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Evolutionary relationships between *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* isolates inferred from mating type, elongation factor-1α and exopolygalacturonase sequences

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ABSTRACT

Fusarium oxysporum is a ubiquitous species complex of soilborne plant pathogens that comprises many different *formae speciales*, each characterized by a high degree of host specificity. In this study, the evolutionary relationships between different isolates of the *F. oxysporum* species complex have been examined, with a special emphasis on the *formae speciales lycopersici* and *radicis-lycopersici*, sharing tomato as host while causing different symptoms. Phylogenetic analyses of partial sequences of a housekeeping gene, the elongation factor-1α (EF-1α) gene, and a gene encoding a pathogenicity trait, the exopolygalacturonase (*pgx4*) gene, were conducted on a worldwide collection of *F. oxysporum* strains representing the most frequently observed vegetative compatibility groups of these *formae speciales*. Based on the reconstructed phylogenies, multiple evolutionary lineages were found for both *formae speciales*. However, different tree topologies and statistical parameters were obtained for the cladograms as several strains switched from one cluster to another depending on the locus that was used to infer the phylogeny. In addition, mating type analysis showed a mixed distribution of the *MAT1-1* and *MAT1-2* alleles in the *F. oxysporum* species complex, irrespective of the geographic origin of the tested isolates. This observation, as well as the topological conflicts that were detected between EF-1α and *pgx4*, are discussed in relation to the evolutionary history of the *F. oxysporum* species complex.

Introduction

Fusarium oxysporum Schlechtend:Fr is a ubiquitous, asexually reproducing fungal species complex that occurs in soil and includes morphologically indistinguishable plant pathogenic strains, as well as strains for which no host(s) have been identified (yet). Collectively, *F. oxysporum* strains can cause wilt or root rot in a very broad range of host plants, among which are

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many economically important crops (Gordon & Martyn 1997). Therefore, *F. oxysporum* strains have been grouped into *formae speciales* based on host specificity, and further into races based on cultivar specificity (Armstrong & Armstrong 1981). In addition, *F. oxysporum* strains have been subdivided into vegetative compatibility groups (VCGs) (Puhalla 1985) based on the ability to establish hyphal anastomosis and form stable heterokaryons. Molecular and genetic analyses have shown that strains within a VCG are genetically similar. In addition, it has been suggested that each VCG may represent a clonal population (Correll 1991; Koenig *et al.* 1997; Kistler *et al.* 1998; Katan & Katan 1999). Nevertheless, examples of molecular variability between strains of the same VCG exist, indicating they do not belong to the same clonal lineage (Leslie 1993; Appel & Gordon 1995, 1996; Kawabe *et al.* 2007).

F. oxysporum is considered a monophyletic, diverse complex of evolutionary lineages (O'Donnell & Cigelnik 1997). An understanding of the evolutionary history of the *formae speciales* and races within *F. oxysporum* requires knowledge of the phylogenetic relationships among isolates (Appel & Gordon 1996). Phylogenetic analyses based on DNA sequences of housekeeping genes such as, for example, the mitochondrial small subunit (mtSSU) ribosomal RNA gene, the rDNA intergenic spacer (IGS) region, and the translation elongation factor (EF)-1α gene have helped to reveal the genetic and evolutionary relationships within and among *formae speciales* of *F. oxysporum* (Lievens *et al.* 2008). Such studies showed that a limited number of *F. oxysporum formae speciales* is monophyletic while many were found to be polyphyletic, including the *formae speciales asparagi*, *cubense*, *cucumerinum*, *dianthi*, *gladioli*, *lini*, *lactucae*, *lycopersici*, *melonis*, *opuntiarum*, *phaseoli*, *radicis-lycopersici* and *vasinfectum* (O'Donnell *et al.* 1998; Alves-Santos *et al.* 1999; Baayen *et al.* 2000; Kistler 2001; Skovgaard *et al.* 2001; Abo *et al.* 2005; Kawabe *et al.* 2005; Wong & Jeffries 2006; Lievens *et al.* 2007; Mbofung *et al.* 2007; van der Does *et al.* 2008), suggesting that pathogenicity towards a specific crop has evolved several times independently. In addition to housekeeping genes, DNA sequences encoding pathogenicity factors are increasingly used to study genetic relationships between isolates within a species (Lievens & Thomma 2005). For example, *endo*polygalacturonase (*pg1*) and *exo*polygalacturonase gene (*pgx4*; Garcia-Maceira *et al.* 2000) sequences have been used to study the genetic diversity of *F. oxysporum* isolates (Kawabe *et al.* 2005; Hirano & Arie 2006).

Recently, the mating type locus, *MAT1*, which regulates sexual reproduction in ascomycete fungi, was cloned from *F. oxysporum* and found to be present in all tested isolates (Arie *et al.* 2000). Each isolate had either of the two idiomorphs (*MAT1-1* or *MAT1-2*). Previous studies have demonstrated that, in addition to phylogenetic analyses based on the previously mentioned genes, *MAT*-based phylogenetic analyses can be useful for studying the evolution of closely related fungi (Pöggeler 1999; Barve *et al.* 2003), including *formae speciales* of *F. oxysporum* (Kawabe *et al.* 2005, 2007).

F. oxysporum forma specialis (f. sp.) *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* are two *formae speciales* that infect tomato. While *F. oxysporum* f. sp. *lycopersici* is the causal agent of vascular wilt only on tomato, *F. oxysporum* f. sp. *radicis-lycopersici* causes crown and root rot on tomato and several other hosts (Menzies *et al.* 1990). For *F. oxysporum* f. sp. *lycopersici*, three races and five VCGs (0030–0033 and 0035) have been reported so far (Katan 1999; Katan & Di Primo 1999; Cai *et al.* 2003); though VCG 0030 was found to include VCG 0032 (Cai *et al.* 2003; Kawabe *et al.* 2005). For *F. oxysporum* f. sp. *radicis-lycopersici* nine VCGs (0090–0094 and 0096–0099) were identified, but no races have been reported (Katan 1999; Katan & Di Primo 1999). Based on the intensity of hyphal complementation, each of the VCGs 0090, 0091 and 0094 can be subdivided into at least two subgroups.

Remarkably, topological discrepancies between IGS and EF-1α or mtSSU trees have been reported for *F. oxysporum*, a species that is often thought of as only occurring in clonal populations (Mbofung *et al.* 2007; O'Donnell *et al.* 2008). We hypothesize that not all genes in the different *F. oxysporum formae speciales* have the same evolutionary history. The objective of this study was to evaluate this hypothesis and, at the same time, determine the evolutionary relationships among isolates that belong to the two *F. oxysporum formae speciales* of tomato, using a phylogenetic analysis of both a housekeeping gene (EF-1α) and a gene encoding a cell wall-degrading enzyme (*pgx4*), as well as by the distribution of the mating type locus (*MAT1*). Together with the random distribution of the mating type idiomorphs MAT1-1 and MAT1-2 the different tree topologies that were obtained are discussed in relation to the evolutionary history of the *F. oxysporum* species complex.

Materials and methods

Fungal isolates and DNA extractions

A worldwide collection of 52 *Fusarium oxysporum* strains, including 16 of *F. oxysporum* f. sp. *lycopersici*, 20 of *F. oxysporum* f. sp. *radicis-lycopersici* and 16 of other *formae speciales*, was used in this study. Isolates were mainly from North America, Asia and Europe, but also from Australasia, Africa and South America (Table 1). For most of these isolates pathogenicity and vegetative compatibility have been assessed in previous studies (e.g. Katan *et al.* 1991; Marlatt *et al.* 1996; O'Donnell *et al.* 1998; Katan & Katan 1999; Cai *et al.* 2003; Balmas *et al.* 2005; Kawabe *et al.* 2005; van der Does *et al.* 2008). Isolates were grown on potato dextrose agar containing 100 ppm streptomycin sulphate, in the dark, at 22 °C. Genomic DNA was extracted using the phenol–chloroform extraction method as described previously (Lievens *et al.* 2003) and the yield was determined spectrophotometrically. As a check for DNA quality, all samples were successfully subjected to PCR analysis using the universal primers ITS5 and ITS4, which anneal to conserved regions of the 18S and 28S ribosomal RNA genes, respectively (White *et al.* 1990; Table 2).

Mating type determination

The mating type of each isolate was determined using a *MAT1*-specific PCR assay as described (Arie *et al.* 1999, 2000). Isolates for which an approximately 370-bp fragment was generated using primers Falpha1 and Falpha2 (Table 2) were classified as MAT1-1, and those for which a ca. 190-bp fragment was obtained using primers FHMG11 and FHMG12

Table 1 – *Fusarium oxysporum* strains used in this study

Isolate[a]	VCG[b]	Race[c]	Host	Origin (source)[d]	Mating type[e]	GenBank Accession No[f]	
						EF-1α	*pgx4*
F. oxysporum f. sp. *lycopersici*							
FOL-24L	0030	1	*Solanum lycopersicum*	Israel (A)	MAT1-1	FJ790383	FJ790332
281	0030	2	*S. lycopersicum*	Spain (B)	MAT1-1	FJ790384	FJ790333
FOL-93H	0030	2	*S. lycopersicum*	Israel (A)	MAT1-1	FJ790385	FJ790334
14844	0030	3	*S. lycopersicum*	Australia (C)	MAT1-1	FJ790386	FJ790335
CA92/95	0030	3	*S. lycopersicum*	California, USA (D)	MAT1-1	FJ790387	FJ790336
FOL-MM59	0030	2	*S. lycopersicum*	Arkansas, USA (A)	MAT1-1	FJ790388	FJ790337
FOL-MM66	0030	2	*S. lycopersicum*	Arkansas, USA (A)	MAT1-1	FJ790389	FJ790338
NRRL 26200	0030	2	*S. lycopersicum*	Ohio, USA	MAT1-1	AF008499	FJ790339
NRRL 26037	0030	3	*S. lycopersicum*	Florida, USA	MAT1-1	AF008498	FJ790340
011093	0031	1	*S. lycopersicum*	Belgium (A)	MAT1-1	FJ790390	FJ790331
E175	0031	1	*S. lycopersicum*	The Netherlands (E)	MAT1-1	FJ790391	FJ790341
OSU-451	0031	2	*S. lycopersicum*	Ohio, USA (F)	MAT1-1	FJ790392	FJ790342
FOL-MM10	0033	3	*S. lycopersicum*	Arkansas, USA (A)	MAT1-2	FJ790393	FJ790343
NRRL 26380[g]	0033	3	*S. lycopersicum*	Florida, USA	MAT1-2	AF008509	FJ790344
NRRL 26383	0033	3	*S. lycopersicum*	Florida, USA	MAT1-2	AF008502	FJ790345
NRRL 26203	Unknown	Unknown	*S. lycopersicum*	Italy	MAT1-2	AF008501	FJ790346
F. oxysporum f. sp. *radicis-lycopersici*							
ATCC 52429	0090 I		*S. lycopersicum*	Canada	MAT1-2	FJ790394	FJ790347
FORL-C709	0090 I		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790395	FJ790348
FORL-C1018F	0090 II		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790396	FJ790349
FORL-C809L	0090 II		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790397	FJ790350
FORL-710B	0090 III		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790398	FJ790351
FORL-C696A	0090 III		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790399	FJ790352
ATCC 60095	0091 I		*S. lycopersicum*	Canada	MAT1-1	FJ790400	FJ790353
FORL-Pt473D	0091 I		*S. lycopersicum*	Israel (A)	MAT1-1	FJ790401	FJ790354
FORL-C434	0091 II		*S. lycopersicum*	Israel (A)	MAT1-1	FJ790402	FJ790355
FORL-C815A	0092		*S. lycopersicum*	Israel (A)	MAT1-1	FJ790403	FJ790356
FORL-C202	0093		*S. lycopersicum*	Israel (A)	MAT1-1	FJ790404	FJ790357
MUCL 39790	0094		*S. lycopersicum*	Belgium	MAT1-1	FJ790405	FJ790358
MUCL 39792	0094		*S. lycopersicum*	Belgium	MAT1-1	FJ790406	FJ790359
FORL-UK3Q	0094 I		*S. lycopersicum*	UK (A)	MAT1-1	FJ790407	FJ790360
FORL-FL418	0094 II		*S. lycopersicum*	Korea (A)	MAT1-1	FJ790408	FJ790361
FORL-C622A	0096		*S. lycopersicum*	Israel (A)	MAT1-2	FJ790409	FJ790362
PB9	0098		*S. lycopersicum*	Florida, USA (G)	MAT1-1	FJ790410	FJ790363
NRRL 26033	Unknown		*S. lycopersicum*	Florida, USA	MAT1-2	AF008507	FJ790364
NRRL 26379	Unknown		*S. lycopersicum*	Florida, USA	MAT1-1	AF008508	FJ790365
NRRL 26381	Unknown		*S. lycopersicum*	Florida, USA	MAT1-1	AF008510	FJ790366
F. oxysporum f. sp. *batatas*							
NRRL 26409	Unknown		*Ipomoea batatas*	Unknown	MAT1-1	AF008484	FJ790367
F. oxysporum f. sp. *canariensis*							
NRRL 26035	Unknown		*Phoenix canariensis*	Tenerife, Canary Islands	MAT1-1	AF008485	FJ790368
F. oxysporum f. sp. *cubense*							
NRRL 25603	0120		*Musa acuminata*	Australia	MAT1-2	AF008487	FJ790369
NRRL 26029	01210		*M. acuminata* × *M. balbisiana*	Florida, USA	MAT1-1	AF008493	FJ790370
NRRL 25609	01214		*M. acuminata* × *M. balbisiana*	Malawi	MAT1-1	AF008490	FJ790371
NRRL 25367	Unknown		*M. acuminata* × *M. balbisiana*	Unknown	MAT1-1	AF008486	FJ790372
NRRL 25605	Unknown		*M. acuminata*	Unknown	MAT1-2	AF008488	FJ790373
NRRL 26024	Unknown		*M. acuminata*	Unknown	MAT1-2	AF008492	FJ790374
NRRL 26038	Unknown		*M. acuminata* × *M. balbisiana*	Unknown	MAT1-1	AF008494	FJ790375
F. oxysporum f. sp. *erythroxyli*							
NRRL 26574	Unknown		*Erythroxylum coca*	Hawaii, USA	MAT1-1	AF008495	FJ790376
F. oxysporum f. sp. *glycines*							
NRRL 25598	Unknown		*Glycine max*	South Carolina, USA	MAT1-1	AF008496	FJ790377
F. oxysporum f. sp. *melonis*							
NRRL 26406	0136		*Cucumis melo*	Mexico	MAT1-2	AF008504	FJ790378
NRRL 26178	Unknown		*C. melo*	China	MAT1-1	AF008503	FJ790379

(*continued on next page*)

Table 1 – (continued)

Isolate[a]	VCG[b]	Race[c]	Host	Origin (source)[d]	Mating type[e]	GenBank Accession No[f]	
						EF-1α	*pgx4*
F. oxysporum f. sp. *passiflorae*							
NRRL 22549	Unknown		*Passiflora edulis*	Brazil	MAT1-2	AF008505	FJ790380
F. oxysporum f. sp. *perniciosum*							
NRRL 22550	Unknown		*Albizia julibrissin*	Iran	MAT1-2	AF008506	FJ790381
F. oxysporum f. sp. *tuberosi*							
NRRL 22555	Unknown		*S. tuberosum*	Iran	MAT1-2	AF008511	FJ790382

a ATCC, American Type Culture Collection, Manassas, VA, USA; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL, Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.
b VCG, vegetative compatibility group. Kawabe *et al.* (2005) showed that the VCG 0030 tester isolate NRRL 26037 and some other isolates of VCG 0030 encompassing NRRL 26200 were weakly compatible with VCG 0032 tester isolate FOL-MM66. In addition, FOL-MM59 (VCG 0032) was found to be a bridging isolate that can also form heterokaryons with some members of VCG 0030 (Cai *et al.* 2003; Kawabe *et al.* 2005).
c Reported for *F. oxysporum* f. sp. *lycopersici*.
d A = T. Katan, Hebrew University of Jerusalem, Jerusalem, Israel; B = M.I.G. Roncero, Universidad de Cordoba, Cordoba, Spain; C = D.J. McGrath, The University of Queensland, St Lucia, Australia; D = D.A. Lawn, Asgrow Seed Company, Sinaloa, Mexico; E = D.M. Elgersma, Willie Commelin Scholten Phytopathological Laboratory, the Netherlands; F = K.S. Elias, Louisiana State University Agricultural Center, Lousiana, US; G = Q. Migheli, University of Sassari, Sassari, Italy.
e Assessed by MAT-specific PCR (Arie *et al.* 1999, 2000).
f GenBank Accession numbers FJ790331–FJ790410 determined in this study.
g Previously misidentified as *F. oxysporum* f. sp. *radicis-lycopersici*; recently classified as *F. oxysporum* f. sp. *lycopersici* (Kawabe *et al.* 2005; van der Does *et al.* 2008).

(Table 2) were determined to be MAT1-2. All PCR reactions were conducted at least twice and generated the same results.

Amplification and sequencing

Segments of the EF-1α (approximately 750 bp) and *pgx4* gene (approximately 1480 bp) were amplified and sequenced using the primers EF1 and EF2 (O'Donnell *et al.* 1998; Table 2) and ExoF and ExoR (Hirano & Arie 2006; Table 2), respectively. PCR amplification was performed in a reaction volume of 20 μl, containing 0.15 mM of each deoxynucleoside triphosphate, 0.5 μM of each primer, 1x Titanium Taq DNA polymerase, 1x Titanium Taq PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 5 ng genomic DNA. PCR conditions consisted of 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at the annealing temperature indicated in Table 2 and 45 s at 72 °C, with a final elongation step at 72 °C for 10 min.

As no *pgx4* fragments could be obtained for the *Fusarium oxysporum* f. sp. *lycopersici* strains belonging to VCG 0030, PCR amplification was performed using ExoF and Exo-FOL-R2 (Table 2) for these isolates, generating a fragment of approximately 1365 bp. Subsequently, amplified products were sequenced in both directions using the primers used for PCR amplification. All sequences obtained in this study were deposited in GenBank (Accession numbers FJ790331–FJ790410) as denoted in Table 1.

Phylogenetic analyses

For all isolates listed in Table 1, phylogenetic analyses were performed on DNA sequences of the EF-1α (between 572 and 577 bp) and *pgx4* gene (1040 bp) that were either retrieved from GenBank or determined in this study (Table 1). To examine *Fusarium oxysporum* interrelationships, *Gibberella zeae*

Table 2 – Primers used in this study

Code	Sequence (5′–3′)	Target	T_{ann}[a]	Reference
ITS5	GGAAGTAAAAGTCGTAACAAGG	18S rDNA	58	White *et al.* (1990)
ITS4	TCCTCCGCTTATTGATATGC	28S rDNA	58	White *et al.* (1990)
Falpha1	CGGTCAYGAGTATCTTCCTG	MAT1-1	58	Arie *et al.* (2000)
Falpha2	GATGTAGATGGAGGGTTCAA	MAT1-1	58	Arie *et al.* (2000)
FHMG11	TACCGYAAGGAGCGTCACC	MAT1-2	58	Arie *et al.* (1999)
FHMG12	TTYWYCTSATCSGCSMKHWSCTTG	MAT1-2	58	Arie *et al.* (1999)
EF1	ATGGGTAAGGARGACAAGAC	EF1-α	67	O'Donnell *et al.* (1998)
EF2	GGARGTACCAGTSATCATGTT	EF1-α	67	O'Donnell *et al.* (1998)
ExoF	CAAGAGAAGTCATCGCAAGG	*pgx4*	65	Hirano & Arie (2006)
ExoR	ACCCCAACCCCCCTCATCT	*pgx4*	65	Hirano & Arie (2006)
Exo-FOL-R2	GCACTAACACAATCCACACC	*pgx4*	65	–

a Annealing temperature (°C).

(anamorph: *F. graminearum*), the only related species for which a *pgx4* homologue is deposited in GenBank, was included as an outgroup (GenBank Accession numbers AJ543600 (EF-1α) and XM387727 (*pgx4*)). *F. oxysporum* phylogenies were constructed using BEAST v. 1.5beta2 (Drummond & Rambaut 2007) that requires only limited assumptions for evolutionary and population biology parameters. Estimations of parameter values in BEAST are mainly based on models employing Bayesian statistics (Huelsenbeck *et al.* 2001) that perform well in studies where genetically diverse taxa are less densely sampled (Holder & Lewis 2003). BEAST input XML files were generated using BEAUti v. 1.5beta2. BEAST output was examined using Tracer 1.4 (Rambaut & Drummond 2007). This program draws frequency distributions and outputs statistical parameters and their statistical support through the so-called effective sample size (ESS) index. The ESS of a parameter reflects the reliability of distributions, such that a small ESS indicates that the estimate of the posterior distribution of that parameter will be poor. Well-supported parameters have ESS > 200; poorly supported parameters have 100 > ESS < 200; parameters with ESS < 100 should not be used in analyses. Phylogenetic trees were generated using TreeAnnotator v. 1.5beta2 and visualized using FigTree v. 1.2 (http://tree.bio.ed.ac.uk/software/figtree/). Multiple BEAST runs were performed for each model, and the suggestions made at the end of a BEAST run were used for later runs until the estimations appeared to be optimized. For all runs, the Hasegawa, Kishino and Yano (HKY) DNA base substitution model was used, with both invariant and variant sites according to a gamma distribution, and a relaxed uncorrelated lognormal molecular clock. These settings gave the most consistent and best-supported results in preliminary BEAST test runs. The standard amount of Markov chains was 10 million, with screen output and tree sampling after each of 1000 chains, according to the default chain settings of BEAUti.

Results

Phylogeny reconstructions

To reliably reconstruct *Fusarium oxysporum* phylogenies the BEAST software tool was used. To find the overall best performance, BEAST was run several times using the HKY model of DNA substitution as well as the generalized time reversible (GTR) model. The HKY model outperformed the GTR model (data not shown). The molecular clock, a hypothesis that mutation and substitution rates do not vary among lineages in a tree, is a fundamental assumption in all BEAST models. We varied two molecular clock models relating to taxon divergence and evolutionary change (Kimura 1968; Drummond *et al.* 2006) and found that the relaxed clock model outperformed the random local clock model (data not shown). Interrelationships were examined relative to *Gibberella zeae* as an outgroup. The statistics calculated by BEAST were examined using Tracer 1.4. Table 3 shows that, using a standard number of Markov chains of 10 million, all statistical parameters in our study are well supported (ESS > 200) and differed depending on the phylogeny used. The resulting cladograms for EF-1α and *pgx4* are shown in Figs 1 and 2, respectively.

EF-1α sequences ranged from 572 to 577 bp containing intron sequences of 370–375 bp. Roughly, the corresponding cladogram displayed three major clades, referred to as E1, E2 and E3 (Fig 1). Whereas clades E1 and E3 represent well-supported clades (posterior probability of 1) (the posterior probability is comparable to bootstrap support; a posterior probability of 1 correlates with 100 % bootstrap support), clade E2 is poorly supported (posterior probability of 0.09) (Fig 1). All *F. oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici* strains were grouped within different subclades belonging to E1 and E2 (Fig 1; referred to as subclades E1-1, E1-2, E1-3, E1-4, E2-1,

Table 3 – Tracer output[a] for population parameters as determined by Bayesian models run using BEAST

Statistic	EF-1α		*pgx4*	
	Mean	ESS	Mean	ESS
Posterior	−1289.87	1518.05	−2071.18	1806.325
Prior	180.929	1440.325	193.026	1352.554
Likelihood	−1470.8	3833.288	−2264.2	1537.659
Clock.rate	1	–	1	–
Treemodel.rootheight	0.117	6107.817	9.17E-02	1311.201
Constant.popsize	1.32E-02	2297.78	1.04E-02	2305.895
Hky.kappa	2.906	7867.884	5.747	2830.319
Sitemodel.alpha	50.68	5364.249	12.874	659.101
Treelikelihood	−1470.8	3833.288	−2264.2	1537.659
Coalescent	177.628	1437.163	190.166	1364.577

a The different values for the parameters show that models run using elongation factor (EF)-1α or exopolygalacturonase (*pgx4*) sequences differ substantially for these parameters. The effective sample size (ESS) values are well above 200, the lower limit for models to have achieved sufficient statistical reliability. The statistics that reflect reliability of the BEAST models are the prior and posterior (that describe the appropriateness of the chosen model parameters, e.g. the speciation model, and the reliability of the outcome of the population parameter statistics), the (model) likelihood and resulting phylogenetic tree likelihood; especially these statistics have ESS values well over 200. The HKY-kappa value describes the transition/transversion ratio for each locus. The sitemodel parameter indicates the gamma distribution of heterogeneity of base substitution rates under a model where DNA nucleotides do not undergo similar rates of substitutions. Since we have assumed similar generation-times lengths (clock.rate = 1), constant.popsize reflects the effective population size *times* the DNA substitution rate per site per generation. The coalescent indicates for each locus the relative (here dependent on the constant.popsize parameter since the age of the isolates is unknown) length to the most recent common ancestor of the taxa used in the BEAST calculations.

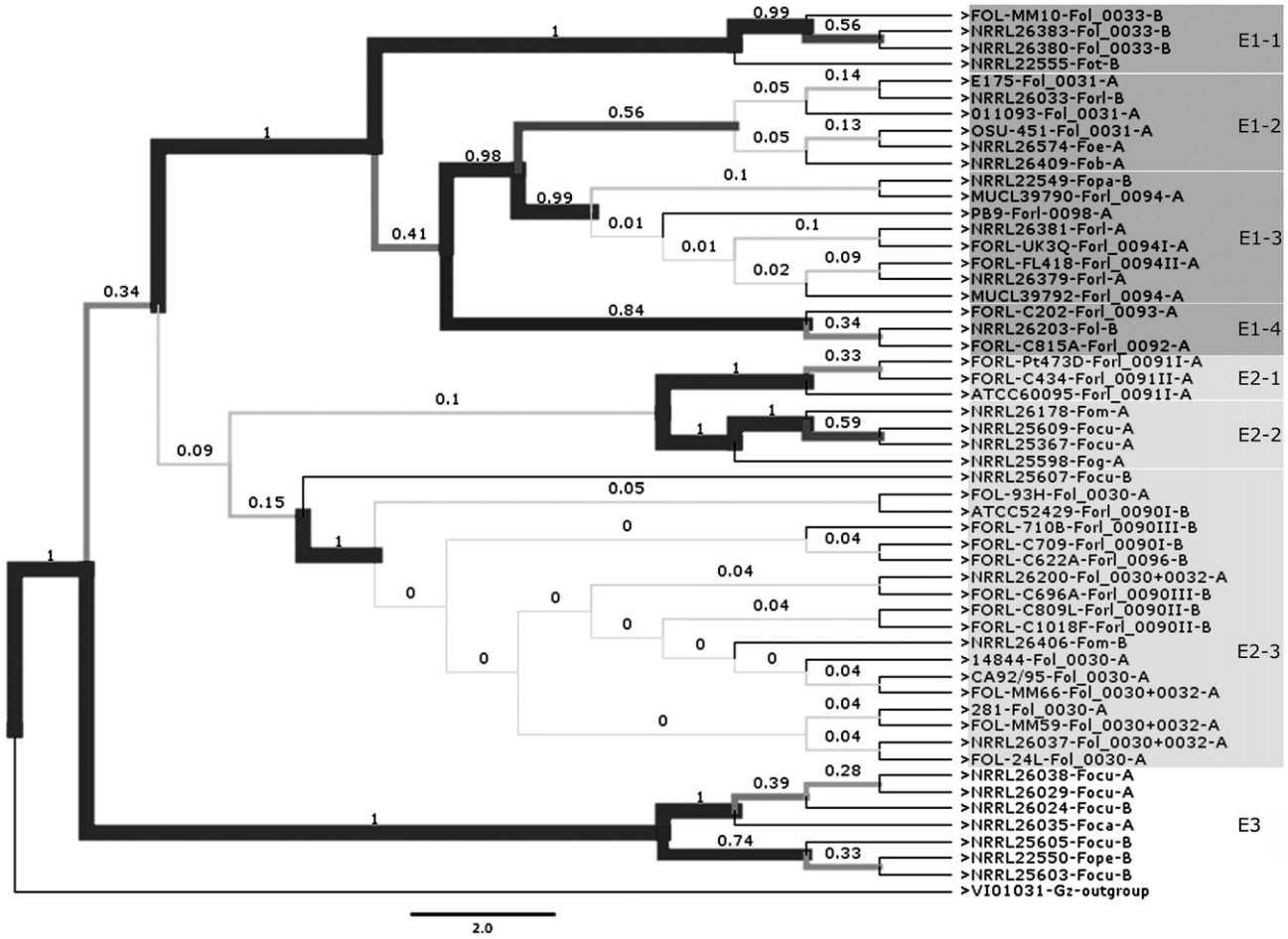

Fig 1 – Cladogram inferred from elongation factor (EF)-1α sequence variation rooted with the *Gibberella zeae* sequence VI01031. Isolate numbers correspond to those listed in Table 1. Strains denoted with the characters 'A' and 'B' correspond to MAT1-1 and MAT1-2, respectively. Vegetative compatibility groups (VCGs) are reported for *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici*. Posterior probabilities are shown on the branches. In addition, thickness of the branches is a measure for support of the clades: the better a clade is supported, the thicker the branch is marked. Fob, f. sp. *batatas*; Foca, f. sp. *canariensis*; Focu, f. sp. *cubense*; Foe, *erythroxyli*; Fog, f. sp. *glycines*; Fol, f. sp. *lycopersici*; Fom, f. sp. *melonis*; Fopa, f. sp. *passiflorae*; Fope, f. sp. *perniciosum*; Forl, f. sp. *radicis-lycopersici*; Fot, f. sp. *tuberosi*.

and E2-3). Subclade E1-1 (posterior probability of 1) was made up of sequences (575 bp) of *F. oxysporum* f. sp. *lycopersici* VCG 0033 isolates (Fig 1) and showed at least five polymorphic sites in comparison with the other subclades within clade E1. Subclade E1-2 contained all *F. oxysporum* f. sp. *lycopersici* strains belonging to VCG 0031 as well as the *F. oxysporum* f. sp. *radicis-lycopersici* isolate NRRL 26033 of unknown VCG. Furthermore, strains NRRL 26409 (f. sp. *batatas*) and NRRL 26574 (f. sp. *erythroxyli*) were localized within this subclade (Fig 1). Subclade E1-3 (posterior probability of 0.99), which is basal to subclade E1-2, was composed of the VCG 0094 and 0098 isolates of *F. oxysporum* f. sp. *radicis-lycopersici* and the *F. oxysporum* f. sp. *passiflorae* isolate (NRRL 22549) (Fig 1), all having an identical sequence of 576 bp. Subclade E1-4 (0.84 posterior probability) consisted of *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0092 (FORL-C815A) and VCG 0093 (FORL-C202) isolates as well as the *F. oxysporum* f. sp. *lycopersici* isolate NRRL 26203 (VCG unknown). Within the second major clade, isolates landed in two, rather poorly supported, groups of which one represented subclades E2-1 and E2-2 and the other one subclade E2-3 and isolate NRRL 25607 (Fig 1). Grouping of this isolate received only low posterior probability support and it may be included in either subclade E2-2 or E2-3. Subclade E2-1 (posterior probability of 1) was composed solely of *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0091 sequences (575 bp) (Fig 1). Isolates belonging to *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0090 and 0096 clustered in subclade E2-3, together with all strains of *F. oxysporum* f. sp. *lycopersici* VCG 0030, and one of the two *F. oxysporum* f. sp. *melonis* isolates (NRRL 26406) (Fig 1). The isolates belonging to this subclade had identical EF-1α sequences (577 bp) and displayed at least six single nucleotide differences with the other *F. oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici* strains. No *F. oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici* strains clustered within subclade E2-2. As expected, polymorphisms in the EF-1α gene were generally located in the noncoding regions. Nevertheless, isolates belonging to the E2-1 and E2-3 subclades contained the same silent single nucleotide polymorphism (SNP)

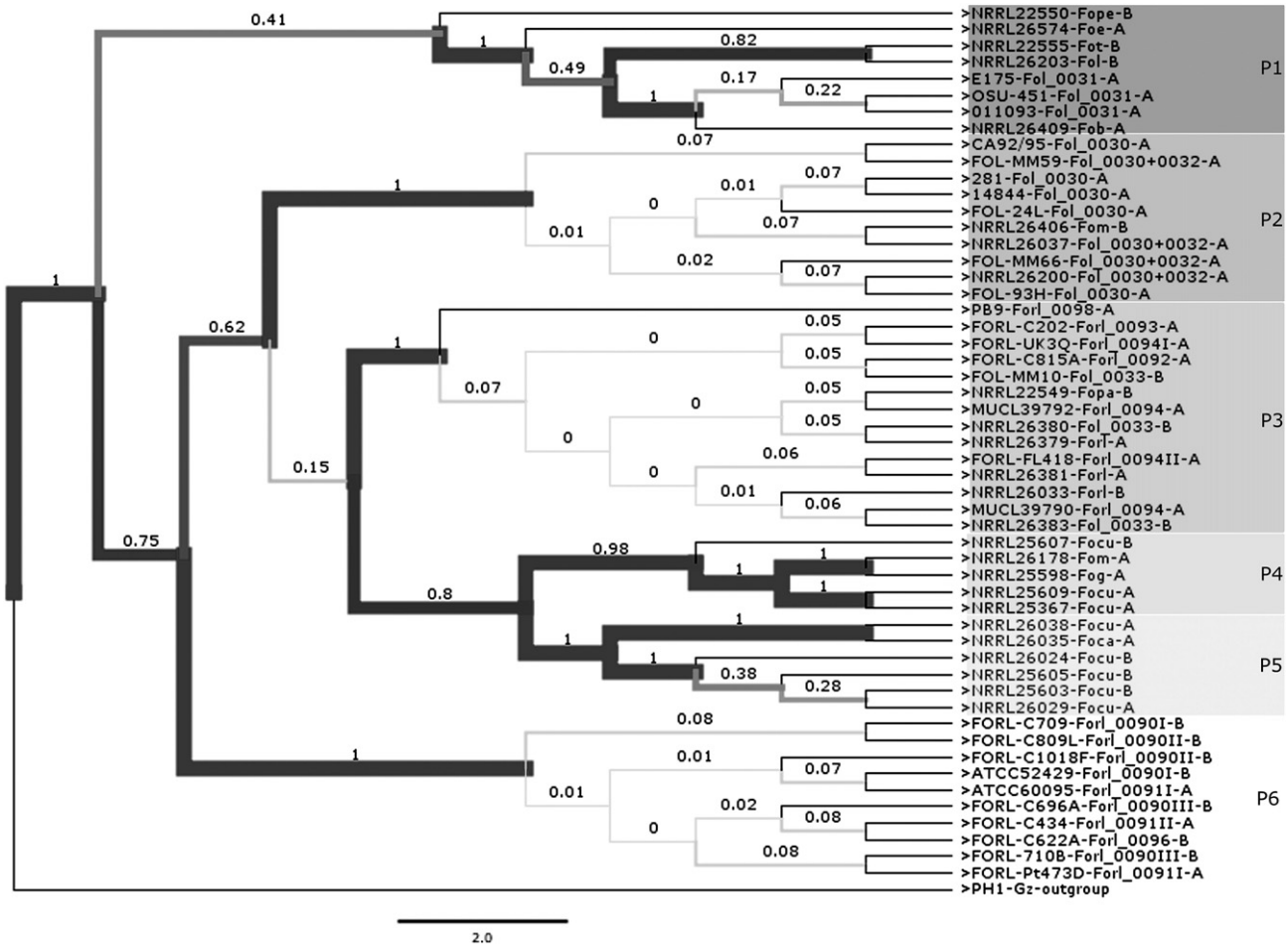

Fig 2 – Cladogram inferred from *exo*polygalacturonase (*pgx4*) sequence variation rooted with the *Gibberella zeae* sequence PH-1. Isolate numbers correspond to those listed in Table 1. Strains denoted with the characters 'A' and 'B' correspond to MAT1-1 and MAT1-2, respectively. Vegetative compatibility groups (VCGs) are reported for *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici*. Posterior probabilities are shown on the branches. In addition, thickness of the branches is a measure for support of the clades: the better a clade is supported, the thicker the branch is marked. Fob, f. sp. *batatas*; Foca, f. sp. *canariensis*; Focu, f. sp. *cubense*; Foe, *erythroxyli*; Fog, f. sp. *glycines*; Fol, f. sp. *lycopersici*; Fom, f. sp. *melonis*; Fopa, f. sp. *passiflorae*; Fope, f. sp. *perniciosum*; Forl, f. sp. *radicis-lycopersici*; Fot, f. sp. *tuberosi*.

in an exon. Also for the E1-1 subclade one silent SNP was found in the coding sequence.

In addition to the EF-1α phylogeny, a phylogenetic tree was assembled using 1040 bp *pgx4* sequences, composed of 194 bp intron and 846 bp exon sequences. For this gene a cladogram was generated displaying six well-supported major clades (P1, P2, P3, P4, P5 and P6; Fig 2), of which four encompassed *F. oxysporum* f. sp. *lycopersici* and/or *F. oxysporum* f. sp. *radicis-lycopersici* strains (P1, P2, P3 and P6). Overall, four polymorphic sites were found in the intron sequences, whereas 11 (silent) SNPs were found in the exon sequences within these *formae speciales*. In contrast to the EF-1α phylogeny, the *F. oxysporum* f. sp. *radicis-lycopersici* isolates grouped into two, well supported, clades: P3 and P6 (both posterior probability of 1) (Fig 2). Clade P6 solely consisted of *F. oxysporum* f. sp. *radicis-lycopersici* isolates, belonging to VCG 0090, 0091 and 0096, with identical *pgx4* sequences. In contrast, clade P3, which differs in at least four nucleotides from clade P6, was composed of VCG 0092, 0093, 0094 and 0098 isolates, the VCG 0033 *F. oxysporum* f. sp. *lycopersici* strains and the *F. oxysporum* f. sp. *passiflorae* isolate NRRL 22549 (Fig 2). The VCG 0031 *F. oxysporum* f. sp. *lycopersici* strains that grouped together within a cluster of multiple *formae speciales* based on EF-1α sequences (subclade E1-2) were now differentiated from the others in an almost unique cluster within clade P1 (Fig 2). *F. oxysporum* f. sp. *lycopersici* strain NRRL 26203 landed in a slightly divergent group within clade P1 (posterior probability of 0.82) (Fig 2). Finally, all *F. oxysporum* f. sp. *lycopersici* isolates belonging to VCG 0030 grouped together in a distinct cluster (clade P2; posterior probability of 1). While these isolates had EF-1α sequences identical to those of *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0090 and 0096 (subclade E2-3), four SNPs were observed in the examined *pgx4* segment between clades P2 and P6. In addition, clade P2 differed in at least eight and four nucleotides from P1 and P3, respectively.

Remarkably, isolate *F. oxysporum* f. sp. *perniciosum* NRRL 22550, which clustered with a number of *F. oxysporum* f. sp.

cubense isolates based on EF-1α sequences (clade E3, Fig 1; O'Donnell *et al.* 1998), now landed rather isolated in a different part of the tree (clade P1, Fig 2). To confirm the accuracy of the sequence, amplicons from different PCR reactions were sequenced using forward and reverse primers, and found to be identical to the original sequence. In addition, the EF-1α sequence of this and a few additional isolates of which sequences were obtained from GenBank was determined and corroborated the sequences retrieved from GenBank, demonstrating that our sequence data were reliable. In summary, both Table 3 and Fig 1 vs. Fig 2 show that tree reconstructions based on EF-1α sequences and *pgx4* sequences clearly differ, both in statistical parameter support and in tree topology.

Mating type distribution

The mating type designation for each isolate is shown in Table 1 as determined by PCR assays. Results revealed the presence of both mating types in the *F. oxysporum* species complex, with overall 63.5 % MAT1-1 and 36.5 % MAT1-2 within the tested isolates (Tables 1 and 2). There was no evidence for geographical isolation of one mating type from the other (Table 4). As expected, all isolates belonging to a given VCG are composed of a single mating type (Table 1), supporting clonality. Consequently, a bias has been introduced in the mating type frequency presented in Table 4 as different numbers of isolates were studied per VCG. Interestingly, both mating type idiomorphs appeared scattered over the phylogenetic trees (Figs 1 and 2, A = MAT1-1 and B = MAT1-2), and both *MAT*-alleles could appear in the same cluster (Figs 1 and 2; see E1-2, E1-3, E1-4, E2-3, P1, P2, P3, P4, and P6). All *Fusarium oxysporum* f. sp. *lycopersici* strains belonging to VCG 0030 and 0031 were found to be MAT1-1. Isolates belonging to VCG 0033 as well as the isolate for which no VCG was known (NRRL 26203) are MAT1-2 (Table 1). For *F. oxysporum* f. sp. *radicis-lycopersici*, the *MAT1-1* idiomorph was found in all isolates belonging to VCG 0091–0094 and 0098. In contrast, the VCG 0090 and 0096 isolates contain the *MAT1-2* allele. Of the three strains for which the VCG was not determined, isolates NRRL 26379 and NRRL 26381 are MAT1-1, whereas isolate NRRL 26033 is MAT1-2 (Table 1). Altogether, a significant dominance of MAT1-1 isolates was found in the tomato affecting *F. oxysporum formae speciales* (representing seven out of ten VCGs that were investigated). Out of the 16 strains that belong to other *formae speciales* nine were determined as MAT1-1 and seven as MAT1-2 (Table 1). For each *forma specialis* for which more than one isolate was studied both mating type alleles were found (f. sp. *lycopersici*, *radicis-lycopersici*, *cubense* and *melonis*) (Table 1). No isolates failed to show a band with one of the primer sets and no isolates yielded PCR products with both sets of primers.

Table 4 – Worldwide distribution of MAT1-1 and MAT1-2 idiomorphs[a] (mating type) among the tested *Fusarium oxysporum* isolates

Continent	Country	Number of tested isolates	Mating type distribution	
			MAT1-1	MAT1-2
Africa	Malawi	1	1	0
Asia	China	1	1	0
	Iran	2	0	2
	Israel	12	6	6
	Korea	1	1	0
Australasia	Australia	2	1	1
Europa	Belgium	3	3	0
	Italy	1	0	1
	Spain	1	1	0
	Tenerife, Canary Islands	1	1	0
	The Netherlands	1	1	0
	UK	1	1	0
North America	Arkansas	3	2	1
	California	1	1	0
	Canada	2	1	1
	Florida	8	5	3
	Hawaii	1	1	0
	Mexico	1	0	1
	Ohio	2	2	0
	South Carolina	1	1	0
South America	Brazil	1	0	1
Unknown	Unknown	5	3	2
	Total	52	33	19

a Assessed by MAT-specific PCR (Arie *et al.* 1999, 2000).

Discussion

Fusarium oxysporum interrelationships, with an emphasis on *F. oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici*, were studied using a set of isolates of different geographic origins and vegetative compatibility groups. Both mating types were determined and a phylogenetic analysis was conducted based on partial EF-1α and *pgx4* sequences. The results suggest more than one evolutionary lineage for both *formae speciales*. In addition, it was shown that both tree topologies were not congruent and that none of them correlated to the distribution of the MAT1-idiomorphs.

Previously, three major clades were delineated within the *F. oxysporum* species complex, based on the grouping of 36 isolates (the majority of which are included in the present study) in a maximal parsimony tree using a combined dataset of EF-1α and mtSSU sequences (O'Donnell *et al.* 1998). Using EF-1α sequences, we obtained the same tree topology as shown by O'Donnell *et al.* (1998), with the difference that in our study clade 2 as identified by O'Donnell *et al.* (1998) landed within the poorly supported clade E2 which consisted of several subclades, representing isolates from O'Donnell *et al.*'s (1998) clades 2 and 3. This topological difference may be explained by the addition of several closely related isolates to the previously used strain collection (O'Donnell *et al.* 1998). Indeed, a BEAST analysis using only the previously used isolates showed a cladogram (data not shown) identical to the one that was reported (O'Donnell *et al.* 1998), supporting the robustness of the basal topology of our EF-1α tree.

In accordance with previous observations (Cai *et al.* 2003; Balmas *et al.* 2005; Kawabe *et al.* 2005), our study reveals that *F. oxysporum* f. sp. *lycopersici* is composed of at least three independent clonal lineages represented by the EF-1α groupings E1-1, E1-2, E1-4 and E2-3 and by the *pgx4* clades defined as P1, P2

and P3. Isolate NRRL 26203 of unknown VCG, which was experimentally demonstrated to cause tomato wilt (van der Does *et al.* 2008), clustered in a distinct, well-supported group based on EF-1α sequences (subclade E1-4, Fig 1) and within the *pgx4* clade P1 separated from VCG 0031 isolates (Fig 2). Together with the observation that this strain is MAT1-2, in contrast to the VCG 0031 isolates, this suggests that isolate NRRL 26203 belongs to another VCG. However, this should still be verified by VCG determination. EF-1α phylogeny revealed several subclades for *F. oxysporum* f. sp. *radicis-lycopersici* isolates, encompassing lineages composed of VCG 0094 and 0098 (E1-3), VCG 0092 and 0093 (E1-4), VCG 0091 (E2-1), and VCG 0090 and 0096 (E2-3) isolates (Fig 1). In addition, isolate NRRL 26033 of unknown VCG grouped in a different cluster (E1-2) and may represent another lineage of *F. oxysporum* f. sp. *radicis-lycopersici*. Remarkably, based on *pgx4* phylogeny, the *F. oxysporum* f. sp. *radicis-lycopersici* strains were clustered in two groups. On the one hand, *F. oxysporum* f. sp. *radicis-lycopersici* members of the EF-1α subclades E1-2, E1-3 and E1-4 were combined with *F. oxysporum* f. sp. *lycopersici* VCG 0033 and one other isolate in the *pgx4* clade P3. On the other hand, *F. oxysporum* f. sp. *radicis-lycopersici* strains of E2-1 and E2-3 were grouped in a cluster uniquely composed of *F. oxysporum* f. sp. *radicis-lycopersici* strains (P6) (Figs 1 and 2). Similarly, *endo*polygalacturonase gene (*pg1*) sequences were found to provide less phylogenetic resolution than the highly variable IGS region when studying the genetic diversity within *F. oxysporum* f. sp. *lycopersici* (Kawabe *et al.* 2005). However, with regard to the latter, *pgx4* seems to display more discriminative power than *pg1*, on the basis of which the *F. oxysporum* f. sp. *lycopersici* strains were subdivided in two groups only (Kawabe *et al.* 2005).

Different tree topologies and statistical parameters were obtained for cladograms based on EF-1α sequences and *pgx4* sequences (Figs 1 and 2; Table 3). Significant topological differences were exemplified by in-between clade strain switching, depending on the used locus. Apart from the examples discussed above, *F. oxysporum* f. sp. *perniciosum* NRRL 22550, for instance, switched from the distinct major clade E-3 to a group of isolates belonging to clade E-1. Incongruency between phylogenetic trees for *F. oxysporum formae speciales* was also observed in other studies. Mbofung *et al.* (2007), for example, detected significant topological discrepancies between IGS and EF-1α or mtSSU trees using sequences of over 40 *F. oxysporum* isolates, representing 20 different *formae speciales*. Several processes may contribute to these reported conflicts.

One explanation for the observed in-between clade switching includes exchange of genetic material. Although sexual reproduction has never been observed in *F. oxysporum*, functional mating type (*MAT1*) genes have been identified that are expressed (Arie *et al.* 2000; Yun *et al.* 2000). Mating type determination of all isolates studied here showed a random distribution of the *MAT1-1* and *MAT1-2* alleles as both idiomorphs appeared scattered over the different clades of both phylogenetic trees, irrespective of the geographic origin of the tested isolates (Figs 1 and 2). Nevertheless, crossing of *F. oxysporum* f. sp. *lycopersici* isolates from opposite mating types did not result in viable offspring (Kawabe *et al.* 2005). Alternative mechanisms potentially leading to genetic recombination are parasexual fusion or horizontal gene transfer. Although the mechanisms involved are still unclear, examples exist for gene transfer across different phylogenetic borders at several taxonomical levels, ranging from species to kingdoms (Thomma 2003; Temporini & VanEtten 2004; Friesen *et al.* 2008; Khaldi *et al.* 2008). In the case of *F. oxysporum*, however, exchange of genetic material *via* direct gene transfer from one fungal isolate to another would be limited by vegetative incompatibility. Nevertheless, in another ascomycete fungus, *Colletotrichum gloeosporioides*, transfer of a chromosome under laboratory conditions has been reported between vegetatively incompatible isolates (He *et al.* 1998), showing that under certain conditions, genetic material can be exchanged across VCGs. Different evolutionary histories of gene lineages may also result from forces other than recombination, including natural selection. Due to such processes, different tree topologies may arise by a differential accumulation of mutations in the tested loci by which polymorphisms in the examined genes evolve at different rates. When mutations accumulate faster in a given genomic region, genes in that region may evolve faster and may appear to be under positive selection. Several genes involved in host defense systems, as well as virulence factors, have been shown to be under positive selection pressure, exhibiting a faster accumulation of mutations compared to genes that regulate the basal physiology of the organism (Johannesson *et al.* 2004; Liu *et al.* 2005; Matute *et al.* 2008). This may also apply to our study. Although several examples of pathogenicity genes that experience positive selection do exist, such and other genes can also be under negative or purifying selection in pathogenic fungi, in order to avoid the accumulation of deleterious mutations (Koufopanou *et al.* 2001). As high mutation rates may mimic recombination further study on genetic relationships is required, preferably, using a larger set of genetic loci (Rokas *et al.* 2003) in order to describe the population structure or evolution of the *F. oxysporum* species complex (Skovgaard *et al.* 2002). In some cases topological conflicts may also be resolved by analyzing additional and more diverse isolates (Rokas *et al.* 2003; O'Donnell *et al.* 2004). However, when selection is the driving force behind the incongruency between gene trees, adding more isolates will not improve the analysis (Rokas *et al.* 2003).

Apart from presenting a view on the evolution of *F. oxysporum*, our findings may also contribute to the development of diagnostic markers. Although no strong phylogenetic signal could be specifically related to the *formae speciales* investigated, specific markers can be developed for certain subgroups (VCGs or a group of certain VCGs) based on the data sets that were generated in this study. This is important since discrimination between pathogenic and nonpathogenic strains towards a specific crop could prevent unnecessary efforts to control harmless populations (Lievens *et al.* 2008). For instance, a PCR method has been developed which distinguishes Japanese *F. oxysporum* f. sp. *radicis-lycopersici* from Japanese *F. oxysporum* f. sp. *lycopersici* strains, based on polymorphisms in the *pgx4* gene (Hirano & Arie 2006). Inclusion of the corresponding sequences in our phylogenetic study revealed that these strains grouped in clade P6 (data not shown). A PCR screen using these primers on a large, worldwide collection of almost 300 *F. oxysporum* isolates (van der Does *et al.* 2008), encompassing 16 *formae speciales* and approximately 75

and 80 strains of *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici*, respectively, showed a 100 % success rate in identifying *F. oxysporum* f. sp. *radicis-lycopersici* strains belonging to the VCGs 0090, 0091 and 0096 (data not shown). No amplicons were generated for strains belonging to other VCGs or *formae speciales*, demonstrating that identification of *F. oxysporum* subgroups based on these sequences could indeed be very robust.

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