‘A new race of *Fusarium oxysporum* f.sp. *lactucae* of lettuce’

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Abstract

Fusarium oxysporum f. sp. *lactucae*, the causal agent of the Fusarium wilt of lettuce (*Lactuca sativa* L.), occurs in most countries in which lettuce is grown and causes serious economic losses. Three races (1, 2, and 3) of the pathogen have previously been identified on the basis of their ability to cause disease on differential lettuce cultivars as well as by means of molecular tools developed to characterize different races of this pathogen. Only race 1 has been detected in Europe so far. In this study, two isolates of *Fusarium oxysporum*, obtained from lettuce plants grown in the Netherlands showing symptoms of wilt, have been characterized by combining the study of pathogenicity with differential cultivars of lettuce and molecular assays to determine whether the isolates were different from the known races of *F. oxysporum* f.sp. *lactucae*. The present study reports the presence of *F. oxysporum* f. sp. *lactucae* for the first time in the Netherlands. The causal pathogen has been identified, using the IRAP-SCAR technique, as a new race of *F. oxysporum* f. sp. *lactucae*. Specific primers have been designed to identify this new race.

INTRODUCTION

Fusarium oxysporum f.sp. *lactucae* (FOL), the causal agent of *Fusarium* wilt of lettuce (*Lactuca sativa* L.), was first found on butter head lettuce in 1955 in Tokyo (Matuo & Motohashi 1967). From there, the pathogen has spread to most lettuce growing regions throughout the world (Matheron & Gullino, 2012). The disease was first described in Europe by Garibaldi *et al.* (2002), who found the pathogen on 'Salad bowl' lettuce grown in northern Italy under intensive cropping systems for the ready-to eat sector. *F. oxysporum* f. sp. *lactucae* is host specific, and causes yellowing of the leaves, wilting and brown or black streaks in the vascular system of lettuce. The colonization of melon, tomato, watermelon, cotton, broccoli, cauliflower and spinach roots has also been reported (Hubbard & Gerik, 1993; Scott *et al.*, 2014).

Three races (1, 2, and 3) have been identified so far: race 1 was first described in Japan in 1967 (Matuo & Motohashi,1967), and was reported in the United States in 1993 (Hubbard & Gerik, 1993), in Iran in 1995 (Millani *et al*.,1999), in Taiwan in 1998 (Huang & Lo, 1998), in Brazil in 2000 (Ventura & Costa, 2008), in Portugal in 2004 (Marques Ramalhete *et al*., 2006) and in Argentina in 2014 (Malbrán *et al.*, 2014). Until recently, races 2 and 3 had only been reported in Japan (Fujinaga *et al.*, 1997; 2001; 2003; 2005; Yamauchi *et al.*, 2001). However, Lin *et al.*, (2014) have recently detected a highly aggressive isolate of FOL in Taiwan, which has been classified as race 3.

It has been suggested that race 1 may be disseminated in other countries through seed transmission (Garibaldi *et al.*, 2004). Mbofung and Pryor (2010) developed a PCR-based assay for the detection of *F. oxysporum* f. sp. *lactucae* from seed lots with infestation rates as low as 0.1%, but the negative results from 88 commercial seed lots supported the hypothesis that a rapid spread in Arizona may have been due to contaminated farm equipment.

Moreover, three vegetative compatibility groups (VCGs) VCG-1, VCG-2, and VCG-3 (VCG-3-1, VCG-3-3) have been reported in the *forma specialis lactucae*, which correspond to races 1, 2 and 3, respectively (Ogiso *et al.*, 2002; Fujinaga *et al.*, 2005).

The Arizona, California and the type 1 isolates from Taiwan all belong to the same VCG as the race 1 isolates from Japan (Pasquali *et al.*, 2008, Mbofung *et al*., 2007), and all the race 1 isolates from Arizona, California, and Japan had identical mtSSU and EF-1α sequences and almost identical

intergenic spacer (IGS) region IGS sequences, which would seem to suggest a common origin.

On the basis of IGS region of the Japanese and Californian FOL isolates, it seems that each race has evolved independently, and a close correlation between VCGs and phylogenetic groupings has been found (Fujinaga *et al.*, 2005).

Several molecular tools have been developed over the years to characterize the different *formae speciales* and the different races, e.g., amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLP) (Chiocchetti *et al.*, 1999; Pasquali *et al*., 2003; Konstantinova & Yli-Mattila, 2004). In addition, different approaches have been followed in order to obtain specific primers. Shimazu *et al.,* (2005) developed race-sequence tagged site (STS) markers to identify races of FOL, using the sequence-characterized amplified region (SCAR) technique, based on RAPD fingerprinting for FOL (FLA0001), FOL race1(FLA0101) and FOL race 2 (FLA0201).

Pasquali *et al.,* (2007) designed two sets of primers to map the variability between the FOL races, using the IRAP technique based on transposable elements (TE) to detect the polymorphisms produced by retrotransposon insertion in the genome (Kalendar & Schulman, 2006). The two classes of TE, class I, or retrotransposons (transposition using an RNA intermediate), and class II, or DNA transposons (transposition via DNA cut and paste), were found in *Fusarium oxysporum*. Several TEs in class II have been reported, such as *Impala* (Hua-Van *et al.,* 2000), *Fot1*(Migheli *et al.,* 1999), *Fot2 , FOt3* (Hua-Van *et al.,* 2000), *Fot4* (Hua-Van *et al.,* 2000) and *Hop* (Chalvet *et al.,* 2003). In class I, two different types have been reported: LTR (long terminal repeat) retrotransposons belonging mostly to the *gypsy* group, such as *skippy* and *Foret* (Julien *et al.,* 1992; Anaya & Roncero, 1994) and non-LTR retrotransposons with LINE (long interspersed nuclear element) characteristics, such as *Palm* in *Fusarium oxysporum* (Mouyna *et al.,*1996). Retrotransposons have long terminal direct repeats (LTR) as retroviruses that flank a region which encodes protein homologies to *gag* and *pol* gene products. The *gypsy* group presents a third ORF in the same position as the retroviral gene *env*, but with no homology to it, and also presents a specific order in the proteins codified by the *pol* gene (protease, reverse transcriptase, RNase H and integrase), while the non- LTR-retrotransposons do not have the LTR regions and instead have a polyA tail at their 3′ end. *Han* retrotransposon

has an estimated high total copy number located in preference regions as RNA polymerase III (Hua-Van *et al*., 2000). These TEs are characterized by some short repeats, flanked by 5´ –TG…CA-3´ terminal sequences (Hua-Van *et al*., 2000), and they share some characteristics with LTR transposons.

In this study, two isolates of *Fusarium oxysporum*, obtained from lettuce plants grown in the Netherlands, which showed symptoms of wilt, were characterized by combining the study of pathogenicity with differential lettuce cultivars and molecular assays in order to determine whether the isolates were different from the known races of FOL, and could thus be classified as a new race.

MATERIAL AND METHODS

Isolate propagation and pathogenicity test

In 2015, the 04750896 and 04750888 fungal isolates with characteristics typical of *F. oxysporum* (Leslie et al. 2006) were isolated from the vascular tissue of wilted head lettuce taken from two different growers in the Netherlands. In both cases, a yield loss of up to 50% was reported. The lettuce was grown in soil, inside greenhouses. The 04750896 isolate was obtained from cv. Carter, while the 04750888 isolate was taken from cv. Wengen. Similar symptoms and loss have been reported for Abeba and Alega cultivars.

The isolates were compared with FOL isolate ATCCMya-3040 belonging to race 1, which was isolated in Northern Italy (Garibaldi *et al.,* 2002), and with a race 2 isolate (9501; Fujinaga *et al.*, 2003), and race 3 isolates (MAFF 744085, MAFF 744086), which have kindly been provided by Japanese colleagues (Table 1).

The tested FOL isolates were grown in potato dextrose broth (Sigma), in a shaken culture at 100 RPMs, for 10 days at 23 °C, with 12 h/day of fluorescent light. Each isolate suspension was filtered through a layer of sterile cheesecloth and centrifuged at 10,000 g for 15 min. The bud cell pellet was suspended in distilled water and counted, by means of a hemocytometer, to produce a concentration of $1x10^6$ CFU/ml. A single-spore culture of each isolate was stored in glycerol at -80°C.

Twenty–30 day old lettuce seedlings, grown in plug trays (100 cells/tray), were removed from the substrate and immediately inoculated with each of

the collected isolates by dipping the roots into 100 ml of the previously described suspension for 5 minutes. The inoculated seedling were transplanted into 15 L pots, filled with a peat mixture (previously steamed at 70°C for 60 minutes), with 10 plants/pot, thus resulting in a total of 40 plants/isolate per trial. The pots were then placed in a greenhouse at air temperatures ranging from 26 to 33 °C. Four experimental trials were carried out with four replicates. A completely randomised block design was adopted.

The isolates shown in Table 1 were tested for pathogenicity on 11 lettuce cultivars (Table 2). Lettuce cultivars, mainly loose-leaf, batavian and romaine types, with a resistant or partially resistant reaction to race 1 of the pathogen, were chosen among those used on the European market (Table 2). The differential lettuce cultivars Costa Rica N.4, which is resistant to race 1, and Banchu red fire, which is resistant to race 2, were also included (Fujnaga *et al.,* 2001; 2003). In the present study, Cavolo di Napoli, which is susceptible to all the three races of the pathogen (Gilardi *et al.,* 2014), has been used instead of 'Patriot'.

The severity of symptoms was assessed according to a disease rating scale that ranged from 0 to 4 (Garibaldi *et al.,* 2004): 0 = healthy plant; 1 = initial symptoms of leaf chlorosis, slight reduction in development, slight vascular browning; 2 = severe leaf chlorosis, evident reduction in development, sometimes asymmetric development of the head, evident vascular browning; 3 = leaf chlorosis and inhibition of growth, evident deformation and initial vascular browning symptoms of wilting during the hottest hours of the day; 4 = plant strongly deformed with leaf chlorosis or completely necrotic leaves, totally wilted. The data were expressed as disease index (DI) 0 to 100, which was calculated using the following formula: $DI= [\sum (i \times xi)]/ (4\times \text{total of plant})$ with i = 0-4 (xi is the number of plants with rating i) x 100.

DNA extraction and PCR assays

Each single-spore culture of the isolates listed in Table 1 was grown in potato dextrose broth (Sigma Aldrich) on a rotary shaker (120rpm) for 10 days at 22°C. The mycelium was collected, by filtration through Whatman No.1 filter paper, and stored at -20°C. The total genomic DNA was obtained by means of an E.Z.N.A. Fungal DNA mini kit (OMEGA Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions.

The DNA isolation was also performed on the mycelium of targeted and non-targeted organisms (Table 1) with the QuickPick SML Plant DNA kit (Bio- Nobile), using a KingFisher 96 flex isolation robot, in the National Plant Protection Organization of The National Reference Centre in Wageningen (The Netherlands).

A specific PCR was conducted for FOL race 1 to check whether the Dutch isolates belonged to race 1, because only race 1 has been found in Europe so far. The PCR reaction was developed using the Hani3′ and Hanilatt3rev primers listed in Table 3. These primers have been designed using a specific band (2337bp), obtained by means of IRAP-PCR, with the Hana primer which amplifies the LTR region of the Han solo-LTR retrotransposon. The two primers were designed considering the IRAP technique, i.e., one was designed to include a 3' sequence of Han solo-LTR and the other to include a 3´ flanking region of these retrotransposons (Pasquali et al., 2007).

Touchdown PCR was tested twice using the non-targeted *Fusarium oxysporum* isolates (isolates:PD 83/816, PD 78/442, PD 88/396,PD 83/808, PD 20036631), and three isolates of FOL race 1 (FusLat 11/10, ATCCMya-3040, FusLat5/12) as control, and in the second attempt the isolates ATCCMya-3040, F9501, and MAFF 744085 or MAFF 744086 were used as control for races 1, 2 and 3 as reported in Table 1.

To determinate if the Dutch isolates belonged to race 1 the PCRs with the primers pairs FOLR1-F and FOL1-RR, FIGS11, FOLR2-RR and FOLR3-F FOLR3-RR (Table 3) were performed with the isolates PD 83/816, PD 78/442, PD 88/396, PD 83/808,PD 20036631, FusLAt 11-10, ATCCMya-3040, FusLAt5-12, MAFF No. 244121 and MAFF No. 244122 (Fujinaga et al., 2014).

PCR reactions were performed to amplify the elongation factor 1α (*EF-1α*) gene and the IGS rDNA, using the specific primers listed in Table 3.

The *EF-1α* –PCR reactions were carried out in a 20 µl reaction volume with 50 ng of genomic DNA and 0.25 mM deoxynucleotide triphosphates, 1.0 mM MgCl2, 0.5 µM for each primer, QIAGEN PCR buffer diluted 1:10 and 1U of Taq DNA polymerase (QIAGEN, Chatsworth, CA, USA) in a T-100 thermal cycler (Bio-Rad Japan, Tokyo, Japan) under the following conditions: 94 °C for 5 minutes followed by 40 cycles: 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min, with a final extension of 10 min at 72 °C.

The amplification products were checked by means of electrophoresis in 1% agarose gel (Eppendorf AG). The amplification products were

sequenced in both directions using the BMR-Genomics service (Padova, Italy, http://www.bmr-genomics.it).
Fifty microlitre reaction volumes and 100 ng of DNA were used for the IGS amplification to develop a PCR reaction using the CNS1 and CNL12 primers with 0.25 mM deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 0.5 µM of each primer, QIAGEN reaction buffer diluted 1:10 and 1U of Taq DNA polymerase (QIAGEN, Chatsworth, CA, USA) in a T-100 thermal cycler (Bio-Rad Japan, Tokyo, Japan) under the following conditions: 94 °C for 5 minutes followed by 40 cycles: 94 °C for 1 min, 60 °C for 90s and 72 °C for 2 min, with a final extension of 10 min at 72°C.
The PCR products were checked by means of gel electrophoresis and were sequenced using the two outer primers and the two inner primers (CNS12 and RU46.67, listed in Table 3) by the BMR- Genomics services.

Restriction digests of the IGS region (RFLP) assay

The Sequences of the race 1 (HL-1) and race 2 (F9501) IGS regions were used to perform a virtual digestion with the pDRAW3 program to select the enzymes that were able to produce different digestion profiles. Five enzymes were selected: *Aat*II, *Nae*I, *Sac*II, *AluI* and *HpyCH4*IV (Life Technologies, Carlsbad, CA, USA). The digestions were carried out individually or in double digestion (*Aat*II and *Sac*II) mode. The digestion was conducted using different enzyme concentrations, from 5U to 15U, in 1 µg of the IGS amplification.
The reaction mixtures were incubated according to the manufacturer's instructions, and the digestion products were shown in 2% agarose gels. The digestion profiles were determined through a comparison with Wide Range Ladder (QIAGEN) or 1 kb plus GelPilot 1 Kb Plus Ladder (QIAGEN).

Phylogenetic analysis of the EF-1α and IGS regions

Contigs were obtained using the DNA BASER program (Heracle BioSoft SRL, Romania). The obtained sequences were utilized for CLUSTALW multiple sequence alignments, using MEGA 6 software set to the default parameters. Manual corrections were performed for each alignment in order to delete any external trimmer regions and discard incomplete sequences: a data set of 603 bp was obtained for *EF-1α* and 1833 bp for the IGS region.

Several isolaes belonging to FOL race 1 and race 2 were used as reference strains (Table1), while different *formae speciales (Fusarium oxysporum* f.sp. *lycopersici* (HM057293.1 and HM057278.1), *Fusarium oxysporum* f.sp *conglutinans* (FJ985443.1 and FJ985678.1), *Fusarium oxysporum* f.sp *vasinfectum* (FJ985318.1 and FJ985551.1), together with *Giberella fujikuroi* (HQ165853.1 and HQ165889.1), were used as out-groups. The Tajima-Nei model was used for the distance matrix, and the phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei 1987). Stability was evaluated using 1000 bootstrap replications.

IRAP PCR

IRAP PCR was carried out using the primers designed by Pasquali *et al.* (2007) within the LTRs of the *skippy* element (GenBank accession number L34658) called Gaga and Gagi; and within the LTRs of the *Han* solo-LTR element (GenBank accession number AF076629) called Hana and Hani. The adopted amplification protocol was that described by Pasquali *et al.* (2007).

All the combinations of these primers (Hana, Hani, Gaga, Gagi, Hana-Hani, Hana-Gaga, Hana-Gagi, Hani-Gaga, Hani-Gagi, and Gaga-Gagi) were used to test the distribution of these LTRs in the different FOL races. The PCR reaction was repeated twice for each isolate.

Each PCR, containing 50 ng of DNA, 0.25 mM NTPs, 1.0 mM $MgCl_2$, 0.5 µM each primer, 1× PCR buffer and 1U of *Taq* DNA polymerase (QIAGEN), was performed in a 20 µl reaction volume using the T-100 thermal cycler (Bio-Rad) set at the following program: 94 °C for 5 min; 30 cycles of 94 °C for 10 s, 60 °C for 50 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C.

The amplification products were checked in 2.5% agarose gel (Eppendorf).

Cloning, Sequencing and PCR assay of the primers designed to establish the new race

One polymorphic band, obtained for 04750888 and 04750896 by means of the Gaga-Gagi amplification, was extracted using the QIAquick Gel Extraction Kit (QIAGEN) and cloned in the pGEM®-T Easy Vector System, according to the manufacturer's instructions. Positive colonies were screened, using the M13F and M13R primers, and sequenced in both

directions using the BMR-Genomics service (Padova, Italy). Two new primers (FUPF and FUPR) were generated using the Perl Primer program (Source Forge) (primers listed in Table 3).
The FUPF and FUPR primers were tested by means of PCR in a 20 µl reaction volume with 50 ng genomic DNA, 0.25 mM dNTPs, 1.0mM $MgCl_2$, 0.5 µM of each primer, QIAGEN reaction buffer diluted 1:10, and 1U of *Taq* DNA polymerase (QIAGEN) in a T-100 thermal cycler (Bio-Rad), according to the following program: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 60.6 °C for 30 s and 72 °C for 30 s, with a final extension of 5 min at 72 °C.
The amplification product was checked in 1% agarose gel (Eppendorf).

RESULTS

Pathogenicity test

The typical symptoms of Fusarium wilt appeared 10 -14 days after inoculation. The plants were checked every 7-10 days for disease development. The final disease rating took place 27-35 days after inoculation.
When the pathogenicity of the Dutch isolates of FOL was tested, the 04750896 and 04750888 FOL isolates showed the same virulence on the different lettuce cultivars. These two isolates were highly virulent on Costa Rica N.4 (DS 98.8-98.1), while Cavolo di Napoli was moderately susceptible (DS 33.5-36.3) and Banchu red fire was partially resistant (DS8.0-12.8). The reaction of Lattughino biondo 32888 to the different Fusarium isolates from lettuce was found, by means of the pathogenicity test, to be comparable with that of Banchu red fire.
In general, it was found that the virulence of the two Dutch isolates on other commercial lettuce cultivars was similar to that shown by MAFF 744086, which belongs to race 3, while differences with races 1 and 2 were evident when the Romabella, Crispilla, Duna, Lattuga gentilina and Riccetto cultivars were considered (Table 2).

PCR Specific for race 1

Amplification with Hani3′ and Hanilatt3rev produced a 183 bp band product that is specific of FOL race 1. In the present tests, this product only

amplified in the FOL race 1 ATCCMya 3040 isolate and in all the Italian isolates belonging to race 1.The Dutch isolates (04750896 and 04750888) and the race 2 and race 3 controls did not produce any amplification. The results obtained from the two repetitions of the test reported only positive results in the control isolates of race 1.
The primers developed by Fujinaga *et al.* (2014) were used to discriminate the race of the Dutch isolates, and results that were consistent with the results of Fujinaga *et al.*, 2014, were obtained. FOL race 1 gave an amplicon in the race 1 and race 3 tests, FOL race 2 gave an amplicon in the race 2 test and weak amplicons with different lengths in the race 1 and race 3 tests, while FOL race 3 only gave an amplicon in the race 3 test. Furthermore, some false positives were obtained in the tested non-targets (not shown).

RFLP

Amplification of IGS with the CNL12 and CNS1 primers (Mbofung *et al.*, 2007) produced a band of between approximately 2.6 and 3.0 kb, which was used to perform restriction fragment length polymorphism analysis with several enzymes. Only the IGS-RFLP patterns obtained with 15 U of *Aat*II and 5 U of *Alu* I in 1 ng of IGS amplification allowed a specific profile to be obtained for each race; the other digestions did not produce specific pattern for each race (data not shown). The ATCCMya-3040 isolate was again used as a control for race 1, while the F9501 isolate was used as a control for race 2, while MAFF 744085 and MAFF 744086 were used as controls for race 3 (Table 1). The digestion pattern of the Dutch isolates was different from that of all known races (Figure 1), while the FL1/14 and FL3/14 isolates produced the same pattern as the ATCCMya-3040 isolate belonging to FOL race 1.

Phylogenetic study

EF1-α phylogeny

Sequence analysis of 603 bp, with the neighbor-joining method, using the Tajima-Nei model for the distance matrix, produced 3 clades (Figure 2). The first clade was subdivided into two subclades, where the main group was composed of FOL race 1 references, the Italian FL1/14 and FL3/14 isolates and the Dutch isolates, supported with a bootstrap of 76%; the

second subclade was formed by *Fusarium oxysporum* f. sp. *conglutinans* clustered with a bootstrap of 63%.
The second clade was composed of two lineages; the isolates belonging to race 3 (MAFF7 44085 and MAFF7 44086) clustered with a bootstrap of 63% with *Fusarium oxysporum* f. sp. *lycopersici*.

The third clade only included the F9501 and FK09701 isolates belonging to race 2, with a bootstrap of 99%, while *Fusarium oxysporum* f. sp *vasinfectum* and *Gibberella fujikuroi* were used as outgroups.

IGS phylogeny
The 1833 bp was used for sequence analysis using the neighbor-joining method and the Tajima-Nei model for the distance matrix and 2 clades were found (Figure 3).
The first clade included FOL race 1, and this was supported by a bootstrap of 99% and it included the FOL race 1 reference isolates FL1/14 and FL3/14 as well as the two Dutch isolates, while the second clade was formed by the race 2 isolates, F9501 and FK09701, and was supported by a bootstrap of 99%.
In this phylogenetic study, unlike the result of *EF-1α*, FOL MAFF 744085 and FOL MAFF 744086, *Fusarium oxysporum* f. sp *lycopersici*, *Fusarium oxysporum* f. sp. *conglutinans* and *Fusarium oxysporum* f. sp. *vasinfectum* were found to be individual lineages, while *Gibberella fujikuroi* was used as an out-group.

IRAP-PCR

The results of all the combinations of primers used in IRAP–PCR (Figure 4) produced specific profiles for each FOL race, and bands ranging from 100 bp to more than 7000 bp were obtained. The profile obtained from the 1.14 and 3.14 isolates was similar to that of the ATCCMya-3040 FOL race 1 isolate, while the two Dutch isolates produced a specific profile that was different from any other race. Curiously, the two reference strains of FOL race 3, MAFF 744085 and MAFF 744086, showed a divergence in their profiles for all of the primer combinations.
In order to design a specific marker for the Dutch isolates, a specific band, obtained with the Gaga-Gagi primer combination, was isolated (Figure 4A).

The 250 bp sequence was used to generate the specific primers FUPF and FUPR (Table 3) using the PERL PRIMER program. The primers were tested, by means of PCR, on all the isolates and amplification was only obtained for the two Dutch isolates, i.e. 04750888 and 04750896 (Figure 5).

DISCUSSION

Recently, three races of FOL have been identified, but only race 1 has been detected in Europe so far (Garibaldi et al., 2002; Gullino et al., 2004), while race 2 and race 3 have been identified in Japan (Fujinaga et al., 2001; 2003). However, race 3 has recently also been found in Taiwan (Lin et al., 2014). A new physiological race has been isolated and characterized in the present investigation.

Differentiating *formae speciales* in the *F. oxysporum* spp. complex usually requires pathogenicity tests. Fujinaga et al. (2001 and 2003) described a useful assay for the determination of the pathological specialization of FOL in different races. They have found that race 1 causes high levels of disease on Patriot and Banchu red fire. Race 2 is pathogenic on Patriot and Costa Rica No. 4, but not on Banchu red fire. Race 3 is pathogenic on all three cultivars.

The results obtained in the present study show that FOL isolates 04750896 and 04750888, which were isolated in the Netherlands, belong to a new race. The assay on differential cultivars shows that 'Banchu red fire' and Lattughino biondo are partially resistant to these Dutch isolates, compared to a susceptible reaction of the isolates belonging to race 3 (Table 4). Moreover, on the basis of the disease severity on the Banchu red Fire, Costa rica N. 4 and Cavolo di Napoli cultivars, the race 2 reference isolate was almost identical to the Dutch isolates. However, this was not the case with Romabella and Crispilla, which showed a susceptible reaction to the Dutch isolates, compared to a resistant one of FOL race 2 (Table 4). Considering the results of the present study, races 1, 2, 3 and the newly detected race 4 can be identified using these new cultivars, as shown in Table 4.

Several studies have reported difficulties in differentiating races of *F. oxysporum* on the basis of differential cultivars, especially when the differences between races are primarily due to alterations in the level on aggressiveness (Armstrong & Armstrong, 1981; Larkin *et al.,* 1990; Zhou

et al., 2010). In fact, the expression of lettuce fusarium wilt symptoms is influenced by the virulence of the pathogen isolates, the genetic background of the plants, and by factors such as soil temperature and age of the plants (Matheron *et al*., 2005; Scott *et al*., 2010a; Gordon & Koike 2015). Moreover, Scott *et al.* (2010 a, b) reported a high correlation with environmental conditions, such as temperature and disease severity, in different lettuce cultivars tested by means of root dip assays.

Different artificial inoculation methods have been used to evaluate lettuce resistance to FOL (Fujinaga *et al.*, 2001 and 2003; Garibaldi *et al*., 2004; Scott *et al.*, 2010a, b; Cabral & Reis, 2013; Lin *et al*., 2014). The use of an appropriate inoculation procedure is crucial to provide consistent results, especially when the resistance is partial or polygenically controlled. Scott *et al.* (2010b) observed that the cultivars considered susceptible were affected more by fusarium wilt than those with intermediate resistance, when they were tested with a higher concentration of inoculum. However, from the results of the present study, even though variability in disease severity has been observed for some isolates and cultivar combination, the used artificial inoculation method has allowed a good resolution to be obtained for the differentiation of the degrees of susceptibility among the cultivars.

Discrepancies between the results of the molecular analysis and biological assays have also been previously observed. According to Lin *et al.* (2014), the virulence of the Taiwanese Fola-40 isolate on differential cultivars was slightly lower than that of the race 3 reference isolate, but was still considered as race 3 on the basis of the phylogenetic tree. However, O'Donnell *et al.* (2009) reported that, despite the fact that the *EF-1α* and IGS region have a sufficient phylogenetical signal, these regions have not enough resolution to discriminate *formae speciales*, and the data can be incongruent, especially if there is not a sufficient representation of the taxa (Rokas *et al.*, 2003). In the present study, the phylogenetic analyses based on *EF-1α* and IGS, grouped the Dutch isolates in the same clade as the race 1 reference strains, and showed results that were contradictory to those shown by the pathogenicity assay with differential cultivars (Table 2 and 4). In order to understand the phylogenetic results, a specific PCR reaction for FOL race 1 was performed with primers designed by Pasquali *et al.* (2007) and a positive results was only obtained for the Italian isolates. The primers developed by Fujinaga *et al.* (2014) were also tested and several false negatives were obtained in the non-target isolates, which prevented the Dutch isolates from being identified.

In order to better characterize the Dutch isolates, two different molecular approaches were used, IGS RFLP and IRAP-PCR to correlate a fingerprint of the bands with each physiogical race, as already reported in *Fusarium oxysporum* f.sp *lycopersici* by Jordão do Amaral *et al.* (2013). The IGS region was chosen to identify RFLP, because the greater length (2.6 Kb) increased the possibility of obtaining different profile increases. In fact, using the specific restriction sites of *Aat*II and *Alu*I, diverse profiles were associated with pathogenic races. The profile of the 04750896 and 04750888 isolates differed from the three known races. Both techniques showed a specific profile for the Dutch isolates that was different from that of the three known races, with a better result being obtained when IRAP-PCR was used.

The polymorphic band obtained with the Gaga-Gagi combination of primers allowed specific SCAR primers, FPUF and FPUR, to be designed for these isolates. A PCR assay was developed with FPUF and FPUR to test the specificity of these primers, and positive results were only obtained in the Dutch isolates.

On the basis of the VCGs and phylogenetic study, it seems that each race of FOL has evolved independently (Fujinaga *et al.*, 2005). The relationship between races and VCG has been demonstrated in several *formae speciales* of *Fuarium oxysporum*. Moreover, a good correlation with the pathological assays and VCG analyses has been evidenced, and the VCGs assigned to FOL races 1, 2 and 3 were VCG-1, VCG-2 and VCG-3, respectively (Ogiso *et al.,* 2002; Fujinaga *et al.,* 2005; Pasquali *et al.,* 2005). In the vegetative compatibility assays carried out by Pintore *et al.* (unpublished data). The Dutch isolates belonged to a different VCG group, because their mutants did not form a heterokaryon with the known FOL races (Correll *et al.,* 1987).

Different techniques have been used in this study to characterize the two Dutch isolates. In light of the results, it is here proposed that these two isolates constitute a new FOL race, i.e. race 4.

However, our data also indicates that combined analysis, pathogenicity with differential lettuce cultivars and molecular assays, is needed in order to establish differences from the known races.

Among the available disease management measures currently adopted, the use of resistant cultivars is the most effective way of controlling lettuce Fusarium wilt, and it can easily be combined with other control methods (Matheron & Gullino, 2014). Lettuce varieties that are resistant, or at least tolerant, to Fusarium wilt are available (Garibaldi *et al*., 2004; Matheron

et al., 2005; Scott *et al.,* 2010 a, b; Matheron & Gullino, 2012; Cabral & Reis, 2013; Gilardi *et al.,* 2014), but their effective use is complicated by the presence of different races of the pathogen.

The presence of a new race of FOL poses a serious threat to growers and breeders, and requires the adoption of preventative measures, such as seed dressing and the use of healthy propagation material (Katan *et al.,* 2012; Lopez *et al.,* 2014), in order to prevent it from spreading quickly to new cultivation areas. A careful monitoring of the race situation in the field would be useful for an efficient use of genetic control means. Specific molecular markers can help to satisfy this goal, by providing a successful detection and identification of *formae speciales* and races of *Fusarium oxysporum* from seeds, plants and soil samples. The discovery of FOL in the Netherlands for the first time in fields that had been cropped with lettuce of various cultivars for many years is not surprising. FOL has also recently spread to other European countries. The new race of FOL may have evolved due to a high selection pressure, although the possibility of its introduction from a foreign source with infected seeds or seedlings, or the evolution from non pathogenic *F. oxysporum,* cannot be excluded. Further studies are needed to elucidate why a new race has developed.

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TABLES

Table 1. Isolates used in this study with their corresponding GenBank accession number.

				GenBank accession	
Strain	Species name	Year of	Geographical	EF-1α	IGS
ATCCMya30-	*Fusarium oxyporum* f.sp.	2002	Lombardy,	KU84091	KU84091
BMP1300[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83765	
BMP1301[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83765	DQ83186
BMP1306[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83186
BMP1307[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83186
BMP1308[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83186
BMP1323[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83186
BMP1324[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83187
BMP1326[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83187
BMP1331[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83187
BMP1333[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83766	DQ83187
BMP1363[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83767	DQ83187
BMP1370[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83767	
BMP1375[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83767	DQ83187
BMP1880[a]	*Fusarium oxyporum* f.sp.	unknown	Wellton, AZ	DQ83767	DQ83187
HL-1[a]	*Fusarium oxyporum* f.sp.	1990	Fresno, CA,	DQ83766	DQ83187
HL-2[a]	*Fusarium oxyporum* f.sp.	1990	Fresno, CA,	DQ83766	
S1[a]	*Fusarium oxyporum* f.sp.	unkonwn	Nagano ,Japan	DQ83765	DQ83186
FL 1/14	*Fusarium oxyporum* f.sp.	2014	Lombardy,	KU84092	KU84091
FL 3/14	*Fusarium oxyporum* f.sp.	2014	Lombardy,	KU84092	KU84091
FK09701[a]	*Fusarium oxyporum* f.sp.	unkonwn	Japan	DQ83769	DQ83189
9501	*Fusarium oxyporum* f.sp.	1995	Nagano ,Japan	DQ83769	DQ83189
6234852	*Fusarium oxyporum* f.sp.	unkonwn	Nagano ,Japan		DQ83769
MAFF744085	*Fusarium oxyporum* f.sp.	unkonwn	Japan	KU84092	KU84091
MAFF744086	*Fusarium oxyporum* f.sp.	unkonwn	Japan	KU84092	KU84091
PD	*Fusarium oxyporum* f.sp.	2015	Netherlands	KU8409	KU8409
PD	*Fusarium oxyporum* f.sp.	2015	Netherlands	KU84092	KU84091
NRRL 53158[a]	*Fusarium oxysporum* f.sp.	unknown	NC,USA	FJ985443.	FJ985678.
PD 83/808	*Fusarium oxysporum* f.sp.	1983	Netherlands		
BE1[a]	*Fusarium oxysporum* f.sp.	unkonwn	unknown	HM05729	
NRRL 32897[a]	*Fusarium oxysporum* f.sp.	unknown	AR,USA	FJ985318.	
PD 83/816	*Fusarium oxysporum* f.sp.	1983	Netherlands		
PD 78/442	*Fusarium oxysporum*	1978	Netherlands		

Strain	Species name	Year of	Geographical	GenBank accession EF-1α	GenBank accession IGS
PD 88/396	*Fusarium oxysporum*	1988	Netherlands		
PD 20036631	*Fusarium oxysporum*	2003	Netherlands		
PUF022 [a]	*Gibberella fujikuroi*	unknown	unknown	HQ16585	

[a]All the EF-1α and IGS sequence were obtained from GenBank

ATCC and MYA codes are deposited at American Type Culture Collection (United States), CBS at Centraalbureau voor Schimmelcultures (NED), and MAFF at the National Institute of Agrobiological Sciences—NIAS Genebank (Japan). All strains preceded by BMP are from B. M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson; all strains preceded by NRRL are from K. O'Donnell (ARS culture collection), National Center for Agricultural Utilization Research, Peoria.

Table 2. Reaction of commercial lettuce cultivars to the Italian, Japanese and Dutch isolates of *Fusarium oxysporum* f. sp. *lactucae* expressed as disease severity (0-100).

Cultivar	Type	Race 1			Race 2			Race 3						New					
		Mya3040		Reaction	9051		Reaction	MAFF 744085		Reaction	MAFF 744086		Reaction	04750896		Reaction	04750888		Reaction
Cavolo di Napoli	Loose-leaf	79.6	±24.5	HS[a]	70.3	±23.6	HS	97.7	±2.4	HS	68.2	±14.2	HS	33.5	±8.8	S	36.3	±2.5	S
Costa Rica N.4	Romaine	0.0	±0.0	R	97.5	±3.1	HS	100.0	±0.0	HS	75.0	±1.2	HS	98.8	±2.2	HS	98.1	±2.4	HS
Banchu red fire	Butthered	50.2	±4.1	S	7.2	±6	R	88.3	±14.0	HS	-[b]			8.0	±5.8	R	12.8	±7.4	PR
Lattughino biondo 32888	Loose-leaf	80.1	±3.8	HS	0.6	±1.1	R	80.9	±19.8	HS	59.9	±14.5	HS	24.7	±17.5	PR	29.8	7.6	PR
Romana Romabella 30CN	Romaine	9.8	±10.5	R	6.7	±7.9	R	92.7	±12.6	HS	75.2	±0.0	HS	92.2	±8.7	HS	95.5	±7.5	HS
Romana Duna 37425	Romaine	0.6	±1.1	R	5.0	±5.3	R	84.7	±8.7	HS	64.3	±16.6	HS	43.6	±6.9	S	63.9	±19.2	HS
Elisa	Batavian	13.1	±13.2	PR	0.0	±0.0	R	43.1	±16.1	S	7.7	±8.9	R	20.3	±16.7	PR	12.2	±7.4	PR
Crispilla	Batavian	99.2	±0.9	HS	24.6	±9.6	PR	100.0	±0.0	HS	75.0	±0.0	HS	100.0	±0.0	HS	100.0	±0.0	HS
Lattuga Gentilina	Batavian	68.8	±11.8	HS	98.8	±1.3	HS	28.5	±0.4	PR	10.8	±8.2	PR	3.8	±1.3	R	20.4	±9.5	PR
Riccetto	Loose-leaf	84.0	±11.5	HS	32.1	±12.6	S	7.9	±3.1	R	15.2	±2.6	PR	6.4	±4.1	R	11.8	±9.9	PR
Foglia di quercia rossa 29350	Loose-leaf	74.3	±19.1	HS	9.9	±6.4	R	48.3	±28.1	S	23.3	±8.3	PR	27.2	±10.3	PR	18.0	±6.7	PR

[a]Reaction: Disease index 0-100; Resistant R=0-10; Partially Resistant PR=11-30; Average Susceptible S=31-60; Highly Susceptible HS=61-100

[b] Not tested

Table 3. Primer pairs for the different regions amplified and sequenced in this article.

LOCUS/NAME	PRIMER SEQUENCE	REFERENCE
IRAP Primers		
Gaga	5′-GGGAACCAACCGTCACA-3′	Pasquali *et al.*, 2007
Gagi	5′-TAACCGCTAGGGTCGTAACA-3′	Pasquali *et al.*, 2007
Hana	5′-	Pasquali *et al.*, 2007
Hani	5′-GAACCCTCCAACATTCAACA-3′	Pasquali *et al.*, 2007
Primer race specific		
Hani3′	5′-CCCTCCAACATTCAACAACTG-	Pasquali *et al.*, 2007
Hanilatt3rev	5′-ATTCACTGTACACCAACCTTTT-	Pasquali *et al.*, 2007
FOLR1-F	5′-GGCTGTGTCTTGGACGGTGTAG	Fujinaga *et al*., 2014
FOLR1-RR	5′-GCCTACCCTAAACCACACAT -3′	Fujinaga *et al*., 2014
FIGS11	5′-GTAAGCCGTCCTTCGCCTCG -3′	Fujinaga *et al*., 2014
FOLR2-RR	5′-GCCTACCCTGTACCTACAGC -3′	Fujinaga *et al*., 2014
FOLR3-F	5′-GGCTGTGTGTTGGACGGTGCAG	Fujinaga *et al*., 2014
FOLR3-RR	5′-GCATACCCTAGACCACATAT-3′	Fujinaga *et al*., 2014
Intergenic spacer region		
CNL12	5′-CTGAACGCCTCTAAGTCAG-3′	Anderson and Stasovski
CNS1	5′-GAGACAAGCATATGACTACTG-	White *et al.*, 1990
CNS12	5′-GCACGCCAGGACTGCCTCGT-3′	Mbofung *et al.*, 2007a
RU46.67	5′-GTGTCGGCGTGCTTGTATT-3′	Mbofung *et al.*, 2007a
Elongation factor		
EF1	5′- ATGGGTAAGGARGACAAGAC -	O'Donnell *et al.*, 1998
EF2	5′- GGARGTACCAGTSATCATGTT -	O'Donnell *et al.*, 1998
Primers designed in this study		
FUPF	5′-GGAACCAACCGTCACAATAAC-	This study
FUPR	5′-GTCGTAACACTAACTCGCTT-3'	This study

Table 4. Determination of pathological races of *Fusarium oxysporum* f. sp. *lactucae* using pathogenicity with differential lettuce cultivars and molecular assays.

		Pathogenicity on differential cultivars[a]												
Race	Isolate	Napoli di Cavolo	No. 4	Red Fire	Biondo	Romabella	Crispilla	EF-1α	IGS	RFLP	IRAP	Race 1-specific primers[b]	Race 4-specific primers[c]
1	ATCCMya-3040	S[d]	R	S	S	R	S	I	I	Race 1	Race 1	+	–
2	F9501	S	S	R	R	R	R	II	II	Race 2	Race 2	–	–
3	MAFF 744085	S	S	S	S	S	S	III	III	Race 3	Race 3	–	–
3	MAFF 744086	S	S	NT	S	S	S	III	III	Race 3	Race 3	–	–
New	04750888	S	S	R	R	S	S	I	I	New race	New race	–	+
	04750896	S	S	R	R	S	S	I	I	New race	New race	–	+

NT, not tested; +, amplification; –, no amplification.

[a]Race classification system developed by Fujinaga *et al.* (2001, 2003). Assignment of disease reaction ratings are based on incidence of fusarium wilt on the differential genotypes, where R = resistant (<30% wilt) and S = susceptible (≥31% wilt), according to the criteria of Zhou *et al.* (2010).

[b]Han3′ and Hanlatt3rev.

[c]New primers designed in this study.

FIGURES

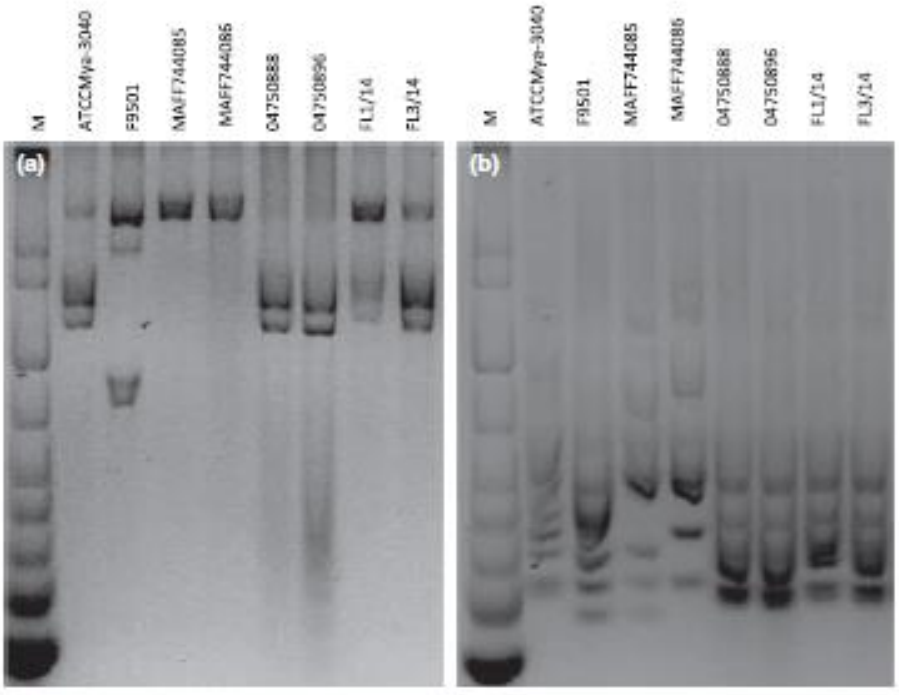

Figure 1. (**a**) Restriction fragment length polymorphism (RFLP) of the intergenic region (IGS) of *Fusarium oxysporum* f. sp. *lactucae* with *Aat*II (15 U for 1 µg of IGS amplification). (**b**) RFLP-IGS with *Alu*I (5 U for 1 µg of IGS amplification). M, Gel Pilot 1 kb DNA Plus ladder (QIAGEN).

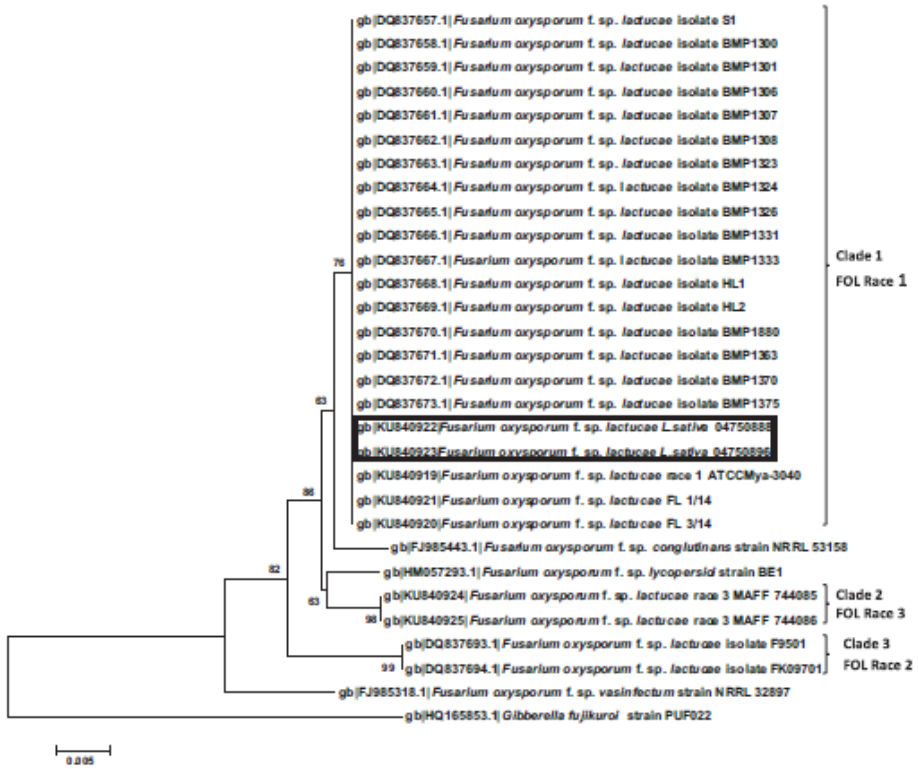

Figure 2. Neighbor–joining tree in which the relations of elongation factor 1 α (EF-1α) with *Fusarium oxysporum*.f. sp *lactucae* are reported. The sequence of several *forma specialis* of *Fusarium oxysporum* and the sequence of *Gibberella fujikuroi* obtained from GenBank have been used as outgroups for the phylogenetic study. The numbers above the nodes indicate Bootstrap values.

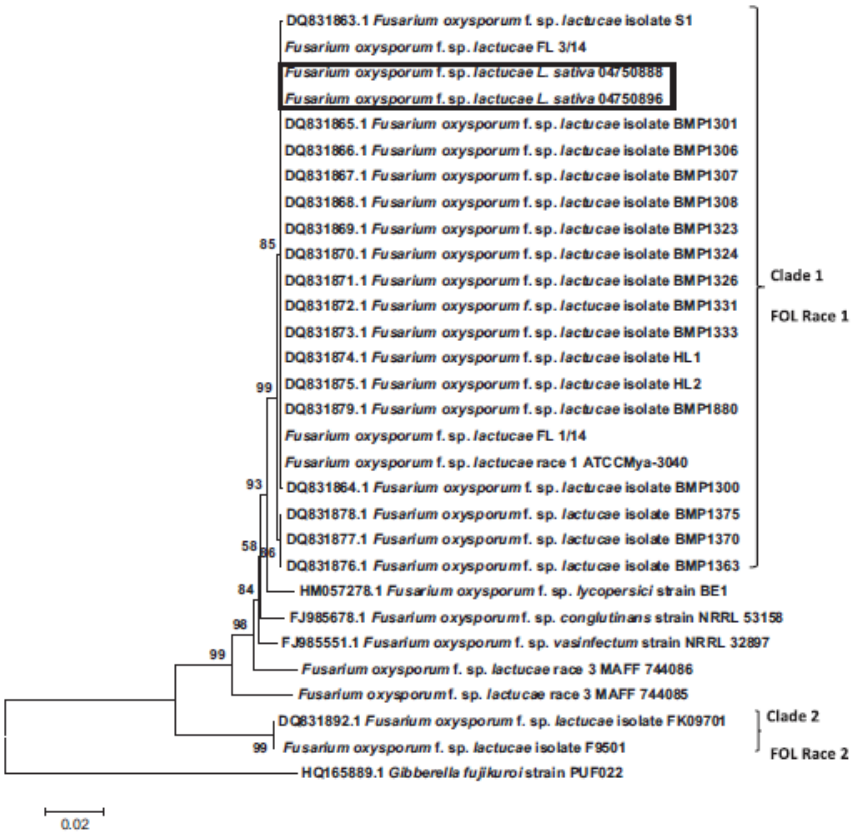

Figure 3. Neighbor –joining tree in which the relations of the intergenic spacer region (IGS) with *Fusarium oxysporum* f. sp *lactucae* are reported. The sequence of several *forma specialis* of *Fusarium oxysporum* and the sequence of *Gibberella fujikuroi* obtained from GenBank have been used as out-groups for the phylogenetic study. The numbers above the nodes indicate Bootstrap values.

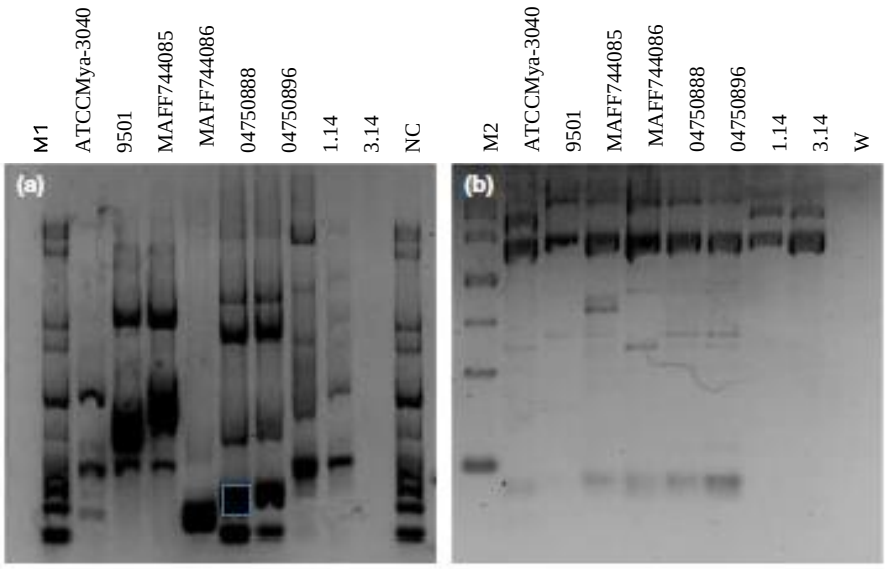

Figure 4. Inter retrotransposon amplified polymorphism (IRAP)-PCR of isolates of Fusarium oxysporum f. sp. lactucae. A.IRAP PCR using a combination of Gaga-Gagi primers. B IRAP PCR using a combination of Gaga-Hana primers.NC Neighbour –joining tree in which the relations of the intergenic spacer region (IGS) with Fusarium oxysporum f. sp lactucae are reported. The sequence of several forma specialis of Fusarium oxysporum and the sequence of Gibberella fujikuroi obtained from GenBank have been used as outgroups for the phylogenetic study. The numbers above the nodes indicate Bootstrap values. Negative control. M1. GelPilot 1-kb DNA plus Ladder (QIAGEN) .M2. GelPilot Wide Range Ladder (QIAGEN)

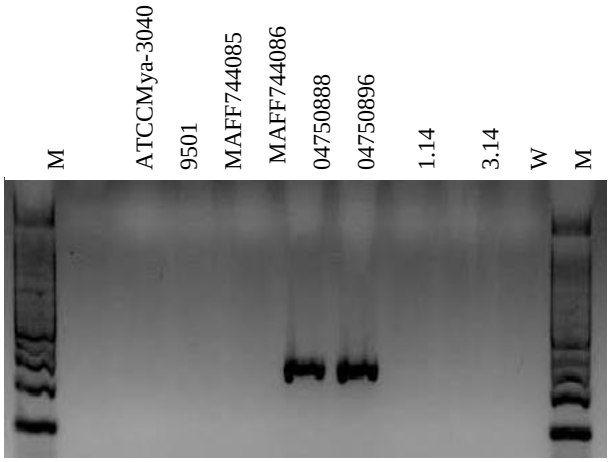

Figure 5. Specific PCR reaction using the FPUF, FPUR primers for the Dutch isolates, which belong to a new race that is different from the three known races. **W** =negative control. **M**. GelPilot 1-kb DNA plus Ladder (QIAGEN).