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Genotypic Identification of *Fusarium subglutinans*, *F. proliferatum* and *F. verticillioides* Strains Isolated from Maize in Austria

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***Gibberella fujikuroi* is species complex. This species complex includes *Fusarium tabacinum*, *F. moniliforme* (= *F. verticillioides*), *F. nygamai*, *F. proliferatum* as well as *F. subglutinans*. Our objective was to develop a technique to differentiate between isolates of *F. subglutinans*, *F. proliferatum* and *F. verticillioides*. Thirty-two strains of *F. subglutinans*, six strains *from F. verticillioides* and five strains of *F. Proliferatum* isolated from maize in Austria were studied using random amplified polymorphic DNA (RAPD). *F. subglutinans* strains clustered very closely, with similarity ranging from 87~100%. On the other hand, all 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*, *F. moniliforme*, *Gibberella fujikuroi*, RAPD-PCR, Phylogenetics**

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 the 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 and Reinking (1935), Booth (1971), Nirenberg (1976), Gerlach and 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 and O'Donnell, 1998), brings the number of described species within this complex to twenty-nine. 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). 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.

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F. 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 (Burgess *et al.*, 1988; Nelson *et al.*, 1983; Nirenberg and O'Donnell, 1998).

F. subglutinans has recently been raised to species status by Nelson *et al.* (1983) and by Nirenberg and 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 (Burgess *et al.*, 1988; Gerlach and Nirenberg, 1982; Marasas *et al.*, 1979). In Austria *F. subglutinans* is the most prevalent *Fusarium* species (about 50% of all isolates) on naturally infected maize ears (Adler, 1993; Lew *et al.*, 1991). The species is noted for the production of toxic secondary metabolites such as moniliformin (Chelkowski *et al.*, 1990; Kriek *et al.*, 1977; Marasas *et al.*, 1979) 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 (Bullerman and Tsai, 1994; Burgess *et al.*, 1988; Gerlach and Nirenberg, 1982). 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 (Bullerman and Tsai, 1994; Gelderblom *et al.*, 1988; Sydenham *et al.*, 1990); isolates from Austrian maize are known to produce fumonisins (Lew *et al.*, 1991).

Morphological key characteristics of *F. proliferatum* within *Liseola* are the presence of chains of microconidia produced from polyphialides and the absence of chlamydospores (Burgess *et al.*, 1988; Gerlach and Nirenberg, 1982; Nirenberg, 1976). 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 (Bullerman and Tsai, 1994; Chelkowski and Lew, 1992; Gerlach and Nirenberg, 1982), 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 and Lew, 1992; Chelkowski *et al.*, 1990; Moretti *et al.*, 1994; Nelson *et al.*, 1992; 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.*, unpublished data).

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 and Leslie, 1991; Viljoen *et al.*, 1997; Voigt *et al.*, 1995), mitochondrial restriction fragment length polymorphisms (RFLP) (Correll *et al.*, 1992), and ribosomal DNA (rDNA) internal transcribed spacer (ITS1 and ITS2) sequences (O'Donnell and 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 and Cigelnik, 1997; Waalwijk *et al.*, 1996). However, a reliable diagnostic technique based on these sequences could not be developed. Alternative regions such as the histone and β-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 β-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 exactly identify the most prevalent *Fusarium*, section *Liseola*, species from naturally infected Austrian maize ears through 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 in 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 plates and incubated for one week at 28°C. Colonies were subcultured as single-spore isolates on synthetic low nutrient agar (SNA)

Table 1. *Fusarium* strains used in this study

IAM	*Fusarium* sp.	Host	Cultivar	Crop	Origin
MA 1248	*F. tricinctum*	Wheat			USA
MA 1745	*F. subglutinans*	Maize			BA Linz, Austria
MA 1746	*F. subglutinans*	Maize		1996	Hartberg, Austria
MA 1749	*F. subglutinans*	Maize		1997	Mogersdorf, Austria
MA 1750	*F. subglutinans*	Maize		1996	Wieselsdorf, Austria
MA 1751	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1752	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1753	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1754	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1756	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1757	*F. subglutinans*	Maize		1996	Mogersdorf, Austria
MA 1759	*F. subglutinans*	Maize		1997	Wieselsdorf, Austria
MA 1799	*F. subglutinans*	Maize			BA Linz, Austria
MA 1837	*F. subglutinans*	Maize ear	Banguy	1998	Grossnondorf, Lower Austria
MA 1838	*F. subglutinans*	Maize ear	Banguy	1998	Grossnondorf, Lower Austria
MA 1839	*F. subglutinans*	Maize ear	Banguy	1998	Breitbrunn, Upper Austria
MA 1840	*F. subglutinans*	Maize ear	Banguy	1998	Breitbrunn, Upper Austria
MA 1841	*F. subglutinans*	Maize ear	DK 300	1998	Gleisdorf, Styris, Austria
MA 1842	*F. subglutinans*	Maize ear	DK 300	1998	Gleisdorf, Styris, Austria
MA 1843	*F. subglutinans*	Maize ear	Prinz	1998	Hörzendorf, Carinthia, Austria
MA 1844	*F. subglutinans*	Maize ear	Prinz	1998	Hörzendorf, Carinthia, Austria
MA 1845	*F. subglutinans*	Maize ear	Costella	1998	Hörzendorf, Carinthia, Austria
MA 1846	*F. subglutinans*	Maize ear	Costella	1998	Hörzendorf, Carinthia, Austria
MA 1847	*F. subglutinans*	Maize ear	Prinz	1998	Grossnondorf, Lower Austria
MA 1848	*F. subglutinans*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
MA 1849	*F. subglutinans*	Maize ear	Raissa	1998	Fluttendorf, Styria, Austria
MA 1850	*F. subglutinans*	Maize ear	Clarisia	1998	Fuchsenbigl, Lower Austria
MA 1851	*F. subglutinans*	Maize ear	DK 300	1997	Eltendorf, Styria, Lower Austria
MA 1852	*F. subglutinans*	Maize ear	Raissa	1997	Fluttendorf, Styria, Austria
MA 1853	*F. subglutinans*	Maize ear	DK 300	1997	Alkoven, Upper Austria
MA 1854	*F. subglutinans*	Maize ear	DK 300	1997	Fuchsenbigl, Lower Austria
MA 1855	*F. subglutinans*	Maize ear		1994	Hartberg, Styria, Austria
MA 1249	*F. subglutinans*	Maize			USA
MA 2526	*F. sacchari* var. *sacchari*				USA
MA 1737	*F. verticillioides*	Maize			BA Linz, Austria
MA 1739	*F. verticillioides*	Maize			BBA Berlin, Germany
MA 1857	*F. verticillioides*	Maize ear		1994	Hartberg, Styria, Austria
MA 1857	*F. verticillioides*	Maize ear		1994	Breitbrunn, Upper Austria
MA 1833	*F. verticillioides*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
MA 1834	*F. verticillioides*	Maize ear	DK 300	1998	Grossnondorf, Lower Austria
MA 1835	*F. proliferatum*	Maize ear	Prinz	1998	Breitbrunn, Upper Austria
MA 1836	*F. proliferatum*	Maize ear	Prinz	1998	Hofing, Upper Austria
MA 1730	*F. proliferatum*	Chinase soil			BBA Berlin, Germany
MA 1758	*F. proliferatum*	Maize		1997	Mogersdorf, Austria
MA 1250	*F. proliferatum*	Maize			USA

with an approx. 1×2 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 and 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 m*l* Erlenmeyer-flasks containing 20 m*l* 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 m*l* 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 5 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 m*l* 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 15,000 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 (10 mM Tris, 1 mM EDTA, pH 7.5) buffer. DNA concentrations were evaluated by agarose gel electrophoresis (Moeller *et al.*, 1992).

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 with the aid of primer V1 (5' d ACGGTCTTGG; Schäfer and Wöstemeyer, 1992); V5 (5' dTGCCGAGCTG; Caetano-Anolles *et al.*, 1992) and M13 (GAGGGTGGCGGTTCT; O'Donnell *et al.*, 1999) respectively following the temperature protocol: denaturation at 98°C for 15s; annealing at 40°C for 90s and extension 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 and Li (1979). Computer analysis of RAPD patterns was 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

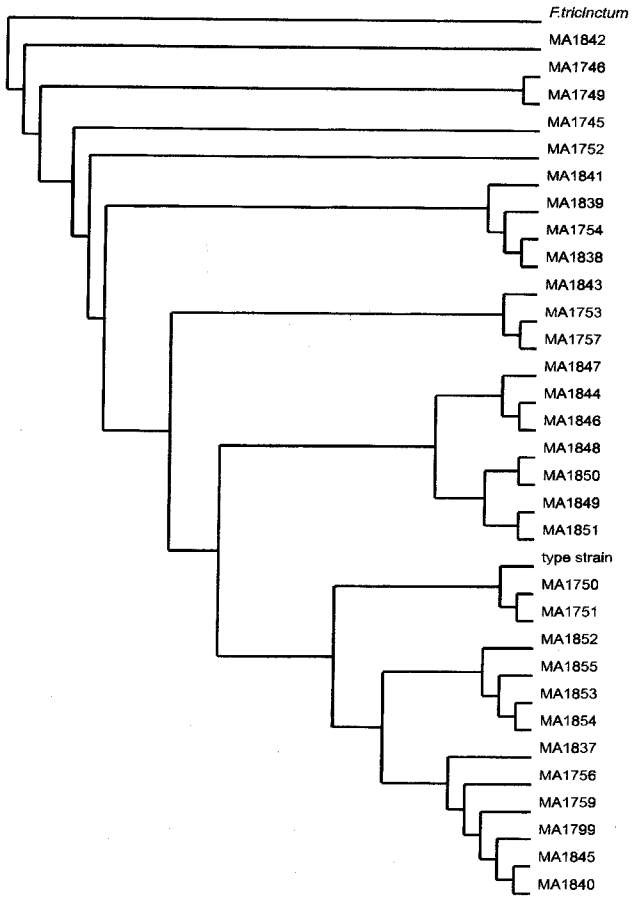

Fig. 2. Dendrogram showing relationships among 32 strains of *Fusarium subglutinans* includes type strain (MA1249). Genetic distances were obtained from random amplified polymorphic DNA analysis with three different primers.

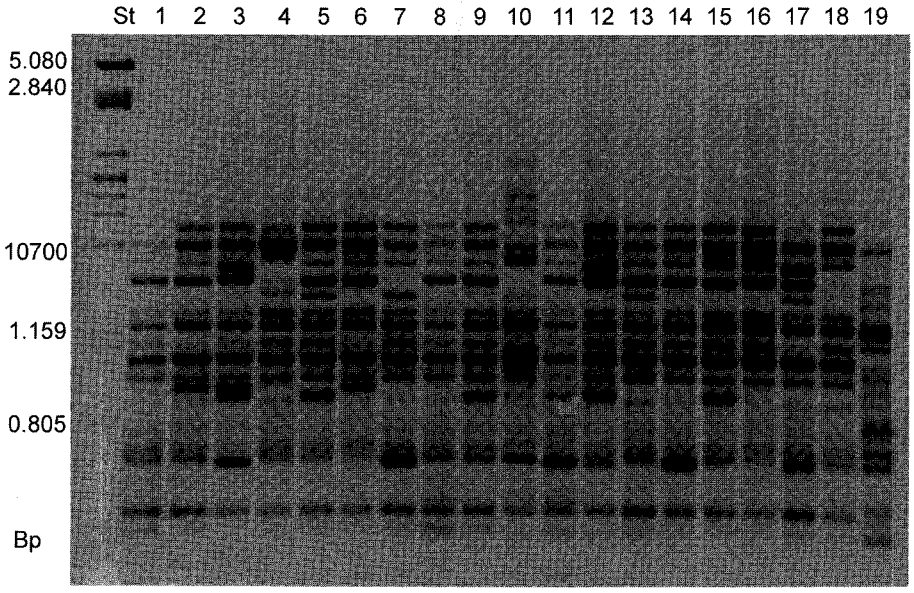

Fig. 1. Pattern of fragments from RAPD analysis of different *Fusarium subglutinans* strains (lanes 1-18) and *F. tricinctum* (lane 19) obtained with primer M13 (5' dGAGGGTGGCGGTTCT, O'Donnell *et al.*, 1999).

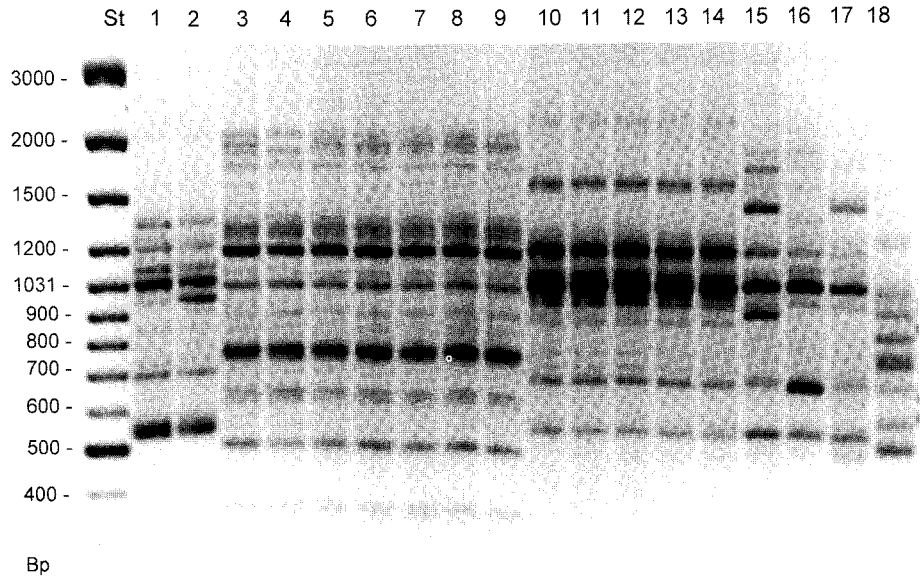

Fig. 3. Random amplified polymorphic DNA patterns obtanied by using the primer M13 (GAGGGTGGCGGTTCT. O'Donnell *et al.*, 1999) from *Fusarium proliferatum* (lanes 1 and 2), *F. verticillioides* (lanes 3-9), *F. subglutinans* (lanes 10-17) and *F. sacchari* var. *sacchari* (lane 18).

had to be converted to Guanine and Thymine in the RAPD data matrix. Complete alignment of data was performed with CLUSTALX software, and then the cluster analysis will be ready by using Treecon programme (van der Peer, 1994).

Results and Discussion

Thirty-two strains of *F. subglutinans*, six strains from *F. verticillioides* and five strains from *F. proliferatum,* which were 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 three primers revealed high similarities among *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.

The combined data from all the isolates of *F. subglutinans* were analyzed to produce a dendrogram (Fig. 2). *F. 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 con-

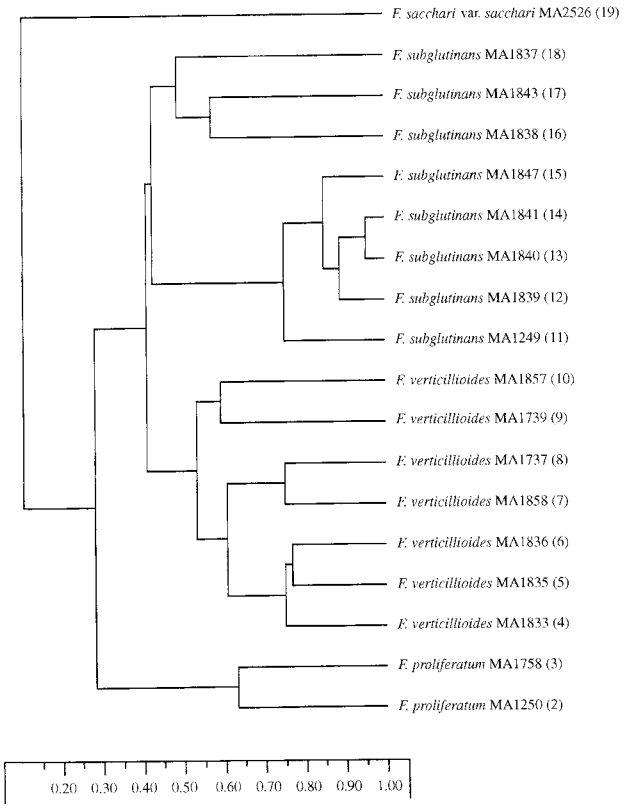

Fig. 4. Dendrogram showing the relationships among the isolates of *Fusarium subglutinans*, *F. verticillioides*, *F. proliferatum* and *F. sacchari* var. *sacchari* based on RAPD results. Number in brackets indicates the position of isolate on the gel according to Fig. 3.

structed 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.

Figure 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 500~2,000 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 *Fusarium sacchari* var. *sacchari* strain to compare 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.

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