Molecular characterization of Fusarium oxysporum f.sp. cichorii pathogenic on chicory (Cichorium intybus)

Molecular characterization of *Fusarium oxysporum* f.sp. *cichorii* pathogenic on chicory (*Cichorium intybus*)

Anna Poli*, Giovanna Gilardi, Davide Spadaro, Maria Lodovica Gullino and Angelo Garibaldi

Agroinnova – Centre of Competence for the Innovation in the Agro-Environmental Sector, University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco, Turin, Italy.

*Corresponding author: Anna Poli, E-mail: anna.poli@unito.it, Phone: +39-011-6708544, Fax: +39-011-6709307

Abstract

Keywords: *Cichorium intybus,* wilt disease, *Fusarium oxysporum, formae speciales,* phylogeny.

Fusarium oxysporum is a ubiquitous soilborne ascomycete responsible for vascular wilt in many plant species worldwide. This species comprises more than 120 putative host-specific *formae speciales* capable of causing remarkable economical losses. In summer 2009, wilt symptoms, including chlorosis and poor development of the root system were observed on cultivars of chicory (*Cichorium intybus)* in northern Italy. The causal agent isolated from symptomatic tissues in this case was identified as *F. oxysporum* on the basis of both morphological features and molecular analyses. In this work, we attempted to characterize the isolates of *F. oxysporum* from *C. intybus* by both biological and molecular approaches. Pathogenicity trials performed on 5 species of the Asteraceae family with isolates of *F. oxysporum* from *C. intybus* indicated that the pathogen has a unique host range, infecting chicory only. Neither lettuce nor endive, lawn daisy and Paris daisy developed the disease. Five cultivars within *C. intybus* species were tested, and the cv Clio was the most susceptible. Phylogenetic analyses relative to the ribosomal intergenic spacer (IGS) and translation elongation factor 1-alpha (EF1-α) assigned isolates pathogenic to chicory to a single cluster, distinct from other pathogenic *F. oxysporum.* In light of these views, we propose to designate this organism as *Fusarium oxysporum* f.sp. *cichorii.*

Introduction

Chicory (*Cichorium intybus* L.) is a wild perennial plant belonging to the family Asteraceae (Compositae), which has been selected, domesticated and cultivated in Europe, as well as endive (*C. endivia* L.), its closest related species. Within *C. intybus*, several cultivars can be distinguished, based on the use of roots or leaves (fresh or cooked) for human consumption (Bremer, 1994; Lucchin et al., 2008).

Chicory crops are subjected to attacks caused by several soilborne fungi, such as *Rhizoctonia solani* (Alfieri et al., 1984), *Phytophthora tentaculata* (Garibaldi et al., 2010), *Sclerotium rolfsii* (Alfieri et al., 1984), and *Sclerotinia sclerotiorum* (Gerlagh et al., 1998). *S. sclerotiorum*, in particular, is the major pathogen of chicory, due to its broad spectrum of virulence on several cultivars, provoking remarkable yield losses up to 32% (Benigni and Bompeix, 2010). In summer 2009, a wilt caused by *F. oxysporum* was observed for the first time in the world on 25-30% of 1 hectare of *C. intybus* cv. Clio plants in northern Italy. Symptoms observed included chlorosis and a poor development of the root system. The causal agent isolated from symptomatic vascular tissues was recently identified as *F. oxysporum* on the basis of morphological features and molecular analyses by direct sequencing of the internal transcribed spacer (ITS) rDNA region and of the translation elongation factor-1α (EF-1α) (Garibaldi et al., 2011).

F. oxysporum is a ubiquitous soilborne ascomycete which causes devastating vascular wilts in many plant species worldwide (Armstrong and Armstrong, 1981). The infection process starts when infection hyphae directly penetrate the specific host roots. Mycelium spreads inter- and intra-cellularly through the root cortex until it reaches the vascular vessels. As the infection spreads, the plant vessels get plugged, also due to production of vascular gels and gums as a plant defense mechanism. As a consequence, water supply is severely disrupted, and wilting, chlorosis, necrosis and eventually death occur. More than 120 different but morphologically undistinguishable *formae speciales* have been identified, based on host specificity (Katan, 1999; Michielse and Rep, 2009). In addition, *formae speciales* are subdivided into physiological races depending on their capability to cause wilt disease on different host cultivars (Correll et al, 1991). *Formae speciales* of *F. oxysporum* can be differentiated using pathogenicity tests with the appropriate host; however, these tests are time consuming and affected by environmental and growth culture changes (Mbofung et al., 2007). Therefore these methods

are often integrated or replaced by different molecular techniques which are rapidly increasing (Lievens et al., 2008), even though the molecular discrimination of *F. oxysporum* isolates is complicated by the polyphyletic nature of many *formae speciales*: some isolates of different *formae speciales* may be more related than isolates belonging to the same *forma specialis* (Kistler, 1997). Restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (PCR-RFLP; Botstein et al., 1980) and amplified fragment length polymorphism (AFLP; Vos et al., 1995) can help to characterize large *F. oxysporum* populations (Edel *et al.*, 1995), although the former is laborious and the latter is relatively costly. Random amplified polymorphic DNA (RAPD) analysis has also been employed (Williams et al., 1990); although the poor reproducibility of results between different laboratories is considered the major disadvantage of this technique, RAPD approach remains a relatively fast and cheap method capable of revealing major genetic variation (Bardakci, 2001) In addition, conserved housekeeping genes, such as the transcription elongation factor 1α (EF-1α ; Amatulli *et al.*, 2010) and the intergenic spacer of the nuclear rRNA gene (IGS; Mbofung *et al.*, 2007) seem to have a phylogenetic meaning (O'Donnell *et al*., 2009). The IGS region, which separates rDNA repeat units, is particularly suitable for studying intraspecific relationships (Mbofung *et al*., 2007; Srinivasan *et al*., 2010).

No study about the genetic diversity of *F. oxysporum* isolates from *C. intybus* has been reported to date. Hence, the aim of our work was to characterize the isolates of *F. oxysporum* from *C. intybus* by both biological and molecular approaches, correlating DNA sequence typing with the pathogenicity.

Materials and methods

***F. oxysporum* isolates.** Table 1 shows details of the isolates, including codes, year of isolation, geographical origin, host source and GenBank accession numbers for IGS and TEF. Isolates from *Cichorium intybus* were maintained on slants of potato dextrose agar (PDA, Merck, Darmstadt, Germany) at 4°C.

Inoculum production and pathogenicity tests. All *F. oxysporum* isolates from infected chicory plants were grown in 100 mL of casein hydrolyzate at 25°C with 12 h of fluorescent light and shaking at 0.1 x g. After 10 days, the cultures were filtered to collect conidia which were adjusted to a final concentration 10^6 CFU (colony forming units)/mL with sterile deionized water.

All single conidium cultures identified as *F. oxysporum* were tested for pathogenicity (Table 2). Cross-inoculations on economically important hosts belonging to Asteraceae were performed in the greenhouse at 26-30°C. Different cultivars of lettuce (*Lactuca sativa:* cvs, Batavia verde), lawn daisy (*Bellis perennis*, cv. Rubyrosa), Paris daisy (*Argyranthemum frutescens*, cv Yellow), endive (*Cichorium endivia:* cvs Riccia Cuor d'oro, Scarola Verde Cuor Pieno), chicory (*C. intybus:* cvs Katia, Clio, Catalogna frastagliata, Selvatica da campo, Puntarelle, Pan di zucchero, Gigante di Chioggia) obtained from different seed companies in Italy were tested. Seeds were sown in a steamed mixture (peat: compost broad bark: sand, 60: 20: 20 vol/vol) in plug trays and maintained at 25°C. Roots of 20-day-old plants were trimmed to a length of 5 cm, and dipped for 10 min in the pathogen spore suspension prepared as described above. Inoculated seedlings were transplanted in trays (10 litres) containing steamed soil (30 min at 80°C). For ornamental species, 40-day old plants were used. Control plants were prepared similarly but soaked in plain deionized water. Five seedlings per pot were transplanted and each pot was considered as a replicate. Four replicates were used in each trial. Plants were maintained in a glasshouse at temperatures ranging between 22°C and 34°C. Symptoms started to be visible 10-12 days after artificial inoculation. Plants were evaluated weekly for disease development and wilted plants were counted. The disease index used throughout the experiments ranged from 0 to 100 (0= healthy plant; 25 = initial symptoms of leaf chlorosis; 50 = severe leaf chlorosis and initial symptoms of wilting during the hottest hours of the day; 75 = severe wilting symptoms and initial symptoms of leaf chlorosis; 100 = plant totally wilted, leaves completely necrotic). Symptomatic plants showed brown or black streaks in the vascular system. The final disease rating took place five weeks after inoculation (Table 2). Data were statistically analyzed by using analysis of variance (ANOVA) and Duncan test ($P<0.05$). The pathogenicity test was performed at least twice.

DNA extraction. Genomic DNA of all studied *F. oxysporum* isolates from chicory and four different *formae speciales* (FR 30/03, ATCC 16600, ATCC 16601, ATCC 16603) was extracted from about 100 mg of mycelium scraped from PDA Petri dishes using the NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer's instructions, adding 10 µl of a solution of Proteinase K (10 mg mL^{-1}) and 10 µl of RNAse A (12 mg mL^{-1}) to the lysis buffer in each tube. DNA concentration was measured with a Nanodrop 2000 (Thermoscientific, Whaltam, MA, USA) and purified DNAs were stored at -20°C.

Random amplified polymorphic DNA (RAPD) conditions

RAPD-PCR was performed using sixteen 10-mer primers, randomly chosen among OPB, OPE OPA and OPL (Operon Technologies Inc., Alameda, CA, USA). Amplification was carried out in 20 µL of a reaction mix containing 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 50 mM KCl, 0.1% triton X-100, 0.01% (wt/vol) gelatin, 60 µM each dATP, dCTP, dGTP, and dTTP, 5 pM primer, 0.2 ng of genomic DNA, and 0.75 U of *Taq* DNA polymerase (Qiagen, Chatasworth, CA, USA). A T-Gradient thermal cycler (Biometra, Gottingen, Germany) was programmed to one cycle of 2.5 min at 94°C, 45 cycles of 30 s at 94°C, 1 min at 36°C, 2 min at 72°C, with no ramping, followed by one cycle of 5 min at 72°C. RAPD analysis was repeated at least twice for each isolate. The whole reaction product (20 µl) was loaded on a 1.5% agarose gel containing 2µL 100 mL^{-1} SYBR safe DNA gel stain (Invitrogen, Eugene, OR, USA). Moreover, the DNA of two species of *Fusarium* (*F. fujikuroi and F. verticilllioides*) was included as positive control of RAPD amplification. Bench top 100 bp DNA Ladder with size ranging from 100 to 1500 base pairs was used in RAPD fingerprinting. Electrophoresis conditions were the following: 2 h 30 min at 3.3 V cm^{-1} in 1× TAE (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0) running buffer. Amplimers were visualized under UV light and images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA).

IGS and EF-1α amplification and sequencing. The IGS sequences were amplified entirely using the primers CNL12 and CNS1in a mixture containing 10 ng genomic DNA, 1 µM each primer, 5 U Fast Start Taq DNA Polymerase (Roche, Basel, Switzerland), 10 µL colourless 10x buffer containing dNTPs and 1X GC-rich solution (Roche). A T-Gradient thermal cycler was programmed to 95°C for 5 min; 38 cycles: 94°C for 1 min, 60°C for 90 sec, 72°C for 3 min; 72°C for 10 min. PCR products were electrophoresed on 0.8% agarose gel (Agarose D-1 LOW EEO Eppendorf, Hamburg, Germany) with 2µL 100 mL^{-1} SYBR safe DNA gel stain (Invitrogen). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced with additional internal primers (Mbofung *et al.*, 2007). For amplification of EF-1α, the primer pair EF-1/EF-2 was employed; the reaction consisted of 10 ng genomic DNA, 1 µM each primer, 1 U Taq DNA Polymerase (Qiagen) 10x buffer containing $MgCl_2$ and 200 µM each dNTP. PCR program was the following: 95°C for 5 min; 35 cycles: 94°C for 45 sec, 60°C for 45 sec, 72°C for 1min; 72°C for 10 min. Purified products were directly sequenced (LMU

sequencing service, Martinsried, Germany). The IGS and EF-1α sequences obtained were deposited in GenBank (accession numbers are reported in Table 1). The primers used in this work are listed in table 3.

Phylogenetic analysis. The complete IGS sequence was re-constructed using DNA Baser software v2.71.0 (Heracle Software, Lilienthal, Germany). Complete IGS and EF-1α sequences were aligned using the ClustalW v.1.6 program (Kumar et al., 2008) included in the MEGA 5 software. Sequences were analyzed to determine the relationships between isolates by neighbour-joining method (Saitou and Mei, 1987). Bootstrap analysis was based on 1,000 re-samplings. A unique consensus phylogenetic tree was obtained using the SpiltsTree4 software version 4.11.3 (Huson and Bryant, 2006). Fourteen IGS and ten EF-1α sequences of other *formae speciales* of *F. oxysporum* were retrieved from GenBank database and used as reference sequences (marked with an asterisk in Table 1).

Results

Pathogenicity trials

Results of the pathogenicity trials carried out on 5 species (12 cultivars) of the Asteraceae family with 11 isolates of *F. oxysporum* from *C. intybus* are presented in Table 2. Typical symptoms of wilt disease on chicory were first observed 10-15 days after inoculation. Symptoms, including chlorosis and a poor development of the root system, were similar to those described by Garibaldi *et al.* (2011). None of the 11 isolates was pathogenic on Paris daisy, lawn daisy, lettuce (two cultivars) and endive (two cultivars). All the isolates showed some level of pathogenicity on the 5 cultivars of *C. intybus* tested. However, the cv Clio was the most susceptible to the 11 isolates with a disease index ranging from 40 to 65, followed by “Cicoria Katia” and “Puntarelle”. Even though the cvs Catalogna frastagliata and Selvatica da Campo showed some disease symptoms, their disease index was never statistically different from the other species tested. Among the 11 isolates, FUSCIC 45A and FUSCIC 45B were obtained in 2009 from *C. intybus* cv. Clio, while the other 9 were obtained in 2010 from *C. intybus* cv. Katia. In any case, no significant differences could be observed on the 5 cultivars tested. *F. oxysporum* was consistently re-isolated from inoculated symptomatic plants at the end of the experiments. The re-isolation was performed by using the semi-selective medium for *Fusaria* (Komada, 1975) from both symptomless and affected plants. Similar results were observed in all the trials performed.

RAPD analysis

RAPDs generated by single-primer PCR were used to compare the relationship between the isolates FUSCIC1-9, FUSCIC45A, FUSCIC45B, and *F. fujikuroi* and *F. verticillioides* as outgroups. A negative control without DNA template was included. Figure 1 shows only three gels obtained from amplifications with three representative primers (OPE-07, OPE-15 and OPL-07), out of the 16 used. Isolates from *C. intybus* presented the same pattern with each primer tested, except with a primer. In effect, a slight difference was observed for FUSCIC45A with the primer OPE-07 (red squared). A red arrow in the three Figures 1A, 1B and 1C, indicates a fragment typical of all the isolates of *F. oxysporum* f. sp. *cichorii*. Each analysis was repeated at least twice, and the same pattern was observed. These results led us to conclude that isolates FUCIC1 to 9 are identical, based on the RAPD analysis carried out. Therefore no phylogenetic tree based on amplimer presence/absence was considered and for further analysis only five of these isolates were considered.

IGS AND EF1-α sequencing and phylogenetic analysis. The PCR reactions performed with IGS primers resulted in an amplicon ranging from 2,000 to 2,500 bp in the isolates tested. The internal primers generated smaller fragments that were assembled with DNA baser in order to obtained IGS contigs. The IGS sequence of each isolate was analyzed to infer the phylogenetic relationship using the Neighbour-Joining method. As for transcription elongation factor, the amplicon obtained was between 650 and 700 bp and the same phylogenetic analysis was applied. Subsequently a consensus supertree was built (Figure 2).

Three different clusters (Groups I, II and III) were identified. The analysis supported the three groups proposed by Neighbor Joining. While Group I comprised isolates of *formae speciales lactucae* (race 2), ATCC16603-*matthioli*, NRRL22545-*matthioli*, BMP1300, BMP1301 and BMP1333 (*lactucae* race 1) clustered in Group II. Five isolates from *C. intybus* fell into group III, significantly separated from the others. Interestingly, FUSCIC45A and FUSCIC45B which were isolated in 2009 were separated from FUSCIC1-5, isolated in 2010.

Discussion

Although five members of the Asteraceae family were inoculated with the 11 isolates in analysis, different levels of susceptibility were scored only on cultivars of *C. intybus*. Therefore, we propose that the organism responsible for Fusarium wilt in *C. intybus* first observed in 2010 (Garibaldi et al., 2010) could be designated as *F. oxysporum* f.sp. *cichorii*. As described above, the fungus shows a unique host range, since vascular wilt developed severely only in *C. intybus* cv Clio and cv Katia, but not in lettuce**,** lawn daisy, paris daisy or endive. This conclusion based on biological tests, is supported by molecular evidences. Although bioassays are very effective, they are time-consuming and laborious. Attempts are made to replace them with molecular identification techniques to group isolates (Lievens *et al.*, 2008; Pasquali *et al.*, 2008). To better understand the genetic relationship between the *formae speciales* of *F. oxysporum* that have been already characterized and our isolates, it was useful to sequence the EF-1α and IGS regions (Srinivasan *et al.*, 2012). Sequence analysis is very precise and reproducible to evaluate genetic similarity or identity, and can separate *formae speciales* of *F. oxysporum* better than RAPD, which was used in this work only to assess the similarity of the eleven FUSCIC.

Because of the high polymorphism rate present in the IGS as well as in the EF 1-α sequences (Fujinaga *et al.*, 2005; Mbofung *et al.*, 2007), these loci allowed us to determine the diversity among *formae speciales* with a different host range. Our analysis indicates that the FUSCIC were phylogenetically distinct from the other isolates, although an interesting closeness with race 1 of *F. oxysporum* f.sp. *lactucae* could be detected. Bearing in mind that *F. oxysporum* f.sp *lactucae* infects lettuce, which is a member of the Asteraceae, that *C. intybus* also belongs to this family, and that the genome of *F. oxysporum* is highly versatile (Ma et al., 2010), the possibility that the new *forma specialis* arouse from multiple recombination events taking place in known *formae speciales* including *lactucae* could be considered. A similar but mirror event has been recently reported, when it was described how *F. oxysporum* f.sp. *raphani* transferred its infection capability from Brassicaceae to lamb's lettuce (*Valerianella olitoria*, Valerianaceae; Srinivasan et al., 2010). In the latter case the plants shared the growing environment and one *forma specialis* acquired the ability to enlarge its host range. Another example is represented by the non pathogenic strains of *Fusarium* which can be easily converted to pathogenic ones by transferring lineage-specific genes (van der Does *et al.*, 2008) or even chromosomes. Finally, the different susceptibility of the five cvs of *C. intybus* evaluated lead to the conclusion that different physiological races might already exist.

Recently, the need and utility of using a multi-locus approach has been demonstrated. The disagreement between the IGS rDNA and data (O'Donnell *et al*., 2009), provides a clear evidence against single-locus data for phylogenetic reconstruction and for inferring species limits within the Fungi (Taylor *et al*., 2000). Although IGS rDNA has become one of the most popular loci for investigating genetic diversity within the *F. oxysporum* species complex (FOSC) and its high levels of nucleotide diversity provide a high degree of discriminatory power useful for isolate identification, sometimes the evolutionary history of this locus could obscure an accurate phylogenetic relationship within this species. The same is extended to the transcription factor EF. Other loci to be considered are for example genes for endo- and exo-polygalacturonases, mating factors and mitochondrial small subunit rDNA (mtSSU; Hirano and Arie, 2009; Wunchs et al., 2009; O'Donnell et al., 2011). In this work we evaluated IGS and EF-1α since many of the sequences used for our analysis were already available in GenBank.

Acknowledgements We gratefully acknowledge the financial support from the project "Safe Food Control – Development of innovative systems and technologies for the production, storage, processing and valorization of Piedmontese fruit and vegetables" funded by the Regione Piemonte.

References

Alfieri, S.A., Langdon, K.R., Wehlburg, C., & Kimbrough, J.W. (1984). Index of Plant Diseases in Florida. *Florida Department of Agriculture and Consumer Service, Division of the Plant Industry* Bulletin, 11, 1-389.

Armstrong, G.M., & Armstrong, J.K. (1981). *Formae speciales* and races of *Fusarium oxysporum* causing wilt diseases. In: P.E. Nelson, T.A. Toussoun, R.J. Cook R.J. (Eds.), *Fusarium: disease, biology and taxonomy*. University Park: State University Press, 391-399.

Amatulli, M.T., Spadaro, D., Gullino, M.L., & Garibaldi, A. (2010). Molecular identification of *Fusarium* spp. associated with bakanae disease of rice and assessment of their virulence. *Plant Pathology, 59,* 839-844.

Bardakci, F. (2001). Random amplified polymorphic dna (RAPD) markers. *Turkish Journal of Biology, 25,* 185-196.

Benigni, M, & Bompeix, G. (2010). Chemical and biological control of *Sclerotinia sclerotiorum* in witloof chicory culture. *Pest Management Science, 66,* 1332-1336.

Bremer, K. (1994). Asteraceae: Cladistics and classification. Portland: Timber Press.

Correll, J.C., Gordon, T.R., McCain, A.H., Fox, J.W., Koheler, C.S., et al. (1991). Pitch canker disease in California, USA: Pathogenicity, distribution and canker development on Monterey Pine, *Pinus radiata*. *Plant Disease, 75,* 676-682.

Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., et al. (2005). Phylogenetic relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. *lactucae* races 1, 2 and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA. *Journal of General Plant Pathology, 71,* 402-407.

Garibaldi, A., Gilardi, G., & Gullino, M.L. (2010). First report of collar and root rot caused by *Phytophthora tentaculata* on witloof chicor**y (***Cichorium intybus*) in Italy. *Plant Disease,* 94, 1504.

Garibaldi, A., Gilardi, G., Poli, A., & Gullino, M.L. (2011). First report of Fusarium wilt of chicory (*Cichorium intybus*) caused by *Fusarium oxysporum* in Italy. *Plant Disease, 95,* 496.

Gerlagh, M., Goossen-van de Geijn, H.M., Fokkema, N. J., & Vereijken, P.F.G. (1999). Long-Term Biosanitation by Application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-Infected Crops**.** *Phytopathology, 89,* 141-147.

Hirano, Y., & Arie, T. (2009). Variation of phylogeny of *Fusarium oxysporum* isolates based on nucleotide sequence of polygalacturonase genes. *Microbes and Environments, 24,* 113-120.

Huson, D.H.. & Bryant. D. (2006). Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology and Evolution, 23,* 254-267.

Katan, T. (1999). Current status of vegetability groups in *Fusarium oxysporum. Phytoparasitica, 27,* 51–64.

Kistler, H.C. (1997). Genetic Diversity in the Plant-Pathogenic Fungus *Fusarium oxysporum. Phytopatology, 87,* 474-479.

Kumar, S, Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics, 9,* 299-306.

Lievens B., Rep M., & Thomma, B.P.H.J. (2008). Recent developments in the molecular discrimination of *formae speciales* of *Fusarium oxysporum*. *Pest Management Science, 64:* 781-788.

Lucchin, M., Varotto, S., Barcaccia, G., & Parrini, P. (2008). Chicory and endive. In: J. Prohen, F. Nuez (Eds.). *Vegetables I.* (pp. 3–48). New-York: Springer.

Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Pietro, A.D. et al. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature, 464,* 367-373.

Mbofung, G.Y., Hong, S.G., & Pryor, B.M. (2007). Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mitochondrial small subunit, elongation factor 1-α and nuclear ribosomal intergenic spacer sequence data. *Phytopathology, 97,* 87-98.

O'Donnell, K., Kistler, H., Cigelnik, E., & Ploetz, R.C. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Science, 95,* 2044-2049.

O'Donnell, K., Gueidan, C., Sink, S., Johnston, P.R., Crous, P.W., et al. (2009). A two-locus DNA sequence database for typing plant and human pathogens within *Fusarium oxysporum* species complex. *Fungal Genetics and Biology, 46,* 936-948.

O'Donnell, K., Humber, R.A., Geiser, D.M., Kang, S., Park, et al. (2011). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Mycologia, Epub ahead of print.*

Pasquali, M., Saravanakumar, D., Gullino, M.L., & Garibaldi, A. (2008). Sequence-specific amplified polymorphism (SSAP) technique to analyse *Fusarium oxysporum* f. sp. *lactucae* VCG 0300 isolate from lettuce. *Journal of Plant Pathology, 90,* 527-535.

Saitou, N., & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4,* 406-425.

Srinivasan, K., Gilardi, G., Spadaro, D., Garibaldi, A., & Gullino M.L. (2010). Molecular characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum* pathogenic on lamb's lettuce. *Phytopathologia Mediterranea, 49,* 309-320.

Srinivasan, K., Spadaro, D., Gilardi, G., Gullino, M.L., & Garibaldi, A. (2012). Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted rocket plants in Italy. *Phytoparasitica, in press.*

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., et al. (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology, 1,* 21-32.

van der Does, H.C., Lievens, L., Claes, L., Houterman, P.M., Cornelissen, B.J.C., & Rep, M. (2008). The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato wilt from other isolates. *Environmental Microbiology, 10,* 1475–1485.

340 **TABLES**

341

342 **TABLE 1.** List of strains used in this work. Codes, *formae speciales*, origin of isolation, specific host,

343 accession numbers relative to IGS and EF-1α sequences. n.d. = mot determined.

Code	***F.oxysporum* f.sp.**	***Geographic origin***	***Year of isolation***	***Specific host***	***IGS GenBank accession number***	***TEF GenBank accession number***
FUSCIC1	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	JQ278594	JQ278587
FUSCIC2	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	JQ278595	JQ278588
FUSCIC3	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	JQ278596	JQ278589
FUSCIC4	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	JQ278597	JQ278590
FUSCIC5	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	JQ278598	JQ278591
FUSCIC6	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	-	-
FUSCIC7	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	-	-
FUSCIC8	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	-	-
FUSCIC9	*Unknown*	Italy	2010	*Cichorium intybus* L cv Katia	-	-
FUSCIC45A	*Unknown*	Italy	2009	*Cichorium intybus* L cv Clio	JQ278599	JQ278592
FUSCIC45B	*Unknown*	Italy	2009	*Cichorium intybus* L cv Clio	JQ278600	JQ278593
FOA50	*asparagi*	Australia	n.d	*Asparagus officinalis*	DQ831886*	DQ837691*
NRRL22536	*callistephi*	Germany	n.d	*Callistephus chinensis*	DQ831897*	DQ837679*
ATCC16600	*conglutinans*	South Carolina	n.d	*Brassica oleracea*	GQ914766*	JQ315229
BMP1300	*lactucae* (race1)	Wellton, Az	n.d	*Lactuca sativa*	DQ831864*	DQ837658*
BMP1301	*lactucae* (race1)	Wellton, Az	n.d	*Lactuca sativa*	DQ831865*	DQ837659*
BMP1333	*lactucae* (race1)	Wellton, Az	n.d	*Lactuca sativa*	DQ831873*	DQ837667*
F9501	*lactucae* (race2)	Japan	n.d	*Lactuca sativa*	DQ831863*	DQ837657*
FK09701	*lactucae* (race2)	Japan	n.d	*Lactuca sativa*	DQ831893*	DQ837693*
FOLR2	*lycopersici*	California	n.d	*Solanum lycopersicum*	DQ831894*	DQ837692*
ATCC16603	*matthioli*	USA	n.d	*Matthiola incana*	GQ914769*	JQ315231
ATCC16603						
NRRL22546	*medicaginis*	SE Asia	n.d	*Medicago sativa*	DQ831901*	DQ837690*
ATCC16601	*raphani*	USA	n.d	*Rhapanus sativus*	GQ914765*	JQ315230
FR-30/3	*raphani*	Italy	2003	*Rhapanus sativus*	GU001853*	JQ315232
NRRL26871	*spinaciae*	Japan	n.d	*Spinacia oleracea*	DQ831888*	DQ837687*

344

TABLE 2. Results of pathogenicity test. Disease index (DI) for the isolates listed. Disease index was assessed on a 0 to 100 scale. 0: healthy plants; 12.5: plants growing regularly with slight vascular discoloration; 25: slight leaf chlorosis and reduced growth, vascular discoloration; 50: chlorosis, 50% growth reduction respect the healthy control, vascular discoloration, initial symptoms of wilting; 75: extended vascular discoloration, strong leaf chlorosis, severe growth reduction and wilting symptoms; 100: plants totally wilted and then dead. Analysis of variance (ANOVA) was performed and Tukey test ($P<0.05$).

		Disease index (0-100%)																	
Species	**Cv**	**FC1**		**FC2**		**FC3**		**FC4**		**FC5**		**FC6**		**FC7**		**FC8**		**FC9**		**FC45A**		**FC45B**	
A. frutescens	**Yellow**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
B. perennis	**Rubyrosa**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
C. endivia	**Riccia Cuor D'oro**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
C. endivia	**Scarola Verde Cuor Pieno**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
C. intybus	**Catalogna Frastagliata**	**16**	**ab**	**25**	**ab**	**18**	**ab**	**20**	**ab**	**20**	**ab**	**15**	**ab**	**26**	**ab**	**24**	**ab**	**19**	**ac**	**30**	**ac**	**31**	**ab**
C. intybus	**Selvatica da campo**	**20**	**ab**	**21**	**ab**	**5**	**ab**	**19**	**ab**	**22**	**ab**	**21**	**ab**	**20**	**ab**	**15**	**ab**	**15**	**ab**	**15**	**ab**	**20**	**ab**
C. intybus	**Puntarelle**	**39**	**b**	**34**	**ab**	**26**	**ab**	**46**	**b**	**40**	**b**	**39**	**ab**	**17**	**ab**	**37**	**ac**	**30**	**bc**	**39**	**bc**	**33**	**ab**
C. intybus	**Clio**	**50**	**b**	**49**	**b**	**65**	**c**	**54**	**b**	**53**	**b**	**45**	**b**	**40**	**b**	**60**	**c**	**63**	**c**	**65**	**c**	**57**	**b**
C. intybus	**Katia**	**41**	**b**	**41**	**ab**	**40**	**bc**	**41**	**b**	**38**	**ab**	**25**	**ab**	**30**	**ab**	**45**	**bc**	**26**	**bc**	**45**	**bc**	**42**	**b**
C. intybus	**Pan di zucchero**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
Cichorium intybus	**Cicoria Gigante Chioggia**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**
L. sativa	**Batavia Verde**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**	**0**	**a**

351

352 **TABLE 3.** List of primers used in this study. The sequence of only three RAPD primers is reported,
353 the others, randomly chosen among OPB, OPE OPA and OPL (OPA02, OPA03, OPA05,
354 OPA15, OPA17, OPA18, OPA19, OPB01, OPB03, OPB04, OPB07, OPB13 and
355 OPE02 can be retrieved from Operon Technologies, (Alameda, CA, USA).
356

Primer	Sequence (5'→3')	Target gene	Reference
CNL12	CTGAACGCCTCTAAGTCAG	IGS fragment	Anderson and Stasovski, 1992
CNS1	GAGACAAGCATATGACTACTG	IGS fragment	White et al., 1990
U46.67	AATACAAGCACGCCGACAC	IGS fragment	Mbofung et al., 2007
RU46.67	GTGTCGGCGTGCTTGTATT	IGS fragment	Mbofung et al., 2007
CN61	GGTTCAATTTGATGTCGGCT	IGS fragment	Mbofung et al., 2007
RCN61	AGCCGACATCAAATTGACC	IGS fragment	Mbofung et al., 2007
RU3	GTGTGAAATTGGAAAGTCGG	IGS fragment	Mbofung et al., 2007
RRU3	CCGACTTTCCAATTTCACAC	IGS fragment	Mbofung et al., 2007
CN34	CCAACACATGGGTGGTACCG	IGS fragment	Mbofung et al., 2007
IGSF4	CCAGACTTCCACTGCGTGTC	IGS fragment	Mbofung et al., 2007
CNS12	GCACGCCAGGACTGCCTCGT	IGS fragment	Mbofung et al., 2007
EF-1 (forward)	ATGGGTAAGGAGGACAAGAC	Translation elongation factor 1α	O'Donnel et al., 1998
EF-2 (reverse)	GGAAGTACCAGTGATCATGTT	Translation elongation factor 1α	O'Donnel et al., 1998
OPE07	AGATGCAGCC		
OPE15	ACGCACAACC		
OPL07	AGGCGGGAAC		

357
358

FIGURE CAPTIONS

FIGURE 1. Random amplified polymorphic DNA (RAPD) pattern obtained from 11 isolates of *F. oxysporum* extracted from *C. intybus* and two reference strains with primers OPE-07 **(A)**, OPE-15 **(B)** and OPL-07 **(C)**. M: molecular weight marker (100 bb DNA Ladder; Qiagen); NC: no template control. 1-9: FUSCIC1- FUSCIC9; 10-11: FUSCIC45A-FUSCIC45B; 12-13: *F. fujikuroi* and *F. verticilliodes*, respectively. Red arrow indicates an amplicon that appears only in the strains of *F. oxysporum* f. sp. *cichorii* isolated in 2010.

FIGURE 2. Consensus phylogenetic tree relative to IGS and EF-1 sequences of strains listed in table 1. Individual trees (Neighbour-Joining method, bootstrap analysis (1000)) were joined in a single consensus tree through consensus Supernetwork using Splitstree4 software.