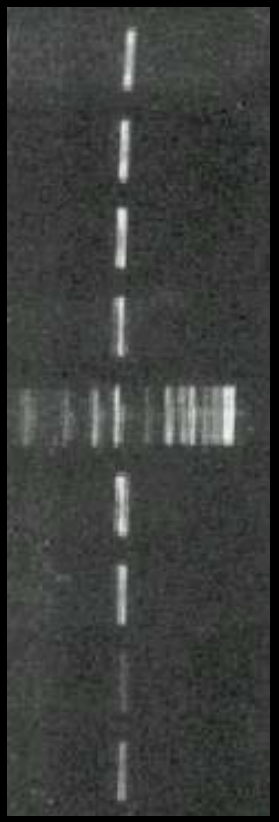
28S D2/D3, 800bp



ITS, variable size



COI, 900bp



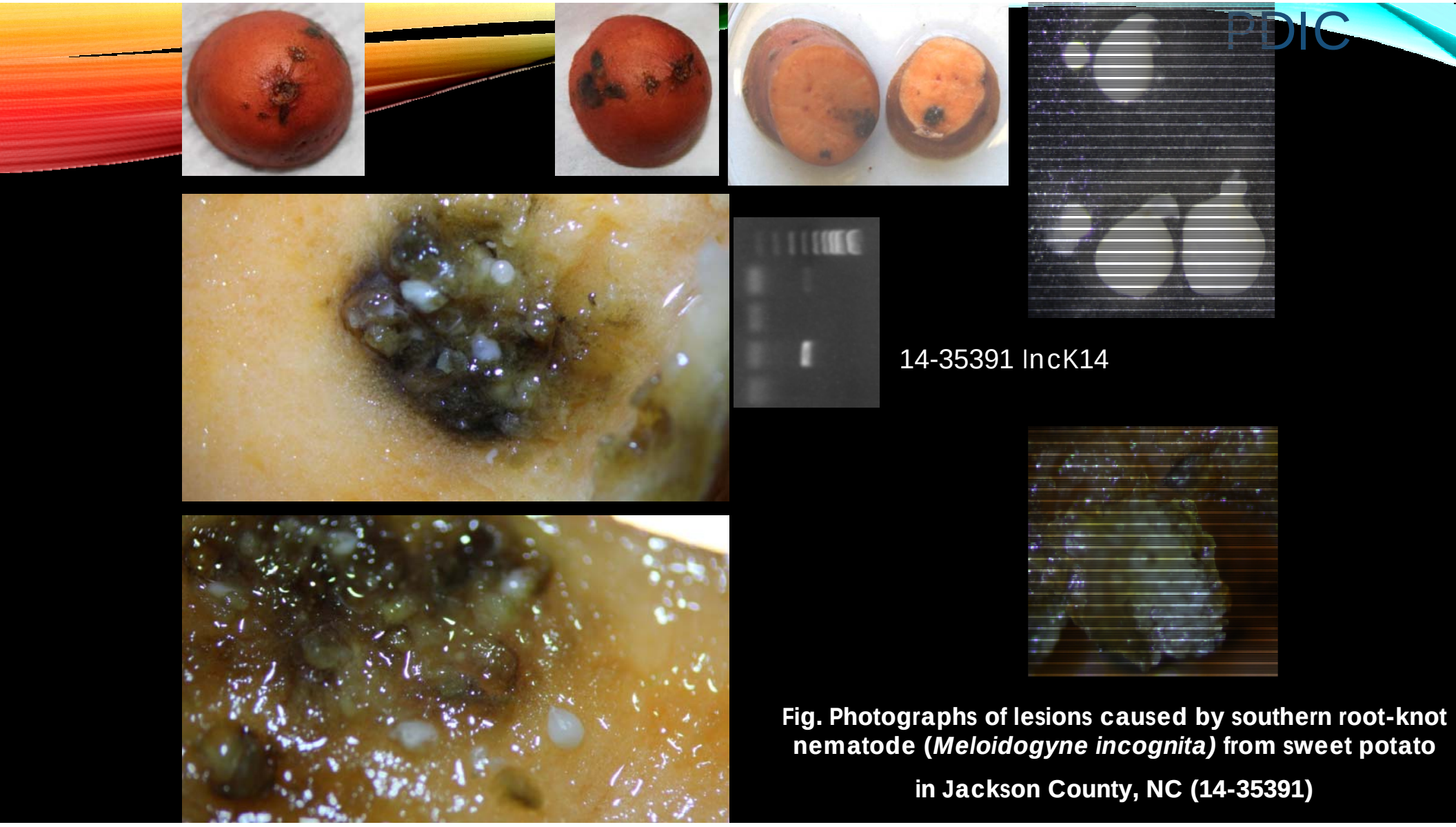


Fig. Photographs of lesions caused by southern root-knot nematode (*Meloidogyne incognita)* from sweet potato in Jackson County, NC (14-35391)

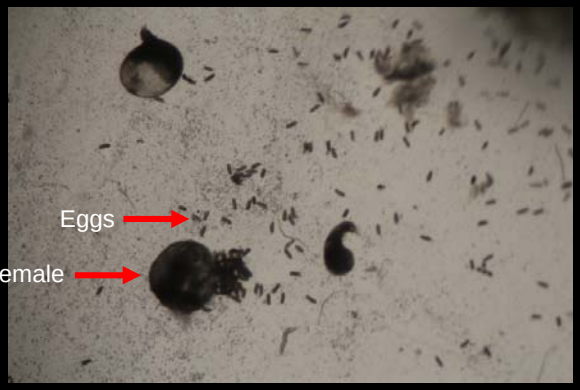
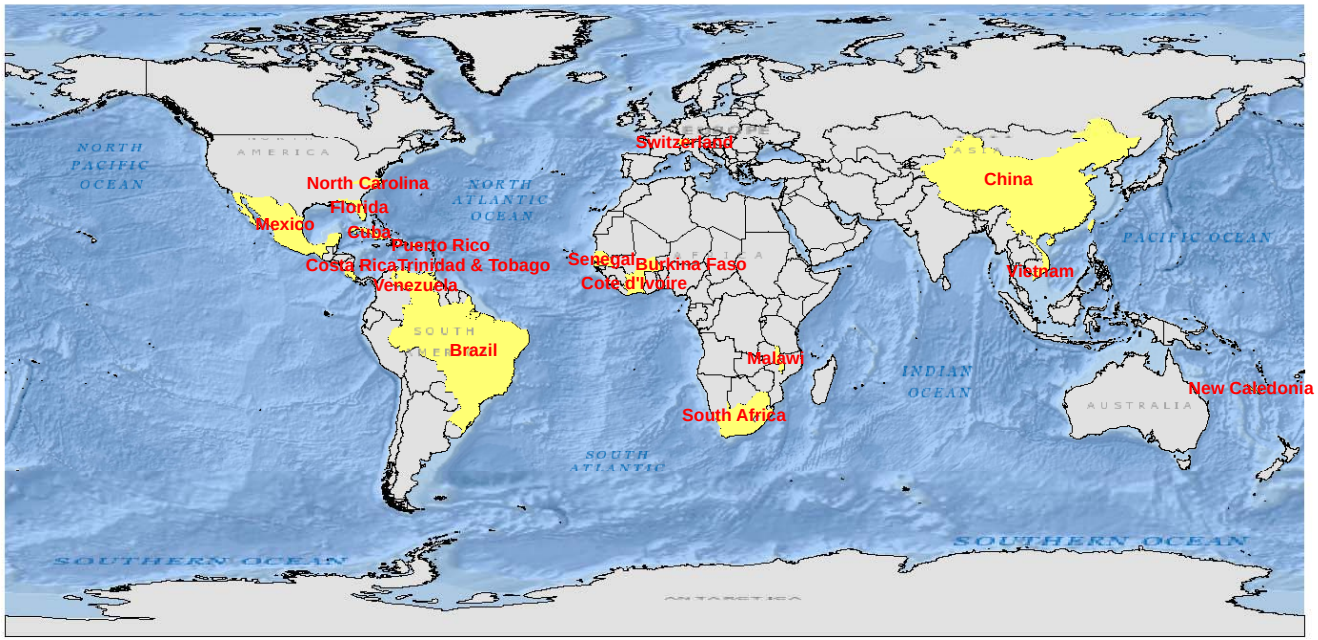


Fig. Photographs of symptom caused by root-knot nematode (*Meloidogyne enterolobii*) on sweet potato in Johnston County, NC (Lab ID: 16-4159)

PCR by *M. enterolobii*-specific Primers

Primer	Amplified gene	Direction	Sequence (5'-3')	Reference
MK7-F	SCAR	F	GATCAGAGGCGGGCGCATTGCGA	Tigano *et al.*, 2010
520bp				
MK7-R	SCAR	R	CGAACTCGCTCGAACTCGAC	Tigano *et al.*, 2010
Me-F	IGS2	F	AACTTTTGTGAAAGTGCCGCTG	Long *et al.*, 2006
236bp				
Me-R	IGS2	R	TCAGTTCAGGCAGGATCAACC	Long *et al.*, 2006

Meloidogyne enterolobii World Distribution



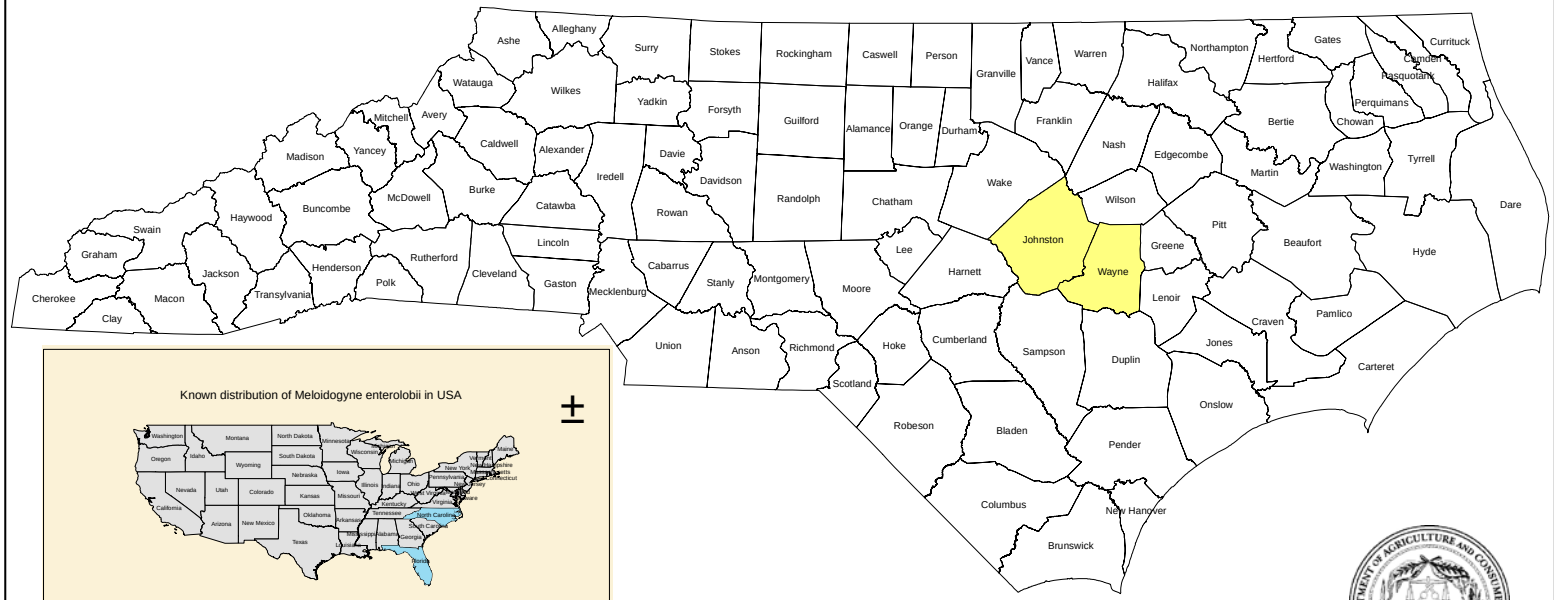
Date: 2/28/2016

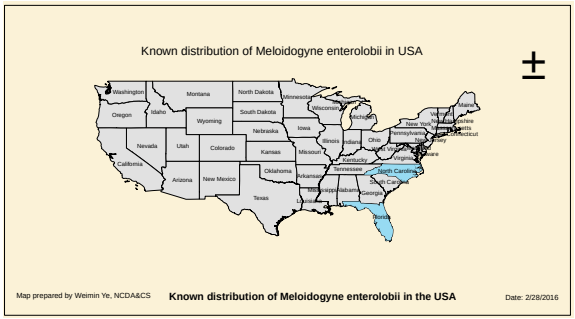
Map prepared by Weimin Ye, NCDA&CS

Esri, DeLorme, GEBCO, NOAA NGDC, and other contributors, Sources: Esri, GEBCO, NOAA, National Geographic, DeLorme, HERE, Geonames.org, and other contributors

Meloidogyne enterolobii distribution in NC

Map updated on Feb. 2016





Legend

M. enterolobii

- Present
- Absent

Map prepared by Weimin Ye, NCDA&CS

Date: 2/28/2016

First Report of *Meloidogyne enterolobii* In North Carolina, United States

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Published by The American Phytopathological Society

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September 2013, Volume 97, Number 9

Page 1262

http://dx.doi.org/10.1094/PDIS-03-13-0228-PDN

Disease Notes

First Report of *Meloidogyne enterolobii* on Cotton and Soybean in North Carolina, United States

W. M. Ye, Nematode Assay Section, Agronomic Division, North Carolina Department of Agriculture and Consumer Services, Raleigh 27607; **S. R. Koenning**, Department of Plant Pathology, North Carolina State University, Raleigh 27695; and **K. Zhuo** and **J. L. Liao**, Laboratory of Plant Nematology, South China Agricultural University,

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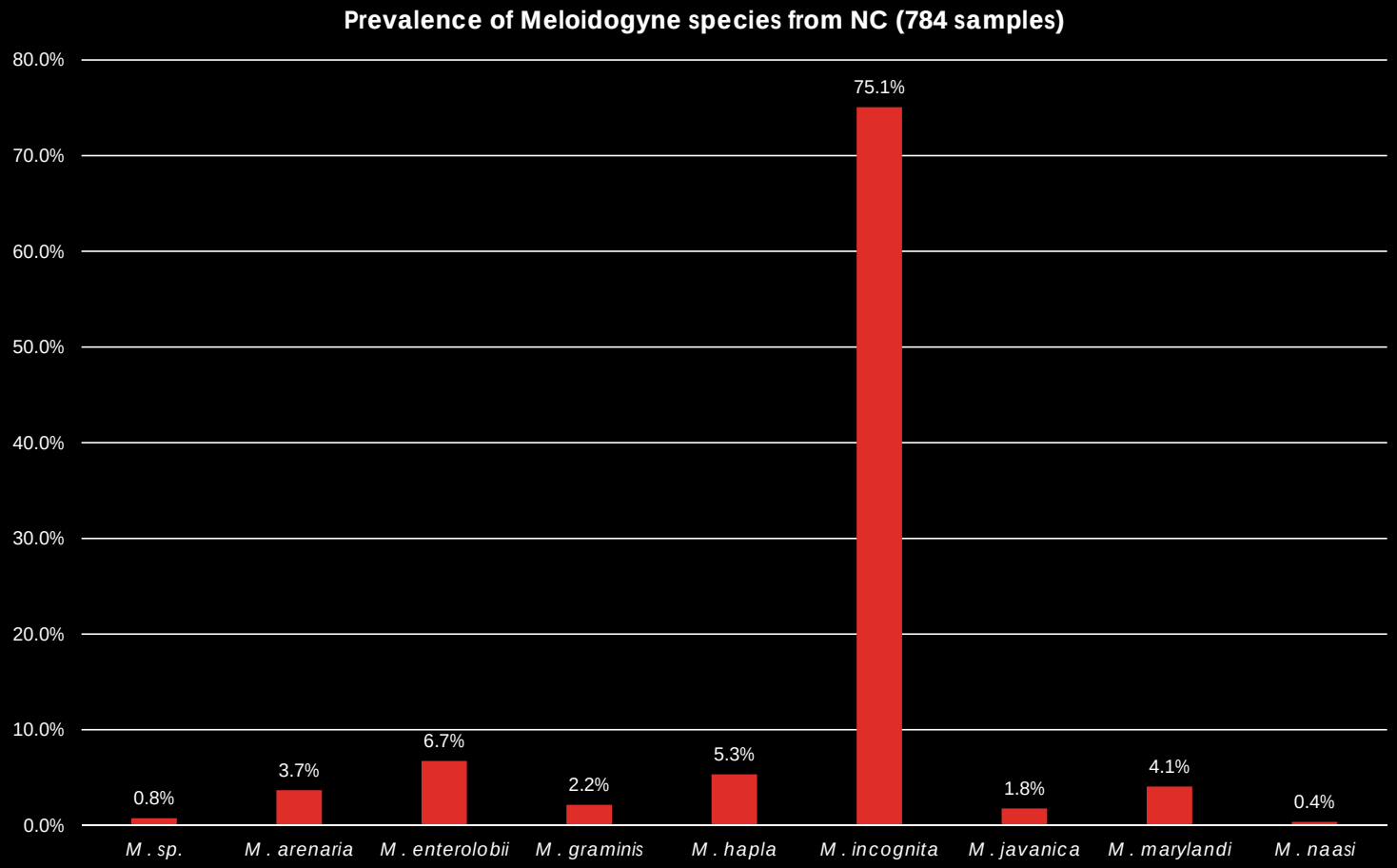
- **Of particular concern is its ability to develop on crop genotypes carrying root-knot-nematode resistance genes (*Mi-1*, *Mh*, *Mir1*, *N*, *Tabasco* and *Rk*) in tobacco, tomato, soybean, potato, cowpea, sweet potato and cotton.**
- **EPPO A2 Alert list in 2010.**

Summary Of Root-knot Nematode Species Composition In NC (786 Samples By Feb 2016)

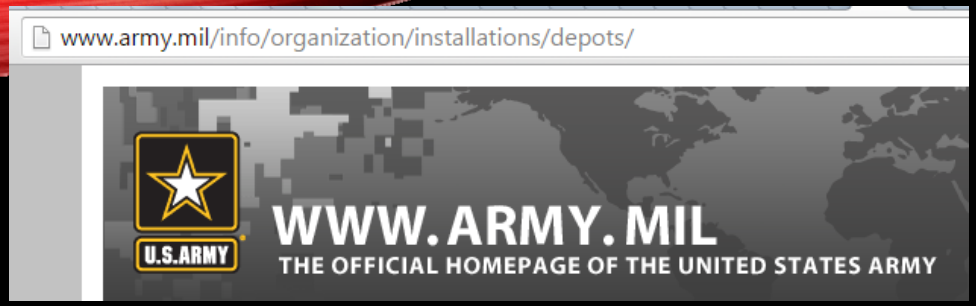


Regulated Nematode Species

- Inspected material
  - Pine wood log
  - Ornamental plants
  - Seeds (soybean, onion, etc.)
  - Seed sweet potato
- Exported to
  - China
  - Canada, Holland, Turkey, South America
  - California, Arizona and other states
- Regulated Nematode Species
  - Pine wood nematode (PWN, ***Bursaphelenchus xylophilus***)
  - Soybean cyst nematode (***Heterodera glycines***)
  - Cyst nematode species
  - Reniform nematode (***Rotylenchulus renifomis***)
  - Burrowing nematode (*Radopholus similis*)
  - Stem and bulb nematode (***Ditylenchus dipsaci***, *D. destructor*)

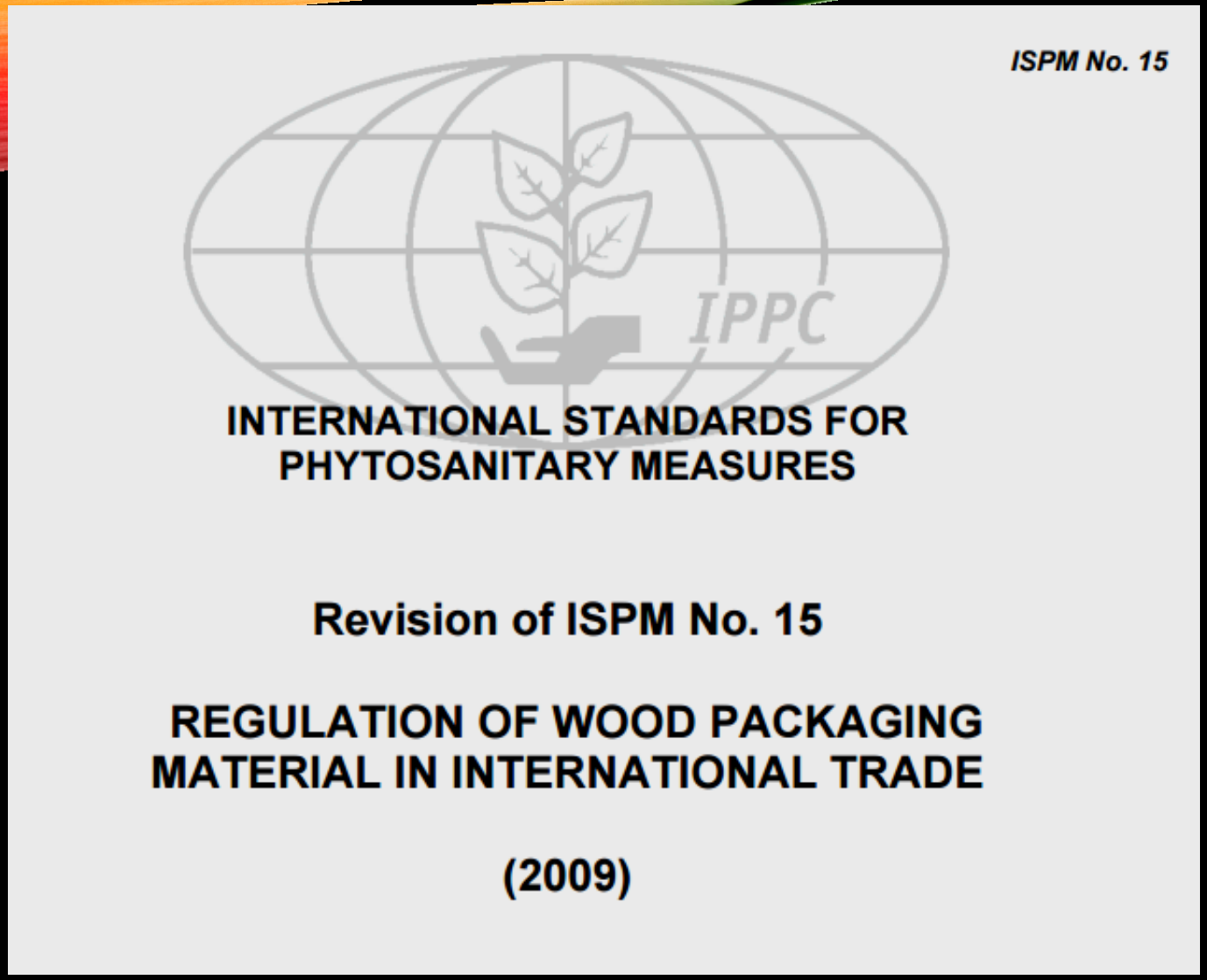
Species in yellow: detected in NC

Pathway analysis and pest detection sampling to achieve EU derogation for DoD ammunition wood packaging material moving into Europe



- **US Army Depots**

- Anniston Army Depot, AL
- Blue Grass Army Depot, KY
- Corpus Christi Army Depot (CCAD), TX
- Deseret Chemical Depot, UT
- Letterkenny Army Depot, PA
- Red River Army Depot (RRAD), TX
- Sierra Army Depot, CA
- Tobyhanna Army Depot, PA
- Tooele Army Depot, UT
- Umatilla Chemical Depot, OR



ISPM 15 stipulates

- Fumigation with methyl bromide
- Heat treatment
- Debarking

Survey for DoD ammunition wood packaging material moving into Europe

DoD softwood characterisation

- The surveyed DoD softwood boxes were at least 5 years old (3 years old for commercial trade).
  - 75%: 7 or more years old
  - 50%: 18 or more years old
- The DoD boxes had been under DoD control
  - stored in DoD storage buildings, with occasional transport between storage locations throughout their lifetime and had never left the United States.

DoD requirements - The boxes were

- dried to a moisture content 12-18%
- treated with one of the following wood preservatives to prevent infestation by wood rot fungi and termites:
  - copper-8-quinolinolate and zinc napthenate (since 1985)
  - copper naphthenate (since 1989)

Survey methods

- The total number of boxes inspected was 630, which - based on a binomial distribution with a 0.95 sensitivity of inspection - provided a 95% confidence of detecting pest presence if 1.5% or more of boxes were infested.

Surveyed only boxes that were

a) made out of softwood,
b) not compliant with ISPM 15,
c) subject to shipment to the EU.

The total number of boxes included in the sampling frame was 215,285, weighing 1,240 metric tons.

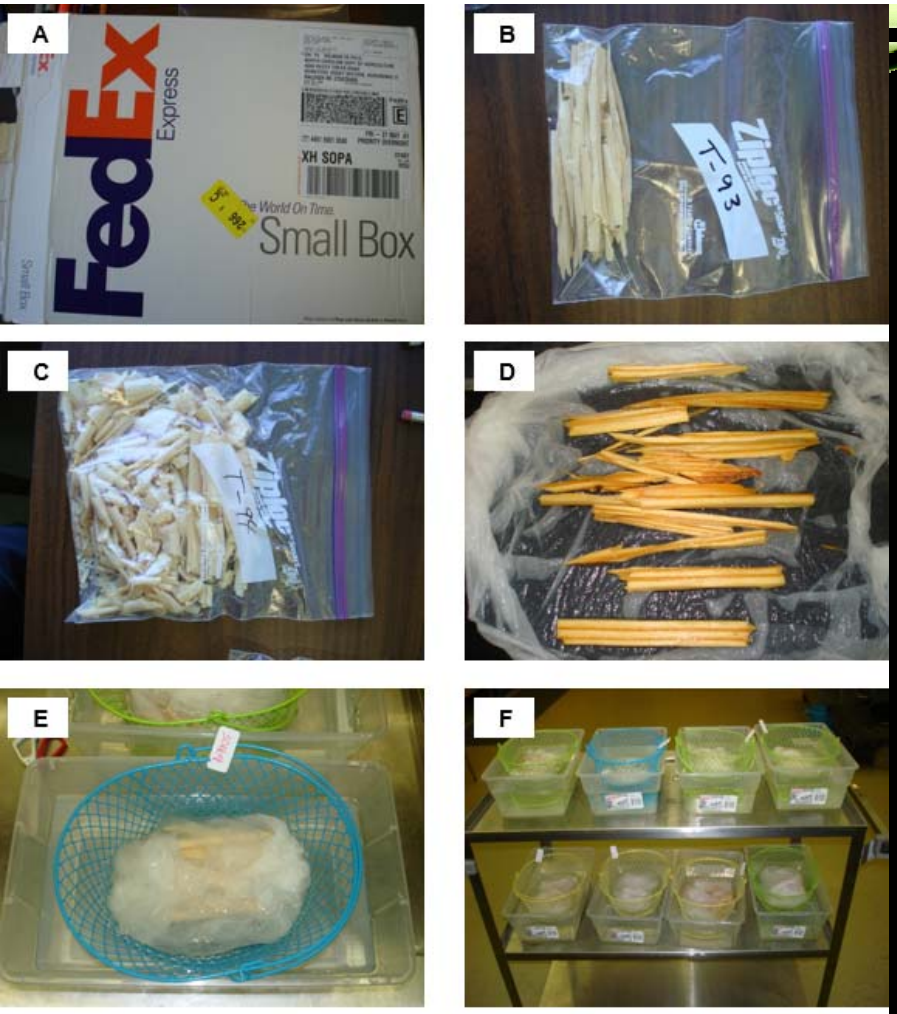
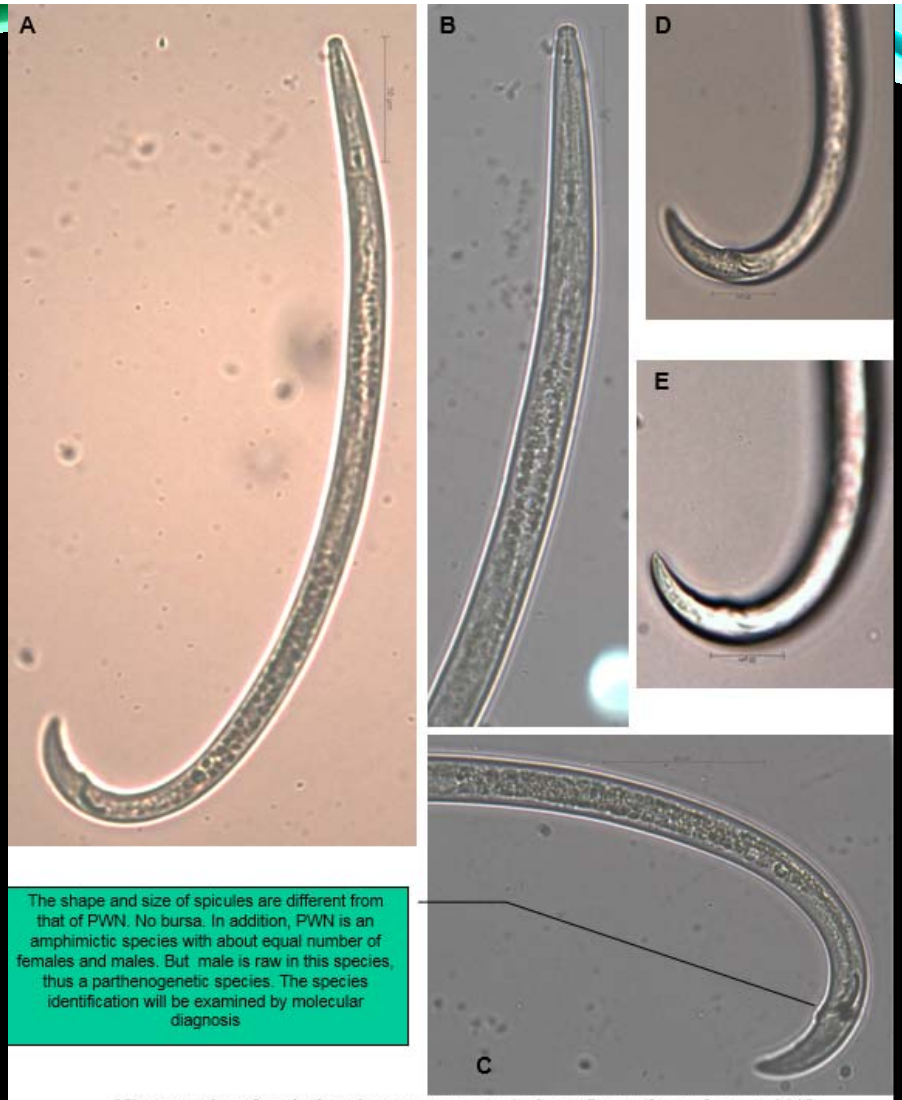


Fig. Samples of wood packaging material from Tooele Army Depot, Utah and nematode extraction in Nematode lab in NCDA

A. Shipping box. B. Wood chop. C. Wood shaving. D. Wood materials for nematode extraction in filter paper. E. Nematode extraction device. F. Nematode extraction.

This is the only suspect of PWN. The population is high with 220 nematodes found from 18.95 gram woods.

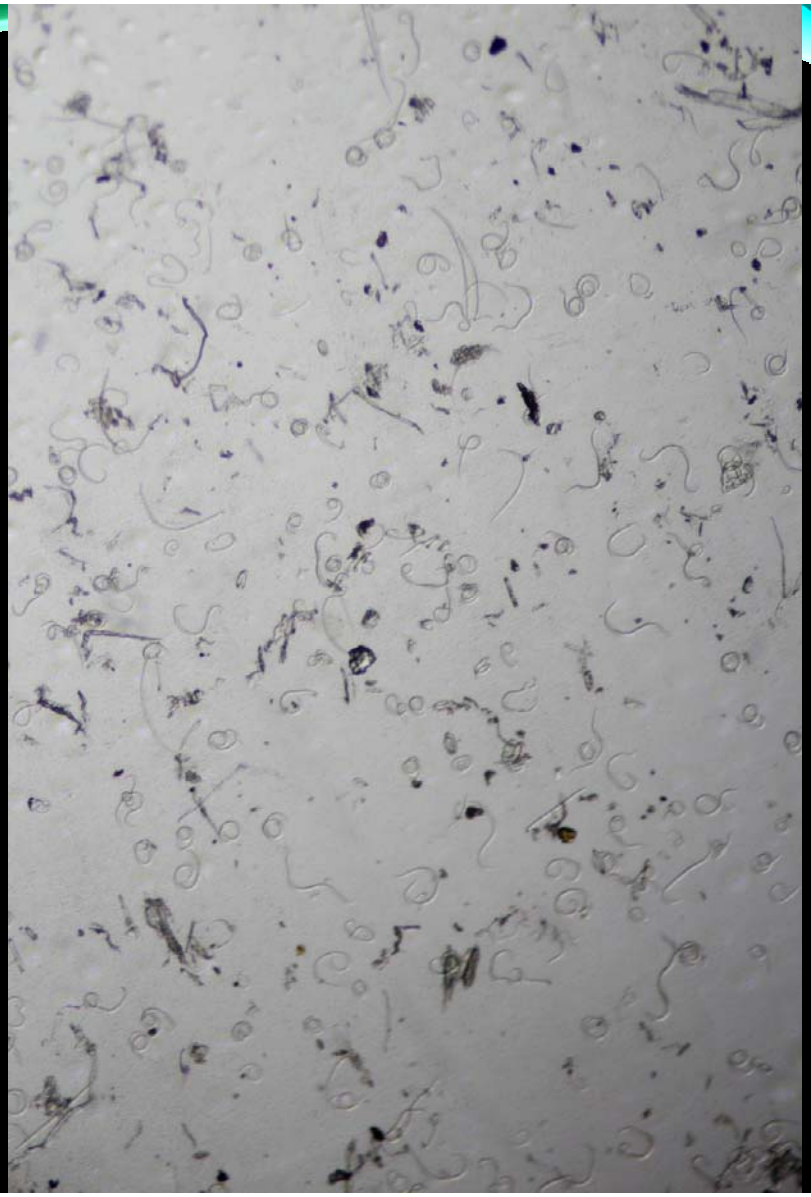
Micrographs of female fungivorous nematode from Crane Army Ammunition Activity (CAAA), Crane, Indiana (ID:12-6370) KC
A. Entire body B. Head region C. Vulva D. Tail region

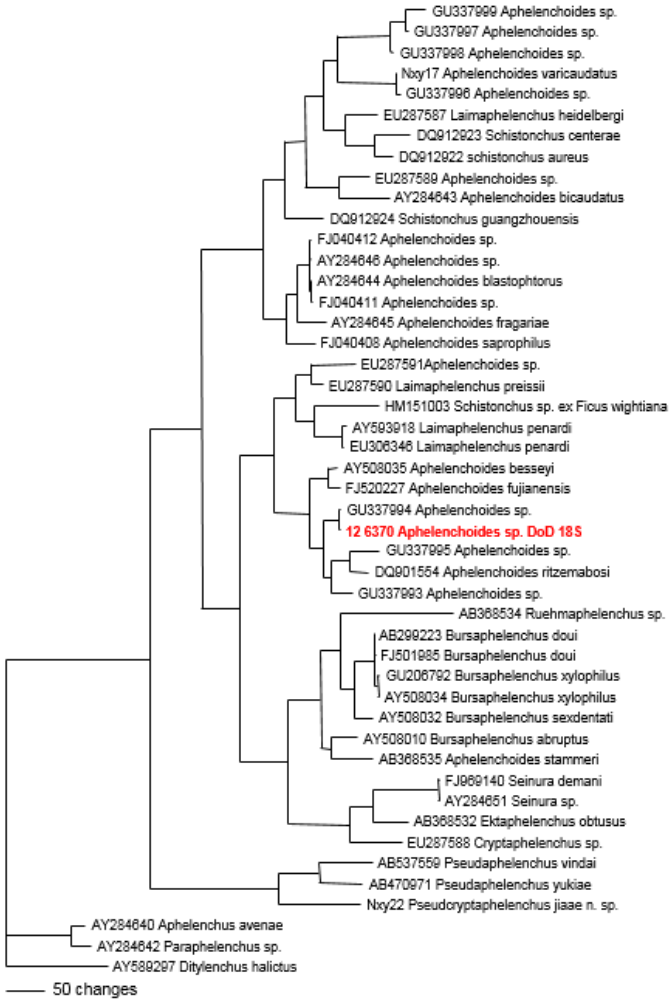


The shape and size of spicules are different from that of PWN. No bursa. In addition, PWN is an amphimictic species with about equal number of females and males. But male is raw in this species, thus a parthenogenetic species. The species identification will be examined by molecular diagnosis

Micrographs of male fungivorous nematode from Crane Army Ammunition Activity (CAAA), Crane, Indiana (ID:12-6370) KC
A. Entire body B. Head region C, D, E. Tail region

Fig. Ammunition wood packaging material from Crane Army Ammunition Activity (CAAA) from Crane, Indiana (Sample ID C167 & C168) to Nematode lab in NCDA&CS on Dec 7, 2011 for retesting suspect of pine wood nematode.





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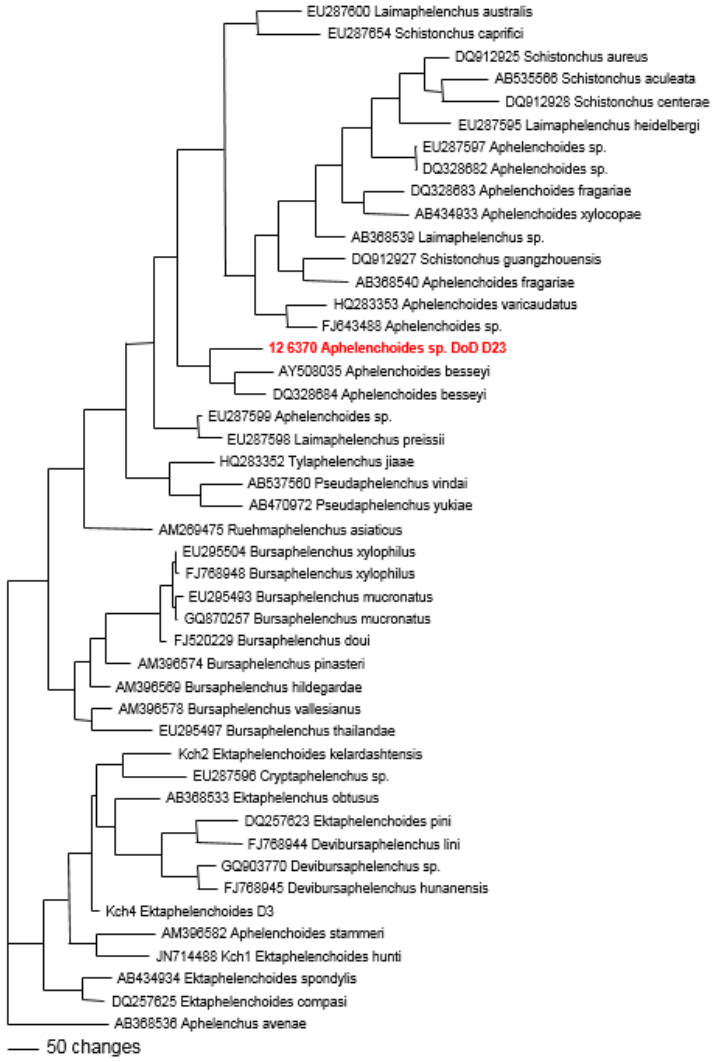
Parsomony tree of molecular phylogenetic relationships of fungivorous Aphelenchid nematode from Crane Army Ammunition Activity (CAAA), Crane, Indiana with other closely related species from the world (ID:12-9861; Sample ID: C168) based on rDNA 18S.



98

Parsomony tree of molecular phylogenetic relationships of fungivorous Aphelenchid nematode from Crane Army Ammunition Activity (CAAA), Crane, Indiana with other closely related species from the world (ID:12-9861; Sample ID: C168) based on rDNA 28S.

Valued Smart Cycler Customer 2012/1/31 09:17 AM

Optics Graph

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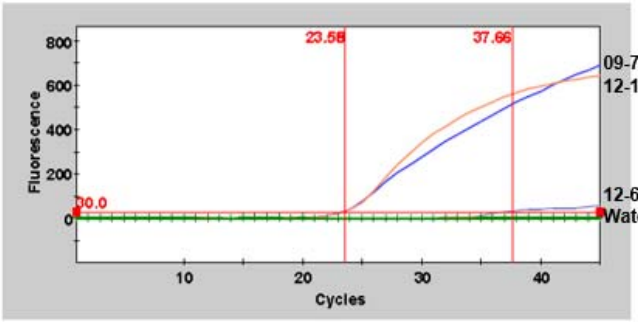
User Name : Default User
Run Date : Jan 31, 2012 08:21 AM



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Bulletin OEPP/EPPO Bulletin (2014) **44** (1), 37–43 ISSN 0250-8052. DOI: 10.1111/epp.12083

Pest survey of softwood boxes, with special emphasis on pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae)

H. Meissner[1], P. DeWald[2], E. Jones[3], A. Lemay[1], L. Millar[1] and W. Ye[4]

[1]*United States Department of Agriculture, Animal and Plant Health Inspection Service, 1730 Varsity Drive, Raleigh, NC, 27606, USA; e-mail: heike.e.meissner@aphis.usda.gov*
[2]*United States Department of Defense, US Army Armament Research Development & Engineering Center (ARDEC), Picatinny Arsenal, NJ, 07806-5000, USA*
[3]*1-alpha Solutions, 751 Heritage Arbor Dr., Wake Forest, NC, 27587, USA*
[4]*North Carolina Department of Agriculture and Consumer Services, Agronomic Services Division, 4300 Reedy Creek Road, Raleigh, NC, 27607, USA*

Species confirmation of pine wood nematode (PWN) by TaqMan Real-time PCR
This test revealed that fungivorous Aphelenchid nematode from Crane Army Ammunition Activity (CAAA), Crane, Indiana (ID:12-6370; Sample ID: C168) is negative for PWN.

Ref. Ares(2014)4208028 - 15/12/2014

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HEALTH AND CONSUMERS DIRECTORATE-GENERAL

Safety of the Food Chain
Plant health

Brussels,
SANCO.E2/FH/svi (2014) 4472774

Dear Dr. Dowdy,

Subject: Wood packaging material used in the transport of ammunition from the United States to the European Union

I am pleased to inform you that a Commission proposal on a temporary derogation from

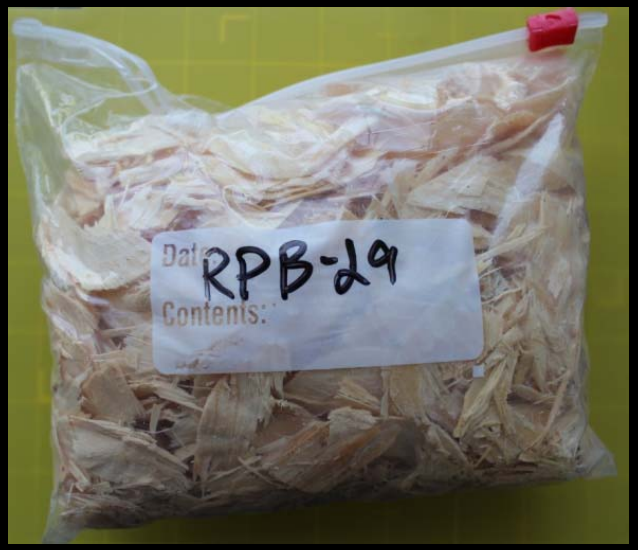
Pine Wood Nematode Inspection

http://permaculturinginportugal.net/blog/pine-wilt-nematode/

Pine Wood Samples

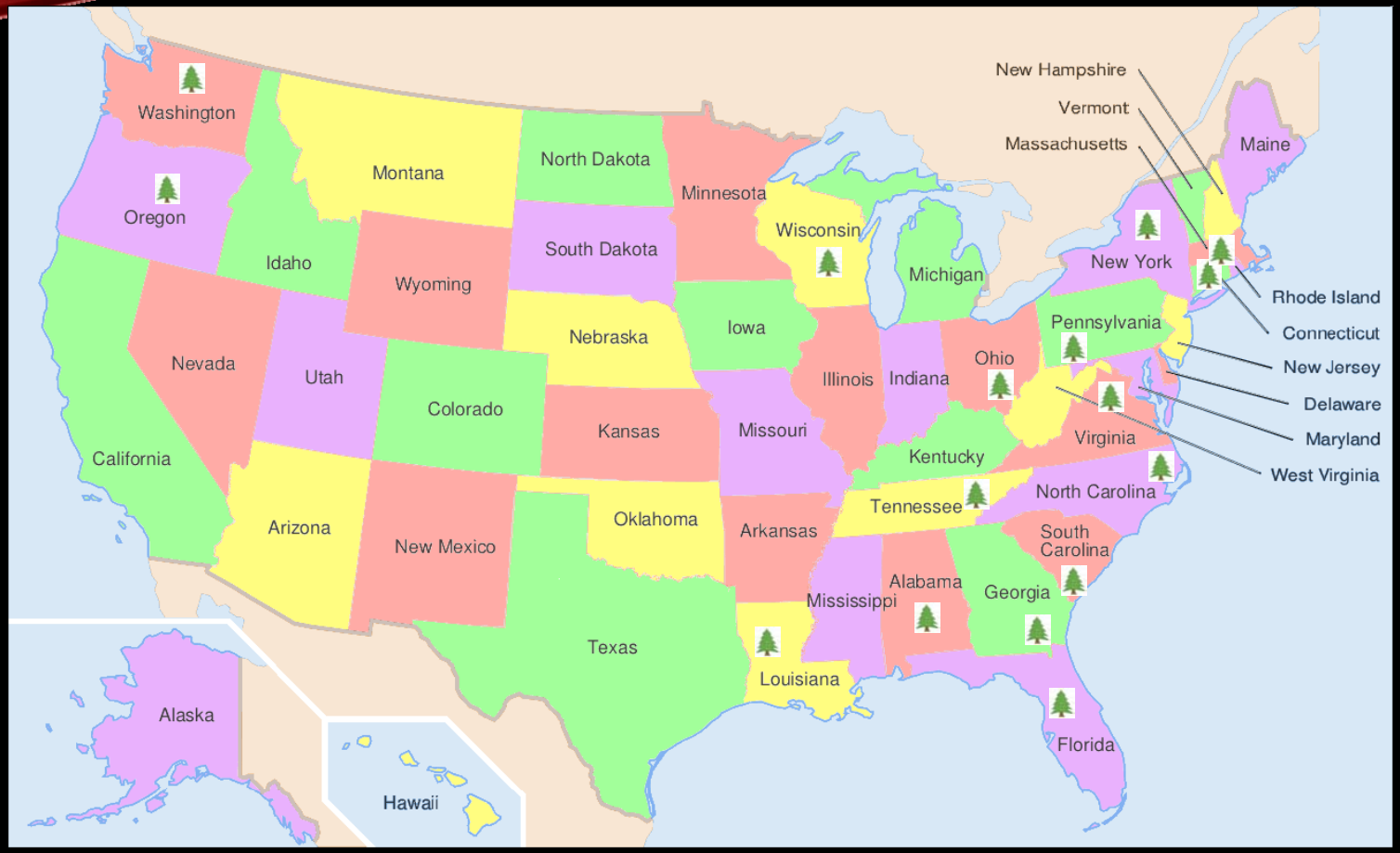
Shipping address:
Weimin Ye, Ph.D
Nematode Assay Section
NCDA&CS Agronomic Division
4300 Reedy Creek Rd
Raleigh, NC 27607-6465

Phone: (919) 733-2655
FAX: (919) 733-2837
Email: Weimin.Ye@ncagr.gov
www.ncagr.gov/agronomi/



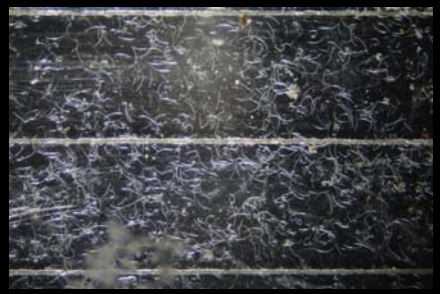
At least 200 g per sample

Pine Wood Log Sample Sources



Pine Wood Samples (FY2014)

Total PWN reports	**703**	
Total PWN samples	**9,825**	
Total nematode samples	**42,383**	**23.18% pine wood**
Total nematode reports	**5,536**	**12.70% pine wood**



Bosch 36618-02 18-Volt 1/2-Inch Compact-Tough Litheon Drill/Driver with 2 Slim Batteries

IRWIN 2-1/8" Self Feed Bit

Item #: 154235 | Model #: 3046011

Pinewood Nematode

Fig. Photographs of female pinewood nematode (*Bursaphelenchus xylophilus*) from pine wood in Georgia (lab ID: 13-662)

NCDA&CS Nematode Report

NCDA&CS Agronomic Division Phone: (919) 733-2655 Website: www.ncagr.gov/agronomi/ Report No. FY13-N000162

Pinewood

Nematode Report

Sampled: 08/09/2012 Received: 08/10/2012 Completed: 08/20/2012

Client:
12750 Center Court DR #460
Cerritos, CA 90703

Advisor: Stephen Davis
5514 Export Blvd.
Garden City, GA 31408

County:

Farm: SAA004

Links to Helpful Information

Nematologist's Comments: Pine wood nematode (Bursaphelenchus xylophilus) was found from sample 002. The other samples are free of pine wood nematode.

Sample Information
Total # Samples on Report: 8

Results

Sample ID	Lab ID	Number of Pine Wood Nematodes (PWN)	Sample Weight (g)	PWN / gram Wood	Number of Fungivores	Number of Bacterivores
001	N000661	0	511	0	0	6
002	N000662	768	412	2	128	300
003	N000663	0	472	0	0	0
004	N000664	0	507	0	0	2
005	N000665	0	398	0	0	12
006	N000666	0	571	0	0	0
007	N000667	0	497	0	0	0
008	N000668	0	504	0	0	0

This report is authorized by law (7 U.S.C. 147a). While you ar not required to respond your cooperation is needed to make an accurate record of plant pest conditions. *See reverse for additional OMB information.*

FORM APPROVED OMB NO. 0579-0010

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE

SPECIMENS FOR DETERMINATION

Instructions: Type or print information requested. Press hard and print legibly when handwritten. Item 1 - assign number for each collection beginning with year, followed by collector's initials and collector's number. Example (collector, John J. Dingle): 83-JJD-001.
Pest Data Section – Complete items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete items 17 and 18 if a trap was used.

FOR IIBIII USE
LOT NO.
PRIORITY

1. COLLECTION NUMBER: ILM-1814-1875-2012

2. DATE: MO 06 | DA 14 | YR 12

3. SUBMITTING AGENCY: ☐ State Cooperator ☒ PPQ ☐ Other _____

SENDER AND ORIGIN

4. NAME OF SENDER: D Brown

6. ADDRESS OF SENDER:
USDA-APHIS-PPQ
1815 GARDNER DRIVE
WILMINGTON, NC ZIP 28405

INTERCEPTION SITE

5. TYPE OF PROPERTY *(Farm, Feedmill, Nursery, etc.)*: NC STATE PORT WILMINGTON

7. NAME AND ADDRESS OF PROPERTY OR OWNER:
NC STATE PORT AUTHORITY
1 SHIPYARD BLVD
WILMINGTON, NC COUNTRY/COUNTY NEW HANOVER

PURPOSE

8. REASON FOR IDENTIFICATION *("X" ALL Applicable Items)*

A. ☐ Biological Control (Target Pest Name)	E. ☐ Livestock, Domestic Animal Pest
B. ☐ Damaging Crops/Plants	F. ☐ Possible Immigrant *(Explain in* REMARKS*)*
C. ☐ Suspected Pest of Regulatory Concern *(Explain in* REMARKS*)*	G. ☐ Survey *(Explain in* REMARKS*)*
D. ☐ Stored Product Pest	H. ☒ Other *(Explain in* REMARKS*)*

9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER "REMARKS".

HOST DATA

10. HOST INFORMATION
NAME OF HOST *(Scientific name when possible)*: PINUS

11. QUANTITY OF HOST
NUMBER OF ACRES/PLANTS
PLANTS AFFECTED *(Insert figure and indicate* ☐ Number ☐ Percent*)*:

12. PLANT DISTRIBUTION
☐ LIMITED ☐ SCATTERED ☐ WIDESPREAD

13. PLANT PARTS AFFECTED

☐ Leaves, Upper Surface	☒ Trunk/Bark	☐ Bulbs, Tubers, Corms	☐ Seeds
☐ Leaves, Lower Surface	☐ Branches	☐ Buds	
☐ Petiole	☐ Growing Tips	☐ Flowers	
☐ Stem	☐ Roots	☐ Fruits or Nuts	

PEST DATA

14. PEST DISTRIBUTION
☐ FEW ☐ COMMON ☐ ABUNDANT ☐ EXTREME

15. ☐ INSECTS ☒ NEMATODES ☐ MOLLUSKS

NUMBER SUBMITTED	LARVAE	PUPAE	ADULTS	CAST SKINS	EGGS	NYMPHS	JUVS.	CYSTS
ALIVE								
DEAD								

16. SAMPLING METHOD: BORING

17. TYPE OF TRAP AND LURE: NA

18. TRAP NUMBER: NA

19. PLANT PATHOLOGY – PLANT SYMPTOMS *("X" one and describe symptoms)*
☐ ISOLATED ☐ GENERAL

20. WEED DENSITY
☐ FEW ☐ SPOTTY ☐ GENERAL

21. WEED GROWTH STAGE
☐ SEEDLING ☐ VEGETATIVE ☐ FLOWERING/FRUITING ☐ MATURE

22. REMARKS
PWN SAMPLING TO MEET EXPORT REQUIREMENTS FOR CHINA (A)573-582

23. TENTATIVE DETERMINATION

24. DETERMINATION AND NOTES *(Not for Field Use)*
Pine wood nematode (Bursaphelenchus xylophilus) was detected from A2581

SIGNATURE Weimenje DATE 6/21/12

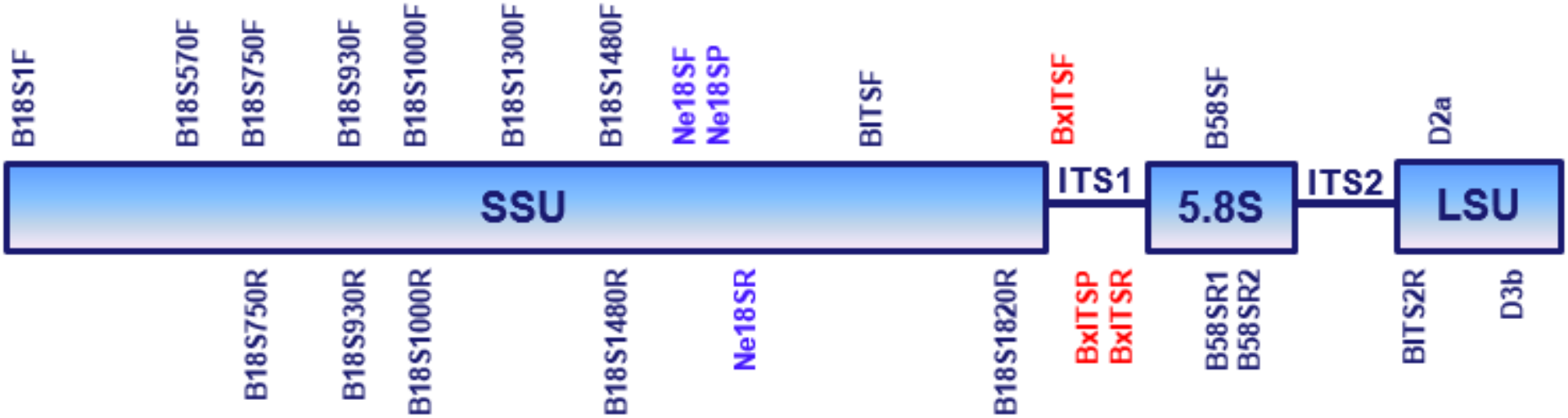
FOR IIBIII USE
DATE RECEIVED
NO.
LABEL
SORTED
PREPARED
DATE ACCEPTED
RR

PPQ FORM 391 (AUG 02) *Previous editions are obsolete.*

This is a 6-Part form. Copies must be disseminated as follows:
☐ PART 1 – PPQ ☐ PART 2 – RETURN TO SUBMITTER AFTER IDENTIFICATION ☐ PART 3 – IIBIII OR FINAL IDENTIFIER
☐ PART 4 – INTERMEDIATE IDENTIFIER ☐ PART 5 – INTERMEDIATE IDENTIFIER ☐ PART 6 – RETAINED BY SUBMITTER

Primer and Probe Locations

for PCR amplification, sequencing and real-time PCR of ribosomal DNA

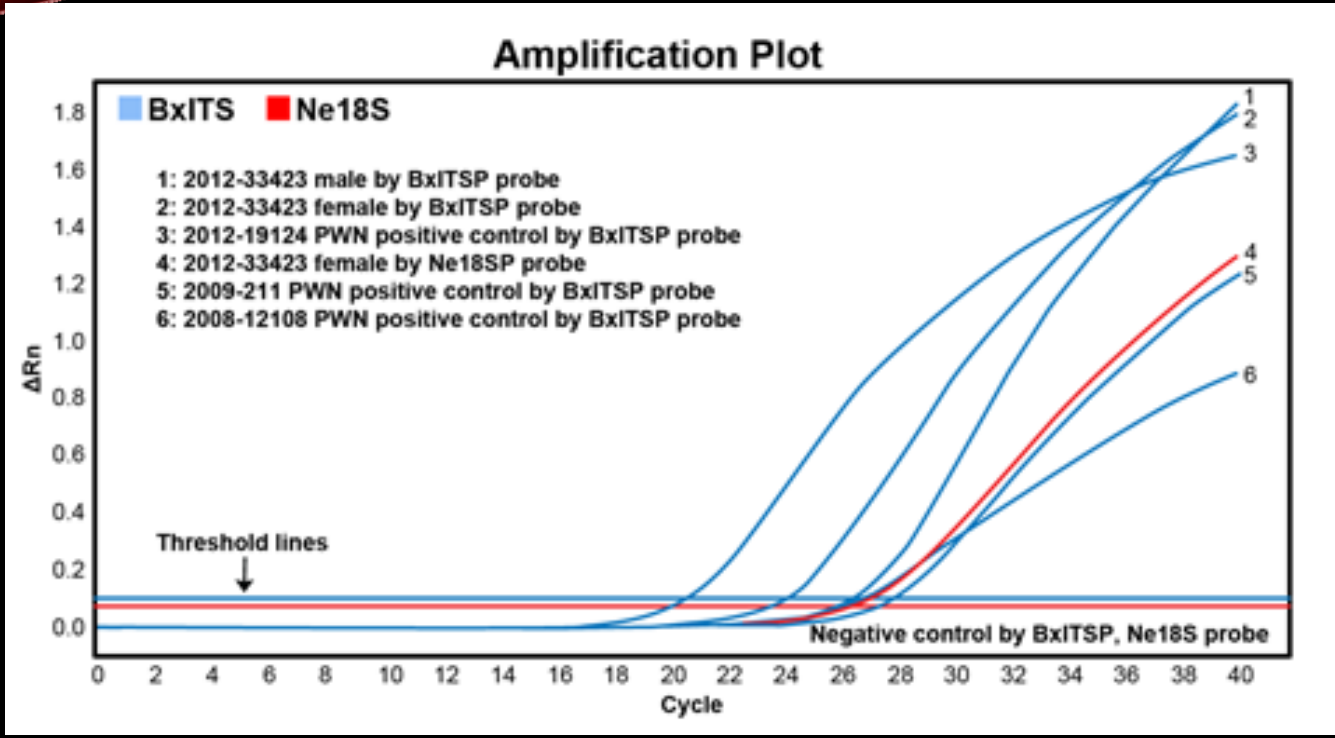


Primer (BxITSF, BxITSR) and Probe (BxITSP) Design

based on multiple alignment of ITS1 DNA sequences of *Bursaphelenchus* species. Data show only two representative species: *B. xylophilus* (EU259322) and *B. mucronatus* (DQ841162).

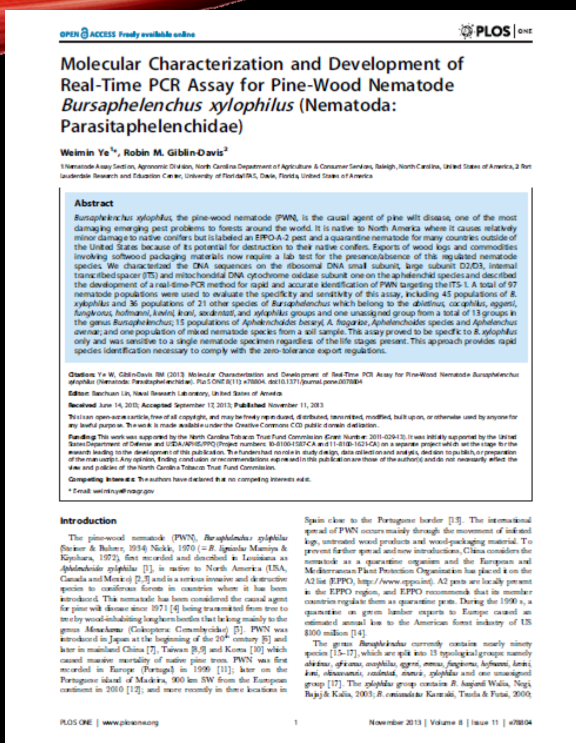
```
DQ841162 Bm   GATGTGGGTTCGATTCG--TCGTCCCCGCTACTGATTGTTCGCACGGAAGCCGAGAGGTGACCGTGCAAC
EU259322 Bx   GATGGCGGTTCGATTCGCGTCGTTCCGCCTACTGATGGTTCGCATGGAAGCCGAGAGGCGACCGTGCAAC
                      BxITSF
DQ841162 Bm   GGTAAAGTCTGGGTTTCTATGCGCTGC-GTTGAGTCGACGTTTTACCGTGTCGACAGATGAGACCAGCCA
EU259322 Bx   GGTAAAGTCTGGGTTTCTACGTGCTGTTGTTGAGTTGGCGTTTTACCGTGCCGACAGATGAGACCAGCCA
                                    BxITSP                                BxITSR
```

Real-time PCR Example



Test result for sample 2013-33423
by PWN-specific and nematode-universal primer/probes

Publication of Molecular Diagnosis



OPEN ACCESS Freely available online   PLOS | ONE

Molecular Characterization and Development of Real-Time PCR Assay for Pine-Wood Nematode *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae)

Weimin Ye[1]*, Robin M. Giblin-Davis[2]

1 Nematode Assay Section, Agronomic Division, North Carolina Department of Agriculture & Consumer Services, Raleigh, North Carolina, United States of America, 2 Fort Lauderdale Research and Education Center, University of Florida/IFAS, Davie, Florida, United States of America

Abstract

Bursaphelenchus xylophilus, the pine-wood nematode (PWN), is the causal agent of pine wilt disease, one of the most damaging emerging pest problems to forests around the world. It is native to North America where it causes relatively minor damage to native conifers but is labeled an EPPO-A-2 pest and a quarantine nematode for many countries outside of the United States because of its potential for destruction to their native conifers. Exports of wood logs and commodities involving softwood packaging materials now require a lab test for the presence/absence of this regulated nematode species. We characterized the DNA sequences on the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer (ITS) and mitochondrial DNA cytochrome oxidase subunit one on the aphelenchid species and described the development of a real-time-PCR method for rapid and accurate identification of PWN targeting the ITS-1. A total of 97 nematode populations were used to evaluate the specificity and sensitivity of this assay, including 45 populations of *B. xylophilus* and 36 populations of 21 other species of *Bursaphelenchus* which belong to the *abietinus, cocophilus, eggersi, fungivorus, hofmanni, kevini, leoni, sexdentati*, and *xylophilus* groups and one unassigned group from a total of 13 groups in the genus *Bursaphelenchus*; 15 populations of *Aphelenchoides besseyi, A. fragariae, Aphelenchoides* species and *Aphelenchus avenae*; and one population of mixed nematode species from a soil sample. This assay proved to be specific to *B. xylophilus* only and was sensitive to a single nematode specimen regardless of the life stages present. This approach provides rapid species identification necessary to comply with the zero-tolerance export regulations.

Citation: Ye W, Giblin-Davis RM (2013) Molecular Characterization and Development of Real-Time PCR Assay for Pine-Wood Nematode *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). PLoS ONE 8(11): e78804. doi:10.1371/journal.pone.0078804

Editor: Baochuan Lin, Naval Research Laboratory, United States of America

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Funding: This work was supported by the North Carolina Tobacco Trust Fund Commission (Grant Number: 2011-029-13). It was initially supported by the United States Department of Defense and USDA/APHIS/PPQ (Project numbers: 10-8100-1587-CA and 11-8100-1621-CA) on a separate project which set the stage for the research leading to the development of this publication. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Any opinion, finding conclusion or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view and policies of the North Carolina Tobacco Trust Fund Commission.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: weimin.ye@ncagr.gov

Introduction

The pine-wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (= *B. lignicolus* Mamiya & Kiyohara, 1972), first recorded and described in Louisiana as *Aphelenchoides xylophilus* [1], is native to North America (USA, Canada and Mexico) [2,3] and is a serious invasive and destructive species to coniferous forests in countries where it has been introduced. This nematode has been considered the causal agent for pine wilt disease since 1971 [4] being transmitted from tree to tree by wood-inhabiting longhorn beetles that belong mainly to the genus *Monochamus* (Coleoptera: Cerambycidae) [5]. PWN was introduced in Japan at the beginning of the 20th century [6] and later in mainland China [7], Taiwan [8,9] and Korea [10] which caused massive mortality of native pine trees. PWN was first recorded in Europe (Portugal) in 1999 [11]; later on the Portuguese island of Madeira, 900 km SW from the European continent in 2010 [12]; and more recently in three locations in Spain close to the Portuguese border [13]. The international spread of PWN occurs mainly through the movement of infested logs, untreated wood products and wood-packaging material. To prevent further spread and new introductions, China considers the nematode as a quarantine organism and the European and Mediterranean Plant Protection Organization has placed it on the A2 list (EPPO, http://www.eppo.int). A2 pests are locally present in the EPPO region, and EPPO recommends that its member countries regulate them as quarantine pests. During the 1990 s, a quarantine on green lumber exports to Europe caused an estimated annual loss to the American forest industry of US $100 million [14].

The genus *Bursaphelenchus* currently contains nearly ninety species [15–17], which are split into 13 typological groups: namely *abietinus, africanus, cocophilus, eggersi, eremus, fungivorus, hofmanni, kevini, leoni, okinawaensis, sexdentati, sinensis, xylophilus* and one unassigned group [17]. The *xylophilus* group contains *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003; *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000;

NORTH CAROLINA
DEPARTMENT OF AGRICULTURE & CONSUMER SERVICES

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Agronomic Services — Nematode Assay

Weimin Ye, Ph.D., section chief

- What the Nematode Assay Section does
- Nematode assay sample forms and information
- Sample turnaround times

Check it out!

The March–April 2012 issue of *Carolinas Green* magazine has the results of a recent survey of plant-parasitic nematodes in turfgrass (sponsored by a Rounds4Research grant from the Carolinas Golf Course Superintendents Association).

CAROLINAS

Palmetto Proud

Recent journal publications

Ye W. 2012. Development of primetime-real-time PCR for species identification of soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) in North Carolina. J Nematol 44(3):284–90.

Ye W, Giblin-Davis RM. 2013. Molecular characterization and development of real-time PCR assay for pine-wood nematode *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). PLoS ONE 8(11): e78804. doi:10.1371/journal.pone.0078804

Soybean Cyst Nematode

Fig. Microphotograph of cysts of soybean cyst nematode (*Heterodera glycines*) from soybean (08-1705)

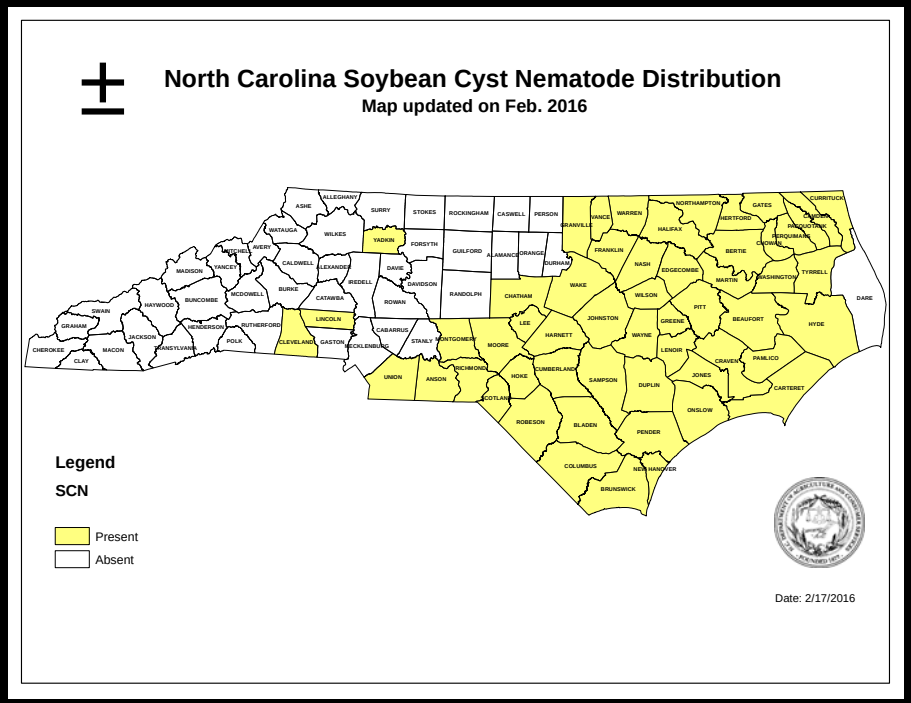
Distribution of Soybean Cyst Nematode

In FY2015-Feb. 2016

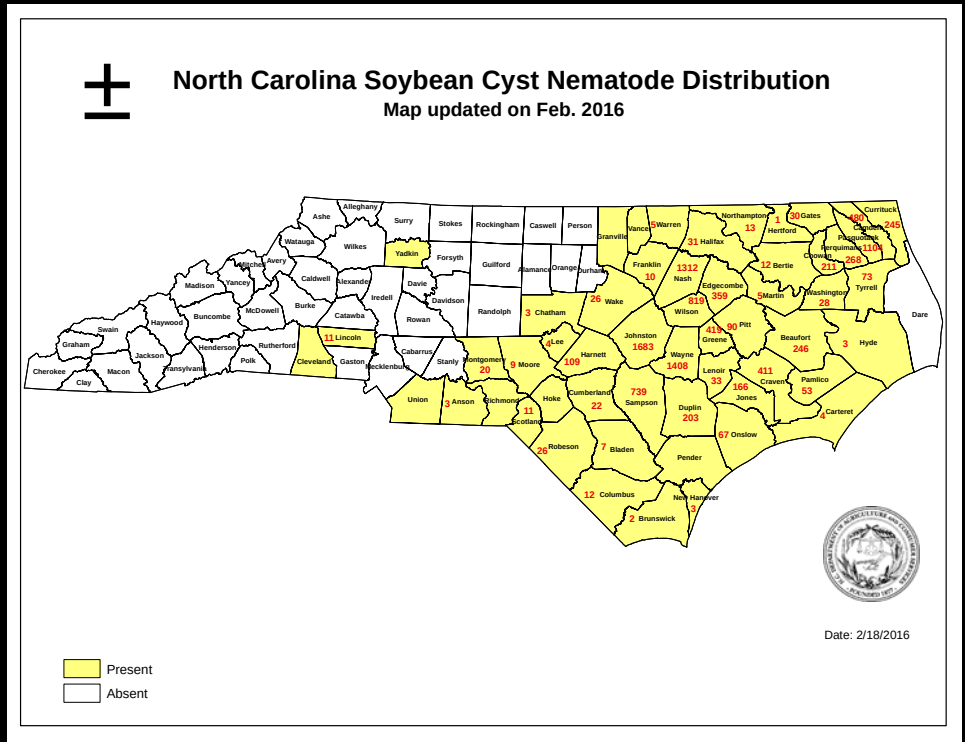
10,799 samples in total with SCN

20.82%

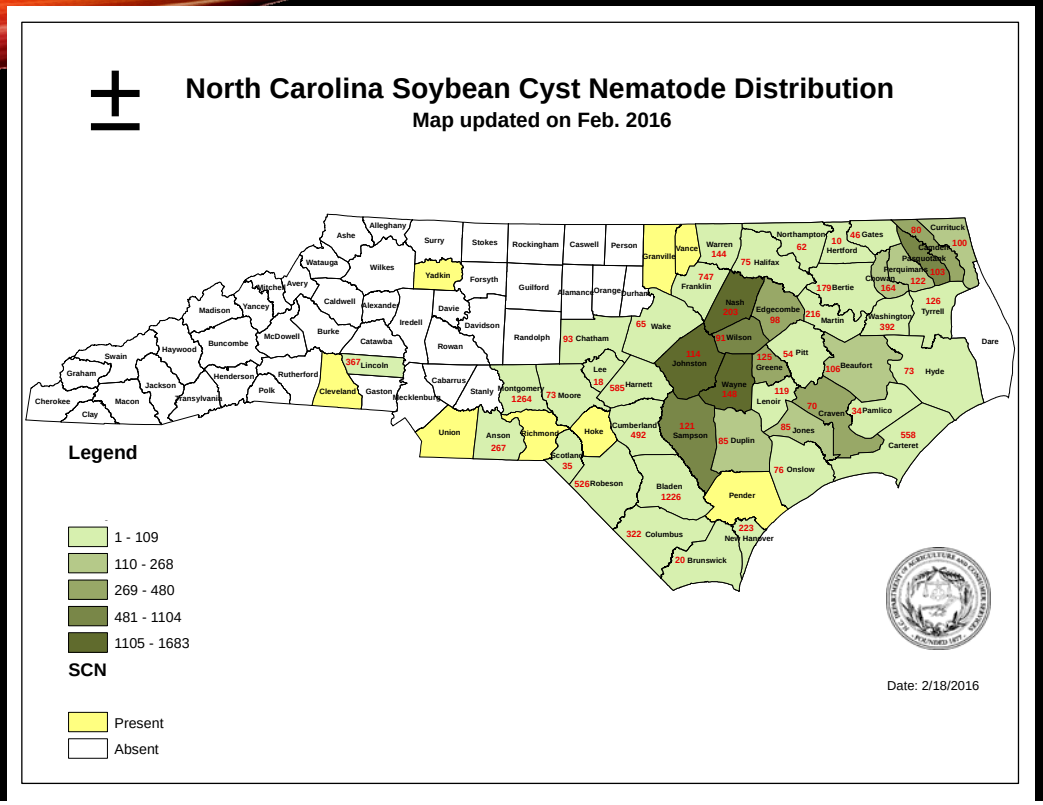
133.3 ± 322.3 (10 - 7930)



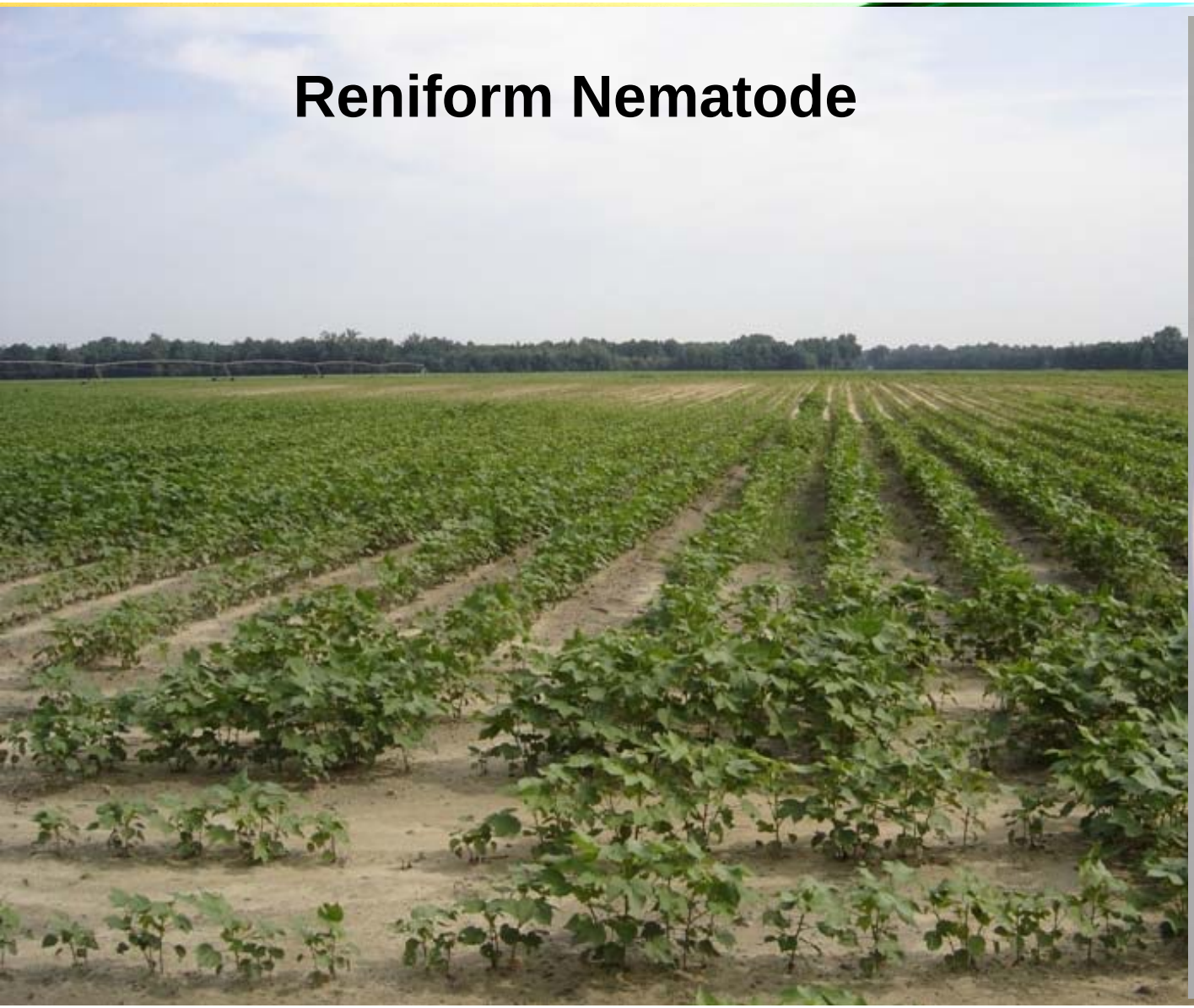
Number of Samples with SCN from Each County in NC in FY2015-Feb. 2016 (10,799 samples in total with SCN)



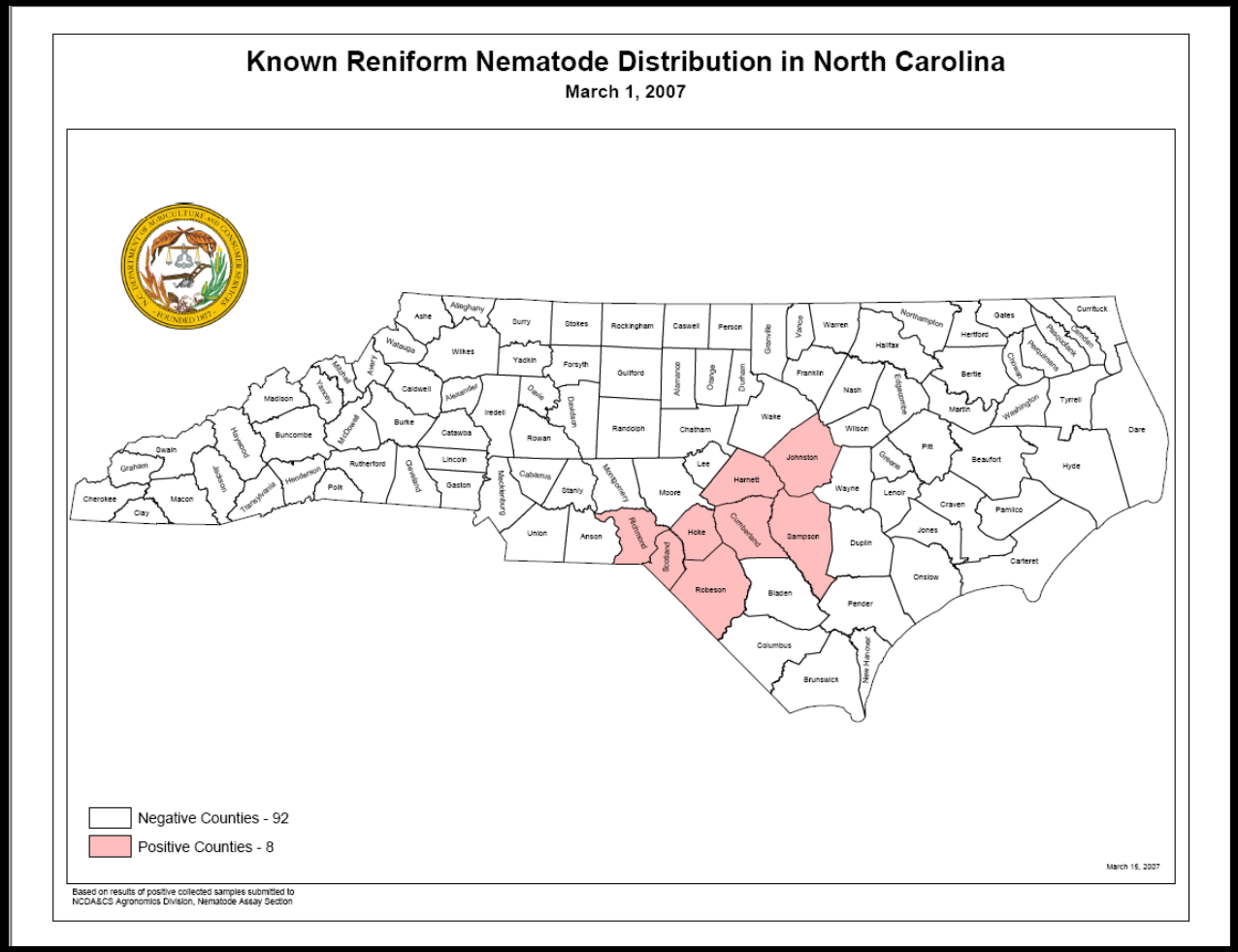
Average SCN per 500 cc Soil from Each County in NC in FY2015-Feb. 2016



Reniform Nematode



Distribution Of Reniform Nematode



Hydrangea macrophylla

Fig. Photographs of symptoms caused by *Ditylenchus dipsaci* on *Hydrangea macrophylla* in Johnston County, NC (14-38639)

NCDA&CS Agronomic Division Phone: (919) 733-2655 Website: www.ncagr.gov/agronomi/ Report No. FY14-N005023

Internal

Nematode Report

Client: Amy Radford
Creech"s Greenhouse
481 W. Braswell Rd
Selma, NC 27576

Sampled County : Johnston

Advisor: PDIC
Campus Box 7211
Raleigh, NC 27695

Sampled: 04/08/2014 Received: 04/11/2014 Completed: 04/14/2014 Farm: 18430

Links to Helpful Information

Nematologist's Comments: This sample (Hydrangea macrophylla) has high number of stem and bulb nematode (Ditylenchus dipsaci), which is one of the most devastating plant-parasitic nematode especially in temperate regions and was recorded in North Carolina occasionally in the past. It is known to attack over 450 different plant species. DNA sequencing was performed on ribosomal DNA 18S, 28S domain 2 and 3, and ITS. These sequences have exact match with some populations of Ditylenchus dipsaci from genBank, thus the species identification is confirmed by combined morphology and molecular data.

Sample Information

Results and Recommendations:

For each species listed, **column S** = # nematodes per 500 cc soil and **column R** = # nematodes per gram of root.

Total # Samples on Report: 1

Sample ID	Lab ID	Crop: Last / Current	Action Code(s)	Nema Notes	StemBulb															
					S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
18430	N038639	flowers	C			196**														

Understanding the Inernal Nematode Assay Report

Action Codes

A: No expected harm to crop production
B: Possible damage; consider chemical treatment
C: Chemical treatment recommended
D: Use of nematode-resistant variety recommended
E: Rotate with nonhost crop(s)

Nema Notes (if present)

contain information about nematodes detected in the assay as well as management options.

Nematode Counts

Asterisks after count numbers indicate a potential problem.
* indicates low hazard. Nematodes are stressing plants, but factors such as disease or moisture stress are probably also present.
** indicates high hazard. Nematodes are likely to be damaging plants severely even in the absence of other stress factors.

North Carolina
Tobacco Trust Fund Commission

Reprogramming of the laboratory-information-management system that makes this report possible is being funded through a grant from the North Carolina Tobacco Trust Fund Commission.

Thank you for using agronomic services to manage nutrients and safeguard environmental quality.
- Steve Troxler, Commissioner of

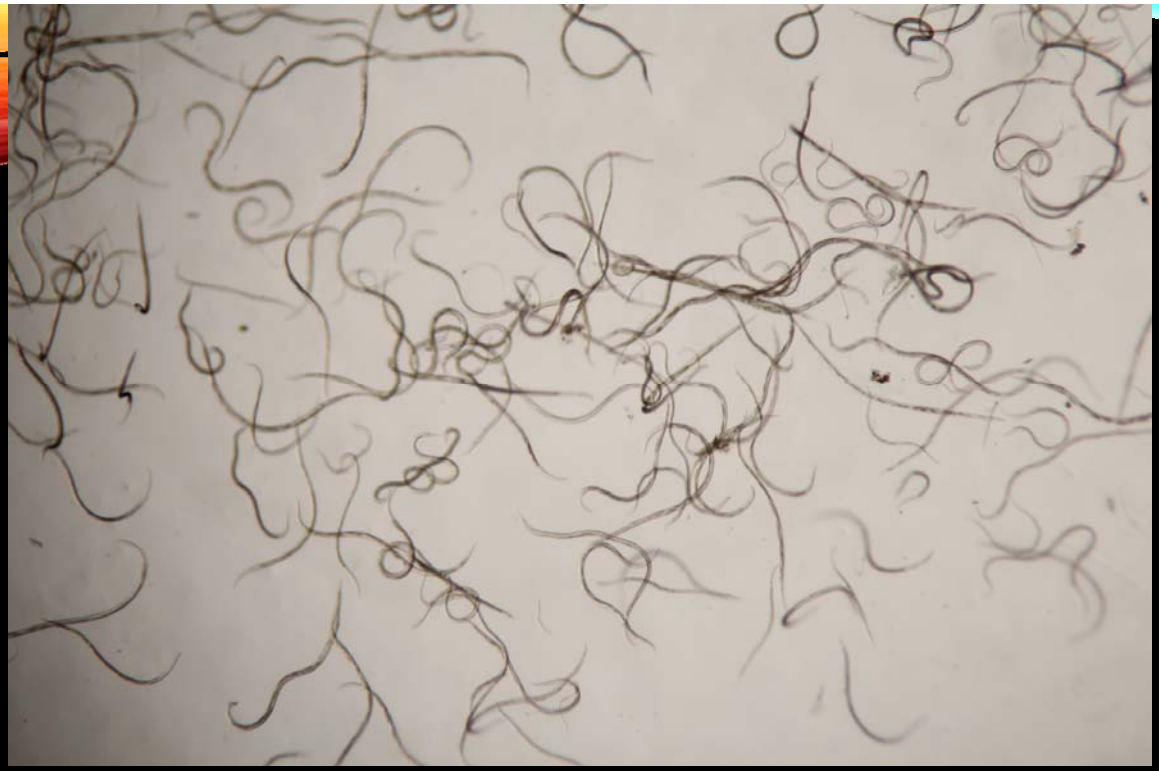


Fig. Photographs of nematodes of *Ditylenchus dipsaci* in a counting dish from *Hydrangea macrophylla* in Johnston County, NC (14-38639)

A
B
C
D
E
20 µm
20 µm
20 µm
20 µm
A
B
C
D
E
20 µm
20 µm
20 µm
20 µm
Ditylenchus dipsaci

DNA Sequencing

>14-38639 Ditylenchus dipsaci 18S ITS

ATGTATAAGTATAAACGATTCTATCGTGAAACCGCGAACGGCTCATTATAACAGCTATAATTTACTTGAT
CTTGACTATCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCACCAAAGCCCTGACCTTACG
GAAAGGGTGCATTTATTAGAACAAAACCAAGCGACTTCGGTCGTTTTTTGTTGACTCAGAATAACTAAGC
TGATCGCATGGTCTTGCACCGGCGACGTGTCTTTCAAGTATCTGCCTATCAACTTTCGATGGTAGTGTA
TCTGACTACCATGGTGGTGACGGGTAACGGAGGATAAGGGTTTGACTCCGGAGAAGGGGCCTGAGAAATG
GCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAGAACGAGGAGGTAGTGACGAAA
AATAACGAGACCGTTCTCTACGAGGCCGGTCATCGGAATGGGTACAATCTAAACCCTTTAACGAGTATCT
ATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAAATGCATAGAATTATTGCTGC
GGTTAAAAAGCTCGTAGTTGGATCTGTGCTGAGGACCTGGTCCACCTTCGGGTGCGTACTAGGCTCCACG
GCTTTCTGCCGGTTTCCCGTCATGGCTTTCATCGGCTGTGGCGGGTGGCTGGTGATTTTACTTTGAAC
AAATCAGAGTGCTTCAAACAGGCGTTTAGCTTGAATGTTTTGCATGGAATAATAGAAGAGGATTTCGGT
TCTATTTTATTGGTTTTATAGACTGAAATAATGGTTAACAGAGACCAACGGGGGCATTCGTACCGCTGCG
TGAGAGGTGAAATTCTTGGACCGCAGCGAGACGAACTACAGCGAAAGCATTTGCCAAGAAGGTCTTCATT
AATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTGACCGTAAACGATGCCA
ACTAGCGATCTGCCGGCGGAATTTTCGCCCTGGTAGGGAGCTTCCCGGAAACGAAAGTCTTCCGGTTCCG
GGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGGAAACTCACCCGGCCCGGACACCATAAGGATTGACAGATTGATAGCT
TTTTCATGATTTGGTGGATGGTGGTGCATGGCCGTTCTTAGTTCGTGGAGCGATTTGTCTGGTTTATTCC
GATAACGAGCGAGACTCTAGCCTACTAAATAGTCTGCACATTGCTTCTGTGATGCAGTACTTCTTAGAGG
GATTTTCGGTGTACAGCCGCACGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTG
CACGCGCGCTACACTGGCGAAATCAGCGTGCTTGTCCTTGCTCGAAAGAGCTTGGTAAACCAATGAAAAT
TTGCCGTGATTGGGATCGGAAATTGCAATTATTTTCCGTGAACGAGGAATTCCAAGTAAATGCGAGTCAT
CAACTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGCCCGGGACTGAGCCATTTCG
AGAAATTCGGGGATTGCCGATTAGCGGTCTTTCGGGATTGCTTTTGGTGAGAACCAACTTAATCGCAGT
GGCTTGAACCGGGCAAAAGTCGTAACAAGGTGGCTGTAGGTGAACCTGCTGCCGGATCATTATCGATCAA
CCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAATTCATTCTTACAGCCAATA
GTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTGATGTCCCCACCGGTTTGCATGCTTATTC
TTGGGCGAAAAAACGGCTCTGTTGGCTTCTATGGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGC
GTTGAAGAGAACTGGCACGTGGTCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACA
AAAACCCCATTTTTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAG
ATCGATGAAGAACGCAGCCAACTGCGA

>14-38639 Ditylenchus dipsaci D23

ACAAGTACCGTGAGGGAAAGTTGCAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAAACCGATAAGA
TCGAAACGGATAGAGCCGACGTATCTAACCCGTATTCAGCTGTTGGGCGGTGCTGGGCGGATTGACTGGT
CCTGCTTCTGGCTAGTTAGTCTGATTCTGGTGCTGTTCGGCAGTGCATTTACAGGTGGAGTGCGCTGAGA
GATACACTTCAGCGGTTGGAATCCAGTCAAGAGGCCTTAACCTTCGGGCAAAGAAACCCAGGCTGGTGGA
AGACTGTTTGTTGTGTTTGAAACTAGGAGTGTGGCTTACGGGTCGGCTAGCTTCTTGCTCTGCGGTGGAT
GGCGGTCGCATGCGACTGTTACTGCCTGTGGGGTAGGTTGTTGGTGCGGACTCTCATGCTCGATCTCGGT
GTAAAAGTTGGTCATCTATCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGGATGCGCGAGTCAT
TGGGTGTTAAAACCTAAAGGCGCAATGAAAGTAAAGGTGTCCTTATGGAGCTGATATGCGATCCCGGGCA
TCTCGGTGCCTGGGCGCAGCATAGCCCCATCCTAATTGCTTGCAATGGGGTGGAGGTAGAGCGTATCCGC
TGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAA
GCGGTTCTGACGTGCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA
GCTG

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Agronomic Services — Nematode Assay

Weimin Ye, Ph.D., section chief

- What the Nematode Assay Section does
- Nematode assay sample forms and information
- Sample turnaround times

Check it out!

The March–April 2012 issue of *Carolinas Green* magazine has the results of a recent survey of plant-parasitic nematodes in turfgrass (sponsored by a Rounds4Research grant from the Carolinas Golf Course Superintendents Association).

Recent journal publications

Ye W. 2012. Development of primetime-real-time PCR for species identification of soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) in North Carolina. J Nematol 44(3):284–90.

Ye W, Zeng Y & Kerns J. 2015. Molecular characterization and diagnosis of root-knot nematodes (*Meloidogyne* spp.) from turfgrasses in North Carolina, USA. PlosOne. DOI: 10.1371/journal.pone.0143556.

Ye W, Giblin-Davis RM. 2013. Molecular characterization and development of real-time PCR assay for pine-wood nematode *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). PLoS ONE 8(11): e78804. doi:10.1371/journal.pone.0078804.

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Questions?

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Thank you!!!