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Assessing and Monitoring the Impacts of Genetically Modified Plants on Agro-ecosystems

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Deliverable 4.2 – Report on baseline soil fungal community structure

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Summary

Fungi are an important biological constituent of soils. They are involved in the degradation of organic substances, support plant growth as mycorrhiza, or also act as plant pathogens. In the rhizosphere, fungi may help to mobilize phosphate or other nutrients and, in turn, receive carbon and energy sources provided by plant root cells. Together and in multiple interactions with bacteria, they contribute to the rhizomicrobiome. In AMIGA, rhizospheres of maize and potato cultivated at different European sites were analyzed for their fungal diversity. The objective was to understand the importance of the biogeographical region for the overall fungal community structure, also related to potential differences between a genetically modified (GM) and a non-modified isogenic version. For maize, four field sites (Slovakia, Sweden, Denmark, and Spain) and for potato two field sites (Ireland, The Netherlands) were sampled. GM and isogenic were cultivated side by side and each site was sampled in two or three subsequent years (2012-2014), except for The Netherlands, sampled only in 2014. The fungal community was characterized from directly extracted rhizosphere DNA independent of cultivation by PCR amplification of a fungal marker sequence (ITS1 region). The PCR amplicons were identified by high-throughput DNA sequencing with Illumina MiSeq. For maize, a total of 137 samples, for potato a total of 119 samples were analyzed. For maize more than 15.5 Million and for potato more than 10.0 Million sequences were obtained. For maize, these could be assigned to 4,459 operational taxonomical units (OTUs), for potato 4,405, respectively. Phylogenetic analyses assigned the ten most dominant fungi of maize and potato to Ascomycota, followed for maize by Basidiomycota or, for potato, by Zygomycota. Independent of the cultivation site, year or sampling, for maize a total of 13 OTUs representing 28.9 % of all sequences (minimum: 8.5 %; maximum 50.1 %) were detected as the “rhizofungal core”. For potato this core consisted of only 6 OTUs which represented a total of 12.9 % of all sequences in the Irish rhizospheres, but only 0.7 % on those from The Netherlands. Multivariate statistical analyses revealed that the field sites and, thus, the soils and environmental conditions had a strong effect on the fungal community structures. A less pronounced but clearly detectable difference also occurred between years, while there was no

strong influence of the genetic background, as represented by the different cultivars. For potato, the application of fungicides did not affect the overall fungal community structure. There was no indication in the datasets for maize or potato that the GM modification had a significant effect on the overall rhizosphere fungal community structure.

Introduction

Approximately 80 % of all terrestrial plants with roots are associated with fungi. These provide nutrients to support plant growth and, in turn, receive carbon and energy sources produced by the autotrophic plants and released into the soil via their root system. The diversity of fungi varies between plant species and is also affected by plant age and soil properties. Considering that plant species differ in root architecture and the composition of root exudates it is likely that these properties act as selective factors to shape the fungal diversity in the rhizosphere. Furthermore, there is also a potential interaction of fungi with the bacterial communities in rhizospheres, since both compete for carbon released from plants but also transform those compounds into metabolites which may serve as substrates for spatially linked microbial cells. Fungal biomass also provides a critical food source for fungi-feeding nematodes, which play an important role in nematode trophic interaction (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2619405/). In addition to metabolic interactions there is also the possibility of exchange of signaling molecules or antibiotics.

One of the main objectives in the AMIGA work package 4 (soil fertility) is to analyze the fungal diversity which lives in the rhizosphere of maize and potato, assess potential differences between field sites located in different biogeographical regions and detect whether the genetic modification of maize or crop has an effect on fungal diversity and, in case yes, whether these differences are the same at different sites or whether they are different. Considering the close linkage between roots and fungi, it is possible that altered properties of a plant genotype, as caused by a genetic

modification, would cause direct or indirect changes in the structural diversity of the fungal communities, existent in the maize or potato rhizospheres.

The overall diversity of fungi cannot be assessed by classical microbiological methods involving cultivation, due to a lack of knowledge about required growth conditions. Microbial community analyses from soil are therefore preferentially done by direct analyses of DNA extracted from soil (or rhizosphere) samples. This DNA can be used as a starting material to PCR-amplify with primers hybridizing to phylogenetically conserved regions within marker genes which then allow the identification of the constituents of the microbial communities. For fungal community analysis primers amplifying the ITS region has been found to be informative in this regard and is now broadly applied in microbial ecology. The diversity of the amplified products can be visualized by genetic profiling techniques, i.e., TRFLP, SSCP or DGGE, or they can be revealed by DNA sequencing. The latter approach is now increasingly preferred in modern soil microbiology due to the huge potential of the newly developed high-throughput DNA sequencing technologies. While genetic profiles typically distinguish between 20 to 50 dominant community members, the new massively parallel sequencing of PCR amplicons can detect and identify several thousand community members in a single process (for a comparable amount of money).

In this deliverable D4.2 we report on the baseline diversity of fungi detected at the different AMIGA field sites in the rhizosphere of maize and potato. We also give an indication about differences which we identified caused by the genetic modifications, the field sites and, for potato, agricultural management by use of fungicides, which are required to control potato late blight disease (causative organism *Phytophtora infestans*). These latter analyses should be regarded as still being preliminary and they will be further elaborated by additional bioinformatic and statistical analyses in advance of peer-reviewed publication.

Materials and Methods

Description of plants and design of the experimental fields

For maize cultivation, field sites were located in Spain, Slovakia, Denmark and Sweden; for potato, field sites were in Ireland and in The Netherlands. The accurate locations and the particular location of the field plots can be found in other AMIGA documents. It should be noted that in the subsequent years, the order of treatments and number cultivars were not altered, though, for potato plot positions at the Irish and Dutch sites were rotated within the respective sites to mitigate against soil-borne diseases. This was also done for maize at the site in Slovakia. For the other maize field sites, i.e., in Sweden, Denmark and Spain, the plots were fixed in position, with the exception of Sweden in 2012 and 2013, where plots 2, 4, 17, and 19 were not identical in regard to growing GM or conventional (for more details see respective field site reports in the AMIGA output documents).

The genetically modified (GM) maize was the event MON810, which confers the capacity to produce the insecticidal Cry1Ab protein of *Bacillus thuringiensis* and provides resistance against the European corn borer *Ostrinia nubilalis*, which was compared to near isogenic non-modified cultivars. For Spain, the GM MON810 was the cultivar DKC6451YG (YG stands for "Yield Guard", a brand name of Monsanto), and its near isogenic cultivar DKC6450, whereas in Slovakia, Sweden and Denmark, the GM variety was DKC3872YG (Yield Guard), and the near isogenic variety was DKC3871. All field trials consisted of a randomized block design including 20 plots with maize and 10 replicates for each variety. The plot size was 10 x 10 m with an interspace of 5 m. The field site was bordered by a 5 m wide strip of conventional maize.

Potato was cultivated at the sites in The Netherlands and Ireland. The GM version was the cisgenic event A15-031 (Desirée + Vnt1), conferring resistance to the fungus *Phytophtora infestans*, the cause of late blight. The cisgenic potato line was generated via the insertion of the *Rpi-vnt1-1* gene, which was originally derived from the wild potato species *Solanum venturii* with its native

promoter and terminator into the genome of *Solanum tuberosum* cv. Desirée. Thus, the gene was only expressed at exposure to *P. infestans*.

The potato field trials in Ireland, with the exception of 2012, and The Netherlands included three potato genotypes: the GM A15-031 (Desire + Vnt1), its conventional susceptible comparator (Desirée) and Sarpo Mira, a conventional resistant cultivar. In 2012, a preliminary field evaluation was completed in Ireland on smaller plots (1m x 1m) and the conventional cultivar included in this evaluation was the variety King Edward. For the 2013 and 2014 potato study, rhizospheres from two treatments were analyzed: No spraying (**NS**) against late blight, and, conventional protection (**CP**) by weekly applications of preventive fungicides for late blight control. The randomized block design relevant to this study included 42 plots with potato, seven replicates for each of the three varieties and the two late blight control strategies. The 2012, preliminary study conducted in Ireland only contained a non-spray (NS) treatment and four replicates, thus a total of 12 plots (see Results section). For 2013 and 2014, the plot size was 3 x 3 m in Ireland, and 6 x 6 m in The Netherlands, both with interspaces of 6 m. Field trials in Ireland were sampled in the years 2012, 2013 and 2014, in The Netherlands, samples for fungal community analyses were only sampled in 2014.

An overview about the variants, date of sampling and number of samples collected is given in the Appendix, Table A1 A (for maize) and A1 B (for potato).

Sampling at field sites

The samples for rhizosphere microbial community analyses were collected during the flowering period of the respective crops in order to minimize the well documented effect that plant age can modify the composition of the microbial communities in the rhizospheres. Plants were carefully dug out of each plot and transferred immediately to the laboratory in sealed plastic bags. Loosely adhering soil was removed by shaking. Microbial cells (bacteria, archaea, fungal mycelia and spores) adhering to the roots of each plant were detached by suspending the fresh root material (8 g) in 30

ml of sterile saline for 30 min at 4 °C in an orbital shaker (Model 3040, GFL, Burgwedel, Germany) at 10 rpm. The microbial cells were collected by centrifugation at 4,100 x *g* for 30 min at 4 °C and the cell pellets were stored at -80°C.

DNA extraction and purification

DNA was extracted from the frozen cell pellets using the FastDNA SPIN kit for soil (MP Biomedicals, Illkirch, France). The extraction included two bead beating steps of 45 s at 6.5 m s^{-1} on a FastPrep-24 system (MP Biomedicals, Eschwege, Germany) and one additional washing step of the binding matrix with 1 ml 5.5 M guanidine thiocyanate (Carl Roth, Karlsruhe, Germany). Extracted DNA was then split into equal volumes with one processed at the Thünen Institute for bacterial sequencing while the other sample was sent to Teagasc (Ireland) who completed the fungal-related sequencing at the Teagasc Sequencing Centre, Moorepark, Cork.

Illumina library generation

For the fungal sequencing, DNA samples were shipped to the Sequencing Unit at Teagasc Moorepark where Libraries were prepared essentially as described in the Illumina 16S metagenomic library preparation protocol, with some exceptions. Firstly, samples were amplified using ITS-specific primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993) and reverse ITS2 (GCTGCGTTCTTCATCGATGC) (White 1990), which target the ITS1 region. The custom primers contained adapters specific to the Illumina flow cell, a unique 8-bp-index to allow for multiplexing, a 10-bp sequence to adjust the sequencing primer melting temperature to about 65°C, a 2-bp link anti-complementary to known sequences and the ITS1 specific primer sequences. The use of different forward and reverse primers allowed for multiplexing all samples by dual-indexing (Kozich et al. 2013) for a single sequencing run. Each PCR mixture contained 10 ng template DNA, 10 µM of each primer, and 25 µl of Kapa2G Robust PCR ReadyMix (Kapa Biosystems) in a 50 µl total volume. The PCR conditions involved an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C

for 45 s, 62°C for 45 s, and 72°C for 1 min and ended with an extension step at 72°C for 7 min. Products were purified using Ampure XP Beads as described in the protocol. Five µl of each purified PCR product was then used as a template for dual-indexing and indexed-PCR products were purified as described in the protocol. Equimolar amounts of the individual PCR products were pooled and sequenced using MiSeq Reagent Kits v3 2x300 with 600cycles for paired-end sequencing.

Sequence filtering and analysis

The ITS sequences were analyzed as follows: Reads were merged with VSEARCH (vsearch v1.9.5_linux_x86_64, 31.4GB RAM, 24 cores) (github.com/torognes/vsearch) asking for a minimum length of the overlap of 50 nt and a minimum length of the merged read of 250 nt. Primer sequences were removed from the sequences with cutadapt 1.9.1 (Martin M, "Cutadapt removes adapter sequences from high-throughput sequencing reads", EMBnet.journal 17.1; http://dx.doi.org/10.14806/ej.17.1.200) and sequences with total expected errors E > 1 were discarded with the fastq_filter command of VSEARCH. Furthermore, the screen.seqs command of mothur (mothur v.1.31.2) (Schloss et al. 2009) was used to remove sequences with any ambiguous base or more than eight homopolymers and to select sequences in the size range of 211 – 308 nt to obtain good quality sequences. VSEARCH was applied to remove sequences that appeared only once (singletons) or chimeras which were identified by de novo chimera detection using the UCHIME algorithm (Edgar et al. 2011). Sequences were clustered in OTUs (operational taxonomic units) with VSEARCH at a threshold of 97% sequence identity whereat a consensus sequence was generated for each OTU. The consensus sequences that represented the nuclear ribosomal internal transcribed spacer 1 (ITS1) were extracted with ITSx (Bengtsson-Palme et al. 2013).

These curated sequences were taxonomically classified with mothur using the reference database UNITE 7 released on 2015-08-01 (http://unite.ut.ee, (Koljalg et al. 2013)). The dataset of curated ITS1 sequences was used as a reference database to map all good quality sequences against it at a threshold of 97% identity applying USEARCH (usearch v8.1.1831_i86linux32) (Edgar 2010) with the

usearch_global command. For phylogenetic comparisons of the fungal communities the curated sequences were aligned with MUSCLE v3.8.31 (Edgar 2004) and FastTree version 2.1.7 SSE3 (Price et al. 2009) was used to calculate a phylogenetic tree. Weighted unifrac distances of the phylogenetic tree and NMDS were calculated with mother. Results of these analyses will be reported in detail in D4.2. True singletons were not allowed to become the seed of an OTU. However, after formation of OTUs, the singleton sequences which could be assigned to existing OTUs were added. This allowed the extraction of valuable sequences from the raw sequences lost during the first filtering steps.

Results

A. Fungal communities associated with maize and description of a maize-specific fungal core community

More than 15.5 Million raw sequences of ITS amplicons were obtained from the 137 maize rhizosphere samples. After quality filtering, the average number of sequences of the samples was 80,181 $\pm$ 26,031, with a minimum of 32,892 and a maximum of 139,161. The quality filtered sequences were distributed among 4,759 OTUs. After rarefaction at 43,320 sequences, a total 4,561 OTUs remained (see also Appendix Table A2). True singletons were not allowed to become the seed of an OTU, thus the dataset does not contain true singletons (see also Materials and Methods). However, after subsampling 429 OTUs were represented by one single sequence in the dataset.

The ten most dominant OTUs represented 34.5 % of all sequences collected for the fungal community analysis, the twenty most dominant 48.5 %, and the fifty most dominant 68.4 %, respectively. The smallest number of different OTUs detected in a sample was 340, the highest number 574; the average was 462 $\pm$ 51.

Phylogenetic analyses revealed that *Ascomycota* was across all maize samples the most dominant phylum, representing 55.8 % of all sequences, followed by *Basidiomycota* with 24.6 % and

Zygomycota with 16.8 %. An "unclassified phylum" followed with 2 % and *Chytridiomycota* with 0.6 %. Surprisingly, *Glomeromycota*, i.e. the phylum comprising all arbuscular mycorrhizal fungi (AMF), including those which may be of relevance for maize, was only represented with 0.002 % of all sequences. These sequences were detected in a total of 10 samples, 9 originating from Spain and one from Denmark. The sequences could be attributed two three OTUs, one of them, OTU1132, a member of the family *Paraglomeraceae*, represented with 105 sequences coming exclusively from Spain, from both years. There was no apparent preference of *Glomeromycota* for MON810 (4 samples) compared to the isogenic version (6 samples). This underlined that mycorrhization was of no importance under the conventional cultivation conditions of these maize cultivars.

The most dominant OTU (OTU1) across all 137 samples analyzed was a *Cryptococcus aerius* (*Basidiomycota*) which represented on average 9.65 % of the total fungal community, followed by an unclassified *Capnodiales*, *Ascomycota* (OTU2, 4.5 %), a member of the *Mortierellaceae*, *Zygomycota* (OTU3, 4.4 %), *Guehomyces pullulan*, *Basidiomycota* (OTU4, 2.7 %), and an unclassified member of *Ascomycota* (OTU5, 2.5 %), respectively).

A total of 13 OTUs were detected across all 137 samples analyzed, among the above mentioned OTU1 to OTU3, but not OTU4 and OTU5. Together, these consistently occurring OTUs in all samples represented on average 28.9 % of all sequences in a given sample, with a minimum of 8.5 and a maximum of 50.1 %.While the size of the core was similar at most sites its size was significantly larger in Denmark (39.3 %). Interestingly, except for the *Basidiomycete* OTU1, representing as indicated above 9.65%, this core only harbored sequences from *Ascomycota* (9 OTUs, together representing of 12.5 % of all sequences) and *Zygomycota* (3 OTUs, 6.7 %), respectively. Thus all three fungal taxa contributed to the "fungal core" in comparable amounts, with the highest proportions associated to the *Ascomycota*.

A total of 17 OTUs were consistently detected with MON810, while 15 consistently occurred with the isogenic cultivars. Comparing both indicated that four OTUs were unique to MON810, however,

the amount of sequences represented by these OTUs was similar with 1.5 % for MON810 and the isogenic cultivars. Two OTUs were only consistent with the isogenic cultivars, although they represented on average 2.8 % of all sequences for both cultivars. Phylogenetic analyses indicated that the four OTUs consistent with BT were an unclassified member of the *Pleosporales* (*Ascomycota*, OTU42, 0.5 %), two members of the *Mortierellales* (*Zygomycota*, together 0.8 %) and *Trichocladium asperum* (*Ascomycota*, OTU97, 0.2 %), respectively. The two OTUs consistent with the non-modified cultivars were OTU8 and OTU26, representing 2.0 % and 0.8 %, assigned to *Mortierella elongata* (*Zygomycota*) and *Cryptococcus oeirensis* (*Basidiomycota*). It should be noted that these OTUs were not exclusive for the respective MON810 or isogenic cultivars as they also occurred (albeit inconsistently) in the respective alternative version (MON810 or isogenic).

NMDS was performed to analyze and visualize the importance of the field sites, years of cultivation and genetic modification for the diversity of the fungal community (Fig. 1)

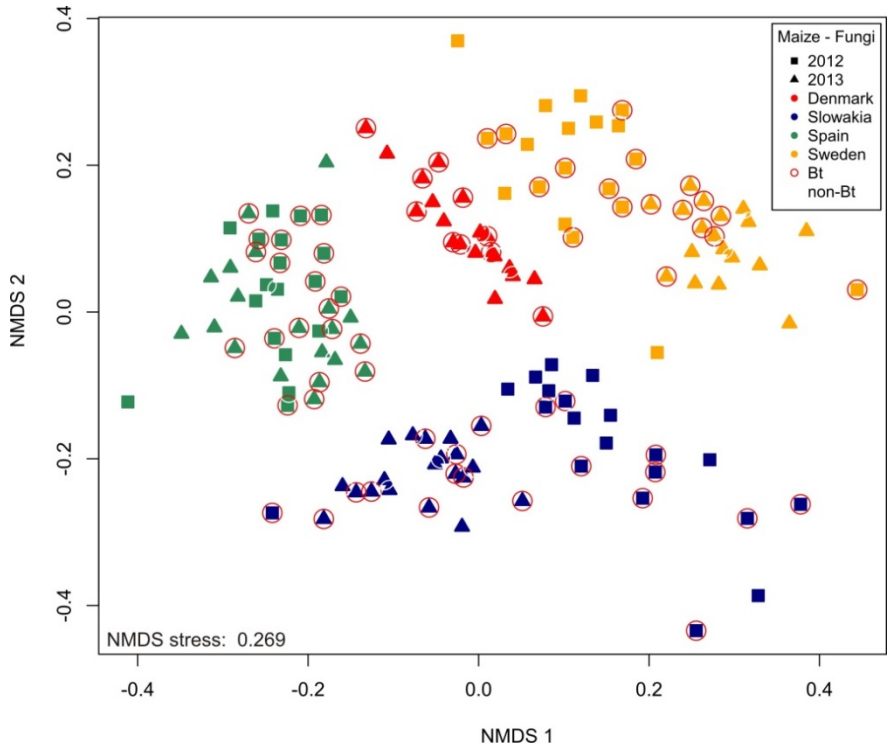

FIGURE 1 NMDS (NONMETRIC MULTIDIMENSIONAL SCALING) OF FUNGAL COMMUNITY STRUCTURES FROM MAIZE RHIZOSPHERES AT THE AMIGA FIELD SITES

NMDS clearly revealed that the field sites and thus the soils and environmental conditions had a strong effect on the fungal community structure, since all data points formed characteristic site-specific clusters. The effect of cultivar (genetic background) was not strong, considering that the same cultivar was used at all sites except for the site in Spain, since the data points representing samples from Spain were not particularly further apart from the other sites, as those among each other. Within each site, annual variability could be seen in the case of Slovakia and Sweden, but not for Spain (data for Denmark only referred to one year). At a more speculative level, one might interpret the relative locations of the data points from the different sites to each other as a reflection of their biogeographical location, considering that Denmark and Sweden were in vicinity of the upper left part of the NMDS plot, while Slovakia was at the button and Spain on the left site. Independent of the sites, there was clear indication by the NMDS plot of Figure 1 that the genetic modification had no detectable effect on the fungal community structure.

ANOSIM analyses indicated that differences between sites were clearly pronounced and significant ($R= 0.713$, $p < 0.001$) and also there were significant, though less clearly pronounced differences between years ($R=0.083$, $p < 0.001$), while no differences were seen between MON810 and their isogenic comparators ($R=-0.011$, $p > 0.5$). It should be noted that these comparisons considered the whole community structure and not each separate OTU, as reported in one of the above sections.

B. Fungal communities associated with potato and description of a potato specific fungal core community

More than 10 Million raw sequences were generated of which 32.5% of sequences were removed during quality filtering. The average number of quality filtered sequences among the 119 rhizosphere samples analyzed was 58,304 + 26,883, ranging from 23,276 to 134,098 (see also Appendix, Table A2). The sequences could be assigned to 4,405 OTUs. After rarefaction at 23,276 sequences, a total

of 4,179 OTUs remained; these were represented by a total of 2,769,844 sequences. True singletons were not allowed to become the seed of an OTU, thus the dataset does not contain true singletons (see Materials and Methods). However, after subsampling, 351 OTUs were represented by one single sequence in the dataset (see also in the Appendix, Table A3).

The ten most dominant OTUs represented 37.8 % of all sequences collected for the fungal community analysis, the twenty most dominant 48.7 %, and the fifty most dominant 65.4 %, respectively. The smallest number of different OTUs detected in a sample was 218, the highest number was 513, and the average was 344 $\pm$ 66.

Phylogenetic analyses revealed that *Ascomycota* was across all potato samples the most dominant phylum, representing 63.0 % of all sequences, they represented a minimum of 36.1 % and a maximum of 94.0 % of all sequences recovered from the rhizospheres. The second most abundant phylum, *Zygomycota* represented 19.7 % of all sequences followed by the *Basidiomycota* with 10.8%. An "unclassified phylum" followed with 6 % and *Chytridiomycota* with 0.25 %. *Rozellomycota* represented on average 0.06 %. *Glomeromycota*, i.e. the phylum comprising all arbuscular mycorrhizal fungi (AMF), were only detected in two of 119 samples in very low abundance (0.1 – 0.2%), demonstrating that mycorrhization of potato was not of importance at the field sites.

The most dominant OTU (OTU1, *Zygomycota*, *Mortierellaceae*) in the samples from Ireland represented on average 12.9 % while it only occurred in 0.7 % of samples from the Netherlands. Similarly, the abundance of OTU2 (*Ascomycota*, *Capnodiales*) was 7.1 % in Ireland and 1.7 % in the Netherlands. The opposite was true for OTU6 (*Basidiomycota*, *Cryptococcus terricola*) which represented 10.6 % in the Dutch samples and only 0.3 % in the Irish samples. In contrast OTU4 (*Ascomycota*, *Plectosphaerella*) was almost equally abundant with 4.1 % in Ireland and 5.1 % in The Netherlands, similarly OTU5 (*Ascomycota*, *Botrytis* cinerea).

A total of 6 OTUs were detected across all 119 samples: Together they represented on average 23.6 % $\pm$ 15.5 % of all sequences found in a respective rhizosphere. With samples only from Ireland,

the core represented 26.9 % ± 15.3 %, while for The Netherlands, these six core OTUs represented 9.8 % ± 5.5 % only. The taxonomic affiliation of OTU1, OTU2 and OTU4 has been indicated above. The other three OTUs were all members of the *Ascomycota*, including *Cladosporium* sp., *Exophilia* sp., and *Fusarium merismoides* var. *merismoides*, respectively.

A total of 10 OTUs were consistently detected in all 64 rhizosphere samples from Desiree cultivated in Ireland over the three years. In addition to the 6 above mentioned OTUs these also included OTU17, OTU20, OTU22, and OTU57, which belonged to four different classes of *Ascomycota*. They were most closely related to *Exophilia*, *Preussia*, a member of the *Helotiales*, and *Volutella ciliata*, respectively. Together these 10 OTUs represented an average of 30.2 % ± 15.7 % of all sequences collated from Desiree. No OTUs were detected which were uniquely synonymous over the three years with either the GM Desiree or the non-modified Desiree cultivar.

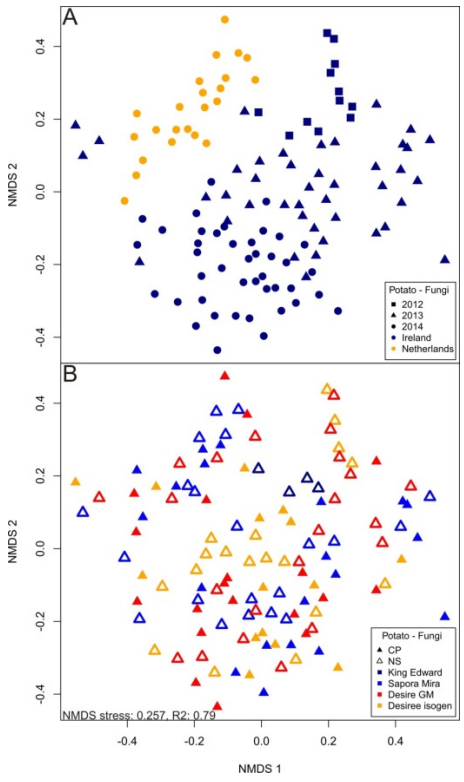

FIGURE 2 NMDS OF FUNGAL COMMUNITY STRUCTURES. PANEL A HIGHLIGHTS THE DIFFERENCES BETWEEN SITES AND YEARS. PANEL B HIGHLIGHTS FOR IRELAND THE DIFFERENT CULTIVARS, INCLUDING GM AND NON GM-DESIREE AS WELL AS THE MANAGEMENT DIFFERENCES, I.E. CONVENTIONAL PROTECTION (CP) COMPARED TO NO SPRAY OF FUNGICIDES (NS).

The multivariate statistical analysis of the potato datasets revealed, as shown by an NMDS plot that the composition of the fungal community in the potato rhizospheres was mainly affected by site location (The Netherland vs. Ireland) and by the year of cultivation (Fig. 2A). In contrast, as visualized for the Irish site, there was no clear effect of the potato and genotypes (including cultivars as well as GM and non-GM) on the overall fungal community structure (Fig. 2B). Additionally, NMDS charts indicated that the overall fungal community did not respond to management differences, i.e. conventional protection compared to no spray of fungicides (data not shown).

Conclusions

The baseline diversity of the fungal communities associated with the maize and potato varieties cultivated in the framework of the AMIGA project, including genetically modified versions, was established on the bases of more the 25 Million DNA sequences. The molecular approach does not allow an unequivocal detection of fungal species, but the phylogenetic analysis on these sequences gives a view on the diversity of the fungal community based on the affiliation of OTUs to known fungal taxa found in the curated public nucleotide database (UNITE).

Ascomycota was the most dominant phylum, in maize and potato rhizospheres as they accounted for 55.8 % and 63.0 % of all sequences, respectively. *Basidiomycota* was the second most dominant phylum for maize (24.6 %) while it was the third most abundant with potato (10.8 %). Vice versa, *Zygomycota* was third dominant with maize (16.8 %), while it was in the second position with potato (19.7 %). All other fungal phyla were poorly presented or assigned to “unclassified” phyla, the latter representing for maize approx. 2 % and for potato 6 %. Surprisingly, the phylum *Glomeromycota*, which includes all known endomycorrhizal taxa, was only present in very low abundance in maize (0.002 % of all sequences) and practically fully absent in potato rhizospheres, underlining that this assumingly plant growth promoting symbiosis was completely irrelevant under the respective cultivation conditions.

The filtered data set allowed the distinction of 4,759 different OTUs from maize rhizospheres and 4,405 OTUs from the respective potato samples. It should be noted that the data set with maize consisted of 137 samples while that of potato was composed of 119 samples, which may have affected the total number of detected OTUs. However, as indicated by Good's coverage more than 99.5 % of the fungal diversity in maize and potato rhizosphere were uncovered, even in the subsampled dataset. The OTU richness of the fungal communities from maize and potato were in the same range, however there were significant differences in the diversity as indicated e.g. by the Shannon diversity index which was 3.9 ± 0.4 for maize and 3.7 ± 0.6 for potato. The evenness of both was also similar, as indicated by the fact that the ten most dominant OTUs of maize and potato represented 35 % and 33 %, and the 50 most dominant OTUs 68 % and 65 %, respectively, hence, resulting in similar metric of community evenness. Thus the diversity of the fungal communities of maize and potato were very similar.

The comparison of the most dominant 50 OTUs of maize and potato revealed that 20 OTUs were shared and on average they contributed to 40 % of all sequences, indicating fungi which preferentially colonized rhizospheres, independent of the plant species or site of cultivation: Representatives with a high abundance were an unclassified member of the *Capnodiales* (*Ascomycota*: 5.3 % of all sequences from potato; 4.5 % of all sequences from maize), a member of the genus *Mortierella* (*Zygomycota*; 1.8 % and 5.4 %), *Cryptococcus terricola* (*Basidiomycota*: 3.2 % and 1.1 %), *Mortierella elongata* (1.4 % and 2.0%), and others. In contrast, the comparison also revealed some taxa with exclusive preference to maize (21 out of 50 OTUs) or potato (also 20 out of 50). The five quantitatively strongest indicators for maize were a member of the *Mortierellaceae* (4.4 % of all sequences), *Guehomyces pullulans* (*Basidiomycota*, 2.8 %), an unclassified member of the *Trichocomaceae* (*Ascomycota*, 2.8%), *Cryptococcus magnus* (*Basidiomycota*, 2.4 %) and an *Alternaria* (*Ascomycota*, 2.3 %). For potato the top five included another unclassified member of the *Mortierellaceae* (9.4 %), an unclassified *Myxotrichaceae* (*Ascomycota*, 4.5 %), an unclassified fungus (3.2 %), *Oidiodendron rhodogenum* (*Ascomycota*, 2.9 %), and *Botrytis cinerea* (*Ascomycota*, 2.8 %),

respectively. Interestingly different members from the same families, i.e., *Mortierellaceae* seem to strongly differ in their preference for host plants, while others show no preference at all.

Strong differences between maize and potato were indicated by the "core fungal rhizobiome", which, for maize was represented by 13 OTUs which contributed, depending on the sample to 9 to 50 % of all sequences found. For potato, even though less samples (see above) and only two and not four sites (like for maize) were analyzed , the core was much smaller with 6 OTUs which represented on average 11 % of all sequences, but at the Irish site 13 % and at the Dutch site less than 1 %. Thus the effect of maize plants on fungal diversity was much stronger and selective than that of potatoes.

Non-metric multidimensional scaling (NMDS) plots allowed the visualization of differences in the overall fungal community structures as affected by field site, year of cultivation, cultivar and genetic modification, and, for potato, spraying of fungicides or not spraying. The most discriminative factor for both crops was the field site, highlighting the variability caused by different soil types which are a product of parental material, pedogenesis, climatic conditions, but also history of soil use and agricultural management. Of interest in this regard was the fact that previous to the AMIGA operations, the Irish site was fallow for ~10 years, which contrasted to the site in the Netherlands which had undergone intensive cultivation in the years previous to the AMIGA studies. Beyond that, the community structures were distinguishable according to the year in which they were cultivated (annual variability), which is a product of the weather conditions, agricultural management (fertilization, pest control), probably resulting in slightly different plant growth in different years. For maize, no differences were seen in NMDS plots between MON810 (the GM version) and the respective isogenic comparator. For potato, which also included an additional conventional cultivar (Sarpo Mira), neither the cultivars nor the cis-genic variants were significantly different from the others.

Overall, the fungal community analyses conducted in the AMIGA project gives an unprecedented deep view into the fungal community structure of maize and potato, it highlights the importance of

the receiving environments and its endemic fungal diversity interacting with the respective crops, but also indicates that, independent of the field sites and thus represented biogeographical regions, that even with the high level of resolution obtained through the protocols adopted here, no differences were detectable between the GM and the non-engineered comparator cultivars.

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Appendix

Table A1 A: Summary of samples collected for the fungal community analyses of **maize** rhizospheres from field sites and cultivars by DNA PCR-amplicon sequencing of fungal ITS1 sequences

Site	Sampling date	Cultivar (BT, ISO)	No. of replicates	Total
Spain	July 24, 2012	DKC6451YG, DKC6450	10, 9	19
	July 24, 2013	DKC6451YG, DKC6450	10, 10	20
Slovakia	July 10, 2012	DKC3872YG, DKC3871	10, 10	20
	July 30, 2013	DKC3872YG, DKC3871	10, 10	20
Sweden	August 15, 2012	DKC4442YG, DKC440	10, 10	20
	August 12, 2013	DKC3872YG, DKC3871	8, 10	18
Denmark	August 26, 2013	DKC3872YG, DKC3871	10, 10	20
Total				**137**

Table A1 B: Summary of samples collected for the fungal community analyses of **potato** rhizosphere from field sites, cultivars and treatments by DNA sequencing of PCR amplified fungal ITS1 sequences. CP refers to "conventional practice", NS to "no spray".

Sampling date	Treatment	Cultivar	Replicates	Total number
Ireland				
October 18, 2012				
	NS	GM, ISO, CON	4, 4, 4	12
August 13, 2013				
	CP	GM, ISO, CON	7, 7, 7	21
	NS	GM, ISO, CON	7, 7, 7	21
July 28, 2014				
	CP	GM, ISO, CON	7, 7, 7	21
	NS	GM, ISO, CON	7, 7, 7	21
The Netherlands				
July 15, 2014				
	CP	GM,ISO, CON	5, 1, 6	12
	NS	GM, ISO, CON	4, 0, 7	11
Total				**119**

Table A2: MAIZE - Illumina ITS1-18S rRNA amplicon sequences – rarefied at 43,320 sequences

Spain

Cultivar	Quality sequences	Number of OTU	Singletons in group of 429	% of OTUs with single sequence	OTUs, more than one sequence
2012 (July 24)					
BT-1	75,899	370	2	9.7	334
BT-2	78,266	404	2	11.1	359
BT-3	82,679	413	3	13.8	356
BT-4	61,761	418	3	11.2	371
BT-5	78,161	433	4	12.0	381
BT-6	84,050	405	4	13.3	351
BT-7	74,704	371	3	17.0	308
BT-8	75,084	408	3	12.7	356
BT-9	72,294	357	1	10.9	318
BT-10	64,295	413	1	15.7	348
ISO-1	74,693	424	6	13.7	366
ISO-2	6,324	Missing due to subsampling at 43,320 sequences			
ISO-3	63,211	428	0	11.7	378
ISO4	59,045	373	0	13.1	324
ISO-5	65,479	418	2	16.5	349
ISO-6	54,760	390	0	10.5	349
ISO-7	54,757	413	1	12.1	363
ISO-8	72,271	437	1	13.0	380
ISO-9	69,088	424	2	14.2	364
ISO-10	64,477	394	4	14.5	337
2013 (July 24)					
BT-1	121,724	520	4	13.1	452
BT-2	124,437	481	12	17.9	395
BT-3	125,659	484	8	16.9	402
BT-4	121,074	454	6	16.3	380
BT-5	135,658	468	8	14.3	401
BT-6	136,043	482	3	13.5	417
BT-7	130,536	431	8	13.0	375
BT-8	110,158	449	7	14.0	386
BT-9	110,541	477	5	13.8	411
BT-10	107,298	491	2	13.6	424
ISO-1	127,261	465	8	16.6	388
ISO-2	131,178	435	11	18.9	353
ISO-3	118,721	476	7	14.3	408
ISO4	115,141	499	8	16.0	419
ISO-5	139,161	487	4	15.8	410
ISO-6	125,328	408	4	12.5	357
ISO-7	52,808	462	1	13.6	399
ISO-8	56,000	453	2	12.8	395
ISO-9	48,750	461	1	9.8	416
ISO-10	51,674	448	1	11.6	396

Slovakia

Cultivar	Quality sequences	Number of OTU	Singletons in group of 429	% of OTUs with single sequence	OTUs, more than one sequence
2012 (July 10)					
BT-1	132,962	421	3	19.2	340
BT-2	94,064	489	6	14.1	420
BT-3	117,793	433	5	17.3	358
BT-4	128,322	482	3	17.8	396
BT-5	107,504	467	3	21.4	367
BT-6	79,767	455	4	18.2	372
BT-7	97,444	491	5	20.4	391
BT-8	50,866	526	3	13.3	456
BT-9	84,294	565	1	13.3	490
BT-10	105,506	517	3	13.5	447
ISO-1	89,172	519	7	15.4	439
ISO-2	102,130	451	4	15.3	382
ISO-3	90,170	555	5	13.3	481
ISO4	94,254	551	5	12.7	481
ISO-5	103,099	561	4	15.5	474
ISO-6	107,150	423	5	24.6	319
ISO-7	99,220	558	6	17.9	458
ISO-8	85,915	561	3	15.2	476
ISO-9	84,346	574	4	16.0	482
ISO-10	76,340	504	2	14.7	430
2013 (July 30)					
BT-1	66,382	399	2	17.0	331
BT-2	62,293	437	0	18.3	357
BT-3	68,052	470	2	16.4	393
BT-4	43,419	361	0	18.0	296
BT-5	58,306	392	1	14.3	336
BT-6	64,012	409	3	14.4	350
BT-7	60,118	395	0	19.7	317
BT-8	50,637	386	1	15.3	327
BT-9	70,358	412	3	19.2	333
BT-10	68,107	438	2	17.1	363
ISO-1	65,594	454	1	19.2	367
ISO-2	65,416	360	0	14.4	308
ISO-3	61,430	429	2	16.8	357
ISO4	60,968	359	2	16.2	301
ISO-5	65,744	409	2	16.9	340
ISO-6	52,243	420	1	16.2	352
ISO-7	57,410	340	1	17.6	280
ISO-8	58,056	405	2	15.6	342
ISO-9	60,606	424	1	14.9	361
ISO-10	57,070	399	1	16.8	332

Sweden

Cultivar	Quality sequences	Number of OTU	Singletons in group of 429	% of OTUs with single sequence	OTUs, more than one sequence
2012					
BT-1	107,914	556	2	19.8	446
BT-2	110,739	497	6	18.3	406
BT-3	125,661	478	1	20.5	380
BT-4	83,756	498	7	22.9	384
BT-5	120,866	527	7	16.1	442
BT-6	110,635	474	8	18.4	387
BT-7	112,156	500	7	17.4	413
BT-8	80,370	529	2	13.4	458
BT-9	108,797	476	2	14.9	405
BT-10	94,081	526	1	17.9	432
ISO-1	116,674	514	5	15.8	433
ISO-2	108,704	514	3	15.4	435
ISO-3	123,018	478	7	23.8	364
ISO4	98,725	505	9	16.6	421
ISO-5	99,394	558	3	16.5	466
ISO-6	87,637	502	4	16.5	419
ISO-7	97,986	537	4	16.8	447
ISO-8	115,805	500	5	19.4	403
ISO-9	100,376	529	8	14.9	450
ISO-10	88,287	496	6	18.3	405
2013					
BT-1	17	Missing due to amplification problems			
BT-2	63,026	462	0	18.8	375
BT-3	58,346	421	1	19.7	338
BT-4	60,658	461	3	21.5	362
BT-5	43,462	434	0	19.8	348
BT-6	59,089	474	1	16.5	396
BT-7	61,571	439	1	16.9	365
BT-8	32,892	Missing due to subsampling at 43,320 sequences			
BT-9	57,531	492	2	16.9	409
BT-10	52,925	478	2	20.9	378
ISO-1	55,033	414	0	18.4	338
ISO-2	56,542	396	1	18.2	324
ISO-3	53,273	473	0	20.5	376
ISO4	58,331	484	2	16.9	402
ISO-5	61,124	427	3	19.2	345
ISO-6	58,093	416	0	19.0	337
ISO-7	63,415	476	4	19.5	383
ISO-8	55,002	458	1	17.0	380
ISO-9	88,275	434	4	20.5	345
ISO-10	57,552	469	2	16.2	393

Denmark

Cultivar	Quality sequences	Number of OTU	Singletons in the group of 429	% of OTUs with single sequence	OTUs, more than one sequence
2013					
BT-1	63,191	504	3	11.7	445
BT-2	62,522	471	1	20.2	376
BT-3	65,845	468	1	19.9	375
BT-4	50,316	455	0	15.8	383
BT-5	62,761	522	5	19.5	420
BT-6	61,091	470	4	19.8	377
BT-7	57,987	506	2	17.6	417
BT-8	67,438	481	3	18.5	392
BT-9	65,603	479	2	18.6	390
BT-10	62,440	483	4	17.0	401
ISO-1	69,046	485	1	13.2	421
ISO-2	43,320	437	0	16.7	364
ISO-3	55,038	525	1	14.9	447
ISO4	61,994	471	1	14.4	403
ISO-5	60,828	530	4	16.8	441
ISO-6	58,014	490	2	20.0	392
ISO-7	57,216	497	1	13.1	432
ISO-8	58,255	481	2	22.0	375
ISO-9	61,475	476	2	19.3	384
ISO-10	72,243	470	6	17.4	388

Table A3: POTATO - ITS1 amplicon sequences (fungal communities) – rarefied at 23,276 sequences

Ireland

Cultivar/ Treatment	Quality sequences	OTU	Singletons in group of 351	% of OTUs with single sequence	OTUs, more than one sequence
2012					
CON1 – NS	37,941	513	3	18.9	416
CON2 – NS	32,650	457	1	17.7	376
CON3 – NS	37,910	505	3	19.4	407
CON4 – NS	25,470	452	1	15.5	382
ISO1 – NS	37,627	444	4	20.5	353
ISO2 – NS	41,728	404	3	27.0	295
ISO3 - NS	38,335	466	1	16.5	389
ISO4 - NS	41,485	438	4	14.8	373
GM1 - NS	41,350	468	0	16.9	389
GM2 - NS	34,263	404	1	17.8	332
GM3 - NS	37,460	382	1	17.3	316
GM4 - NS	28,874	498	1	14.9	424
2013					
GM_CP-1	102,720	328	1	13.4	284
GM_CP-2	116,227	344	6	13.4	298
GM_CP-3	98,150	345	4	14.5	295
GM_CP-4	124,586	322	3	14.9	274
GM_CP-5	134,098	270	5	21.1	213
GM_CP-6	112,826	288	4	12.5	252
GM_CP-7	121,127	331	3	16.3	277
ISO_CP-1	77,816	351	3	12.8	306
ISO_CP-2	63,332	421	3	14.7	359
ISO_CP-3	94,369	409	4	16.4	342
ISO_CP-4	106,598	419	3	15.5	354
ISO_CP-5	98,231	291	2	18.2	238
ISO_CP-6	120,970	310	5	20.6	246
ISO_CP-7	98,177	247	4	23.9	188
CON_CP-1	79,809	371	3	21.0	293
CON_CP-2	100,406	253	1	17.8	208
CON_CP-3	100,306	338	4	15.4	286
CON_CP-4	79,559	324	1	18.5	264
CON_CP-5	95,163	334	4	15.6	282
CON_CP-6	72,639	437	6	17.2	362
CON_CP-7	75,105	373	5	13.7	322
GM_NS-1	83,813	391	3	13.0	340
GM_NS-2	101,746	334	4	18.6	272
GM_NS-3	87,608	377	4	13.0	328
GM_NS-4	80,091	323	3	16.4	270
GM_NS-5	75,769	292	1	19.5	235
GM_NS-6	56,716	322	1	13.7	278
GM_NS-7	102,237	395	4	18.0	324

ISO_NS-1	73,165	367	6	11.7	324
ISO_NS-2	52,943	284	5	12.7	248
ISO_NS-3	60,135	361	4	14.1	310
ISO_NS-4	77,042	355	4	12.7	310
ISO_NS-5	54,232	321	2	13.1	279
ISO_NS-6	68,225	287	0	12.5	251
ISO_NS-7	57,288	371	7	15.4	314
CON_NS-1	65,001	336	1	20.8	266
CON_NS-2	83,780	385	5	17.9	316
CON_NS-3	121,946	308	3	18.2	252
CON_NS-4	80,405	359	4	13.6	310
CON_NS-5	101,750	447	3	13.6	386
CON_NS-6	84,072	339	5	17.1	281
CON_NS-7	95,769	389	7	16.7	324
2014					
GM_CP-1	24,751	273	0	12.5	239
GM_CP-2	46,229	410	8	13.2	356
GM_CP-3	48,533	366	4	10.9	326
GM_CP-4	44,390	247	1	13.4	214
GM_CP-5	45,256	220	1	8.6	201
GM_CP-6	36,491	229	0	11.8	202
GM_CP-7	43,542	248	2	11.7	219
ISO_CP-1	34,328	218	2	12.8	190
ISO_CP-2	36,767	352	0	10.5	315
ISO_CP-3	36,604	263	4	12.9	229
ISO_CP-4	39,977	436	5	11.0	388
ISO_CP-5	46,873	273	0	14.3	234
ISO_CP-6	34,588	278	1	11.2	247
ISO_CP-7	36,663	278	0	10.4	249
CON_CP-1	38,322	314	6	14.3	269
CON_CP-2	47,824	382	6	15.7	322
CON_CP-3	32,866	343	2	13.7	296
CON_CP-4	41,775	402	3	17.2	333
CON_CP-5	23,276	257	0	10.9	229
CON_CP-6	31,494	268	0	14.6	229
CON_CP-7	40,566	368	1	15.2	312
GM_NS-1	43,564	393	4	17.3	325
GM_NS-2	44,595	368	0	15.2	312
GM_NS-3	46,424	422	1	14.9	359
GM_NS-4	34,021	388	1	13.9	334
GM_NS-5	50,136	271	3	12.2	238
GM_NS-6	44,032	284	0	15.8	239
GM_NS-7	37,646	320	2	9.7	289
ISO_NS-1	30,062	351	1	8.8	320
ISO_NS-2	25,353	235	0	9.8	212
ISO_NS-3	38,165	290	0	8.3	266
ISO_NS-4	33,278	287	1	18.5	234
ISO_NS-5	38,292	287	1	13.6	248
ISO_NS-6	45,496	269	2	13.0	234

ISO_NS-7	40,464	330	2	14.2	283
CON_NS-1	27,197	285	0	8.8	260
CON_NS-2	33,482	390	3	11.3	346
CON_NS-3	35,804	441	6	13.8	380
CON_NS-4	26,747	418	0	11.5	370
CON_NS-5	31,393	248	0	13.3	215
CON_NS-6	34,646	359	2	12.8	313
CON_NS-7	36,709	317	2	14.5	271

The Netherlands

Cultivar/ Treatment	Quality sequences	OTU	Singletons in group of 351	% of OTUs with single sequence	OTUs more than one sequence
2014					
GM_CP-1	46,108	295	3	14.6	252
GM_CP-2	Missing				
GM_CP-3	52,752	275	1	16.0	231
GM_CP-4	54,820	368	4	16.8	306
GM_CP-5	45,758	255	1	15.3	216
GM_CP-6	Missing				
GM_CP-7	61,662	328	3	13.4	284
ISO_CP-1	Missing				
ISO_CP-2	Missing				
ISO_CP-3	Missing				
ISO_CP-4	Missing				
ISO_CP-5	Missing				
ISO_CP-6	Missing				
ISO_CP-7	64,423	321	0	13.1	279
CON_CP-1	45,407	254	2	9.4	230
CON_CP-2	47,884	274	1	20.4	218
CON_CP-3	41,694	333	4	18.9	270
CON_CP-4	43,788	399	3	17.8	328
CON_CP-5	46,593	392	7	18.4	320
CON_CP-6	59,556	325	5	17.5	268
CON_CP7	Missing				
GM-NS-1	56,299	294	3	16.0	247
GM_NS-2	49,885	324	4	17.6	267
GM_NS-3	44,455	267	0	18.4	218
GM_NS-4	52,795	376	3	20.7	298
GM_NS-5	Missing				
GM_NS-6	Missing				
GM_NS-7	Missing				
ISO_NS-1	Missing				
ISO_NS-2	Missing				
ISO_NS-3	Missing				
ISO_NS-4	Missing				

ISO_NS-5	Missing				
ISO_NS-6	Missing				
ISO_NS-7	Missing				
CON_NS-1	56,078	400	12	18.0	328
CON_NS-2	70,095	363	10	28.9	258
CON_NS-3	54,360	340	9	17.1	282
CON_NS-4	65,289	393	11	24.7	296
CON_NS-5	38,436	321	2	18.1	263
CON_NS-6	40,787	418	7	18.9	339
CON_NS-7	39,552	394	6	16.0	331