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RESEARCH ARTICLE

"Cryptic" group-I introns in the nuclear SSU-rRNA gene of *Verticillium dahliae*

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Abstract Group-I introns are widespread—though irregularly distributed—in eukaryotic organisms, and they have been extensively used for discrimination and phylogenetic analyses. Within the *Verticillium* genus, which comprises important phytopathogenic fungi, a group-I intron was previously identified in the SSU-rRNA (18S) gene of only *V. longisporum*. In this work, we aimed at elucidating the SSU-located intron distribution in *V. dahliae* and other *Verticillium* species, and the assessment of heterogeneity regarding intron content among rDNA repeats of fungal strains. Using conserved PCR primers for the amplification of the SSU gene, a structurally similar novel intron (sub-group IC1) was detected in only a few *V. dahliae* isolates. However, when intron-specific primers were used for the screening of a diverse collection of *Verticillium* isolates that originally failed to produce intron-containing SSU amplicons, most were found to contain one or both intron types, at variable rDNA repeat numbers. This marked heterogeneity was confirmed with qRT-PCR by testing rDNA copy numbers (varying from 39 to 70 copies per haploid genome) and intron copy ratios in selected isolates. Our results demonstrate that (a) IC1 group-I introns are not specific to *V. longisporum* within the *Verticillium* genus, (b) *V. dahliae* isolates of vegetative compatibility groups (VCGs) 4A and 6, which bear the novel intron at most of their rDNA repeats, are closely related, and (c) there is considerable intra-genomic heterogeneity for the presence or absence of introns among the ribosomal repeats. These findings underline that distributions of introns in the highly heterogeneous repetitive rDNA complex should always be verified with sensitive methods to avoid misleading conclusions for the phylogeny of fungi and other organisms.

Keywords Inter-specific molecular differentiation · rDNA copy number · rDNA heterogeneity · Real-time quantitative PCR · VCG discrimination

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Introduction

Group-I introns comprise one of the four major classes of introns and catalyze their own cleavage from precursor transcripts by a two-step splicing pathway that relies on an exogenous guanosine cofactor (Haugen et al. 2005). Although conservation at the sequence level is limited, the core of group-I introns is characterized by a conserved structural architecture consisting of 10 paired segments (P1–P10), which are organized in three domains: a structural or scaffolding domain (P4–P6), a substrate domain (P1, P10) and a catalytic domain (P3, P7 and P8) (Michel and Westhof 1990; Woodson 2005; Vicens et al. 2008; Nielsen and Johansen 2009). Based on secondary structure characteristics, group-I introns are classified into five groups (IA–IE), which are further sub-divided into 14 subgroups (Li and Zhang 2005; Vicens et al. 2008). Group-I introns are frequently encountered in the nuclear and organelle genomes of eukaryotes, particularly of fungi, plants, and algae, whereas they are rare in viruses, bacteria,

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and phages, and absent from the Archaea (Haugen et al. 2005). Nuclear group-I introns of eukaryotic organisms are limited to ribosomal RNA genes, sporadically inserted in at least 47 and 44 different sites in the small (SSU) and large (LSU) subunit rRNA genes, respectively (Cannone et al. 2002). The distribution of group-I introns is generally scattered and irregular, even within a species or among phylogenetically closely related taxa, possibly resulting from the combination of vertical transmission, horizontal transfer (through homing or reverse splicing), and loss events. Particularly in ascomycete fungi, four positions in the SSU (namely Ec516, Ec943, Ec989, and Ec1199 with respect to the *Escherichia coli* SSU-rRNA gene sequence) and four positions in the LSU (namely Ec1921, Ec2066, Ec2449, and Ec2563) harbor, almost exclusively, the identified group-I introns within the rRNA ribosomal repeat (Pantou et al. 2003; Wang et al. 2003). The distribution patterns and the sequences of group-I introns have been widely used in numerous studies aiming at the reconstruction of phylogenetic relationships of fungi and other organisms (Dujon 1989; Bhattacharya et al. 1996, 2005; Hibbett 1996; Nikoh and Fukatsu 2001; Haugen et al. 2005; Hafez et al. 2012).

Verticillium dahliae is an important soil-borne asexual ascomycete causing vascular wilt disease in over 400 plants, including high-value crops, landscape, fruit, and ornamental trees, and shrubs (Pegg and Brady 2002). Isolates of this fungus that are vegetatively compatible are placed in the same vegetative compatibility group (VCG). According to this classification system, VCGs 1, 2, 3, 4, and 6 have been described, and of these, VCGs 1, 2, and 4 have been sub-divided depending on the vigor of compatibility reactions. These compatibility groups have been traditionally regarded as genetically distinct intra-specific sub-groups, which may differ in ecology, physiology, and virulence (Leslie 1993; Katan 2000; Bhat et al. 2003). Apart from *V. dahliae*, the phytopathogenic genus *Verticillium* also encompasses the relative species *V. albo-atrum*, *V. tricorpus* and *V. nubilum* (Klosterman et al. 2009), as well as the 'nearly diploid' ascomycete *V. longisporum* (Karapapa et al. 1997). The latter has been recently proposed to have originated by three inter-specific hybridization events (Inderbitzin et al. 2011).

In a previous study, a 839-bp-long group-I intron was found inserted in a highly conserved position (Ec943) of the nuclear SSU gene in 36 of the 38 *V. longisporum* isolates tested, whereas it was apparently absent from *V. dahliae* and the other *Verticillium* species (Karapapa and Typas 2001). The presence of this intron in 27 of 29 *V. longisporum* isolates was later confirmed by Collins et al. (2003), who also used specific primers and detected another *V. longisporum* isolate bearing the same intron in a minor repeat number, and a single *V. dahliae* isolate that had a smaller intron inserted at the same position in its SSU gene. It was presumed that introns were absent from the remaining *V. dahliae* and *V. albo-atrum* isolates tested. Consequently, the detection of the 839-bp-long intron has been used thereafter as a *V. longisporum*-specific molecular marker for discrimination between this and its relative *Verticillium* species (Johansson et al. 2006; Banno et al. 2011; Ikeda et al. 2012; Inderbitzin et al. 2013). Furthermore, it was suggested that the presence of this intron may also be a discriminatory character between distinct *V. longisporum* hybrid lineages that descend from different combinations of parental species and exhibit different pathogenic abilities (Inderbitzin et al. 2013; Tran et al. 2013). In these works, the intron was again reported to be absent from *V. dahliae* and *V. albo-atrum* representative isolates.

The objective of this work was principally to test a diverse collection of isolates of *V. dahliae*—covering all VCGs—and related species for the presence of group-I introns in the nuclear SSU-rRNA gene, and to assess the extent of heterogeneity regarding intron presence among rDNA repeats. Detected introns were isolated, sequenced, and structurally characterized, and their inter- and intra-specific distributions were studied in detail. Heterogeneity of these introns within the rDNA of fungal individuals was demonstrated by conventional PCR with intron-specific primer pairs as well as by quantitative real-time PCR (qRT-PCR). In vivo splicing of these introns from precursor ribosomal transcripts was shown with reverse transcription PCR. Furthermore, in the light of intron distribution and recent molecular data, a re-examination of VCG 6 with traditional vegetative compatibility assessment was undertaken, and an appropriate revision is proposed.

Materials and methods

Fungal strains and culture conditions

A collection of 114 *Verticillium* isolates from diverse geographic origins and original hosts (102 *V. dahliae*, 6 *V. longisporum*, 4 *V. albo-atrum*, and a single representative isolate from each of *V. tricorpus* and *V. nubilum*) were used in this study (Table 1). Isolates from all VCGs of *V. dahliae* were included in an attempt to achieve a broad coverage of the known genetic diversity of the species. Monoconidial cultures of all isolates were used. Isolates were maintained as conidial suspensions in potato dextrose broth (PDB; Scharlau, Barcelona, Spain) with 20 % glycerol, at −80 °C, and active cultures were obtained on potato dextrose agar (PDA) plates with incubation at 24 °C, in the dark.

Table 1 *Verticillium* isolates used in this study, with original hosts, geographic origins, VCGs, and the results from the PCR screenings with all primer pairs tested

Isolate	Host	Origin (source)[a]	VCG[b]	18SVDF/R[c]	18Sa/b[c]	18Sc/d[c]
V. dahliae						
T9[f]	Cotton	USA, CA (A)	1A	1.7	0.4	0.8
V44[d,f]	Cotton	USA, TX (A)	1A	1.7	0.4	0.8
V138I[d]	Cotton	Spain (B)	1A	1.7	0.4	0.8
cotVd03[d]	Cotton	Turkey (C)	1A	1.7	0.4 + 0.7	0.8 + 1.2
cotVd11	Cotton	Turkey (C)	1A	1.7	0.4	0.8
cotVd47[d]	Cotton	Turkey (C)	1A	1.7	0.4	0.8
V661I (328 v-1)[d]	Cotton	Greece (B)	1B	1.7	0.4	0.8
V666I (347 v-1)[d]	Cotton	Greece (B)	1B	1.7	0.4	0.8
V607I (R04)[d]	Green ash	USA, MN (B)	1B	1.7	0.4	0.8
Dvd-T5	Tomato	Canada (D)	2A	1.7	0.4 + 0.7	0.8 + 1.2
PH[d,f]	Pistachio	USA, CA (A)	2A	1.7	0.4	0.8
179-4	Melon	Greece (E)	2A	1.7	–	–
164-1[d]	Tomato	Greece (E)	2A	1.7	–	–
98-1[d]	Tomato	Greece (E)	2A	1.7	–	–
113-1	Eggplant	Greece (E)	2A	1.7	–	-
140-2	Tomato	Greece (E)	2A	1.7	–	–
247b-3	Tomato	Greece (E)	2A	1.7	–	–
V320I[f]	Cotton	USA, CA (B)	2A	1.7	0.4	0.8
V720I (V39)[d]	Olive	Italy (B)	2A	1.7	0.4	0.8
V800I[d]	Olive	Spain	2A	1.7	0.4	0.8
cotVd04	Cotton	Turkey (C)	2A	1.7	0.4	0.8
egpVd17[d]	Eggplant	Turkey (C)	2A	1.7	0.4	0.8
136-3[d]	Tomato	Greece (E)	2A (2B)	1.7	–	–
998-1	Eggplant	Greece (E)	2A (2B, 4A)	1.7	–	–
V54	Pepper	Austria (F)	2B	1.7	0.4	0.8
115[d,f]	Cotton	Syria (A)	2B	1.7	0.4	0.8
cot274	Cotton	Turkey (C)	2B	1.7	0.4	0.8
449-2	Broccoli	Greece (E)	2B	1.7	0.4	0.8
V38[d]	Sunflower	Germany (F)	2B	1.7	0.4 + 0.7	0.8 + 1.2
V42	Turnip	Russia (F)	2B	1.7	–	–
V49	Pepper	Austria (F)	2B	1.7	0.4	0.8
V57[d]	Strawberry	Germany (F)	2B	1.7	0.4	0.8
V16	Potato	Germany (F)	2B	1.7	0.4	0.8
V357I (JY)	Cotton	China (B)	2B	1.7	0.4	0.8
V510I (tom20)	Tomato	Israel (B)	2B	1.7	0.4	0.8
V613I	Artichoke	Spain (B)	2B	1.7	0.4 + 0.7	0.8 + 1.2
V702I[d,f]	Artichoke	Spain (B)	2B	1.7	0.4	0.8
Ls.17[d,f]	Lettuce	USA, CA (G)	2B	1.7	0.4	0.8
124-8	Tomato	Greece (E)	2B (1)	1.7	–	–
577-1	Lamb's quarters	Greece (E)	2B (2A)	1.7	–	–
997-1[d]	Eggplant	Greece (E)	2B (2A)	1.7	–	–
525-1	Eggplant	Greece (E)	2B (2A, 4A)	1.7	–	–
150-5[d]	Tomato	Greece (E)	2B (2A, 4A)	1.7	–	–
999-1[d]	Eggplant	Greece (E)	2B (4A, 4B)	1.7	0.4	0.8
302-1[d]	Tomato	Greece (E)	2B (2A)	1.7	–	–
530-1[d]	Pepper	Greece (E)	2B (2A)	1.7	0.4 + 0.7	0.8 + 1.2

Table 1 continued

Isolate	Host	Origin (source)[a]	VCG[b]	18SVDF/R[c]	18Sa/b[c]	18Sc/d[c]
25V (SS4)	Cotton	USA (H)	2AB (1, 4A)	1.7	0.4	0.8
70-21[d,f]	Pepper	USA, AZ (A)	3	1.7	0.4	0.8
PCW[d,f]	Pepper	USA, CA (A)	3	1.7	0.4	0.8
BB[d,f]	Potato	USA, ID (A)	4A	2.2	0.4	0.8
30-6[f]	Potato	Canada (D)	4A	1.7 + 2.2	0.4	0.8
21-18	Potato	Canada (D)	4A	2.2	0.4	0.8
90	Potato	USA, SD (D)	4A	2.2	0.4	0.8
111	Potato	USA, MT (D)	4A	2.2	0.4	0.8
129	Potato	USA, WA (D)	4A	2.2	0.4	0.8
Dvd-E6[d]	Eggplant	Canada (D)	4A	2.2	0.4	0.8
P103	Potato	USA, OH (D)	4A	2.2	0.4	0.8
83-1	Potato	USA, WI (D)	4A	2.2	0.4	0.8
86	Potato	USA, WA (D)	4A	2.2	0.4	0.8
66-13	Potato	USA, ME (D)	4A	2.2	0.4	0.8
170	Potato	Canada (D)	4A	1.7	0.4	0.8
108	Potato	USA, WY (D)	4A	2.2	0.4	0.8
131-M[d]	Potato	USA (B)	4A	1.7	0.4 + 0.7	0.8 + 1.2
171-4A[d]	Potato	USA (B)	4A	1.7 + 2.2	0.4	0.8
V830[d]	Potato	USA, OH (F)	4A	2.2	0.4	0.8
S39[f]	Potato	USA, OH (A)	4B	1.7	0.4 + 0.7	0.8 + 1.2
pn4[d]	Peanut	Israel (I)	4B	1.7	0.4	0.8
149	Potato	Canada (D)	4B	1.7	0.4	0.8
16-1	Potato	Greece (E)	4B	1.7	–	–
455-1	Chicory	Greece (E)	4B	1.7	–	–
44-3	Potato	Greece (E)	4B	1.7	–	–
273-1[d]	Black nightshade	Greece (E)	4B	1.7	–	–
413-5	Romaine lettuce	Greece (E)	4B	1.7	–	–
453-1	Vetch	Greece (E)	4B	1.7	–	–
461-3	Tomato	Greece (E)	4B	1.7	–	–
463-1[d]	Tomato	Greece (E)	4B	1.7	–	–
464-3	Tomato	Greece (E)	4B	1.7	–	–
473-1	Summer squash	Greece (E)	4B	1.7	–	–
un1-1[d]	Wild sweet pea	Greece (E)	4B	1.7	–	–
V684I[d,f]	Artichoke	Spain (B)	4B	1.7	0.4	0.8
V39[d]	Sunflower	Germany (F)	4B	1.7	0.4	0.8
egpVd02[d]	Eggplant	Turkey (C)	4B	1.7	0.4	0.8
451-1	Romaine lettuce	Greece (E)	4B (4A)	1.7	–	–
465-2	Marigold	Greece (E)	4B (4A)	1.7	0.4	0.8
554-1	Chicory	Greece (E)	4B (4A)	1.7	–	–
469-1	Cauliflower	Greece (E)	4B (4A)	1.7	–	–
578-1	Lamb's quarters	Greece (E)	4B (4A)	1.7	0.4	0.8
802-1	Olive	Greece (E)	4B (4A)	1.7	–	–
466-1	Black nightshade	Greece (E)	4B (4A)	1.7	–	-
456-1[d]	Anise	Greece (E)	4B (4A)	1.7	–	–
467-2[d]	Whitetop	Greece (E)	4B (4A)	1.7	–	–
478-1	Chicory	Greece (E)	4B (4A)	1.7	–	–
235-1	Tomato	Greece (E)	4B (4A)	1.7	–	–
lt1-1	Romaine lettuce	Greece (E)	4B (3, 4A)	1.7	–	–

Table 1 continued

Isolate	Host	Origin (source)[a]	VCG[b]	18SVDF/R[c]	18Sa/b[c]	18Sc/d[c]
Cf.38[d,f]	Chile pepper	USA, CA (G)	6	1.7	0.4	0.8
Cf.162[d,f]	Chile pepper	USA, CA (G)	6	1.7	0.4	0.8
Ca.146[d,f]	Bell pepper	USA, CA (G)	6	2.2	0.4	0.8
Ca.148[d,f]	Bell pepper	USA, CA (G)	6	2.2	0.4	0.8
Ca.83[d,f]	Bell pepper	USA, CA (G)	6	2.2	0.4	0.8
V13[f]	Cotton	Spain (F)	HSI[e]	1.7	0.4 + 0.7	0.8 + 1.2
CA26	Cabbage	Japan (J)	–	1.7	0.4	0.8
Vd76	Cotton	Greece (K)	–	1.7	–	–
V. longisporum						
G19[d]	Oilseed rape	Germany (L)	–	1.7	0.7	1.2
K12[d]	Oilseed rape	Russia (M)	–	2.5	0.7	1.2
81[d]	Oilseed rape	Denmark (F)	–	2.5	0.7	1.2
86207	Wild radish	Japan (L)	–	2.5	0.7	1.2
161	Sugar-beet	Sweden (L)	–	2.5	0.7	1.2
G22	Oilseed rape	Germany (L)	–	2.5	0.7	1.2
V. albo-atrum						
M33	Hop	UK (L)	–	1.7	0.7	1.2
220[d]	Alfalfa	UK (L)	–	1.7	0.7	1.2
V90	Cotton	Middle Asia (M)	–	1.7	0.7	1.2
T2[d]	Hop	Slovenia (N)	–	1.7	0.4 + 0.7	0.8 + 1.2
Verticillium spp.						
V. tricorpus 22	Potato	Isreal (I)	–	1.7	0.4 + 0.7	0.8 + 1.2
V. nubilum 278734	Potato	UK (IMI)	–	1.7	0.4 + 0.7	0.8 + 1.2

[a] Provided by: A = R. Rowe, OARDC, The Ohio State University, USA; B = M. Jiménez-Gasco, The Pennsylvania State University, USA; C = S. Dervis, University of Mustafa Kemal, Turkey; D = K. Dobinson, University of Western Ontario, Canada & Agriculture and Agri-Food, Canada; E = E. Ligoxigakis, Plant Protection Institute, N.AG.RE.F., Greece; F = A. von Tiedemann, University of Göttingen, Germany; G = K. Subbarao, University of California, Davis, USA; H = E. Paplomatas, Agriculture University of Athens, Greece; I = T. Katan, The Volcani Center, Israel; J = T. Usami, Chiba University, Japan; K = E. Tjamos, Agricultural University of Athens, Greece; L = J. Heale, University of London, UK; M = O. Strunnikova, All-Russian Research Institute for Agricultural Microbiology, Russia; N = S. Radišek, Slovenian Institute for Hop Research and Brewing, Slovenia; IMI = International Mycological Institute, UK (presently Centre for Agricultural Bioscience International, CABI, UK)

[b] Data on "bridging" behavior (i.e., complementation to varying degrees with tester strains of more than one VCG sub-groups) were available for several *V. dahliae* isolates that were included in this study; these secondary VCG interactions are provided in brackets

[c] Length (in kilobase pairs) of PCR amplicons yielded with the corresponding primer pairs, as estimated with agarose gel electrophoresis

[d] Isolates tested for the presence of insertions in the nuclear LSU gene

[e] *HSI* heterokaryon self-incompatible

[f] Isolates used in VCG classification experiments

DNA extraction, PCR, reverse transcription PCR, cloning, and sequencing

Total DNA of all isolates was extracted according to previously described procedures (Typas et al. 1992), quantified using standard spectrophotometric and agarose electrophoresis methods (Sambrook and Russell 2001), and subjected to PCR with primer pairs listed in Table 2. All PCR analyses were performed in a PTC-200 Gradient Peltier Thermal Cycler (MJ Research Inc., St. Bruno, Quebec, Canada) with KAPA Taq DNA polymerase (Kapa Biosystems, Woburn, MA, USA). The reaction mixture consisted of KAPA Taq Buffer C (without $MgCl_2$) at 1× concentration, 1.5 mM $MgCl_2$ (Kapa Biosystems), DMSO (Stratagene Products Division, Agilent Technologies, La Jolla, CA, USA) at 5 % concentration, 200 μM of each dNTP (Kapa Biosystems), 0.4 μM of each primer, 30 ng of genomic DNA, 0.5 U of the KAPA Taq DNA polymerase, and molecular-grade water (Invitrogen RNA products, Ambion, Life Technologies, Carlsbad, CA, USA) up to a final volume of 25 μl. The amplification program included a 3-min step of initial denaturation at 95 °C, followed by 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at the temperatures indicated in Table 2 for the different

Table 2 PCR primers used throughout this study

Primer	Sequence (5′–3′)	Paired with primer	T_a (°C)[a]	Reference
18SVDF	GCGAAACTGCGAATGGCT	18SVDR	60	Karapapa and Typas 2001
18SVDR	GTAATGATCCCTCCGCTG			Karapapa and Typas 2001
18Sa	CCGAACACATGGCTAGTCTCCTC	18Sb	57	This study
18Sb	ATTCGATTGCAAAGCTACCTATGG			This study
18Sc	GGGACAGTCGGGGGCATCAGTATT	18Sd/e	58/58	This study
18Sd	CAGGCTCCCGTTTTGG			This study
18Se	CTAAGAACGGCCATGCACCACCAC			This study
28SF	GTTCGGCGGCGGGAGGTC	28SR	58	This study
28SR	GTCGCTTTCTGGCACGGATTC			This study
Quantitative (real-time) PCR primers				
tef1F	GGTTCGTCACCCTGTGTATATC	tef1R	57	This study
tef1R	GTGCATGGTCTGCATCAAAG			This study
18SF	CCTGCGGCTTAATTTGACTC	18SR	57	This study
18SR	AACTAAGAACGGCCATGCAC			This study
intuniF	AAAGCCGTCTGTGAAAGCAG	intuniR	57	This study
intuniR	GTCCATTGTTGCATCTCACG			This study
intspF	TGTCCCATTCTTCTCCCTCT	intspR	57	This study
intspR	ATTCTCGTTCCTTATGCCTGAC			This study

[a] Annealing temperature used during the PCR program

primer pairs), and extension (2 min at 72 °C), and a final extension step of 5 min at 72 °C. All reactions were repeated at least twice and all experiments included negative controls (no DNA). Amplification products were separated on ethidium bromide-stained agarose gels (1.0 % w/v) and visualized under UV light. Total RNA was extracted from 50 mg of lyophilized mycelium of each of three selected isolates with the NucleoSpin RNA II kit (Macherey–Nagel, Düren, Germany), and treated with TURBO DNAse (Invitrogen RNA products) for the complete removal of genomic DNA. About 1 µg of DNAse-treated total RNA was used as template for first strand cDNA synthesis with the RevertAid Reverse Transcriptase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions; cDNA was then subjected to PCR with specific primer pairs (Table 2). Prior to sequencing, the desired PCR products were recovered from agarose gels with the NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel) and the purified fragments were directly sequenced or first cloned into pBluescript II KS vector (Stratagene) according to standard procedures (Sambrook and Russell 2001). Plasmids were purified with the NucleoSpin Plasmid kit (Macherey–Nagel) before sequencing. Sequencing reactions were carried out according to Papaioannou et al. (2013b). DNA sequences with overlapping ends were assembled in contigs with the program SeqMan of the software package Lasergene 6 (DNAstar, Madison, WI, USA). DNA similarity searches were performed with Basic Local Alignment Search Tool (BLAST 2.2.27+; Altschul et al. 1997).

Quantitative (real-time) PCR

Primer pairs for qRT-PCR (Table 2) were designed using the online tools *PrimerQuest* (http://eu.idtdna.com/PrimerQuest/Home/Index; Integrated DNA technologies, Coralville, Iowa, USA) and *Primer3Plus* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Untergasser et al. 2007) and were subjected to in silico validation according to the criteria proposed by D'haene et al. (2010). All qRT-PCR analyses were performed in three repetitions for each isolate and run in duplicate in an Mx3000P real-time PCR system (Stratagene) with the Brilliant SYBR Green qRT-PCR Master Mix kit (Stratagene), according to the manufacturer's recommendations. Optimization of primer concentrations was carried out for each primer pair and the optimal concentrations were 50 nM for primers tef1F/R, 18SF, and intuniF, and 150 nM for primers 18SR, intuniR, and intspF/R (Table 2). The thermal cycling protocol consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C for denaturation, 1 min at 57 °C for annealing, and 30 s at 72 °C for extension. Fluorescence was recorded at the end of both the annealing step and the extension step of each cycle. Amplification was followed

by a dissociation curve analysis, starting with a 1-min incubation at 95 °C, followed by incubation at 55 °C for 30 s, and finally a ramp (at 0.1 °C s^{-1}) up to 95 °C with continuous fluorescence data collection. Control reactions with no template were included in all experiments for the confirmation of the absence of contaminating DNA and primer dimers. The absence of non-specific products was further confirmed by melting curve analysis and visualization of end products on 2 % agarose gels.

The absolute quantification method was used for the real-time PCR determination of rRNA gene and intron copy numbers, according to Lee et al. (2008). For this, primer pairs tef1F/R, 18SF/R, intuniF/R, and intspF/R (Table 2) were used, targeting the transcription elongation factor 1 (*tef1*) gene of *V. dahliae*, a conserved intronless portion of the SSU-rRNA gene of *V. dahliae*, a common fragment of all group-I introns identified in this work, and a fragment specific to the 839-bp-long group-I intron, respectively. The *tef1* gene was used as a reference gene for copy number calculations, as it has been previously shown to be present in the genome of *V. dahliae* in a single copy with southern hybridization analyses (Pantou and Typas 2005; locus number VDAG_08296 at the *Verticillium* group database of Broad Institute: http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). The PCR products obtained from isolates *V. dahliae* BB with primer pairs tef1F/R, 18SF/R, and intuniF/R, and *V. longisporum* K12 with primer pair intspF/R were cloned into plasmid vectors as described above and a single bacterial clone exhibiting the anticipated insert size was selected for each PCR amplicon and verified with sequencing. Consequently, plasmids were purified, quantified spectrophotometrically, and a tenfold dilution series was prepared for each one, in the range of 4.0×10^8–4.0×10^2 copies μl^{-1} (according to calculations summarized by Lee et al. 2008). These dilution series were then used for the construction of real-time PCR standard curves for the four DNA targets. All curves were highly linear ($R^2 > 0.999$) in the ranges tested. Amplification efficiencies of the primer sets tef1F/R, 18SF/R, intuniF/R, and intspF/R were 96.7, 95.4, 89.2, and 91.8 %, respectively. Based on the corresponding standard curve of each target, the calculated threshold cycle values of different samples were converted into numbers of copies per sample. The copy number of the SSU-rRNA gene was finally determined by dividing the number of copies of the SSU fragment in each sample with the number of copies of the single-copy reference gene (*tef1*) in the same sample. Similarly, the copy ratio of the two intron types described in the present study was calculated for each sample by dividing the numbers of copies of products yielded with primers intuniF/R and intspF/R, respectively, for each sample.

Prediction of secondary structures of introns

Introns were named according to the nomenclature guidelines proposed by Johansen and Haugen (2001) and their insertion position was determined with respect to the *E. coli* SSU-rRNA gene sequence (GenBank accession number AB035922). The prediction of their secondary structures was based on the *mfold* RNA folding online software (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form; Zuker 2003), with the generally accepted constraints and conventions for group-I introns (Michel and Westhof 1990; Cech et al. 1994; Cannone et al. 2002; Li and Zhang 2005). For the accurate identification of the P1 and P10 helices, parts of the flanking exon sequences were included in the models. The predicted structures were finally drawn with graphics design software CorelDRAW Graphics Suite X6 (Corel Corporation, Ottawa, Canada).

Vegetative compatibility grouping

The production of chlorate-resistant *nit* mutants of selected *V. dahliae* isolates (Table 1 and Online Resource 1), their classification into *nit1* and nitM mutant classes, and their use in complementation tests for VCG classification were performed according to standard procedures (Correll et al. 1987; Joaquim and Rowe 1990), with the specifications described by Papaioannou et al. (2013b). All pairings were repeated at least twice.

Results

PCR screening with SSU-specific primers: group-I introns in *V. longisporum* and in VCGs 4A and 6 of *V. dahliae*

DNA from each of the 114 *Verticillium* isolates listed in Table 1 was used in PCR analysis with primer pair 18SVDF/R (designed to amplify nearly the full-sized SSU rDNA; Table 2), and the products were analyzed for detectable size differences. In agreement with all previous studies, the screening of five of six isolates of *V. longisporum* produced a single PCR product of 2.5 kb (Fig. 1a shows the representative amplicon of isolate K12; Table 1), presumably due to the presence of the previously described 839-bp-long intron in the SSU gene (Karapapa and Typas 2001). To verify this assumption, the amplicon of *V. longisporum* K12 was sub-cloned and sequenced; the sequence of the insertion matched perfectly the previously deposited intron sequence (GenBank accession number AF153421). This 839-bp-long group-I intron is referred to hereafter as intron Vert.S943-1. The sixth isolate of *V. longisporum* (G39) was characterized by a single band of 1.7 kb, which is indicative of the absence of any insertions in this region (Fig. 2a).

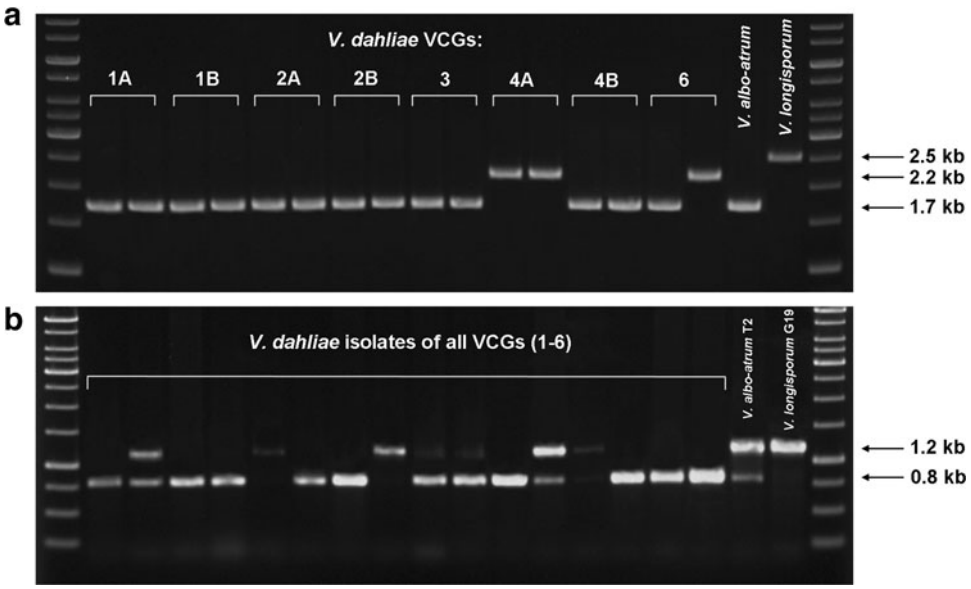

Fig. 1 **a** SSU PCR screening with primer pair 18SVDF/R of representative *V. dahliae* isolates T9, cotVd03 (VCG 1A), V661I, V607I (VCG 1B), Dvd-T5, PH (VCG 2A), V54, 115 (VCG 2B), 70-21, PCW (VCG 3), BB, 21-18 (VCG 4A), S39, 461-3 (VCG 4B), Cf.38, Ca.146 (VCG 6), *V. albo-atrum* M33, and *V. longisporum* K12 (corresponding to lanes 2–19 of the agarose gel, respectively). The 1 kb DNA ladder (Fermentas) has been run on the first and the last lanes of the gel for determination of approximate size of PCR products. **b** PCR screening with intron-specific primer pair 18Sc/d of selected *V. dahliae* isolates of all VCGs, exhibiting variability in their SSU-located intron content: *V. dahliae* T9, cotVd03, V661I, V607I, Dvd-T5, PH, 115, V38, V613I, 530-1, 70-21, 131-M, S39, pn4, Cf.38, Ca.146, *V. albo-atrum* T2, and *V. longisporum* G19, respectively. For isolates Dvd-T5, V38, and S39, the 0.8 kb-sized band is hardly visible on this agarose gel. Considerable heterogeneity in the yield of different PCR products among isolates is observed. The 1 kb DNA ladder has been run on the first and the last lanes

As expected, for the majority of *V. dahliae* isolates, amplification resulted in a single 1.7-kb-long product. However, in 14 of 16 VCG 4A isolates and in the 3 VCG 6 isolates from bell pepper, a PCR amplicon of 2.2 kb was produced (representative amplicons are shown in Fig. 1a; Table 1). The corresponding PCR product of isolate BB (VCG 4A) was isolated, sub-cloned and sequenced (GenBank accession number KF318969). Analysis of this sequence demonstrated the presence of a single insertion of 475 bp, at exactly the same position as in *V. longisporum* (SSU nt position 1165, which corresponds to Ec943; Fig. 2a). Interestingly, this insertion was highly similar to intron Vert.S943-1, with the exception of two deleted internal regions (180 and 186 bp in length, respectively), whereas all conserved sequence and structural features of group-I introns (including the IGS, P, Q, R, S, and 3′ end elements) were identical between the two insertions. On the basis of these characteristics and their comparison with available members of all group-I intron classes, the 475-bp-long insertion in the SSU of *V. dahliae* BB was characterized as a typical group-I intron (sub-group IC1), and designated as Vert.S943-2 intron. When the putative structures of the two introns were compared, it was clear that the two indels, which accounted for the size difference between the two introns, lie within the sequence stretches forming the P6 and P8 loops, respectively, while all other putatively functional elements of the introns remain unaffected (Fig. 2b). Isolates 30-6, 171-4A and V830 (VCG 4A), and Ca.146, Ca.148 and Ca.83 (VCG 6) were further subjected to nested PCR with primers 18Sc/e (Table 2) and amplicons were cloned and sequenced. All these isolates contained the same intron (Vert.S943-2), inserted at the same SSU position, and no sequence variation regarding intron sequence was detected among them (GenBank accession numbers KF318970-75). It should be noted that a double DNA band of 1.7 and 2.2 kb was consistently amplified from genomic DNA of isolates 30-6 and 171-4A (VCG 4A), suggesting that the ribosomal repeats of these isolates are heterogeneous regarding the presence or absence of the Vert.S943-2 intron. Finally, PCR of all representative isolates of *V. albo-atrum*, *V. tricorpus*, and *V. nubilum* yielded a single 1.7-kb-sized product with primer pair 18SVDF/R (Table 1).

Among the five available isolates of *V. dahliae* VCG 6, intron Vert.S943-2 was detected in all three originating from bell pepper, as opposed to the other two, from chili pepper (Table 1). To test whether this discrepancy was possibly due to previous VCG misclassification, the vegetative compatibility behavior of the five isolates was re-examined, against 14 international testers of all VCGs (Table 1 and Online Resource 1). Compatibility tests among 125 chlorate-resistant *nit* mutants of VCG 6 isolates

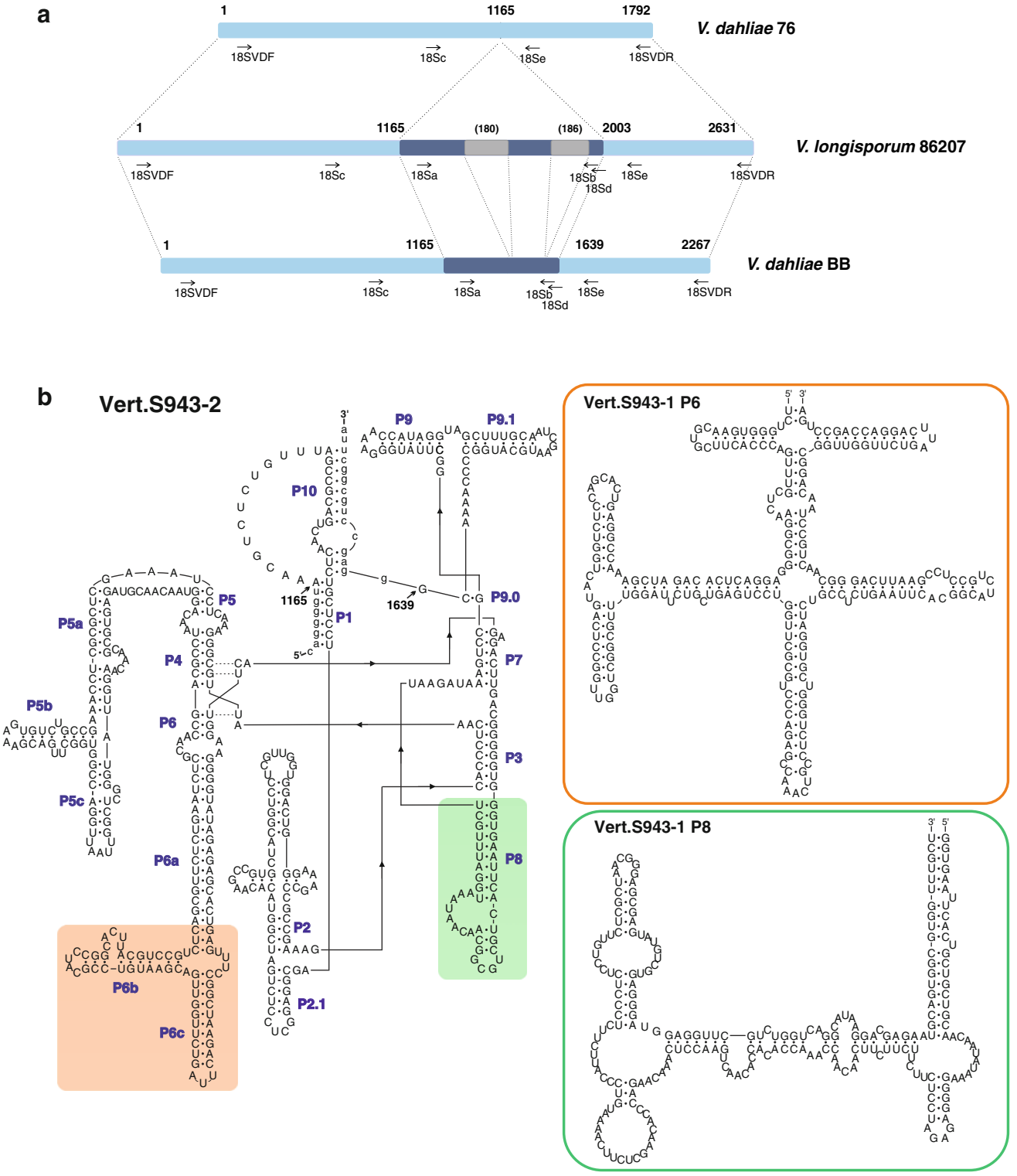

Fig. 2 **a** Structural organization of the SSU-rRNA gene of the intronless *V. dahliae* isolate 76 (GenBank accession number AF104926), *V. longisporum* 86207 (AF153421), and *V. dahliae* BB, determined in this study (GenBank accession number KF318969). The positions of group-I introns are shown above the corresponding *bars* and the lengths of two indels of significant sizes between the two introns are provided in *parentheses*. The positions of primers used in this study are also indicated with *arrows below each bar*. **b** The predicted secondary structure of group-I intron Vert.S943-2. Parts of the flanking exon sequences are included in the model for the accurate determination of the P1 and P10 helices; these nucleotides are displayed as *lower-case letters*. Parts of the peripheral P6 and P8 stem-loop structures that are variable between the two introns are shown in *boxes* and the corresponding structures of intron Vert.S943-1 are provided on the *right* of the figure

and selected mutants from an extended collection of over 650 complementary *nit* mutants of the testers used (I.A. Papaioannou and M.A. Typas, unpublished data) demonstrated that the three isolates from bell pepper (Ca.83, Ca.146, and Ca.148) were readily compatible with each other, yielding heterokaryons with abundant aerial hyphae and extended pigmentation, but incompatible with all other tester strains (Online Resource 1). On the contrary, one of the two isolates from chili pepper, Cf.38, produced heterokaryons with all three testers of VCG 2B but failed to complement the three bell pepper VCG 6 isolates, thus being re-classified into VCG 2B. The second chili pepper isolate, Cf.162, was characterized as heterokaryon self-incompatible, since all its 28 *nit* mutants that were checked for self and non-self compatibility, in all possible combinations, consistently failed to complement each other or other tester mutants. Thus, the inclusion of only the three bell pepper isolates in VCG 6 is substantiated by the compatibility data.

PCR screening with intron-specific primers and qRT-PCR: both introns are frequently encountered in *Verticillium* species, in variable ribosomal repeat numbers

Intron-specific primer pair 18Sa/b was designed to anneal to conserved regions of both introns (Table 2; Fig. 2a), allowing at the same time differentiation between the two by different sizes of the corresponding PCR products (i.e., 0.7 kb for Vert.S943-1 and 0.4 kb for the Vert.S943-2 intron, respectively). Remarkably, the screening of the majority of *V. dahliae* isolates produced DNA bands of one or both characteristic sizes (Table 1; Fig. 1b), indicating that the Vert.S943-2 intron is widespread in *V. dahliae* populations, while Vert.S943-1 was also detected in eight isolates of the species. All isolates were further subjected to PCR screening with primer pair 18Sc/d (Table 2; Fig. 2a), designed to amplify a 1.2- or a 0.8-kb-long region only if the Vert.S943-1 or Vert.S943-2 intron, respectively, is located at the same SSU position (Ec943). It was shown that the position of all insertions was identical since the results of the two PCR screenings were fully congruent (Table 1). Sequencing of the PCR products of randomly selected *V. dahliae* isolates with Vert.S943-2-characteristic amplicons (cotVd03, cotVd47, and 115; GenBank accession numbers KF318976 and KF318978-79, respectively) and isolates with Vert.S943-1-characteristic amplicons (cotVd03 and 131-M; GenBank accession numbers KF318977 and KF318980, respectively) also confirmed the identity of these insertions as introns Vert.S943-1 and -2, and their SSU position. All *V. longisporum* isolates, including G19 that failed to produce a Vert.S943-1-characteristic amplicon with SSU-specific primers, were characterized by the presence of Vert.S943-1 introns with intron-specific primer pairs, similarly to three of four *V. albo-atrum* isolates. The fourth *V. albo-atrum* isolate (T2), together with the *V. tricorpus* and *V. nubilum* representatives, exhibited the presence of PCR products characteristic of both intron types (Table 1). The presence or absence of one or both intron types was further tested by Southern hybridization experiments in 13 *Verticillium* isolates (10 *V. dahliae* strains from different geographic origins, including representatives of all VCGs, 2 *V. longisporum*, and 1 *V. albo-atrum* strains), which fully confirmed the results by the specific PCR screenings (data not shown).

Interestingly, significant differences in the yield of PCR amplicons were systematically observed among different isolates in each experiment (Fig. 1b), suggesting the presence of the two intron types in different copy numbers within the rDNA complex of various isolates. To further examine this observed heterogeneity, qRT-PCR was used for the determination of the SSU-rRNA gene copy numbers and the copy ratios of the two intron types in selected isolates. The SSU copy number in the genomes of five *V. dahliae* isolates varied significantly, ranging from 39 to 70 copies, with an average of 52 iterations (Table 3). Concerning intron detection, the results of qRT-PCR tests were fully congruent with the conventional PCR screenings, i.e., when the two representative intronless and the two isolates that did not contain the Vert.S943-1 intron were examined by qRT-PCR, tests failed to provide amplicons under any conditions (Table 3). For the *V. dahliae* isolates (30-6 and

Table 3 Copy numbers of the SSU-rRNA gene and copy ratios of group-I introns, determined with quantitative real-time PCR for selected *Verticillium* isolates

Isolate	SSU copy number[a]	SSU-located intron content[b]	Copy ratio of introns[c]
V. dahliae T9	48.24 ± 0.23	Only Vert.S943-2	No amplification of Vert.S943-1
V. dahliae V607I (R04)	38.65 ± 0.14	Only Vert.S943-2	No amplification of Vert.S943-1
V. dahliae V613I	70.40 ± 0.28	Both intron types	0.278
V. dahliae cotVd03	48.95 ± 0.19	Both intron types	0.208
V. dahliae 273-1	55.36 ± 0.24	No introns	No amplification of any introns
V. longisporum K12	ND[d]	Only Vert.S943-1	0.957

[a] Mean ± standard deviation (three samples, run in duplicate)

[b] Determined by conventional PCR screenings presented in Table 1

[c] Copy ratio of Vert.S943-1 introns to total introns, determined by qRT-PCR experiments

[d] *ND* not determined

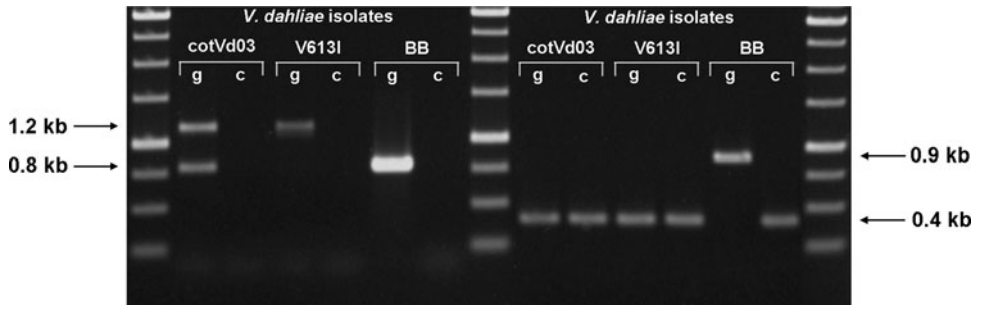

Fig. 3 Reverse transcription polymerase chain reaction (RT-PCR) products of *V. dahliae* isolates cotVd03, V613I, and BB with primer pairs 18Sc/d (between the first and the middle run of 1 kb DNA ladder) and 18Sc/e (between the middle and the last run of 1 kb DNA ladder). For each isolate and primer combination, PCR products with genomic DNA (designated as "*g*") or cDNA ("*c*") as the template, are shown next to each other on the agarose gel

171-4) found to harbor both introns in their SSU gene copies, the two introns were detected by qRT-PCR in a ratio of approximately 25 % Vert.S943-1 to 75 % Vert.S943-2, indicating that the former intron, whenever present, is only found in a minor ribosomal repeat number in the *V. dahliae* isolates tested. On the other hand, all ribosomal repeats of *V. longisporum* K12 seemed to uniformly bear the Vert.S943-1 intron in the SSU gene (Table 3).

Excision of introns from precursor SSU-rRNA transcripts of *V. dahliae*

Total RNA from three *V. dahliae* isolates (cotVd03, V613I, and BB) was extracted and subjected to reverse transcription PCR with primer pairs 18Sc/d and 18Sc/e (Fig. 3; annealing positions of primers are illustrated in Fig. 2a). No amplification was detected from cDNAs with the former primer pair, which demonstrates the absence of introns from mature rRNAs. When the same cDNAs were tested in PCR with the latter primer pair surrounding the insertion position (both primers annealing at regions of the SSU gene), products of intronless-characteristic size (0.4 kb) were obtained, confirming the excision of both introns from precursor rRNA transcripts.

Absence of introns in the LSU-rRNA (28S) gene of *Verticillium* species

Numerous studies of the LSU gene of various fungi have previously reported that group-I introns are usually inserted at highly conserved positions within an approx. 1-kb-long area of the gene nearer to its 3′ end (Pantou et al. 2003; Wang et al. 2003). Based on this observation and the complete sequence of the nuclear ribosomal complex of *V. dahliae* (GenBank accession number AF104926), a primer pair (28SF-28SR; Table 2) was designed to specifically amplify this region, with an expected length of amplicons without any insertions of 1145 bp. Fifty *Verticillium* isolates of all *V. dahliae* VCGs and from various original hosts and geographic origins (Table 1) were tested with this primer pair for detectable size polymorphism of the amplified LSU region. Screening of all isolates invariably yielded a 1.1-kb-long amplicon, suggesting the absence of introns or other detectable sources of heterogeneity in the 3′ area of the LSU gene.

Discussion

Group-I introns have been detected in the nuclear ribosomal RNA genes of various fungi and have been used extensively for discrimination and phylogenetic analyses, as their distribution is highly irregular, even at the intraspecific level (Hibbett 1996; Pantou et al. 2003; Hafez et al. 2012). In *Verticillium*, a group-I intron (Vert.S943-1) has been previously detected by PCR in the majority of *V. longisporum* isolates tested, and was generally assumed to be absent from the other *Verticillium* species (Karapapa and Typas 2001; Collins et al. 2003; Inderbitzin et al. 2013; Tran et al. 2013). In this work, contrary to the previous reports, a group-I intron (named Vert.S943-2) that is highly similar to Vert.S943-1 was found located at the same SSU position of most *V. dahliae* isolates of VCGs 4A and 6. Presumably, the presence of group-I introns in the SSU gene of such *V. dahliae* isolates has not been detected during previous investigations due to the absence or underrepresentation of isolates from these VCGs in the fungal collections tested.

The common intron content for VCGs 4A and 6 is in agreement with a recent phylogenetic analysis of the IGS region (Papaioannou et al. 2013a) and, taken together, these results suggest a close genetic relationship between the two groups. Notably, the two VCGs share a common geographic distribution, i.e., both are limited to North America, which might account, at least in part, for their

common genetic characteristics as a result of common descent or co-evolution. Within VCG 6, isolates originating from bell pepper differed in intron distribution from those from chili pepper. Similarly, the two groups of members of this VCG have been found to differ in additional molecular traits (Papaioannou et al. 2013a, b). Remarkably, the re-evaluation of VCG classification for the five VCG 6 isolates tested here justified the preservation in this group of only the bell pepper isolates, which were identical to VCG 4A members regarding intron content. From the remaining chili pepper isolates, one (Cf.38) was clearly a VCG 2B member and the other (Cf.162) was generally unable to form heterokaryons. It should be noted that globally only few members of VCG 6 are available to date, and therefore, a larger number of such isolates should be obtained and studied before comprehensive conclusions on the genetic homogeneity and the significance of this VCG can be reached.

Importantly, when intron-specific primers were used instead of SSU-specific primers for the PCR screening of the fungal collection, a multitude of insertions with variable distributions was revealed at the same position of the SSU gene of all *Verticillium* species. These insertions were found to correspond to introns Vert.S943-1 and -2, with the former prevailing in *V. longisporum* and *V. albo-atrum*, the latter in *V. dahliae*, and both being present in the unique *V. tricorpus* and *V. nubilum* representative strains. The failure of conventional SSU-based PCR to detect the existing introns can be attributed to the biased nature of amplification when a multi-copy region is targeted, since PCR is expected to preferentially amplify the majority of dissimilar targets, especially when these yield a shorter, intronless product. Thus, introns present in less repeat numbers might often elude detection by SSU PCR screenings, which probably accounts for the failure of previous studies to identify SSU-located introns in *V. dahliae* and its relative species. This finding raises serious concerns about the accuracy of methods that are based on the detection of DNA multi-copy targets for use in population studies and underlines the care that must be taken in interpreting results.

In the case of *Verticillium*, SSU-specific primers are expected to identify not only *V. longisporum* but also *V. dahliae* VCGs 4A and 6. Naturally, this is mostly important for North America, where these VCGs are known to be present (Dobinson et al. 2000; Bhat et al. 2003), while it may be of little relevance for other geographic areas which lack these VCG groups. However, given the widespread occurrence of the two ribosomal intron types in all *Verticillium* species, the results from all such screenings should still be treated with caution. The same rationale should be applicable to the use of the Vert.S943-1 intron for differentiation between *V. longisporum* lineages (Tran et al. 2013), as an isolate (G19) which appeared to be intronless with general SSU PCR-testing was actually found to bear Vert.S943-1 intron in a minor repeat number. Taking into consideration the distributions of the two intron types, it could be hypothesized that Vert.S943-1 is the parental type within the *Verticillium* species complex, with its "shortened" version, Vert.S943-2, probably having originated from it by losing two DNA regions that were nonessential for splicing activity. Similarly, related group-I introns that differ in the length of various loops have been described for other organisms (Michel and Westhof 1990; Müller et al. 2001; Wang et al. 2003).

The rDNA complex of eukaryotic organisms is known to comprise multiple copies of ribosomal repeats arranged as tandem iterations separated by noncoding spacers in the nuclear organizer region of one or more chromosomes (Rooney and Ward 2005). The recovery of more than one PCR amplicon types (differing in their intron content) from the majority of isolates examined in this study clearly demonstrates the presence of more than one types of ribosomal repeats in the genomes of *Verticillium* isolates. Heterogeneity or "heterozygosity" among repeats of the nuclear ribosomal complex and, more specifically, intragenomic variability for the presence or absence of group-I introns have been reported in the past for several fungi (Hibbett 1996; Lickey et al. 2003). This might be explained by transience in the process of slow homogenizing concerted evolution of multi-copy genes, recombination between distinct rDNA types following (para-)sexual recombination and/or intron mobility, i.e., movement of introns into different genes and species through the mechanisms of homing or reverse splicing (DePriest 1993; Haugen et al. 2005; Simon et al. 2005).

Using mainly pulsed-field gel electrophoresis and hybridization-based techniques, the rDNA copy number of various yeasts and few filamentous fungi has been previously reported to vary, in the range of approx. 30 to more than 200 copies per haploid genome (Garber et al. 1988; Maleszka and Clark-Walker 1993; Howlett et al. 1997). Recently, the rDNA copy number of few *V. dahliae* isolates was estimated by a qRT-PCR relative quantification method that was based on the IGS region, to range from approximately 24 to 73 copies per genome (Bilodeau et al. 2012). However, the authors pointed out that these values should be considered only as an estimate because the amplification efficiencies for the IGS region and each of the single-copy genes used in their calculations were different. A more accurate method is the qRT-PCR absolute quantification method, which was used for *Aspergillus fumigatus*, defining the SSU-rRNA gene copy number among different strains of the fungus between 38 and 91 copies per genome (Herrera et al. 2009). In our work, we adopted this method for the accurate determination of the rDNA copy

number of *V. dahliae*, and this was found to vary among different isolates in a similar range, from 39 to 70 copies per haploid genome. Moreover, we demonstrated here that qRT-PCR-based detection methods are appropriate for the sensitive and quantitative identification of insertions present in a few copy numbers within the ribosomal repeat. This must be applicable in many other organisms and may prove very useful in future adaptation studies addressing the rate of change of the ratios between dissimilar copies in response to different environmental stimuli imposing varying levels of selective pressure.

Our results altogether underline that the nuclear rDNA complex of *Verticillium* species can be highly heterogeneous regarding both the identity of insertions (i.e., different group-I introns in the SSU gene) and their copy numbers. It was further demonstrated that insertions that are present in a minor repeat number (i.e., in only a few rDNA repeats) can remain undetected by conventional screenings. Thus, it was shown that more sensitive methods such as Southern blots, PCR screenings with intron-specific primers, and qRT-PCR can be successfully employed and should be used for the elucidation of the presence and the distribution of insertions in the rDNA complex, to avoid incomplete or even misleading results. These conclusions are predicted to be relevant to discrimination and phylogenetic studies of many fungi and other eukaryotic organisms.

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