# Novel methods for detection of pathogens on seeds

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# Two new initiatives with elements of pathogen detection



SpectraSeed



- development of robust and standardized detection methods
- testing of 'generic' methods that detect 'all' pathogens





# Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health

Budget: 3 m€

- Seed transmission
- Sampling strategies
- Detection/diagnosis
- Disinfection methods
- Validation of methods

- FERA, UK
- PLANT RESEARCH INTERNATIONAL, NL
- INRA, F
- UNIVERSITA DEGLI STUDI DI TORINO, I
- UNIVERSITY OF PRETORIA, SA
- SASA, UK
- AARHUS UNIVERSITET, DK
- GEVES, F
- NIAB, UK
- NAK TUINBOUW, NL
- UNIVERSITA DEGLI STUDI DI MODENA E REGGIO
  EMILIA
- VIDEOMETER, DK
- EPPO





- Brassica:
  - Xanthomonas campestris pv. campestris
  - Phoma lingam
  - Fusarium oxysporum (raphani, conglutinans, matthioli)
- Legumes
  - Xanthomonas axonopodis pv. phaseoli
  - Aphelecoides
  - Ditylenchus dispaci
- Barley, wheat, rice
  - Fusarium spp
  - Tilletia spp
- Maize
  - Pantoea stewartii
- Tomato
  - Pepino mosaic virus
  - Pospiviroids
  - Clavibacter michiganensis subsp. michiganensis
  - Pseudomonas syringae pv. tomato
  - Xanthomonas spp.
- Cucurbits
  - Viruses: CGMMV etc
  - Acidovorax avenae subsp. citrulli



## What is needed?

- Lots of PCR assays already exist for almost all pathogens
  - Different protocol for each assay
  - Not easy to transmit protocol from one lab to another
  - Ease of use is not prioritized

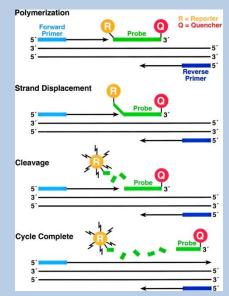




- Diagnosis/detection
  - Make existing protocols more robust and standardized

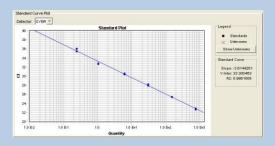
- Sampling methodology (WP2)
  - Representative samples
- Sample extraction (DNA)
  - extraction methods that are automatable
- Test conditions
  - PCR SYBR/TaqMan







## Species specific Real-Time PCR assays



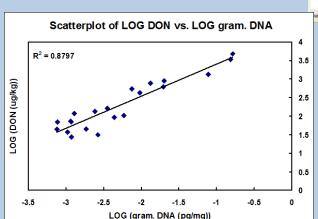
- F. graminearum
- F. culmorum
- F. avenaceum
- F. tricinctum
- F. poae
- F. langsethiae
- F. equiseti
- F. sporotrichioides
- F. proliferatum
- F. verticilloides

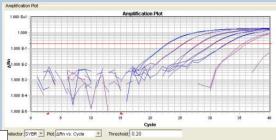
#### > Microdochium nivale

Detects both M. nivale var. nivale and M. nivale var. majus

#### > Tilletia caries, T. indica

> Assays already published







## Test improvements

- DNA extraction
  - Try several methods
    - Test for i) ease of use ii) yield iii) reproducibility
    - MoBio Seed kit
    - LGC Genomics
    - CTAB
- TaqMan assays
  - Use same kits and conditions for all assays
  - Test assays in partner laboratories



# Testing of 'generic' methods that detect 'all' pathogens

"Next generation sequencing can be used to identify a huge number of individuals in a sample"

Diagnostic tool or research tool?

- Culture based methods
  - 100-1000 isolates



- Next generation sequencing
  - 100.000 to 1.000.000 individuals identified





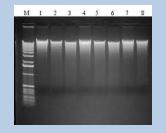
## Workflow

#### Sample



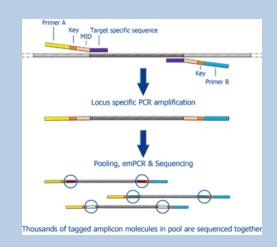


#### **DNA** extraction



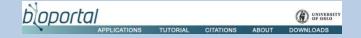


#### PCR amplification





#### Identification of species (BLAST)



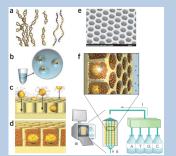
#### Clustering



~300.000 sequences



#### NGS sequencing





## Wheat grain fungal communities

- Wheat grain samples from 2004, 2005, 2006
- 30 samples from each year from a previous *Fusarium* survey

Year

Previous crop

Tillage

Wheat cultivar (including Fusarium 'resistance')

Fusarium biomass (Q-PCR data)

**Toxins** 

e-Xtra\*

#### Fusarium Head Blight of Cereals in Denmark: Species Complex and Related Mycotoxins

L. K. Nielsen, J. D. Jensen, G. C. Nielsen, J. E. Jensen, N. H. Spliid, I. K. Thomsen, A. F. Justesen, D. B. Collinge, and L. N. Jørgensen

First, fifth, seventh, and ninth authors: Authus University, Faculty of Agricultural Science, Department of Integrated Pest Management, Research Centre Flakkebjerg, Denmark; second and eighth authors: University of Copenhagen, Faculty of Life Sciences, Department Plant Biology and Biotechnology, Frederiksherg, Denmark; third and Gouth authors: Knowledge Centre for Agriculture, Crop Production, Aarhus, Denmark; and sixth author: Aarhus University, Faculty of Agricultural Science, Department of Agroecology and Enviro

Nielsen, L. K., Jensen, J. D., Nielsen, G. C., Jensen, J. E., Spliid, N. H., Thomsen, I. K., Justesen, A. F., Collinge, D. B., and Jørgensen, I. N. 2011. Fusarium head blight of cereals in Denmark: Species complex and related mycotoxins. Phytopathology 101:960-969.

Quantitative real-time polymerase chain reaction differentiating 10 Faurimi spp, and Microdrobiam risules or M. majar was applied to a reactive committee of the second of the control of

Faurium species complex among the five cereals as well as great yearly variation were seen. Faurium grantinearum, F. calinorum, and F. oreact were decinitant in wheat, with 100% at the deminine responsine, F. contained to the control of the contr

Fusarium head blight (FHB) in small grain cereals is caused by a complex of toxigenic pathogens belonging to the genus Fu-sarium (Gibberella) and the nontoxigenic genus Microdochium. The result of FHB worldwide is lower grain yield and reduced quality due to mycotoxins. The most common Fusarium spp. in this complex found throughout Europe are Fusarium graminea-rum (Gibberella zeae), F. avenaceum (G. avenacea), and F. culnorum (5). However, F. pone, E. tricinclum, F. sportrichioides, F. equiseti, and F. langsethiae are also frequently found (5,21,44). The distribution of the pathogens causing FHB is believed to be determined, in part, by climatic factors such as temperature and determined, in part, by climatic tactors such as temperature and moisture (41). F. grainiearum has traditionally been found to dominate in regions with warm and humid growth conditions; F. avenaceum and F. culmorum are both associated with cool, wet, and humid conditions; and Microdochium nivale and M. majus are associated with regions of relatively cool temperatures and frequent short rain showers (42).

In addition to climatic factors, agronomic factors are also very important for the occurrence of FHB. Soil tillage and previous crops have been shown to have a significant influence on FHB or mycotoxin contamination (7,10,27). The Fusarium spp. produce several mycotoxins which pose a health risk to humans and animals through food and feed prepared from contaminated crops (11). The mycotoxins produced by the Fusarium spp. include the

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trichothecenes, zearalenone (ZEA), moniliformin, fumonisins, and the enniatins. Trichothecenes constitute the largest group of mycotoxins produced by Fasarium spp. in cereal grain; these can be divided into two groups: type A and type B (24). The type B trichothecenes are the most common trichothecenes and include deoxynivalenol (DON) and the closely related nivalenol (NIV)
DON is produced by F. graminearum and F. culmorum, and NIV is produced by some isolates of these two species as well as by F. poae and F. equiseti (24). F. graminearum and F. culmorum are also producers of ZEA, which occurs commonly along with DON and similar derivates (5). Type A trichothecenes includes T-2 mycotoxin, HT-2 mycotoxin, and diacetoxyscirpenol (DAS) and are considered to be more toxic to humans and animals than type B trichothecenes (22). The mycotoxins T-2 and HT-2 are produced primarily by F. langsethiae and F. sporotrichioides, also, a few isolates of F. poae have been reported as HT-2 and T-2 producers (35). DAS is produced by E poae and E equiseti whereas E avenaceum and E tricinctum produce, among other metabolites, the mycotoxins monilformin, beauvericin, and

ennialins (35). Due to differences in pathogenicity, toxigenicity, and fungicide sensitivity, it is important to obtain detailed knowledge of the occurrence of individual Fusarium spp., and a percequisitie for this is the correct identification and quantification of individual species. However, the classical morphological methods for identi-fication of Fusarium spp. in grain samples are both laborious and require a high level of expertise in order to distinguish closely related species, and will only provide information on incidence of the pathogen infection in seed. In contrast to conventional tests, polymerase chain reaction (PCR)-based methods have the advantage of species specificity, sensitivity, and speed, Furthermore

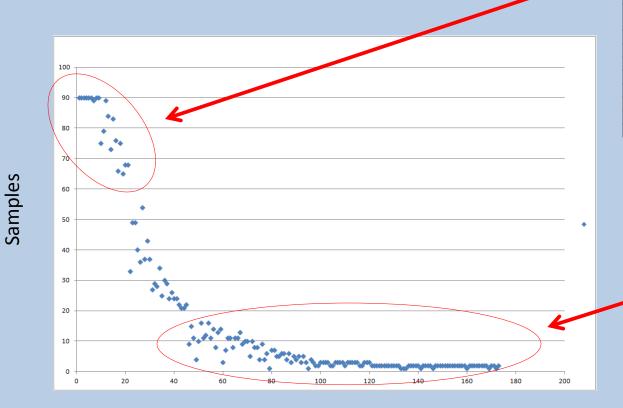


## Raw data from sequencing

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Total					
89	2409	826	38	36	348	48161	Cluster 1	DQ459835.1	Fusarium cul	morum grp	
872	376	368	1262	981	1660	45980	Cluster 2	GU584953.1	Lewia infectoria strain I45		
515	534	211	418	644	587	36072	Cluster 3	HQ540688.1	Davidiellace	Davidiellaceae sp. PIMO_9	
59	40	87	76	164	25	22066	Cluster 4	EU167564.1	Didymella ex	itialis strain C	
13	71	250	275	22	139	16215	Cluster 5	JF340258.1	Gibberella avenacea strair		
55	38	70	250	156	180	9959	Cluster 6	JF440590.1	Epicoccum ni	grum 18S ribo	
76	20	46	40	20	4	9327	Cluster 7	AM502260.1	Microdochiu	m nivale 18S r	
2	4	10	23	82	76	2607	Cluster 8	FJ025231.1	Fungal endop	hyte sp. B16-	
21	23	15	68	28	119	2241	Cluster 9	JF440587.1	Cladosporiur	n tenuissimur	
11	24	48	3	10	418	2139	Cluster 10	FJ907535.1	Pyrenophora	tritici-repent	
7	11	0	0	10	26	1870	Cluster 11	FR732053.1	Fusarium sp.	IMM16 genon	
12	0	13	8	21	1	1822	Cluster 12	AF181696.1	AF181696 Sep	otoria passeri	
15	8	6	20	3	17	1421	Cluster 13	HQ171053.1	Botryotinia f	uckeliana isola	
64	4	5	12	2	2	1404	Cluster 14	AF181710.1	AF181710 Ph	aeosphaeria r	
24	27	11	4	47	68	1209	Cluster 15	FR717837.1	Cryptococcus	victoriae gen	
9	17	4	0	7	41	745	Cluster 16	FJ210642.1	Sporobolomy	ces sp. JJP-20	
2	3	1	0	11	6	493	Cluster 17	HQ533146.1	Stemphylium	sp. CNU0940	
2	7	5	1	11	12	374	Cluster 18	AM160648.1	Cryptococcus	sp. HB 1222 1	
2	1	0	4	5	7	327	Cluster 19	HQ166323.1	Fungal sp. FL-	-2010d isolate	



### Incidence



### Core species

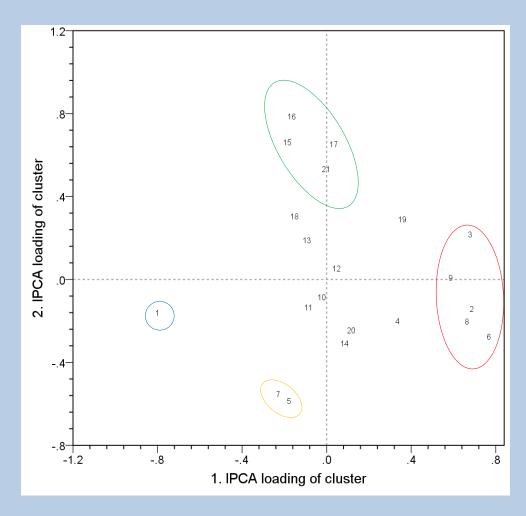
Fusarium culmorum 'group'
Lewia infectoria (Alternaria)
Cladosporium sp
Didymella exitialis (Ascochyta, leaf scorch)
Gibberella avenacea
Epicoccum nigrum
Microdochium nivale
Alternaria alternata
Cladosporium cladosporioides
Pyrenophora tritici-repentis (tan spot, DTR)
Fusarium poae
Mycosphaerella graminicola (Septoria tritici
blotch)
Sclerotinia sclerotiorum/Botryotinia
fuckeliana
Phaeosphaeria nodorum (Septoria)
Cryptococcus sp
Sporobolomyces sp
Stemphylium/Pleospora sp (teleomorph)
Cryptococcus sp
Lewia infectoria
Phaeosphaeria sp
Dioszegia hungarica

### Rare biosphere



#### Fungal 'interactions'

Cluster	Best identification					
1	Fusarium culmorum 'group'					
2	Lewia infectoria (Alternaria)					
3	Cladosporium sp					
4	Didymella exitialis (Ascochyta, leaf scorch)					
5	Gibberella avenacea					
6	Epicoccum nigrum					
7	Microdochium nivale					
8	Alternaria alternata					
9	Cladosporium cladosporioides					
10	Pyrenophora tritici-repentis (tan spot, DTR)					
11	Fusarium poae					
12	Mycosphaerella graminicola (Septoria tritici blotch)					
13	Botryotinia fuckeliana					
14	Phaeosphaeria nodorum (Septoria)					
15	Cryptococcus sp					
16	Sporobolomyces sp					
17	Stemphylium/Pleospora sp (teleomorph)					
18	Cryptococcus sp					
19	Lewia infectoria					
20	Phaeosphaeria sp					
21	Dioszegia hungarica					





## Agronomic factors and fungal communities

ΟΤU		Mean amount of DNA	95% CI lower bound	95% CI upper bound	Mean comparison (Bonferroni- corrected) <sup>a)</sup>			
		2003		normal	16.1	12.5	20.7	а
	Year	2003	Tillage	reduced	30.1	16.4	54.7	а
		2004		normal	23.1	18.0	29.5	а
				reduced	25.5	14.0	45.7	а
		2005		normal	25.7	19.3	33.9	а
				reduced	12.4	6.1	24.2	а
Mean across OTU 1	Previous crop	Barley  Corn  Rape  Wheat		normal	24.0	16.9	33.9	b
- 21				reduced	4.8	0.8	17.7	а
				normal	27.4	19.0	39.4	а
			Tillage	reduced	45.1	22.3	90.1	а
				normal	18.2	12.5	26.1	•
				reduced normal	19.8	15.3	25.6	- a
				reduced	55.9	40.5	25.6 77.2	a b
				2003	453.0	268.9	762.6	ab
Fusarium culmorum Year				2004	541.9	321.8	911.3	b
	2				188.0	111.5	317.0	a
	medium				301.2	197.5	458.9	а
	Fusarium susceptibility			high	503.2	339.4	746.0	b
		2003	Tillage	normal	122.7	81.7	183.9	а
	Year			reduced	252.4	93.6	677.6	а
		2004		normal	629.2	420.6	941.9	а
				reduced	713.8	271.9	1871.4	а
		2005		normal	974.5	616.7	1541.3	а
Landa tata da ta				reduced	380.5	129.6	1113.3	а
Lewia infectoria	Previous crop	hade.	Tillage	normal				
		barley		reduced				
		corn		normal	457.5	251.6	830.3	а
				reduced	1375.1	433.8	4353.7	а
		rape wheat		normal	358.2	198.5	645.1	-
				reduced	b)			-
				normal	444.9	294.0	672.8	а
				reduced	1578.7	923.3	2699.0	b

## Conclusions

NGS reveals a high diversity of fungi in wheat

 Fungal 'interactions' and the influence of environmental factors can be investigated at high level of resolution

Potential for diagnostic tool?



# Thank you!

- Annemarie F. Justesen
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- Lotte Olesen

