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Genotypic identification of *Fusarium subglutinans, F. proliferatum* and *F. verticillioides* strains isolated from maize in Austria

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ABSTRACT

Gibberella fujikuroi is species complex. This species complex includes *F.tabacinum, F. monilifore* (= *F. verticillioides*), *F. nygamai, F. proliferatum* as, well as *F. subglutinans*. Our objective is to develop a technique to differentiate between isolates of *F. subglutinans* , *F. proliferatum* and *F. verticillioides*. Thirty -two strains of *F. subglutinans*, 6 strains *from F. verticillioides* and 5 strains from *F. Proliferatum* isolated from maize in Austria and studied using random amplified polymorphic DNA (RAPD). *Fusarium subglutinans* strains clustered very closely, with similarity ranging from 100-87%. On the other hand the amplification patterns of *F. verticillioides* were identical, as well as in the case of *F. proliferatum*. Our results indicated that these *Fusaria* species are distinct species and hence RAPD markers can be quick and reliable for differentiating them.

KEYWORDS: *Fusarium*, *Gibberella fujikuroi,F. moniliforme,* RAPD-PCR

INTRODUCTION

The systematics of *Fusarium* have been subject to debate for several decades, due to the remarkable degree of variation in phenotypic, physiological, biological and ecological traits among and between species. Traditional classification is based on morphological criteria of isolates grown on special media. In order to identify species confidently by morphological methods not only do the culture conditions have to follow the classification scheme used in detail, but also experience and extensive knowledge of the differentiating morphological features of a species is an inevitable prerequisite.

Fusarium section *Liseola* is recognised in most morphologically based classification schemes for *Fusarium.* Wollenweber & Reinking (1935), Booth (1971), Nirenberg (1976), Gerlach & Nirenberg (1982), Nelson *et al*. (1983) and Burgess *et al*. (1988) accepted 6, 2, 10, 10, 4, and 4 species and varieties, respectively within the section *Liseola*. A recent systematic account of the *Gibberella fujikuroi* (Sawada) Wollenweber. species complex of *Fusarium*, section *Liseola* (Nirenberg & O´Donnell 1998), brings the number of described species within this complex to 29. On the one hand, the different numbers of taxa recognised within the genus *Fusarium* and the section *Liseola* respectively, might reflect the variability of the strains in nature and culture (Nirenberg 1990). On the other hand, an exact identification of species boundaries could greatly increase the accurate prediction of host range, environmental and climatic adaptation, and the mycotoxin potential of each species.

Fusarium subglutinans (Wollenw. and Reinking) Nelson, Toussoun and Marasas sensu Nirenberg and O´Donnell, *F. proliferatum* (Matsushima) Nirenberg and *F. verticillioides* (Saccardo) Nirenberg (syn. *F. moniliforme* Sheldon sensu strictu) are species within the *Gibberella fujikuroi* (Sawada) Wollenw. species complex of *Fusarium*, section *Liseola*, and are recognised in most recent identification schemes based on morphological methods (Nelson *et al*. 1983; Burgess *et al*. 1988; Nirenberg & O´Donnell 1998).

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F. subglutinans has recently been raised to species status by Nelson *et al*. (1983) and by Nirenberg & O´Donnell (1998) and is equivalent to *F. sacchari* (Butler) W. Gams var. *subglutinans* (Wollenw. and Reinking) Nirenberg. Morphological key characters of the species within section *Liseola* are the formation of microconidia in false-heads from polyphialides and the absence of chlamydospores.

F. subglutinans is widespread in cooler temperate zones where it is frequently associated with stalk rot and cob rot of maize (Marasas *et al*. 1979; Gerlach & Nirenberg 1982; Burgess *et al*. 1988). In Austria *F. subglutinans* is the most prevalent *Fusarium* species (about 50% of all isolates) on naturally infected maize ears (Lew *et al*. 1991; Adler 1993). The species is noted for the production of toxic secondary metabolites such as moniliformin (Kriek *et al.* 1977; Marasas *et al*. 1979; Chelkowski *et al*. 1990) and beauvericin mycotoxins (Logrieco *et al*. 1993). Austrian isolates from maize have been proven to produce moniliformin (Lew *et al*. 1991) and beauvericin (Krska *et al.* 1997).

F. verticillioides (Sacc.) Nirenberg is equivalent to *F. moniliforme* Sheldon sensu strictu. The species is morphologically characterised within *Liseola* by the presence of long chains of microconidia produced from monophialides and the absence of chlamydospores. The Experience of Burgess *et al*. (1988) indicates that the chains of microconidia formed by *F. proliferatum* are usually shorter than those of *F. verticillioides.*

While the fungus is widely distributed it appears that it is more abundant in warmer areas where it causes a wide range of economically important plant diseases. *F. verticillioides* is one of the most important pathogens of maize in warmer climates, causing stalk and cob rot (Gerlach & Nirenberg 1982; Burgess *et al*. 1988; Bullerman & Tsai 1994). Isolates of *F. verticillioides* have been proven to be highly toxic to experimental animals and to produce the carcinogenic fumonisins B_1 and B_2 (Gelderblom *et al*. 1988; Sydenham *et al*. 1990; Bullerman & Tsai 1994); isolates from Austrian maize are known to produce fumonisins (Lew *et al*. 1991).

Morphological key characters of *F. proliferatum* within *Liseola* are the presence of chains of microconidia produced from polyphialides and the absence of chlamydospores (Nirenberg 1976; Gerlach & Nirenberg 1982; Burgess *et al*. 1988). This taxon was distinguished only recently by Nirenberg (1976) from what may now be considered the *F. moniliforme*-complex. *F. proliferatum* is similar to *F. verticillioides* in many respects. The formation of polyphialides by *F. proliferatum* is the primary criterion for separating the two species, and it usually has relatively short conidial chains (Burgess *et al*. 1988).

The species occurs world-wide on a broad variety of economically important plants, including maize (Gerlach & Nirenberg 1982; Chelkowski & Lew 1992; Bullerman & Tsai 1994), although in the past the occurrence of the fungus has often been underestimated owing to its resemblance to the species *F. verticillioides*. In Austria *F. proliferatum* plays a minor role as pathogen of maize (Adler 1993; Lew 1995).

Isolates of *F. proliferatum* have been proven to be highly toxic to experimental animals and to produce several mycotoxins, including fumonisins, fusaroproliferin, beauvericin, and moniliformin (Chelkowski *et al*. 1990; Chelkowski & Lew 1992; Nelson *et al*. 1992; Moretti *et al*. 1994; Ritieni *et al.* 1997). A recent report describes the production of fumonisins together with moniliformin and beauvericin by *F. proliferatum* isolated from Austrian maize (Krska *et al*. 1997).

There are indications, mainly from publications in North America (Koehler 1959; Shurtleff 1980), which are in line with our own observations, that corn borer damage of maize plants or ears increases susceptibility to infection by *Fusarium* species of the *Liseola* section (Lew *et al*. 1991). In Austria *F. subglutinans* is the most prevalent *Fusarium* species on naturally infected maize ears (about 50% of all isolates), but there are some indications that under recent changing climatic conditions – characterised by milder winter and warmer and

dryer summer months - may favour fungi like *F. proliferatum* and *F. verticillioides* which occur more frequently in Austrian maize (Lew *et al*., publication forthcoming).

The control of fusarioses of maize is important not only to reduce yield losses caused by *Fusarium* infection, but also to decrease the risk of mycotoxin contamination in human and animal nutrition. An exact identification of the species could greatly increase the accurate prediction of environmental and climatic adaptation and the mycotoxin potential of each species.

Molecular tools such as random amplification of polymorphic DNA (RAPD) (DuTeau & Leslie 1991; Viljoen *et al*., 1997; Voigt *et al*. 1995), mitochondrial restriction fragment length polymorphisms (RFLP) (Correll *el al*., 1992), and ribosomal DNA (rDNA) internal transcribed spacer (ITS1 and ITS2) sequences (O' Donnell & Cigelnik 1997; Waalwijk *et al*. 1996) have been used for differentiating *F. subglutinans* from other related *Fusarium* species. Two different copies of the ITS2 regions were identified in the same isolate within some species (O' Donnell & Cigelnik 1997; Waalwijk *et al*. 1996) but a reliable diagnostic technique based on these sequences could not be developed. Alternative regions such as the histone and ß-tubulin genes might be used more effectively. Steenkamp *et al*. (1999) used alternative regions of the genome, the histone H3 gene to distinguish *F. subglutinans* f. sp. *pini* isolates from other isolates of *F. subglutinans*. O' Donnell *et al*. (1998) used the DNA sequences of the nuclear rDNA large subunit, mitochondrial small subunit, and ß-tubulin to develop a phylogeny that included 36 taxa in the *G. fujikuroi* species complex. These sequences may potentially be useful for diagnostic purposes.

The primary objective of the present study therefore, was to enable exact identification of the most prevalent *Fusarium*, section *Liseola*, species from naturally infected Austrian maize ears by morphological and molecular analysis and to study the genetic diversity of the strains isolated from different locations.

MATERIALS AND METHODS

Strains: *Fusarium* strains were isolated from infected maize ears during the years 1994, 1997 and 1998 from crops in the main maize growing regions of Austria. *Fusaria* were isolated from the ears according to the method of Lew *et al*. (1991). Mycelium from ears with visible infection was transferred to Potato Dextrose Agar (Oxoid CM 139; Basingstoke, GB) plates and incubated for 1 week at 28°C. Colonies were subcultured as single-spore isolates on synthetic low nutrient agar (SNA) with an approx. 1 x 2 cm piece of sterile paper placed on the hardened agar (Nirenberg 1976). The cultures were incubated for 10 - 14 days at 20°C and identified according to Nirenberg & O´Donnell (1998). All strains examined are listed in Table 1 and are maintained at the culture collection of Institute of Applied Microbiology, IAM, University of Agricultural Science, Vienna, Austria.

DNA extraction: Fungal strains were cultured in 100 ml Erlenmeyer-flasks containing 20 ml Mandles Andreoti-Medium (per litre: 10 g glucose; 2 g peptone; 2.8 g ammonium sulphate; 4 g KH_2PO_4; 10 g; Na_2HPO_4, 10 ml of a simplified Czapek conc. 7 g $MgSO_4$; 0.05 g $CuSO_4$ $5H_2O$; 0.1 g $FeSO_4$ $7H_2O$; 0.1 g $ZnSO_4$ $7H_2O$; final pH adjusted to 5.0) for five days using a rotary shaker (30 °C, 150 rpm).

The mycelium was collected by filtration and ground to fine powder in liquid N_2. Fifty mg of the powder was transferred to a 1.5 ml Eppendorf tube and mixed with 700 µl 2×CTAB buffer. Eppendorf tubes were incubated at 65 °C for 30 min., then 700 µl of chloroforme was added and mixed briefly. After centrifugation at 15000 rpm for 30 min, the supernatant was transferred into a new tube mixed with 600 µl isopropanol and chilled to –20°C, followed by another centrifugation step for 5 min at maximum speed. The supernatant was discarded and the remaining pellet was twice washed with 1 ml of 70% ethanol, followed by drying under vacuum

and thereafter dissolved in 100 µl TE (10mM Tris , 1 mM EDTA, pH 7.5) buffer. DNA concentrations were evaluated by agarose gel electrophoresis (Moeller *et al*. 1992).

Table 1**:** *Fusarium* strains used in this study

No	IAM	*Fusarium* sp.	Host	Cultivar	Crop	Origin
1	MA 1248	*F. tricinctum*	Wheat	-		USA
2	MA 1745	*F.subglutinans*	Maize		-	BA Linz, Austria
3	MA 1746	*F.subglutinans*	Maize		1996	Hartberg, Austria
4	MA 1749	*F.subglutinans*	Maize		1997	Mogersdorf, Austria
5	MA 1750	*F.subglutinans*	Maize		1996	Wieselsdorf, Austria
6	MA 1751	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
7	MA 1752	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
8	MA 1753	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
9	MA 1754	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
10	MA 1756	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
11	MA 1757	*F.subglutinans*	Maize		1996	Mogersdorf, Austria
12	MA 1759	*F.subglutinans*	Maize		1997	Wieselsdorf, Austria
13	MA 1799	*F.subglutinans*	Maize	-	-	BA Linz, Austria
14	MA 1837	*F.subglutinans*	Maize ear	Banguy	1998	Grossnondorf, Lower Austria
15	MA 1838	*F.subglutinans*	Maize ear	Banguy	1998	Grossnondorf, Lower Austria
16	MA 1839	*F.subglutinans*	Maize ear	Banguy	1998	Breitbrunn, Upper Austria
17	MA 1840	*F.subglutinans*	Maize ear	Banguy	1998	Breitbrunn, Upper Austria
18	MA 1841	*F.subglutinans*	Maize ear	DK 300	1998	Gleisdorf, Styris, Austria
19	MA 1842	*F.subglutinans*	Maize ear	DK 300	1998	Gleisdorf, Styris, Austria
20	MA 1843	*F.subglutinans*	Maize ear	Prinz	1998	Hörzendorf, Carinthia, Austria
21	MA 1844	*F.subglutinans*	Maize ear	Prinz	1998	Hörzendorf, Carinthia, Austria
22	MA 1845	*F.subglutinans*	Maize ear	Costella	1998	Hörzendorf, Carinthia, Austria
23	MA 1846	*F.subglutinans*	Maize ear	Costella	1998	Hörzendorf, Carinthia, Austria
24	MA 1847	*F.subglutinans*	Maize ear	Prinz	1998	Grossnondorf, Lower Austria
25	MA 1848	*F.subglutinans*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
26	MA 1849	*F.subglutinans*	Maize ear	Raissa	1998	Fluttendorf, Styria, Austria
27	MA 1850	*F.subglutinans*	Maize ear	Clarisia	1998	Fuchsenbigl, Lower Austria
28	MA 1851	*F.subglutinans*	Maize ear	DK 300	1997	Eltendorf, Styria, Lower Austria
29	MA 1852	*F.subglutinans*	Maize ear	Raissa	1997	Fluttendorf, Styria, Austria
30	MA 1853	*F.subglutinans*	Maize ear	DK 300	1997	Alkoven, Upper Austria
31	MA 1854	*F.subglutinans*	Maize ear	DK 300	1997	Fuchsenbigl, Lower Austria
32	MA 1855	*F.subglutinans*	Maize ear	-	1994	Hartberg, Styria, Austria
33	MA 1249	*F.subglutinans*	Maize			USA
34	MA 1737	*F.verticillioides*	Maize			BA Linz, Austria
35	MA 1739	*F.verticillioides*	Maize			BBA Berlin, Germany
36	MA 1857	*F.verticillioides*	Maize ear		1994	Hartberg, Styria, Austria
37	MA 1857	*F.verticillioides*	Maize ear		1994	Breitbrunn, Upper Austria
38	MA1833	*F.verticillioides*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
39	MA 1834	*F.verticillioides*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
40	MA 1835	*F. proliferatum*	Maize ear	Prinz	1998	Breitbrunn, Upper Austria
41	MA 1836	*F. proliferatum*	Maize ear	Prinz	1998	Hofing, Upper Austria
42	MA 1730	*F. proliferatum*	Chinase soil			BBA Berlin
43	MA 1758	*F. proliferatum*	Maize		1997	Mogersdorf, Austria
44	MA 1250	*F. proliferatum*	Maize			USA

RAPD analysis: PCR conditions and separation of RAPD-PCR fragments were done according to the techniques of Messner *et al.* (1994). PCR´s were carried out by aid of primer V1 (5´ d ACGGTCTTGG; Schäfer & Wöstemeyer 1992); V5 (5´ dTGCCGAGCTG; Caetano – Anolles *et al*. 1992) and M13 (GAGGGTGGCGGTTCT K:O'Donnell *et al*. 1999) respectively following the temperature protocol: denaturation at 98°C for 15s; annealing at 40°C for 90s and extenuation at 72°C for 100s for a total of 40 cycles. The levels of

similarity between individual lanes were calculated as previously described by Nei & Li (1979).

Computer analysis of RAPD patterns were performed as given by Halmschlager *et al* (1994). Basically, the formation obtained from agarose gel electrophoresis was digitalized by hand to a two - discrete - character - matrix (0 and 1 for absence and presence of RAPD - bands). Dendrogram was calculated by using the Jukes - Cantor option in the DNADIST program and application of the FITCH program to the computed distance matrix (PHYLIP package; Felsenstein 1989). For running DNADIST, the two discrete characters of 0 and 1 had to be converted to Guanine and Thymine in the RAPD data matrix. Complete alignment of data was performed with CLUSTALX software, and then cluster analysis will be ready by using Treecon programme (van der Peer 1994).

RESULTS AND DISCUSSION

Thirty -two strains of *F. subglutinans*, 6 strains from *F. verticillioides* and 5 strains from *F. proliferatum,* which well isolated from maize in Austria, were used in this study (Table 1). The three primers used in this study, V1, V5 and M13, generated a considerable number of amplification products for comparison. A different DNA banding pattern was present in almost every strain. Comparison of each profile for each of the primers was based on the presence (1) versus absence (0) of RAPD amplimers that migrated to the same position in the gel. Bands of the same size obtained by the same primer were scored as identical, but only bands repeatable in at least two experiments with the same primer at different times were evaluated. All 3 primers revealed high similarities between *F. subglutinans* strains (Fig. 1). Our results showed that RAPD profiles of isolates of *F. subglutinans* from maize were similar but not identical; these findings are similar to those of Viljoen *et al*. (1997), who reported that RAPD profiles of isolates *of F. subglutinans* from pine were similar but not identical.

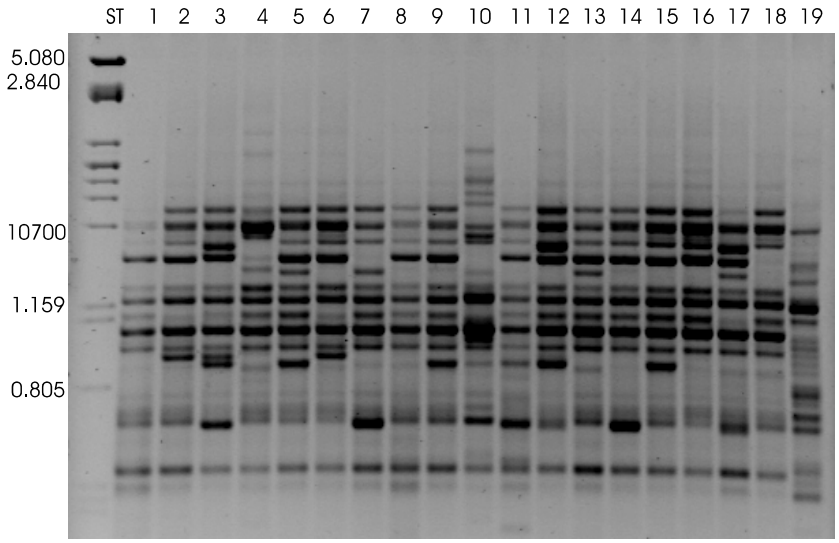

Fig.1. Pattern of fragments from RAPD analysis of different *Fusarium subglutinans* strains (lanes 1-18) and *F. tricinctum* (lane 19) primed by M13 (5' dGAGGGTGGCGGTTCT O'Donnell *et al*. 1999).

The combined data from all isolates of *F. subglutinans* were analyzed to produce a dendrogram (Fig.2). *Fusarium tricinctum* was included as an out-group strain to create a rooted tree in cluster analysis. Dendrogram revealed no correlation between clusters and geographical origin or the type of maize cultivar. Viljoen *et al*. (1997) also constructed a dendrogram that represented similarity among some *F. subglutinans* isolates. They reported

that the clustering patterns in the dendrogram showed that isolate clusters correlate to host, hence isolates of *F.subglutinans* from maize clustered together.

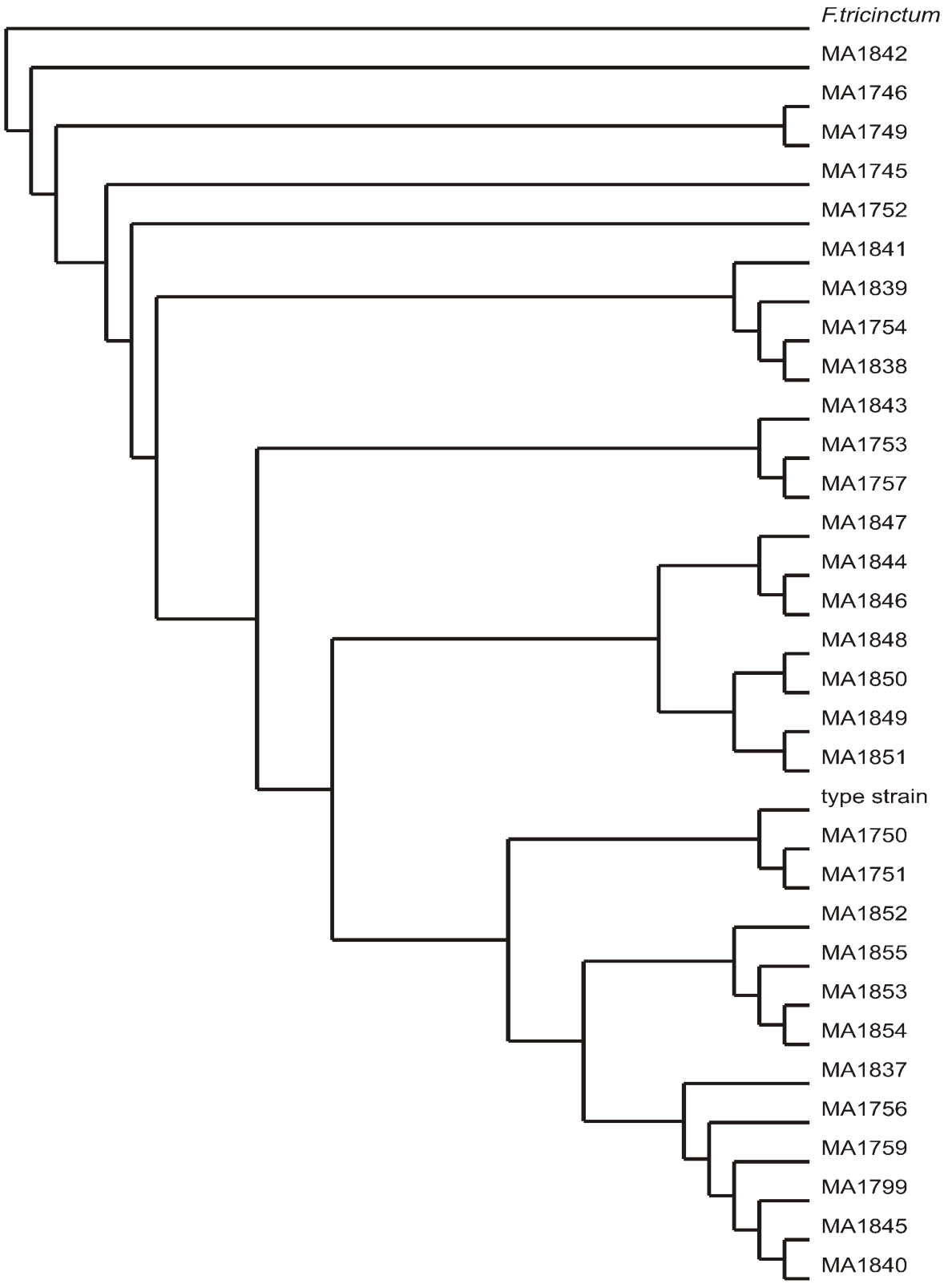

Fig. 2: Dendrogram showing relations among 32 strains of *Fusarium subglutinans* . Genetic distances were obtained by random amplified polymorphic DNA analysis with 3 different primers

Fig. 3 shows the amplification products generated with a primer M13 for some representative strains of all *Fusarium* species under investigation. The molecular size of amplimers ranged from 500-2000 bp and all the primers tested revealed at least one polymorphic band, which could be used to define homogeneous groups among the different isolates. In this part of study we also used a strain from *Fusarium sacchari* var. *sacchari* to compare it with our *F. subglutinans* strains. According to the dendrogram (Fig.4) constructed from these results, the strains of each species of *Fusaria* under investigation clustered together. We can conclude that these strains represented distinct species within *Gibberella fujikuroi*. Also, *F. subglutinans* is a distinct species from *F. sacchari.* var. *sacchari.*

This study has shown that there is considerable genotypic and phenotypic variability among *Fusarium* isolates belonging to three *Fusarium* species (*F. proliferatum, F. subglutinans* and *F. verticillioides*) obtained from different geographic regions in Austria. RAPD pattern analysis proved to be powerful and could be used to describe the *Fusarium* isolates individually.

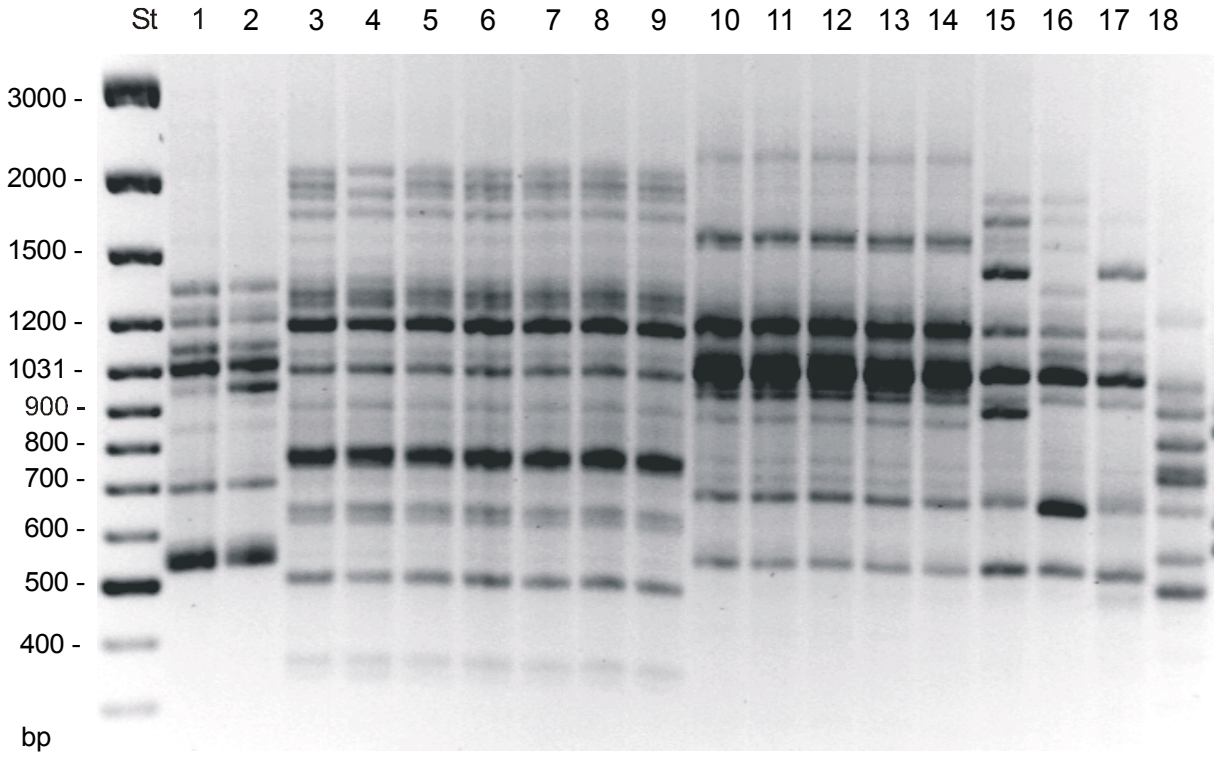

Fig. 3: Random amplified polymorphic DNA patterns obtanied by using the primer M13 (GAGGGTGGCGGTTCT. O'Donnell *et al*., 1999) from *Fusarium proliferatum* (lanes 1 and 2), *F. verticillioides* (lanes 3-9), *F. subglutinans* (lanes 10-17) and *F. sacchari* var. *sacchari* (lane 18).

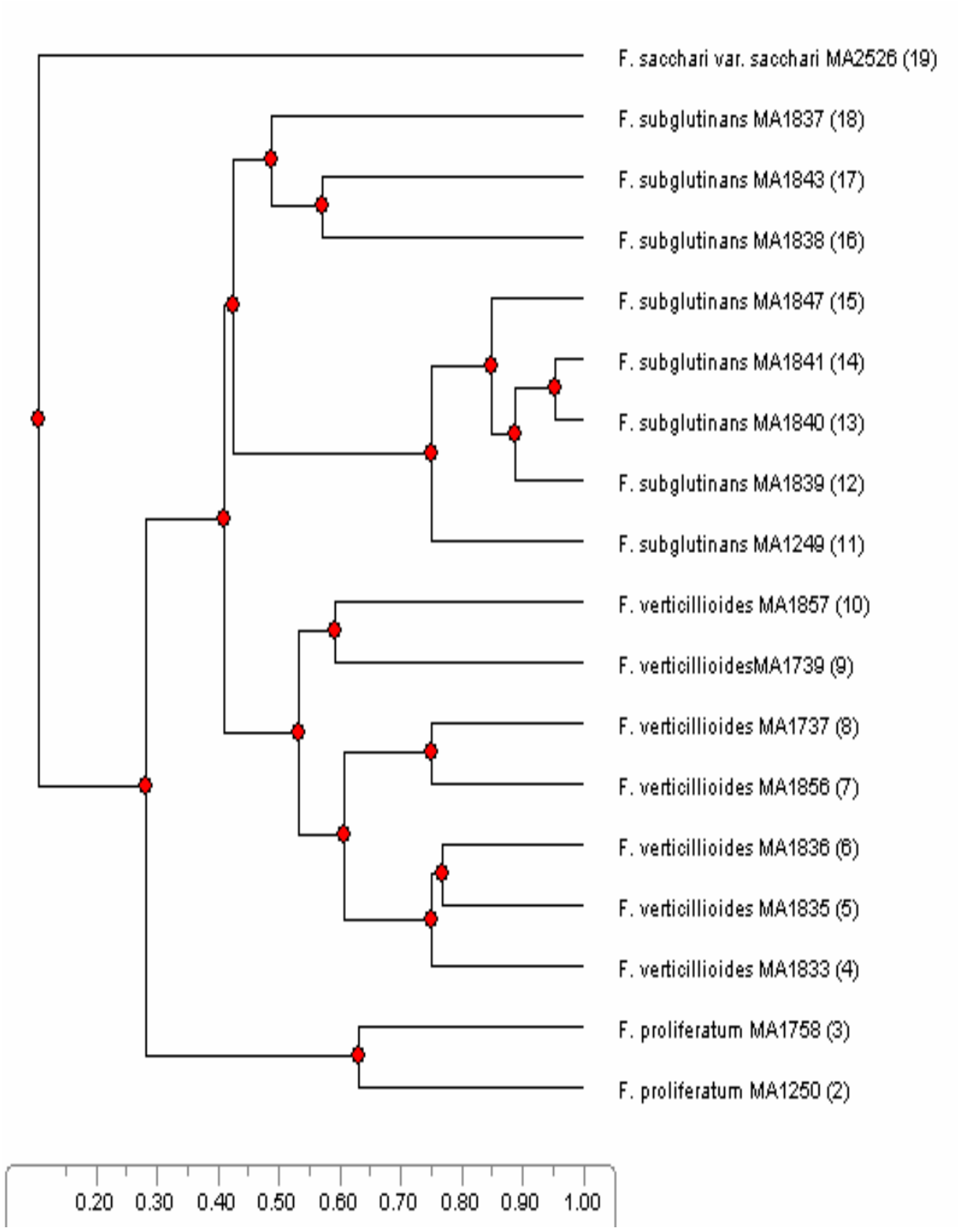

Fig. 4: Dendrogram showing the relationship among some strains of *F. subglutinans, F. verticillioides, F. proliferatum* and *F. sacchari* var. *sacchari* depending upon RAPD results.

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الملخص العربى

التعريف الجينى لفطريـات فيوزاريـوم سـبجليوتينانس , فيوزاريـوم بروليفـراتم و فيوزاريـوم فيرتسلويدس المعزوله من نباتات الذرة المنزرعة بالنمسا

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هدفت هذه الدراسة إلى تطوير تقنية تمكن الدارسين لعلوم الفطريات من التمييز بين العـزلات المختلفـة مـن فطـر *فيوزاريوم سبجليوتينانس* , *فيوزاريوم بروليفراتوم* و *فيوزاريوم فيرتسلويدس* التى تنتمي الى النوع المركـب جيبـريلا *فيجيكورى*، 43 عزله من من الانواع الثلاثة (32 عزله من *فيوزاريوم سبجليوتينانس* ,5 عـزلات مـن *فيوزاريـوم بروليفراتوم* و6 عزلات من *فيوزاريوم فيرتسلويدس*) تم عزلها من نباتات الذرة بالنمسا استخدمت فى هـذه الدراسـة. استخدام تقنية البصمة الجينيه (RAPD) أوضح أن درجة التشابه بين العزلات المختلفـة مـن فطـرة *فيوزاريـوم سبجليوتينانس* تتراوح بين 87-100%. و كذلك باستخدام التحليل الحزمى لتوضيح العلاقات الوراثية بين الأنواع الثلاثة قيد الدراسة ثبت أن كل نوع من هذه الانواع مستقل بذاته وبالتالى تكون هذه التقنية من الوسائل الفعالة لتعريف وتمييز هذه الأنواع من بعضها البعض.