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Phylogenetic relationships and genome organisation of *Colletotrichum acutatum* causing anthracnose in strawberry

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Abstract *Colletotrichum acutatum* is a major plant pathogen which infects a broad range of host plants. Extensive research has been carried out on *C. acutatum* populations affecting various hosts in different geographical locations, showing a considerable genotypic and phenotypic diversity. Anthracnose, caused by *Colletotrichum* spp., is the major disease of cultivated strawberry, *Fragaria x ananassa*. In the present study, the phylogenetic relationships within a worldwide sample of fifty-two *C. acutatum* isolates collected from different strawberry cultivars have been established, by using ITS sequence analyses. Twenty-nine isolates clustered in the molecular group A2, in which seventeen out of eighteen Spanish isolates were included; this may indicate that the group A2 is the key group in Spain. The molecular polymorphism among *C. acutatum* isolates was determined by southern-blot hybridisation using a telomeric DNA probe. Results indicated that the minimum number of estimated chromosomes ranges between six and nine. The molecular characterisation of *C. acutatum* isolates was completed using the Pulsed-Field Gel Electrophoresis (PFGE) technique that resolved from six to nine chromosomal bands, this number being coincident with the number of chromosomes obtained by telomeric fingerprinting. The minimum total genome size was estimated to range from 29 to 36 Mb. Comparison of karyotypes patterns and southern-blot analysis demonstrated a high level of molecular polymorphism among *C. acutatum* isolates from different origins.

Keywords Anthracnose · *Colletotrichum* · Fingerprint · PFGE · Telomere

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Introduction

Colletotrichum acutatum (teleomorph: *Glomerella acutata* (Guerber and Correll 2001)) is a major worldwide plant pathogen that infects a broad range of host plants (Whitelaw-Weckert et al. 2007). It is recognised as a cosmopolitan pathogen that causes anthracnose on a number of economically important crops, including woody and herbaceous crops, ornamentals, fruits, conifers and forage plants (Sreenivasaprasad and Talhinhas 2005). This species was first described by Simmonds (1965) in a survey of fruit-rot pathogens in Australia, and is being currently a major pathogen in various disease complexes, in which more than one

Colletotrichum species are associated with a single host (Sreenivasaprasad and Talhinhas 2005).

Strawberry (*Fragaria ananassa*) is one particularly susceptible host on which three species, *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (Denoyes-Rothan et al. 2003; Mass 1987; Smith and Black 1990), can lead to a variety of diseases, including wilts, rots and anthracnose. *Colletotrichum gloeosporioides* causes up to 80% plant death in nurseries and yield losses of >50% (Sreenivasaprasad and Talhinhas 2005), being a major disease of cultivated strawberry (Buddie et al. 1999; Denoyes-Rothan et al. 2004; Leandro et al. 2001). Anthracnose strawberry symptoms produced by *C. acutatum*, *C. fragariae* and *C. gloeosporioides* are similar (Denoyes-Rothan et al. 2003), and although *C. acutatum* mainly causes fruit rots, it can also infect other parts of the plants such as crowns, petioles, leaves and even roots (Sreenivasaprasad and Talhinhas 2005).

Colletotrichum acutatum can be found throughout the world. In the USA, *C. acutatum* is the major species present in the southwest region (California). In the European Union (EU), it was first observed in 1981 (France), and is now widespread (Denoyes-Rothan and Baudry 1995). It is not clear how the pathogen was introduced into production fields in Europe, although there are several possible routes, including infected mother plants from the American nurseries (Freeman and Katan 1997), contaminated soil associated with strawberry crowns at planting (Leandro et al. 2001), and quiescent infections on strawberry leaves or fruits (Leandro et al. 2003). *Colletotrichum acutatum* was classified as an organism of quarantine significance in Canada from 1991 to 1997 (PPD directive D-97-03) and in the UK and the EU since 1993 (EC directive 77/93) (EPPO/CABI 1997; Mertely and Legard 2004).

Investigations of *Colletotrichum* spp. have been focused on just one or two aspects of the disease: traditionally, with cultural or morphological studies (Afanador-Kafuri et al. 2003; Denoyes-Rothan and Baudry 1995; Smith and Black 1990), and nowadays, using molecular techniques including isoenzyme comparisons, Restriction Fragment Length Polymorphisms (RFLP) analyses of mitochondrial DNA, Amplified Fragment Length Polymorphism (AFLP), AT rich analyses, Random Amplified Polymorphic DNA (RAPD), and ITS sequence analyses for specific PCR identification (Buddie et al. 1999; Garrido et al. 2007, 2009; Freeman et al. 1993; Sreenivasaprasad et al. 1996; Talhinhas et al. 2005). These techniques have improved the accuracy and reduced the time necessary for the identification and classification of *Colletotrichum* spp.

Colletotrichum acutatum populations, affecting various hosts in different geographical locations, were classified by Sreenivasaprasad and Talhinhas (2005) in to molecular groups based on sequence analysis of the internal transcribed spacers (ITS) of ribosomal DNA polymorphic regions. This classification established eight molecular groups for *C. acutatum* and one group for *C. gloeosporioides*, and has been widely used to study the genotypic and phenotypic diversity of this fungus and to classify isolates from different origin (Whitelaw-Weckert et al. 2007). In spite of these investigations, no extensive work has been developed to obtain information about the structure of the genome of *C. acutatum*. Genome organisation has been studied during the last decades in other pathogenic fungi such as *Beauveria bassiana* (Viaud et al. 1996), *Botrytis cinerea* (Vallejo et al. 1996; 2002), and *Verticillium dahliae* (Pantou and Typas 2005), showing a high level of chromosome length polymorphism among fungal isolates. Regarding the genus *Colletotrichum,* only two studies have been reported carried out by using Pulse-Field Gel Electrophoresis (PFGE) for analysing the genome organization of both *C. gloeosporioides* and *C. lindemuthianum* (Masel et al. 1993; O'Sullivan et al. 1998), but the protocols were time-consuming because karyotypes were resolved in two different steps.

The purpose of the present study was: firstly, to classify a worldwide sample of *C. acutatum* isolates collected from different strawberry cultivars, within previously described molecular groups established by Sreenivasaprasad and Talhinhas (2005), and study the phylogenetic relationships among the isolates. Secondly, to describe the genomic organisation of *C. acutatum* regarding both the number of chromosomes and the chromosome length polymorphism (CLP) among isolates, by using three different approaches: (i) RFLP, using four different restriction enzymes; (ii) Optimising a PFGE protocol for *C. acutatum*, and (iii) Southern blot hybridisation of both RFLPs and karyotypes obtained by PFGE, with a telomeric DNA sequence.

Materials and methods

Fungal origin and maintenance

Colletotrichum acutatum isolates used in this study were obtained from: (i) a previous survey carried out in Spain from strawberry anthracnose fruit rot (Garrido et al. 2007); (ii) the International Mycological Institute (IMI), and (iii) the Central Science Laboratory (CSL) collection, which includes isolates from Australia, Canada, France, Germany, Japan, The Netherlands, New Zealand, Norway, Portugal, Switzerland, USA and UK, isolated from strawberry; isolates from lupin, rose and phlox were also included (Table 1). Isolates from this collection were kindly supplied by Dr. Neil Boonham from CSL.

Isolates were grown on potato dextrose agar medium (Becton Dickinson,) for routine culturing; potato dextrose broth medium (Becton Dickinson, Sparks, USA) was used for fungal mycelium production and liquid Czapek-Dox medium (Vallejo et al. 2002) for preparation of protoplasts.

Extraction of DNA from cultured isolates

Fungal DNA was extracted from freeze-dried ground mycelium. Weighed samples (80 – 100 mg) were placed in extraction tubes with two 1/4 inch Ceramic Spheres (Q-Biogene, Valencia, USA) and 1 ml of CTAB lysis buffer (Garrido et al. 2009) supplemented with 2% of antifoam B emulsion (Sigma-Aldrich, St. Louis, USA). Tissue samples were then homogenised using a FastPrep Instrument (Q-Biogene). The resultant lysated samples (600 µl) were transferred to fresh 2 ml tubes and incubated for 30 min at 65ºC; 600 µl of chloroform:isoamyl alcohol (24:1, vol/vol) were then added to the tubes that were then mixed by vortexing, and spun for 10 min at 15,000 *g*. The aqueous layer (500 µl) was collected and the washing step was repeated twice; 400 µl of the upper aqueous layer were then transferred to a clean tube containing isopropanol (1 vol) and 5 M NaCl (0.5 vol), mixed gently, and spun for 10 min at 15,000 *g*. Supernatant was discarded and the pellet diluted in 100 µl of sterile distilled water (SDW). The obtained DNA was precipitate in 90% ethanol (2.5 vol) and sodium acetate 3 M (0.1 vol); the pellet was re-suspended in SDW and samples stored at −20ºC.

PCR amplification with specific primers

The strains used in this research were identified by conventional PCR in order to ensure their correct identity before the remaining assays. Genus-specific primers Col1 (5′-AAC CCT TTG TGA ACR TAC CTA-3′) and Col2 (5′-TTA CTA CGC AAA GGA GGC T-3′) were used for identification of the genus *Colletotrichum* (Garrido et al. 2007; Martínez-Culebras et al. 2003); species-specific primers CaInt2 (5′-GGG GAA GCC TCT CGC GG-3′) for *C. acutatum* (Garrido et al. 2007; Sreenivasaprasad et al. 1996) and CgInt (5′-GGC CTC CCG CCT CCG GGC GG-3′) for *C. gloeosporioides* (Mills et al. 1992) were each coupled in PCR reactions with the universal primer ITS4 (White et al. 1990). Two independent experiments of PCR amplification were performed with each pair of primers.

5.8 S-ITS sequences analysis

Sequencing of rDNA ITS region The internal transcribed spacer regions, including the 5.8 rDNA, were amplified using universal primers ITS1 and ITS4 (White et al. 1990). PCR assays were performed in 30 µl volumes, each containing 100–200 ng of DNA. The amplicons (approximately 600 bp in size) were purified using a JetQuick purification kit (Genomed, Genycell Biotech, Granada, Spain), according to the manufacturer´s instructions. Purified products were quantified by visualising alongside a quantifiable marker (Hyperladder I, Bioline) on 1% agarose gels in 1 × TBE buffer. Quantified products were diluted to 30 ng µl^{-1} and sent to the Genomic Unit of the University of Córdoba (Spain) for sequencing. The PCR products were sequenced in both directions. Nucleotide sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/); the accession numbers are shown in Table 1.

Sequence data management and analysis The files with the sequences were assembled and edited to resolve ambiguities using the CHROMAS v2.24 (Conor McCarthy, University Gold Coast, Australia) and EditSeq software v4.05 (DNAStar Inc., Madison, USA). The sequences of complementary strands were compared and aligned using ClustalW software (http://align.genome.jp/) and a neighbour-joining phylogenetic analysis was estimated using *MEGA* v3.1

Table 1 Isolates of *Colletotrichum* spp. used in this study

Isolate[a]	Identified as[b]	Host	Geographic location	5.8S-ITS Sequences		Telomeric fingerprints[g]				
				EMBL Accession[c,d,e]	Molec. group[f]	*Bam*HI	*Eco*RI	*Hin*dIII	*Pst*I	Minimun number of chromosomes
CSL1182	*C. acutatum*	*Lupinus* spp.	Germany	EF622187[c]	A1	10	14	12	11	7
CSL601	*C. acutatum*	*Lupinus* spp.	Germany	EF622191[c]	A1	8	10	14	12	7
CSL1435	*C. acutatum*	*Lupinus* spp.	The Netherlands	EF622192[c]	A1	9	12	0	12	6
CSL1434	*C. acutatum*	*Lupinus* spp.	Australia	EF622193[c]	A1	8	10	0	12	6
CSL1179	*C. acutatum*	*Lupinus* spp.	Australia	EF622194[c]	A1	8	11	0	12	6
CSL500	*C. acutatum*	*Lupinus* spp.	UK	EF622195[c]	A1	7	11	0	12	6
CSL502	*C. acutatum*	*Lupinus* spp.	UK	EF622196[c]	A1	10	13	10	6	7
CSL513	*C. acutatum*	*Lupinus* spp.	UK	EF622197[c]	A1	9	11	0	12	6
CSL501	*C. acutatum*	*Lupinus* spp.	The Netherlands	EF622198[c]	A1	9	10	0	14	7
UCA1005	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF694673[d]	A2	8	14	18	13	9
UCA1015	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF694674[d]	A2	9	14	18	13	9
UCA1018	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF622177[c]	A2	9	14	18	12	9
UCA1025	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF694675[d]	A2	9	15	18	15	9
UCA1026	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF622178[c]	A2	9	13	18	0	9
UCA1028	*C. acutatum*	*F. ananassa*	Huelva, Spain	EF694676[d]	A2	9	14	18	15	9
UCA1070	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF694677[d]	A2	10	13	18	13	9
UCA1072	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF622179[c]	A2	10	13	18	13	9
UCA1076	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF694678[d]	A2	9	14	18	15	9
UCA1077	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF694679[d]	A2	9	14	18	14	9
UCA1078	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF694680[d]	A2	9	15	18	16	9
UCA1083	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EU109737[c]	A2	9	15	18	15	9
UCA1084	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EF622180[c]	A2	14	16	16	12	8
UCA1088	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EU109738[c]	A2	14	16	16	12	8
UCA1089	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EU109739[c]	A2	12	16	16	12	8
UCA1090	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EU109740[c]	A2	12	16	16	12	8
UCA1091	*C. acutatum*	*F. ananassa*	Cádiz, Spain	EU109741[c]	A2	12	16	16	12	8
CSL721	*C. acutatum*	*F. ananassa*	Switzerland	EF622182[c]	A2	14	12	12	15	8
CSL891	*C. acutatum*	*F. ananassa*	Portugal	EF622184[c]	A2	14	16	14	12	8
CSL874	*C. acutatum*	*F. ananassa*	Portugal	EF622186[c]	A2	12	16	14	12	8
IMI324994	*C. acutatum*	*F. ananassa*	USA	EF622189[c]	A2	15	16	16	14	8
IMI324995	*C. acutatum*	*F. ananassa*	USA	EF622190[c]	A2	14	16	14	14	8
CSL1090	*C. acutatum*	*F. ananassa*	UK	EF622205[c]	A2	13	15	16	13	8
IMI348487	*C. acutatum*	*F. ananassa*	France	EF694681[c]	A2	10	10	10	12	6
UCA-ST17	*C. acutatum*	*F. ananassa*	UK	EF622203[c]	A2	13	16	16	12	8
UCA-ST8	*C. acutatum*	*F. ananassa*	UK	EF622202[c]	A2	13	16	16	12	8
IMI345027	*C. acutatum*	*F. ananassa*	France	AJ536199[e]	A2	12	16	14	13	8
IMI360086	*C. acutatum*	*F. ananassa*	Japan	AJ536205[e]	A2	8	0	2	2	4
IMI360928	*C. acutatum*	*F. ananassa*	Switzerland	AJ536201[e]	A2	14	16	15	15	8
IMI324993	*C. acutatum*	*F. ananassa*	USA	AJ536214[e]	A3	12	16	10	13	8
UCA89	*C. acutatum*	*F. ananassa*	France	EF694682[d]	A3	2	10	12	16	8
CSL720	*C. acutatum*	*F. ananassa*	Switzerland	EF622181[c]	A3	11	12	11	9	6
CSL725	*C. acutatum*	*F. ananassa*	New Zealand	EF622183[c]	A3	3	6	6	4	3
IMI345583	*C. acutatum*	*F. ananassa*	New Zealand	EF622199[c]	A3	2	3	6	1	3
IMI345026	*C. acutatum*	*F. ananassa*	Spain	AJ536209[e]	A4	9	8	14	6	7

Table 1 (continued)

Isolate[a]	Identified as[b]	Host	Geographic location	5.8S-ITS Sequences		Telomeric fingerprints[g]				
				EMBL Accession[c,d,e]	Molec. group[f]	*Bam*HI	*Eco*RI	*Hin*dIII	*Pst*I	Minimun number of chromosomes
IMI351255	*C. acutatum*	*F. ananassa*	UK	AJ536211[e]	A4	3	4	5	3	3
CSL2063	*C. acutatum*	*Rosa* spp.	The Netherlands	EF622188[c]	A5	16	14	12	10	8
CSL1206	*C. acutatum*	*Phlox* spp.	The Netherlands	EF622200[c]	A5	14	10	9	11	7
IMI345581	*C. acutatum*	*F. ananassa*	New Zealand	AJ536212[e]	A7	6	10	14	8	7
IMI345034	*C. acutatum*	*F. ananassa*	Australia	AJ536207[e]	A9	14	16	16	13	8
CSL735	*C. gloeosp*	*F. ananassa*	UK	EF622201[c]	B	10	9	11	14	7
CSL736	*C. gloeosp*	*F. ananassa*	UK	EF622204[c]	B	10	10	7	14	7
IMI345051	*C. gloeosp*	*F. ananassa*	Canada	EF622185[c]	B	8	8	12	14	7

[a] Name of the isolate from University of Cádiz (UCA), Central Science Laboratory (CSL) and International Mycological Institute (IMI)

[b] Identification on the basis of the genus-specific pair of primers, Col1-Col2, and species-specific pairs of primers CaInt2-ITS4 and CgInt-ITS4

[c] GenBank accession numbers for isolates sequenced in this study

[d] GenBank accession numbers for isolates sequenced in previous work (Garrido et al. 2009)

[e] GenBank accession numbers for isolates sequenced by Martínez-Culebras et al. (2003)

[f] Molecular group for *Colletotrichum* spp. described by Sreenivasaprasad et al. (2005)

[g] Minimum number of chromosomes estimated from DNA digested by restriction enzymes and hybridised with a telomeric probe

software. In order to allocate the strains within molecular groups previously described for *C. acutatum* by Sreenivasaprasad and Talhinhas (2005), other sequences of *C. acutatum* and *C. gloeosporioides* from GenBank were included in the phylogenetic tree (GenBank accession numbers, molecular groups and hosts are shown in Table 1).

Telomeric fingerprinting

Genomic DNA of 52 isolates was digested to completion with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I restriction enzymes, (Fermentas Inc., Hanover, USA) in independent experiments. The standard protocol for RFLP described in Sambrook et al. (1989) was followed to digest 1.5 µg of genomic DNA in a total volume of 50 µl. Digestions were then separated on 1.5% agarose gel using 1 × TBE buffer. Band sizes were calculated by regression analysis using phage lambda digested with *Hin*dIII as the molecular marker. Two independent experiments were performed.

Probe Telomeric (TTAGGG)n probes were generated by PCR in the absence of a template (Ijdo et al. 1991), using $(TTAGGG)_5$ and $(CCCTAA)_5$ as primers. The probes were labelled using a PCR procedure with the following dNTP concentrations: 200 µM of dATP, dCTP, dGTP, 150 µM dTTP and 50 µM dig-11-dUTP (Roche Molecular Biochemicals).

Southern transfer and hybridisation Total DNA, cut by restriction enzymes, was transferred to Hybond-N membrane (Hybon NX; Amersham International, Bucks, USA) as described by Sambrook et al. (1989). The membranes were allowed to hybridise with the telomeric probes at 40ºC overnight, according to the recommendations of the manufacturer. The membranes were washed twice in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (wt/vol) sodium dodecyl sulphate (SDS) at room temperature for 5 min and then twice with 0.5 × SSC, 0.1% (wt/vol) SDS at 65ºC for 15 min. Inmunological detection of the hybridisations was performed as recommended by the manufacturer, and membranes were exposed to X-ray film overnight at room temperature. Randomly

selected samples were included to demonstrate the reproducibility of the results.

Fingerprinting analysis Films were digitalised using a GS-800 calibrated densitometer (Bio-Rad). Digital images were edited, individual bands located and size of bands assigned using Fingerprinting II Software v3.0 (Bio-Rad). Telomeric profiles were also analysed to study the polymorphism present among *C. acutatum* isolates.

PFGE analysis

Protoplast and chromosome preparation Protoplasts of *Colletotrichum* spp. were obtained following a modified protocol previously described by Vallejo et al. (1996; 2002). Young mycelial growth in Czapek-Dox liquid medium for 20 h, was suspended (1 g wet weight in 20 ml) in TPP isotonic buffer (0.7 M KCl, 50 mM KPO_4 potassium phosphate buffer, pH 5.8) and treated with lysing enzyme (10 mg ml^{-1}) (Sigma-Aldrich). The mixture was incubated at 25ºC, 50 rpm for 5 h, filtered through 30 µm filters (Nytal) and washed with 0.7% KCl to remove the undigested mycelia. Protoplasts were pelleted by spinning at 800*g* for 5 min and the pellet washed three times with TCE isotonic solution (0.7 M KCl, 50 mM EDTA, pH 8.0).

The protoplast suspensions were mixed with 1 vol of 1% (wt/vol) low melting point agarose (Bio-Rad, Hercules, USA) in TCE for a final concentration of 1×10^8 protoplast ml^{-1}. The mixture was solidified in plug moulds (Bio-Rad) and incubated in ESP buffer (0.5 M EDTA, pH 9.0, 1% lauroylsarcosine, 1 mg ml^{-1} proteinase K) at 50ºC for 48 h to release the DNA. Afterwards, the plugs were washed three times with 50 mM EDTA (pH 8.0), and stored at 4ºC in the same buffer.

CHEF gel electrophoresis Karyotypes were determined using the Bio-Rad Contour-Clamped Homogeneous Electric Field electrophoresis (CHEF) DR-III system. The running buffer was 1 × TAE buffer (40 mM Tris-acetate, 90 mM Boric acid, 2 mM EDTA, pH 8.0) at 13ºC. Running conditions were optimised: 0.8% PFGE ™Megabase agarose (Bio-Rad); 1,200-s pulses for 24 h at 2 V/cm were followed by 1,500-s pulses for 24 h at 2 V/cm, and then by 1,800-s pulses for 24 h at 2 V/cm. Gels were stained with 0.5 µg ml^{-1} ethidium bromide for 45 min and DNA visualised using UV light (Gel doc, XR System; Bio-Rad). Chromosomes of *Hansenula wingei* and *Schizosaccharomyces pombe* were used as markers for calculation of band sizes (Vallejo et al. 2002). Band patterns were analysed using the Fingerprinting II software (Bio-Rad). In order to confirm that all the bands represented chromosomes, PFGE gels were transferred to Hybond-N membranes and hybridised with the telomeric probe, following the above described protocol. Films were digitalised using a GS-800 calibrated densitometer (Bio-Rad), and images edited and studied by using Fingerprinting II Software v3.0 (Bio-Rad) as described previously.

Results

5.8S-ITS Sequence analysis

Forty-nine isolates of *C. acutatum* and three isolates of *C. gloeosporioides* were successfully identified on the basis of genus and species-specific primers by PCR amplification. The nucleotide sequences of the ITS1, 5.8S RNA gene, and ITS2 were studied for each isolate. Thirty-four isolates were sequenced in this work, ten isolates had been previously sequenced (Garrido et al. 2007), and the sequences of the eight remaining isolates had been previously reported by Martínez-Culebras et al. (2003) (GenBank database accession numbers are given in Table 1).

A neighbour-joining phylogenetic tree, with the 5.8S-ITS sequences, was estimated using the Kimura two-parameter model and a bootstrap test of 5000 runs. Thirty-five sequences of *C. acutatum* and one of *C. lindemuthianum* (AB087222, used as the out-group) were downloaded from the GenBank database, and included in the phylogenetic tree, allowing us allocate the 52 samples into molecular groups previously described by Sreenivasaprasad and Talhinhas (2005) (Fig. 1).

The phylogenetic tree showed a large cluster with a strong bootstrap support (90%) containing the subgroups A1, A2, A5, A8 and A9, and clustering 41 isolates (78% of the studied isolates). Twenty-nine sequences of *C. acutatum,* which differed in 13 bp

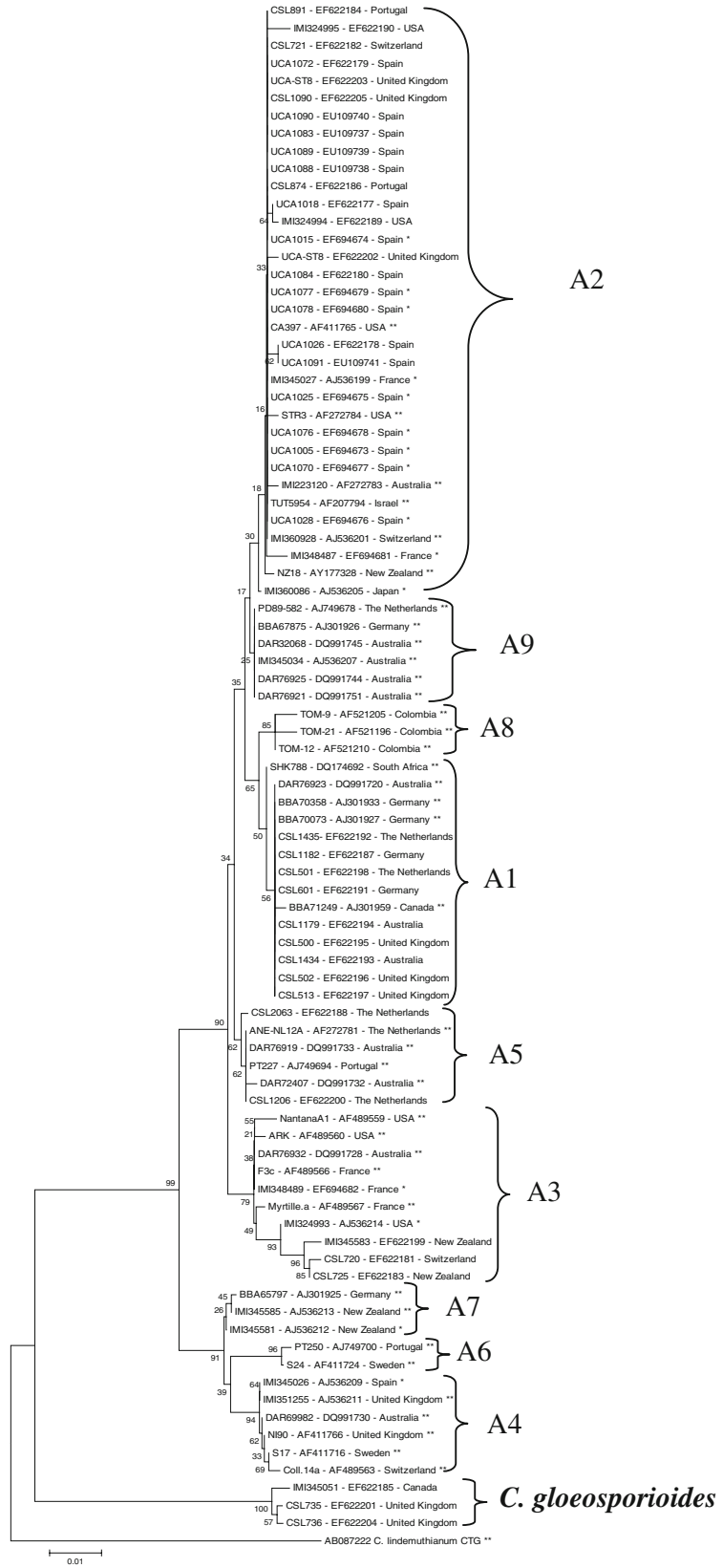

Fig. 1 Neighbour-joining tree derived from 5.8S-ITS gene sequences of sequenced isolates and published sequences, showing separation into *Colletotrichum acutatum* groups of Sreenivasaprasad and Talhinhas (2005). Numbers on nodes are bootstrap values, i.e. the frequency (%) with which a cluster appeared in a bootstrap test of 5000 runs with Kimura 2-parameters and pairwise deletion. *Colletotrichum lindemuthianum* (AB087222) was included as an outgroup, ●— Sequences obtained from previous work (Garrido et al. 2009; Martínez-Culebras et al. 2003), **—Sequences obtained from the GenBank database to establish the separation into *C. acutatum* groups of Sreenivasaprasad and Talhinhas (2005)

(positions 10th, 21st, 55th, 56th, 61st, 67th, 70th, 91st, 371st 372nd, 411th, 466th and 475th of the sequence EF694676), clustered within the molecular subgroup A2. The cluster showed a weak bootstrap support of 30% (Fig. 1). All the obtained isolates were isolated from *F. ananassa* and they belonged to seven countries: France, Japan, Portugal, Spain, Switzerland, UK and USA (Table 1 and Fig. 1). The subgroup A1 clustered all the *C. acutatum* isolates obtained from *Lupinus* spp. and also isolates from Australia, Germany, The Netherlands and UK. Sequences differed in 4 bp (positions 10th, 21st, 440th and 453rd of the sequence EF622187). One isolate clustered in the subgroup A9, and other two isolates of *C. acutatum*, obtained from *Rosa* spp. and *Phlox* spp., clustered within the subgroup A5 (CSL2063 and CSL1206 from The Netherlands).

The 5.8S-ITS sequence of five *C. acutatum* isolates, all of them obtained from strawberry (from USA, Switzerland, New Zealand and France), clustered within group A3, with a bootstrap support of 79%. One *C. acutatum* isolate from New Zealand (IMI345581) clustered in subgroup A7 and two isolates, from Spain (IMI345026) and UK (IMI351255), clustered within the subgroup A4 with a strong bootstrap support (94%). The isolates of *C. gloeosporioides* included in this study clustered in a separate group based on the 5.8S-ITS sequences, and named *C. gloeosporioides* group by Whitelaw-Weckert et al. (2007).

Telomeric fingerprinting

Telomere profiles The telomeric probe, obtained by PCR, was used to fingerprint *C. acutatum* isolates after digestion of total DNA with four restriction enzymes in independent experiments. Each band represents a physically distinct telomere extremity. Therefore, the number of telomeric extremities was evaluated taking into consideration the restriction enzyme which produces the highest number of fragments for each isolate. This number was then divided into two to determine the number of chromosomes (Table 1). When the number of bands was odd, it was rounded up to the next upper number (Viaud et al. 1996), since one of the bands may be composed of two overlapped fragments of similar size. Among the fifty-two isolates analysed, the number of bands or telomeres oscillated between six and eighteen. Forty-eight isolates showed a minimum number of estimated chromosomes between six and nine, and only four isolates (IMI351255, IMI360086, IMI345583 and CSL725) showed a lower number of estimated chromosomes (between three and four) (Table 1). The size of the bands ranged from 24.3 kb to 0.74 kb in the *Bam*HI blots, from 11.1 kb to 0.42 kb in the *Eco*RI blots, from 13.2 kb to 0.36 kb in the *Hin*dIII blots, and from 9.16 kb to 0.53 kb in the *Pst*I blots (Fig. 2).

Fingerprinting analyses of telomeric profiles The telomeric profiles obtained for each isolate of *C. acutatum* were analysed using the Fingerprinting II software v3.0 (Bio-Rad). A cluster analysis was performed based on Dice coefficients. An average matrix was created from the similarity matrices obtained for each individual experiment. Each value was corrected for internal weights proportional to the number of bands present in the individual enzyme profiles. The average matrix was then used to construct a dendogram by the unweighted pair-grouped methods by arithmetic averages (UPGMA). The UPGMA dendogram showed a representative grouping among the isolates, which is coincident with the grouping in the neighbuor-joining phylogenetic tree based on sequences of rDNA ITS regions (Fig. 3). Isolates IMI351255, IMI360086, IMI345583 and CSL725 were not analysed since they showed between six and eight telomeric bands, considered a low number of bands.

Twenty-seven isolates clustered in a group with >70% similarity. All of these isolates belonged to the molecular group A2, with the exception of isolate IMI345034, which belonged to the group A9. The cluster could be divided in three sub-groups, with >80% similarity: subgroup A2-1 clustered isolates from Spain and showed nine estimated chromosomes; subgroup A2-2 included isolates from UK and Spain, and showed eight estimated chromosomes; and subgroup A2-3 grouped together isolates from Switzerland, USA, Australia, France and Portugal, together all of them showing eight estimated chromosomes (Fig. 3).

Seven isolates from Australia, The Netherlands, UK and Germany clustered with >75% similarity. All of them were isolated from *Lupinus* spp. and belonged to the molecular group A1. This cluster included isolates which showed between six and seven estimated chromosomes (Fig. 3). The eleven remaining isolates

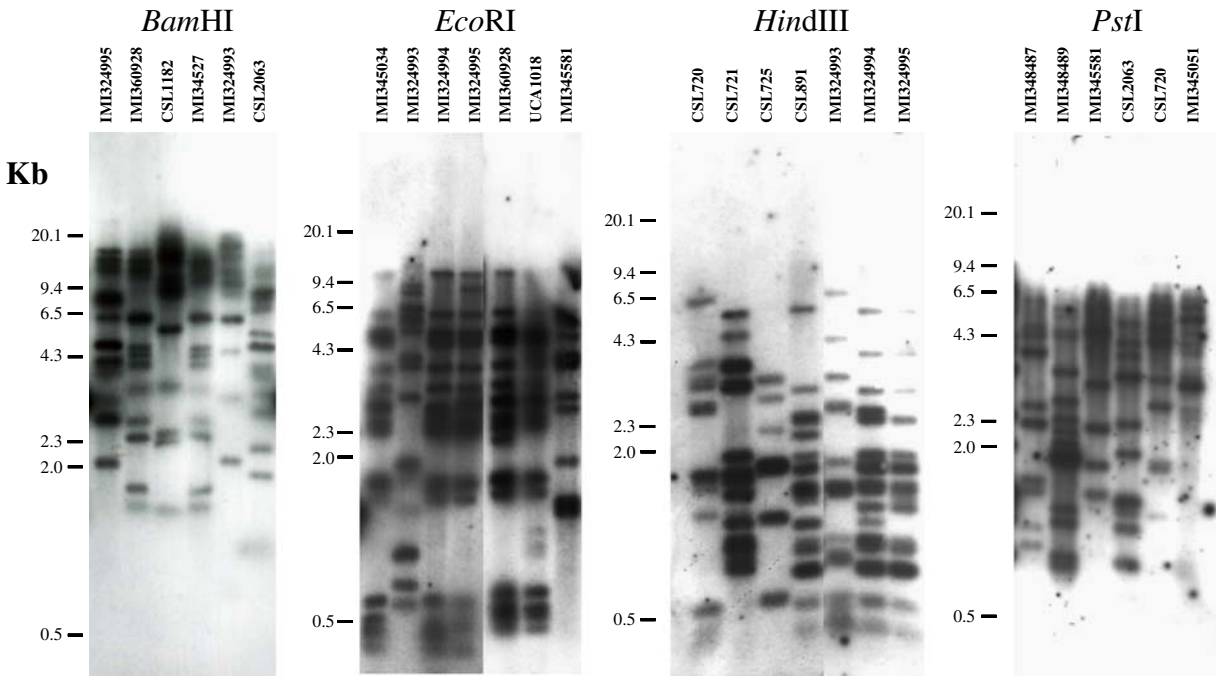

Fig. 2 Telomeric fingerprinting patterns obtained by telomeric hybridisation of Southern blots from *Bam*HI-, *Eco*RI-, *Hin*dIII-, and *Pst*I-DNA digestions.

clustered in different groups, and included isolates from USA, The Netherlands, Germany, Switzerland, New Zealand, France, UK and Spain.

PFGE analysis

The karyotype of *Colletotrichum* spp. was studied among isolates from: (a) close origins in the same country (Huelva and Cádiz in south-western Spain), and (b) different countries (France and Canada) (Table 2). CHEF running conditions were optimised to separate bands between approximately 0.1 and 9 Mb. Fingerprinting software II v3.0 (Bio-Rad) was used to estimate the size of the separated bands, using chormosomes of *S. pombe* (5.7, 4.6 and 3.5 Mb) and chromosomes of *H. wingei* (3.1, 2.7, 2.3, 1.8, 1.6, 1.3 and 1 Mb) as molecular markers. Between six and nine chromosomal bands were resolved in the CHEF gels. The largest band was approximately 8.7 Mb in size, (present in isolates from Spain and France), and the smallest band was approximately 0.1 Mb in size (only present in isolates from Spain) (Fig. 4). The minimum total genome size was estimated to range from 29.4 to 37.5 Mb (Table 2). To determine whether other smaller chromosomes were present, running conditions for small-sized chromosomes, optimised by O'Sullivan et al. (1998) for *C. lindemuthianum* were used, and no additional chromosomal bands were resolved in *C. acutatum*.

CHEF gels were additionally transferred to Hybond-N membranes and they hybridised with the above described telomeric probe to confirm that all the bands represented chromosomes. Hybridisation was detected for each band (between 6 and 9), so that all of them were considered as chromosomes (Fig. 4). The number of chromosomes among isolates was also identical to that obtained from telomeric fingerprinting (Tables 1 and 2).

Fingerprint analyses of karyotypes The karyotype profiles obtained for each isolate were analysed using Fingerprinting II software. A cluster analysis was performed based on Dice coeficients. The distance matrix generated was used to construct a dendogram by, UPGMA (Fig. 5). *Colletotrichum acutatum* isolates showed <40% similarity with those of *C. gloeosporioides*. *Colletotrichum acutatum* isolates

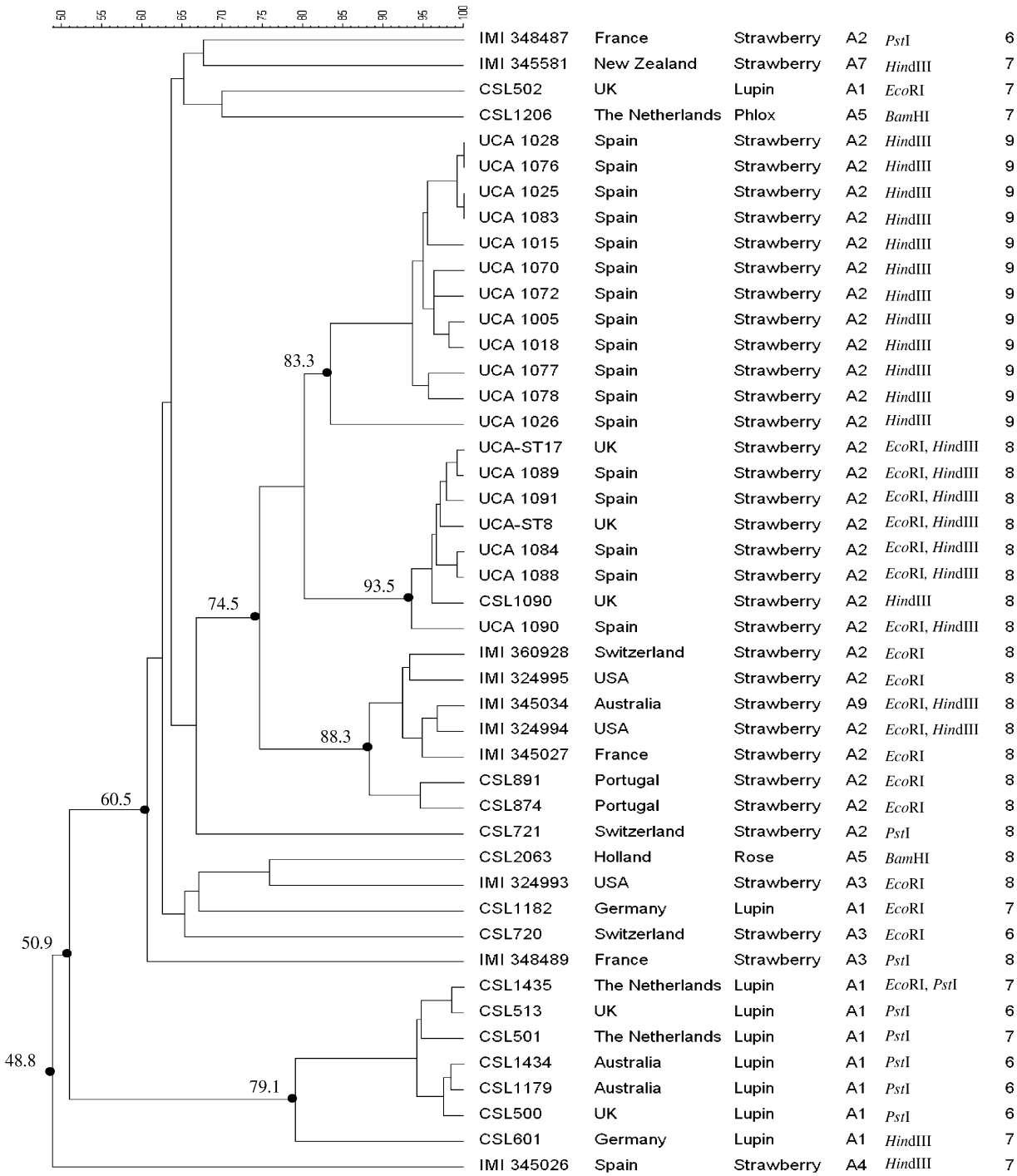

Fig. 3 Combined UPGMA dendograms, based on Dice coefficients generated using a composite data set from individual experiments of each enzyme digestion (*Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I) hybridised with a telomeric probe. The dendogram includes isolate, origin, host, molecular group of Sreenvisaprasad and Talhinhas (2005), restriction enzyme with the highest number of fragments obtained and the minimum number of estimated chromosomes for each isolate. Numbers on nodes are percentages of similarity among isolates.

Table 2 Genome organisation summary including results of 5.8S-ITS sequences, PFGE patterns and telomeric fingerprinting results

Isolate	Identified as	Geographic location	ITS-Seq	PFGE		Telomeric Fingerprinting		
			Molecular group	Number of chromo-somes	Genome size (Mb)	Telomere bands	Number of estimated chromosomes	Enzyme
UCA1070	*C. acutatum*	Cádiz, Spain	A2	9	35.38	18	9	*Hin*dIII
UCA1076	*C. acutatum*	Cádiz, Spain	A2	9	36.02	18	9	*Hin*dIII
UCA1077	*C. acutatum*	Cádiz, Spain	A2	9	35.95	18	9	*Hin*dIII
UCA1078	*C. acutatum*	Cádiz, Spain	A2	9	36.15	18	9	*Hin*dIII
UCA1005	*C. acutatum*	Huelva, Spain	A2	9	34.75	18	9	*Hin*dIII
UCA1015	*C. acutatum*	Huelva, Spain	A2	9	37.5	18	9	*Hin*dIII
UCA1025	*C. acutatum*	Huelva, Spain	A2	9	30.05	18	9	*Hin*dIII
UCA1028	*C. acutatum*	Huelva, Spain	A2	9	30.04	18	9	*Hin*dIII
IMI348487	*C. acutatum*	France	A2	6	32.33	12	6	*Pst*I
IMI348489	*C. acutatum*	France	A3	8	36.61	16	8	*Pst*I
IMI345051	*C. gloeosp*	Canada	B	7	29.43	14	7	*Pst*I

from France and three isolates from Spain (UCA1015, UCA1025 and UCA1028) grouped in a cluster with 56% similarity. The isolates UCA1015 and UCA1025 showed identical karyotypes (100% similarity). The remaining isolates from Spain showed >80% similarity in their karyotype profiles. The isolates UCA1076, UCA1077 and UCA1078 showed 100% similarity. The principal difference among the patterns of the isolates from Spain was the size of the largest chromosomal band. Isolates from France showed similar karyotypes, but the smallest chromosome was lacking in isolates IMI348489 and IMI348487, which displayed only six chromosomes. These results were confirmed when the membranes were hybridised with the telomeric probe, and agreed with the estimated chromosomes obtained by the telomeric fingerprinting analysis.

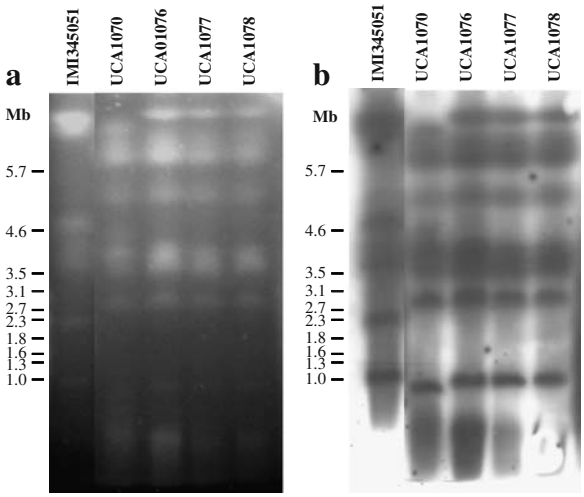

Fig. 4 **a** PFGE chromosomal separation of selected isolates using CHEF. **b** Southern-blot hybridisation using a telomeric DNA probe to hybridise the PFGE separated chromosomal bands. The molecular sizes on the left are those of *Schizosaccharomyces pombe* and *Hansenula wingei* chromosomes

Discussion

The principal purpose of the current study was to classify a worldwide collection of *C. acutatum* isolated from twelve countries (Australia, Canada, France, Germany, Japan, The Netherlands, New Zealand, Norway, Portugal, Switzerland, USA and UK), and to describe the genomic organisation of *C. acutatum*, regarding number of chromosomes and chromosome length polymorphism (CLP).

Although traditional characterisation of *C. acutatum* has been performed on the basis of the variability in both colony growth and morphological characteristics (Denoyes-Rothan and Baudry 1995; Smith and Black 1990), several authors have recently used molecular markers to group and classify populations of *C. acutatum* from different geographical origins and host plants. Sreenivasaprasad and Talhinhas (2005) established eight molecular groups (A1 – A8) for *C. acutatum*, based on sequences such as the internal transcribed spacers (ITS) of the 5.8S ribo-

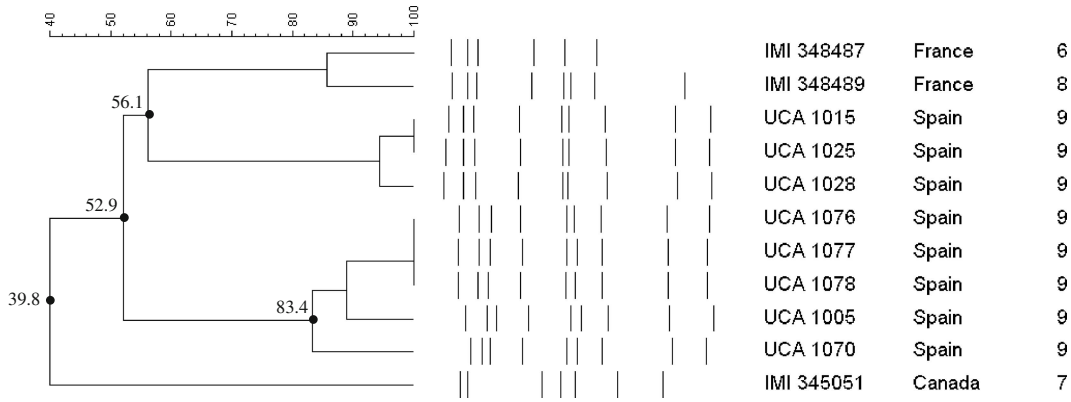

Fig. 5 UPGMA dendograms, based on Dice coefficients generated using chromosomal sizes obtained from PFGE patterns. The dendogram includes isolate, origin and the number of chromosomal bands. Numbers on nodes are percentages of similarity among isolates

somal DNA polymorphic region and the β-tubulin-2 gene. Subsequently, Whitelaw-Weckert et al. (2007), studying a *C. acutatum* population isolated from grapes in Australia, established a new group (A9), completing the molecular groups for this species to date.

In the present work we analysed the sequences of the internal transcribed spacers (ITS) of the 5.8S ribosomal DNA polymorphic region, and completed the molecular description and characterisation of *C. acutatum* isolates with two additional strategies: PFGE and telomeric fingerprinting. Regarding the genus *Colletotrichum,* only two studies have been reported using PFGE for analysing the genome organisation of both *C. gloeosporioides* and *C. lindemuthianum* (Masel et al. 1993; O'Sullivan et al. 1998). As far as we know, *C acutatum* has not been analysed by PFGE to date. The telomeric fingerprinting analysis of *C. acutatum* isolates was performed using Southern blot hybridisation of RFLPs with a telomeric DNA probe. Although similar studies have been performed with *Botrytis cinerea* (Viaud et al. 1996) and *C. lindemuthianum* (O'Sullivan et al. 1998), this is the first study of telomeric fingerprinting carried out with *C. acutatum*.

The phylogenetic study carried out with eighty-eight 5.8S-ITS sequences (fifty-two used in this work and thirty-five sequences of reference isolates from databases), allowed us to allocate the isolates into *C. acutatum* molecular groups. However, although Sreenivasaprasad and Talhinhas (2005) and Whitelaw-Weckert et al. (2007) described nine molecular groups, the analysis of bootstrap support in the neighbour-joining phylogenetic tree indicated that A1, A2, A5, A8 and A9 subgroups could be included in one large group (bootstrap support of 90%). Thus, the previously described nomenclature could represent different subgroups inside one large grouping. The same result was observed for subgroups A6 and A4, since these subgroups clustered together with a strong bootstrap support of 91%. However, A3 and A7 subgroups were clearly different from each other, and the analysis clustered them with a bootstrap support of 90% and 91%, respectively. We believe that our results support a classification into four molecular groups instead of the nine previously described groups for this species. The neighbuor-joining phylogenetic tree obtained in this research had a similar resolution to that obtained by previous authors and was also similar to other analyses of the β-tubulin-2 gene sequences (Sreenivasaprasad and Talhinhas 2005; Whitelaw-Weckert et al. 2007).

In the neighbuor-joining phylogenetic tree, twenty-nine sequences of *C. acutatum* clustered within molecular subgroup A2, being seventeen out of eighteen isolates collected in Spain clustered in this group. Also, when a UPGMA dendogram was generated using a composite data set from each restriction enzyme experiment followed by hybridisation with a telomeric probe, all the A2 isolates clustered in a large group with >70% similarity. These results support the hypothesis that isolates of the A2 subgroup form the key group in Spain, as suggested by Talhinhas et al. (2005). Ten *C. acutatum* isolates collected from *Lupinus* spp., clustered into subgroup A1 when the 5.8S-ITS sequences were studied; they

clustered in a unique group in the UPGMA dendogram, showing >79% similarity in the fingerprinting analysis, and being clearly different from the remaining isolates. Although Nirenberg et al. (2002) described *C. lupini* as the lupin anthracnose pathogen, other studies based on molecular data and cultural characteristics suggested that *C. acutatum* isolates did not show differences between both species, and therefore *C. acutatum* could be considered the causal agent of lupin anthracnose (Talhinhas et al. 2005). *Colletotrichum acutatum* isolates causing lupin anthracnose have been previously described as a very homogeneous group, suggesting that the molecular homogeneity of the majority of *C. acutatum* isolates from lupin may be related to some degree of host preference (Sreenivasaprasad and Talhinhas 2005; Talhinhas et al. 2005). Our results support this hypothesis.

Telomeric fingerprinting analysis also made it possible to determine the number of chromosomes for each isolate dividing into two the number of telomeric bands obtained after hybridisation. The minimum number of estimated chromosomes was from six to nine among isolates. Four isolates showed a very low number of telomeric bands. This may be related to the fact that certain restriction enzymes produce bands as smears of great intensity, and are impossible to resolve, as previously reported by Inglis et al. (2005), who observed the same results when analysing *Paecilomyces lilacinus*. To study the karyotypes of this species, we optimised a protocol to obtain *C. acutatum* protoplasts and the PFGE conditions to separate chromosomes between approximately 0.1 and 9 Mb after only 72 h of running. This protocol improved substantially those previously described for *Colletotrichum* spp. which took longer due to the two steps needed to resolve the complete karyotypes (68 + 168 h) (Masel et al. 1993; O'Sullivan et al. 1998). This study is the first in which a PFGE protocol is optimised to obtain the karyotype of *C. acutatum* and therefore the first report on the genomic organisation of *C. acutatum* regarding number of chromosomes and chromosome length polymorphism (CLP) among isolates.

The PFGE karyotypes were resolved for eight Spanish isolates of subgroup A2 and two French isolates belonging to subgroups A2 and A3, respectively (Table 2 and Fig. 4). The karyotypes showed between six and nine chromosomal bands with different sizes ranging from 0.1 to 8 Mb. The number of chromosomes obtained by PFGE agreed with the number of estimated chromosomes calculated after hybridisation with the telomeric probe and suggests that no additional chromosomes were unresolved by PFGE or telomeric fingerprint. The total genome size for the *C. acutatum* isolates ranged between 29 and 36 Mb, which is similar to that previously described for other pathogens such as *Beauveria bassiana* with a total genome size ranging from 34 to 44 Mb and six to eight chromosomes (Viaud et al. 1996), and *B. cinerea* with a total genome size from 13.09 to 22.8 Mb and between five and eight chromosomes (Vallejo et al. 1996; 2002). For *C. lindemuthianum* between nine and eleven chromosomes were resolved but no data about genome size were found (O'Sullivan et al. 1998).

Colletotrichum gloeosporioides isolates included in this study clustered in a separate group when analysing their 5.8S-ITS sequences. This resulted in a segregated group from those in which *C. acutatum* isolates were clustered, as previously found by other authors when analysing isolates belonging to both species (Whitelaw-Weckert et al. 2007). An approach to determine the karyotype of this pathogen was also performed with the protocol optimised in this work. PFGE resolved seven chromosomes with a total genome size of 29 Mb (Table 2). This result was confirmed by telomeric fingerprinting patterns, which resolved the same number of estimated chromosomes. Nevertheless, further studies should be performed to gain an insight into the genome organisation of this species.

The aims of this work were to establish phylogenetic relationships and to obtain a more extensive knowledge about the genomic organisation in *C. acutatum* isolates responsible for anthracnose on strawberry. As a result, a large volume of data on molecular phylogenetic relationships has been accumulated on *C. acutatum* and two new protocols for PFGE and Southern blot hybridisation with a telomeric probe, never before used for *C. acutatum* species have been optimised. Also, in the present study, we demonstrated that the genotypic diversity of the isolates was not directly related to their geographical origins. Further investigations at the molecular level are needed for a complete understanding of pathogen biology, which is essential to develop strategies for the improvement of an efficient disease management strategy.

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