

MATERIALS AND METHODS

Amplified fragment length polymorphism (AFLP) analysis

We used an AFLP Microbial Fingerprinting kit (Life Technologies, Carlsbad, CA, USA) for AFLP analysis, according to manufacturer's instructions. T4 DNA ligase, EcoRI, and MseI (New England Biolabs, Beverly, MA, USA) were used in the simultaneous restriction–ligation reaction. Preselective and selective polymerase chain reactions (PCRs) were performed in an iCycler thermal cycler (Bio-Rad Laboratories Hercules, CA, USA). A fluorescent dye-labelled EcoRI primer with two-base selection (FAM-EcoRI +AC, NED-EcoRI +AT, or JOE-EcoRI +AG) and an unlabelled MseI primer with two-base selection (MseI +CA) were used in the selective PCR. The FAM-EcoRI +AC, NED-EcoRI +AT, or JOE-EcoRI +AG reaction mixture was mixed in a 1:3:1 (v:v:v) ratio after the selective PCR. Hi-Di formamide (9.95 μ L; Life Technologies) and standard size GeneScan 500 (ROX; 0.05 μ L; Life Technologies) were added to 1.5 μ L of each mixed sample. The samples were analysed with the modified conditions of injection, voltage 2.0, and time 44 using a 3100 Genetic Analyser with a 36-cm 16-capillary array, a Performance Optimized Polymer 6 (POP6) separation matrix, and GeneScan collection software (Life Technologies). The obtained

data were processed using GeneMapper software (Life Technologies) to produce the binary matrix for phylogenetic analysis.

Phylogenetic analysis

The binary data for a total of 66 AFLP markers of EACMCAB (FAM-EcoRI +AC), EATMCAY (NED-EcoRI +AT), and EAGMCAG (JOE-EcoRI +AG) from 95 taxa were compiled into a single data matrix. Neighbour-joining (NJ) analysis (Saitou and Nei 1987) of the binary data was performed using PAUP* (version 4.0 β 10) (Swofford 2002). Ties were broken randomly when encountered.

The sequence datasets combined the data for *TEF1 α* and *RPB2* from 35 taxa including an outgroup (*Fusarium hostae* NRRL29889). The sequences obtained in the present study were aligned with MAFFT (version 7) using the E-INS-i option online (Kato et al 2002, Kato et al 2005, Kato et al 2009). The sequences were truncated at the 5' - and 3' -ends, and manually aligned when necessary using ChromasPro. Phylogenetic trees were obtained using maximum parsimony (MP), maximum likelihood (ML), and Bayesian phylogenetic analyses. The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via the corrected Akaike information criterion (AICc) (Akaike 1974, Sugiura 1978) for MP and ML analyses, and the Bayesian information criterion (BIC) (Schwarz 1978) for the Bayesian analysis.

Kakusan4 (Tanabe 2007) and PAUP* were used for likelihood calculations. As a result of the calculations, the TEF1 α regions were fitted to a J2 model with a gamma-shaped distributed rate. RPB2 regions were fitted to a TN93ef model with a gamma-shaped distributed rate. The combined dataset of the TEF1 α and the RPB2 regions was fitted to a J2ef model with a gamma-shaped distributed rate. MP analysis with the selected evolutionary model was carried out using PAUP*. MP analysis was performed for 1,000 replications with different random starting points using the step-wise addition option to increase the likelihood of finding the most parsimonious tree. Alignment gaps were treated as new state data, and all characters were unordered and had equal weight. The branch-swapping algorithm was tree bisection and reconstruction (TBR). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The best tree topology of the MP trees was established using the Kishino–Hasegawa likelihood test (Kishino and Hasegawa 1989) on PAUP*. We calculated the tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC). The ML analysis was performed by the likelihood ratchet method (Vos 2003). For the ML tree search, 1,000 sets of 25% site-upweighted data were created using the pgresampleseq command in Phylogears 1.0.2009.07.17 (Tanabe 2007), and ML trees with upweighted data were estimated using Treefinder (June 2008

release) (Jobb et al 2005) by applying the best-fit model. The strength of the internal branches from the resulting tree was tested by bootstrap (BS) analysis (Felsenstein 1985) using 10,000 replications in NJ, MP, and ML analysis. Bayesian phylogenetic analyses with the selected evolutionary model were carried out using MrBayes (version 3.2.5) (Ronquist and Huelsenbeck 2003). It was launched with random starting trees for 5×10^6 generations, and the Markov chains were sampled every 500 generations. To ensure that the Markov chain did not become trapped in local optima, we used the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm, performing the estimation with four incrementally heated Markov chains. In each of these analyses, the first 461,000 generations were discarded as burn-in. The remaining trees were summarized in a 50% majority-rule consensus tree, yielding the probabilities of each clade being monophyletic.

Single nucleotide polymorphism analysis

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) experiments for TEF_C447A, FUM1_G423A, FUM78_C41T, FUM8_G2834A, FUM18_G51T, and RPB2_C3250T were performed using the following primers/restriction enzymes: HS434/HS435/NspI, HS482/HS483/NruI,

HS576/HS577/NruI, HS576/HS577/HincII, HS506/HS519/MseI, and HS833/HS834/BmrI, respectively, under the same conditions used for HIS PCR-RFLP (Suga et al 2014) except that the annealing temperature for HS576/HS577 was 58°C, 2% Metaphor agarose gel (Cambrex Bio Science) was used for HS434/HS435/NspI and HS482/HS483/NruI, and 2.5% agarose gel was used for HS833/HS834/BmrI. The derived cleaved amplified polymorphic sequence (dCAPS) primers (HS435, HS482, and HS834) for PCR-RFLP were designed using dCAPS Finder 2.0 (Table S1) (Neff et al 2002). The PCR-sequencing for CPR_C1152A and P4504_C842T were performed as PCR and sequencing in MATERIALS AND METHODS in the original paper.

A multiplex PCR was performed to prepare template DNA for allele-specific primer extension (ASPE) reactions. The PCR components for *TEF1 α* and *FUM* amplification comprised 0.025 units of rTaq polymerase (TaKaRa Bio Inc.) in a 10 μ L reaction mixture containing 1 \times reaction buffer, 200 μ M dNTPs, 1 μ M of each HS438 and HS439 primer (Table S1) (Suga et al 2014), 0.5 μ M of each HS576 and HS577 primer (Table S1), and 5 ng of genomic DNA. The PCR components for CPR and P450-4 amplification comprised 1 μ M of each P138-5, HS556, HSP450-4-GD1, and P450-4-GD2 primer (Table S1) (Malonek et al 2005). The PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories) using the following cycling parameters: 94°C for

2 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Each 2.5- μ L PCR mixture comprising *TEF1 α /FUM* and *CPR/P450-4*, 0.2 μ L of ExoSAP-IT (GE Healthcare Life Sciences, Uppsala, Sweden), and 1.8 μ L of water was kept at 37°C for 30 min, then at 80°C for 15 min. The ASPE reactions were performed according to the manufacturer's instructions using Platinum Genotype Tsp DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA) and HS641, HS642, HS704, HS705, HS700, HS7001, HS557, HS558, HS559, and HS560 primers (Table S1) constituting a volume of 20 μ L and with a 1-min extension step at 50°C. The biotin-labelled products were sorted by hybridization with polystyrene microspheres coated with the following anti-tag sequences: LUA-12, -65, -87, -97, -67, -76, -16, -28, -2, and -14. The hybridizations were performed in 45- μ L volumes using 1 \times T_m hybridization buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100) at pH 8.0 with 20 μ L of the extension product. The samples were incubated for 90 s at 96°C, followed by 30 min at 37°C, and were then transferred to a 96-well filtration plate (MultiScreen HTS; Millipore Corp., Billerica, MA, USA). The liquid was removed using a vacuum manifold, and 100 μ L of 1 \times T_m hybridization buffer was added; this procedure was repeated twice, and the liquid was removed again by vacuum manifold. The microspheres were resuspended in 100 μ L of 1 \times T_m hybridization buffer containing 2 μ g/mL streptavidin-R-

phycoerythrin (Invitrogen Life Technologies). The median fluorescence intensity (MFI) of 100 microspheres was measured with a Luminex 100 flow cytometer (Luminex Corporation, Austin, TX, USA) after incubation for 15 min at 37°C. SNP was determined from more than 100 MFI values after subtracting the background MFI value obtained using water instead of the extension product. SNP was determined based on ratios of more than twice the MFI value between the paired microspheres corresponding to SNPs: LUA-12/-65, -87/-97, -67/-76, -16/-28, and -2/-14. The SNPs of the *F. fujikuroi* strains used by Niehaus et al. (2017) were obtained from the BioProject at the NCBI, as described above.

Fumonisin analysis

A mycelial plug was transferred in sterile rice medium. Sterile rice medium was prepared as follows; 5 g of rice grain was soaked with 4 ml water in an Erlenmeyer flask for 3 hr and then autoclaved. After fungal growth for 10 days at 25°C, fumonisins were extracted with 25 mL methanol/water (3:1, v/v) by reciprocal shaking for 30 min. The extract filtered through paper was purified by a strong anion exchange column (Sep-Pak Accell Plus QMA Short Cartridge (360 mg), Waters, Milford, MA, USA). A Visiprep™ SPE Vacuum manifold (Supelco Inc., Bellefonte, PA, USA) was used for

the purification. Five mL of the extract was loaded onto the column that is previously washed by 5 mL of methanol and then 10 mL of methanol/water (3:1, v/v). Then 5 mL methanol/water (3:1, v/v) and 5 mL methanol were successively loaded on the column for washing. The fumonisins were eluted with final 5 mL of a solution methanol/acetic acid (99:1, v/v). The eluent 1.4 mL was transferred into a glass tube and the solvent was evaporated under a nitrogen gas stream at 50 °C. The residue was dissolved in 0.2 mL of acetonitrile/water (1:1, v/v) and subjected to ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis. It was performed using an ACQUITY UPLC™ system coupled to a Xevo QToF mass spectrometer (Waters, Milford, MA, USA). An ACQUITY UPLC consisting of a binary pump, an auto-sampler, and a column heater was used. Chromatographic separation was performed at 40 °C on a AQUITY UPLC BEH C18 column, 100 x 2.1 mm i.d., 1.7 µm particle size (Waters). Eluents were composed of formic acid/water (1:999, v/v) (eluent A), and methanol/acetonitrile (1:1, v/v) (eluent B), respectively. Sample injection was conducted at a volume of 2 µL. Elution was conducted at the flow rate of 0.3 mL/min with a linear gradient. After starting with the initial portion of B at 60 %, it was hold for 0.5 min and then increased linearly to 100 % B within 4.5 min. Thereafter, the portion of B was decreased linearly to 60 % within 0.1 min, and hold for 1.9 min. It was kept at

60 % prior to the next sample injection. Ionization was conducted with an electro-spray ionization (ESI) probe in positive polarity. The operating parameters were optimized under the following conditions: capillary voltage, 1.0 kV (positive mode)); sample cone voltage, 50 V; extraction cone voltage 4 kV; ion source temperature, 120 °C; desolvation temperature, 400 °C; cone gas flow 50 L/h; desolvation gas flow, 1000 L/h (both gases were nitrogen); and argon gas was used for collision. Conditions of collision energies and multiple reaction monitoring (MRM) transitions were set for each FBs: 30 eV and precursor ion 722/ product ion 705 (m/z); FB₁, 30 eV and 706/689 (m/z); FB₂ and FB₃. Data acquisition was performed for 7 min after injection. Under the above conditions, fumonisins were detected at the retention time of 1.0 min (FB₁), 1.5 min (FB₃), and 1.9 min (FB₂), respectively. The fumonisins FB₁ (Enzo Life Sciences, Lausen, Switzerland), FB₂ (Enzo Life Sciences) and FB₃ (Iris Biotech, Marktredwitz, Germany) were used as standard. Working solutions containing FB₁, FB₂ and FB₃ at concentrations between 0.05-5.0 mg/L (FB₁/ FB₂: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 mg/L, FB₃: 0.05, 0.1, 0.2, 0.4, 0.6, 1.0 mg/L, respectively in 6 bottles) were prepared in acetonitrile/water (1:1, v/v), and used for calibration curve. The concentration (X) (FB₁, FB₂ and FB₃) and corresponding peak area ratio (Y) were plotted for the 6 bottles of the working solution. The limits of quantification (LOQs) for the fumonisin analysis was

the lowest concentration (FB₁/ FB₂: 0.1 mg/L, FB₃: 0.05 mg/L) on the calibration curves for FB₁, FB₂, and FB₃. The concentration of the analyte was calculated from the corresponding Y value with the linear regression line. In cases in which the concentration of the sample exceeded the range given by the calibration curve, the sample was diluted 10 times and re-analyzed.

Gibberellin analysis

A mycelial plug was transferred in 20 mL of 10% ICI medium (Geissman et al 1966) and grown for 7 days on a reciprocal shaker (123 rpm) at 25°C with 0.02% of the trace elements reported by Correl et al. (1987) instead of 0.2% of the original trace elements. After removing the mycelium using filter paper, the gibberellins in the culture filtrate were analysed by thin-layer chromatography (TLC) or by UPLC-MS/MS. The gibberellins GA₃ (Wako Pure Chemicals Ind., Ltd., Osaka, Japan), GA₁, GA₄, and GA₇ (Olchemim Ltd., Olomouc, Czech Republic) were dissolved in 10% ethanol and used as standard solutions, and GA₃ and GA_{4/7} were analysed by TLC according to the method described by Linnemannstons et al. (1999). The culture filtrate (10 µL) was applied to a 60F254 TLC silica gel plate (Merck KG, Darmstadt, Germany) and separated using a mobile chloroform/ethyl acetate/acetic acid phase (90:60:5). After air drying, HCl was sprayed to the TLC plate and left for 30 min. The GAs were visualized in UV light (356

nm) after heating at 120°C for 10 min. GA₃, GA₄, and the GA₇ mixture (5 µL, 100 mg/L each) were used as standards.

The concentrations of the gibberellins (GA₁, GA₃, GA₄, and GA₇) were quantified by UPLC-MS/MS analysis with the same equipment as fumonisin analysis. The experimental cultures were repeated in triplicate, and the resulting broths were subjected to UPLC-MS/MS analysis. Chromatographic separation was performed at 40°C on a AQUITY UPLC BEH C18 column (Waters). The eluents comprised water containing 5 mM ammonium acetate (eluent A) and methanol containing 5 mM ammonium acetate (eluent B). Both solvents (water and methanol) were LC/MS or high-performance liquid chromatography (HPLC) grade. The injection samples were 5 µL in volume, and the elution was conducted at a flow rate of 0.3 mL/min with a linear gradient of methanol. Starting with 10% eluent B, the proportion of B was increased linearly to 65% within 11.5 min, further increased to 95% within 1.5 min, and held for 1.5 min at 95%. Thereafter, the proportion of B was decreased to 10% within 0.1 min, and kept at 10% prior to the next sample injection. Ionization was conducted with an electro-spray ionization (ESI) probe with negative polarity. The optimum operating parameters were: capillary voltage, 0.5 kV (negative mode); sample cone voltage, 40 V; extraction cone voltage, 4 kV; ion source temperature, 120°C; desolvation temperature, 400°C; cone gas

flow, 50 L/h; desolvation gas flow, 1000 L/h (both gases were nitrogen); and argon gas was used for collision. The collision energies and multiple reaction monitoring (MRM) transitions were set for each GA: GA₁, 25 eV and precursor ion 347/product ion 229 (m/z); GA₃, 30 eV and 345/227 (m/z); GA₄, 15 eV and 331/257 (m/z); and GA₇, 30 eV and 329/211 (m/z). The data were acquired for 17 min after injection. Under the conditions described above, the gibberellins were detected at the retention times of 4.6 min (GA₁/GA₃), 9.0 min (GA₇), and 9.3 min (GA₄). Working solutions containing GA₁, GA₃, GA₄, and GA₇ at concentrations of 0.5–5.0 mg/L (GA₃/GA₇: 0.5, 1.0, 2.0, 5.0 mg/L; GA₁/GA₄: 0.5, 1.0, 1.5, 2.0 mg/L, in four bottles) were prepared in ethanol/water (1/9, v/v), and used to create a calibration curve. The concentration (X) (GA₁, GA₃, GA₄, and GA₇) and corresponding peak area ratio (Y) were plotted for the four bottles of the working solution. The limit of quantification (LOQ) for the gibberellin analysis was the lowest concentration (0.5 mg/L) on the calibration curves for GA₁, GA₃, GA₄, and GA₇. The concentration of the analyte was calculated from the corresponding Y value from the linear regression line. In cases in which the concentration of the sample exceeded the range given by the calibration curve, the sample was diluted 4 and 10 times and re-analyzed..

Table S1 Polymerase chain reaction (PCR) primer list

Primer sequence (5' - 3')		Objective	Reference		
Forward	Reverse				
P138-5	AACCCCTACATTGCCCTATC	P138-6	TCGGCAACCAAGAACAAGAGTG	PCR and sequence of <i>CPR</i>	Malonek et al 2005
HS438	GTGTCAAACATAAATTCGACAATAGGAAG	HS439 ^a	ATGATATGTTAGTATGAATAAGTAGAAT	PCR and sequence of <i>TEF</i> and PCR for ASPE of <i>TEF_T618G</i>	Suga et al 2014
EF-11	GTGGGGCATTACCCCGCC			Sequence of <i>TEF</i>	O'Donnell et al 1998
EF-21	GAGTGGCGGGGTAAATGCC			Sequence of <i>TEF</i>	O'Donnell et al 1998
HS398	TTGGAAGTGGCCTACGAGTGT	HS399	GAAGATGGCATTGATTGCCT	PCR and sequence of <i>FUM1</i>	
HS482 ^a	TATGCAATCTTCGGGCCAAAACCGATTTCGCG	HS483	ATGGCAAAGTCATGCCACCGGAGACGC	PCR-RFLP (<i>Nru</i> I) of <i>FUM1_G423A</i>	
HS576	TTCCATCAGATAGCTGATAA	HS577	GACAGCAGGGTCTTGGAAA	PCR for Luminex ASPE of <i>FUM78_C41T</i> and <i>FUM8_G2834A</i>	
P138-5	see above	HS556	GGTTCATCTCAGCCTTGATA	PCR for Luminex ASPE of <i>CPR_C1152A</i>	
P450-4-GD1	TTTCTCGGTCCAGAGCACTGCCGC	P450-4-GD2	CGTGGTCTTCCCTTCCCATCTGGC	PCR and sequence of <i>P450-4</i> , PCR for Luminex ASPE of <i>P4504_C842T</i>	Malonek et al 2005
HS641	TACACTTCTTCTTCTTCTTCTTGTATGAATAAGTAGAATTAC			Luminex ASPE of <i>TEF_T618G</i> , signal detection with <i>LUA-12</i> (FlexMAPxtag)	
HS642	CTTTTCATCAATAATCTTACCTTTGTATGAATAAGTAGAATTAA			Luminex ASPE of <i>TEF_T618G</i> , <i>LUA-65</i>	
HS704	AAACTAACATCAATACTTACATCATATAACTATATCTTARCATCG			Luminex ASPE of <i>FUM78_C41T</i> , <i>LUA-87</i>	
HS705	AATCTCATAATCTACATACACTATTATAACTATATCTTARCATCA			Luminex ASPE of <i>FUM78_C41T</i> , <i>LUA-97</i>	
HS700	TCATTTACTCAACAATTACAAATCACAAATCAGGATGTCCCTCGC			Luminex ASPE of <i>FUM8_G2834A</i> , <i>LUA-67</i>	
HS701	AATCTAACAAACTCATCTAAATACACAATCAGGATGTCCCTCGT			Luminex ASPE of <i>FUM8_G2834A</i> , <i>LUA-76</i>	
HS557	AATCAATCTTCATTCAAATCATCATCATCGTTGGGAGCAAATGCC			Luminex ASPE of <i>CPR_C1152A</i> , <i>LUA-16</i>	
HS558	CTACAAACAACAACATTATCAATCATCGTTGGGAGCAAATGCT			Luminex ASPE of <i>CPR_C1152A</i> , <i>LUA-28</i>	
HS559	CTTTATCAATACATACTACAATCATCCGCGTTGTCCCCCATATC			Luminex ASPE of <i>P4504_C842T</i> , <i>LUA-2</i>	
HS560	CTACTATACATCTTACTATACTTTCCGCGTTGTCCCCCATATT			Luminex ASPE of <i>P4504_C842T</i> , <i>LUA-14</i>	
7cf	ATGGGYAARCAAGCYATGGG	11ar	GCRTGGATCTTRTCRTCSACC	PCR and sequence of <i>RPB2</i>	O'Donnell et al 2010
HS833	GTACGAACCACCAAGATTCTCAA	HS834 ^a	TGCTAAGAAGACACTCAATCAACTG	PCR-RFLP (<i>Bmr</i> I) of <i>RPB2_C3250T</i>	
HS576	see above	HS577	see above	PCR and sequence of <i>FUM8</i> including <i>FUM8/FUM7</i> intergenic region, PCR-RFLP (<i>Hinc</i> II) of <i>FUM8_G2834A</i> or (<i>Nru</i> I) for <i>FUM78_C41T</i>	
HS434	GAGAAGGTTAGTCACTTCCCTTCGATC	HS435 ^a	CCGAGCTCAGCGGCTTCTATGTGCGCA	PCR-RFLP (<i>Nsp</i> I) of <i>TEF_C447A</i>	
HS506	ATGCGGCCGCGRCCGAAAGAAGCATCCGA	HS519	AGCCAAAGAACTTGGCAGT	PCR and sequence of <i>FUM18</i> including <i>FUM18/FUM19</i> intergenic region, PCR-RFLP (<i>Mse</i> I) of <i>FUM18_G51T</i>	

^aPrimers were designed with dCAPS (derived cleaved amplified polymorphic sequence) Finder 2.0 (Neff et al 2002)

Table S2 GenBank accession numbers of translation elongation factor 1 α (*TEF1 α*) and RNA polymerase II second largest-subunit (*RPB2*) used in the present study

Species	Strain	GenBank accession No. for gene		Reference
		<i>TEF</i> ^a	<i>RPB2</i> ^b	
<i>F. verticillioides</i> (MP-A)	NRRL22172	AF160262	EF470122	
<i>F. sacchari</i> (MP-B)	NRRL13999	AF160278	JX171580	
<i>F. proliferatum</i> (MP-D)	NRRL22944	AF160280	HM068352	
	MAFF236459	AB725609	LC133068 (obtained in this study)	
<i>F. subglutinans</i> (MP-E)	NRRL22016	AF160289	JX171599	
<i>F. thapsinum</i> (MP-F)	NRRL22045	AF160270	JX171600	
<i>F. nygamai</i> (MP-G)	NRRL13448	AF160273	EF470114	
<i>F. circinatum</i> (MP-H)	NRRL25331	AF160295	JX171623	
<i>F. concentricum</i>	NRRL25181	AF160282	LC133069 (obtained in this study)	
<i>F. fractiflexum</i>	NRRL28852	AF160288	LC133070 (obtained in this study)	
<i>F. globosum</i>	NRRL26131	AF160285	KF466406	
<i>F. mangiferae</i>	NRRL25226	AF160281	JX171622	
<i>F. hostae</i>	NRRL29889	AY329034	JX171640	used as outgroup of phylogenetic analysis of <i>F.fujikuroi</i> species complex based on <i>TEF</i> and <i>RPB2</i> sequence

^a *TEF* is translation elongation factor 1 α .

^b *RPB2* is RNA polymerase II second largest-subunit.

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