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Phylogenetic analysis of Alternaria spp. associated with apple core rot and citrus black rot in South Africa

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Dry core rot of apple DCRand Alternaria black rot of citrus ARhae in the past respectiely been ascribed to Alternaria alternata and A. citri. In recent years, howeer, it has been speculated that seeral other species of Alternaria could also be associated with these diseases. In an attept to elucidate the identity of these taa, 25 isolates associated with DCR, and 26 isolates associated with AR were selected for olecular characterisation. Nucleotide seuences of 1116 sites including the histone gene section and the internal transcribed spacers ITS 1 and 2of the rRNA gene were deterined for these isolates. The gene trees generated frothe indiidual and cobined data sets using aiuparsiony, aiu likelihood and neighbour-oining analysis ethods distinguished e clades with strong bootstrap support, naely Alternaria sp., A. arborescens, A. infectoria, A. tenuissia , and a clade containing isolates ariable in orphology, referred to as the Alternaria group. In the alignent of the cobined ITS and histone data set, uniue transitiontransersion substitutions, as well as positional insertions and deletions were obsered for each of the aboe clades. In addition, key seuences in the forof serial coposing nucleotides in both the ITS and histone sections of the alignent were also discoered for the olecular identication of A. arborescens, A. infectoria and A. tenuissia . The nal phylogeny also indicated that no host specicity eisted aong the species associated with these two post-harest disease coplees. Contrary to the host specicity obsered on leaf diseases of these hosts in the eld, it appears that the post-harest diseases are the result of adverse storage conditions and opportunism of different small-spored **Alternaria spp.**

INTRODUCTION

Core rot of apple and black rot of citrus are two well-known post-harest diseases attributed to sall-spored species of Alternaria Serdani et al. 1998, Sions 1999a. Dry core rot DCRof apple, which ainly infects the Red Delicious arieties, has in the past been linked to a single species, A. alternata Carpenter 1942, Miller 1959, Cobrink et al. 1985b, while seeral Alternaria spp. hae been associated with ouldy core of apples Heald 1921, Ellis arrat 1983. DCR is a worldwide proble, occurring in ost countries where apples are grown Serdani et al. 1998, including South Africa Cobrink et al. 1985a. Annual losses of between 6 and 8are generally attributed to this disease Cobrink, isagie Grobbelaar 1984. A recent study on DCR that characterised isolates based on etabolite production, cultural characteristics, sporulation patterns, pathogenicity and DNA seuence analysis of the internal transcribed spacers ITS1 and 2 of the nuclear-encoded rRNA gene rDNA, concluded that representaties of seeral species-groups, including A. arborescens, A. infectoria and A. tenuissia were inoled Serdani et al. 2002. Results froinoculation studies suggested, howeer, that the A. tenuissia species-group was the aor pathogen associated with DCR of Top Red apples in South Africa Serdani et al. 2002. It has been reported in basidioycetes, howeer, that DNA seuence data frothe ITS region are insufficient to distinguish all species, as some taxa could **eole ore uickly than utations could accuulate in their ITS regions Gardes et al. 1991, Anderson Stasoski 1992. Furtherore, the sae has also been found in soe ascoycete genera, where seuence data of the b-tubulin and histone regions were reuired to distinguish species in the Cylindrocladiuspathiphylli and C. oridanu species coplees that otherwise appeared siilar based on their ITS data sets Kang, Crous Schoch 2001.**

Ste-end rot SERor Alternaria black rot AR affects virtually all citrus varieties during prolonged **periods of cold storage rown McKornack 1972 and is also a serious problein South Africa. Syptos include ste-end browning as well as rot of the**

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central axis (Shiffmann-Nadel et al. 1981. arious researchers hae reported species of Alternaria to be associated with SER Joly 1967, Pelser La Grange 1981, Prusky, Fuchs auberan 1981as well as with nael-end rot Schutte, eeton Pelser 1994, of which A. citri has been identied as the ain culprit (Brown & McKornack 1972, Shiffmann-Nadel et al. 1981. Although pathotypes of A. alternata hae been linked to arious diseases Kusaba Tsuge 1995, A. citri was originally described frointernal dry rot syptos of oranges Sions 1999a. Additional Alternaria diseases associated with citrus are A. liicola on Meican lie Citrus aurantiifoliaand other species of citrus, Alternaria brown spot on tangerine C. reticulataand related hybrids, and Alternaria leaf spot of rough leon C. abhiri Sions 1999a. Sions 1990, 1999afound that the Alternaria spp. associated with citrus brown spot were neither A. citri not A. alternata, but represented 10 new species of Alternaria, which were associated with these syptos throughout different parts of the world. ABR, which is the citrus disease focused on in the present study, has traditionally been linked to A. citri. No e-type cultures are aailable of A. citri, and its identity reains rather obscure. Howeer, Sions 1999astated that, based on the ariation that he has encountered froAR isolates, species morphologically different from the type specien of A. citri were also responsible for the disease. ased on seuence data of the ITS region, as well as RFLPs of tDNA, Kusaba Tsuge 1994, 1995, 1997) reported that there was little differentiation between host-specic toin-producing citrus isolates, and isolates frohosts such as apple, strawberry and pear. The obecties of the present study were rstly to copare the Alternaria spp. causing DCR and AR using aiuparsiony and likelihood analyses of ulti-locus DNA seuence data of the histone and ITS regions, secondly to establish if any of the taa were uniue to either, or shared by both hosts, and lastly to deterine if the histone data set could distinguish ore species of Alternaria than delineated based on the ITS seuence data.

MATERIALS AND METHODS

Fungal cultures and growth conditions

Twenty-si Alternaria isolates frocitrus species with AR syptos 4 Nael oranges, 14 Minneola tangelo and 8 Cleentineswere plated onto 9 cdiaPetri dishes containing potato carrot agar PCA 20 g iolab agar, 20 g carrot, 20 g potato, 1000 l H $_2$O. Twenty-four Alternaria isolates obtained froa Top Red apple orchard with DCR, as well as one reference isolate of A. infectoria EGS 27-193 CR31were also included and plated onto PCA Table 1. Plates were placed at 23 C under an alternating 816 h cool-white uorescent lightdark cycle Sions Roberts 1993to induce sporulation. A single spore froeach isolate was transferred to Petri dishes containing PCA, and nally aseptically transferred to and stored on potato detrose agar PDA 12 g iolab agar, 200 g potatoes, 20 g sucrose, 1000 l H $_2$Oslants at rooteperature as well as in 20 l bottles containing sterile water for the purpose of long-terstorage. All fungal cultures are held at the culture collection at the Departent of Plant Pathology, Uniersity of Stellenbosch STE-U.

Aplication and seuencing

Genoic DNA was isolated frofungal ycelia collected frothe plates using the isolation protocol of Lee Taylor 1990. Teplate DNA 20 ngwas aplied in a 25 l PCR reaction iture consisting of 10 M KCl, 10 M $NH_4{}_2SO_4$, 20 M TrisHCl pH 8.8, 6 M $MgCl_2$, and 500 M each of dATP, dCTP, dGTP, and dTTP, with 60 pols ITS1 and ITS4 priers White et al. 1990or H3-1a and H3-1b Glass Donaldson 1995priers, and 2.5 units iota ioline, LondonDNA polyerase. The reaction was set up as followsinitial denaturation at 96 for 2 in, followed by 30 cycles of denaturation at 96 for 15 s, annealing at 55 for 30 s, etension at 75 for 35 s and nal etension at 75 for 2 in in a Rapidcycler Idaho Technology, Idaho. A negatie control using water instead of teplate DNA was set up for each eperient. PCR products were analysed by electrophoresis at 75 for 2 h in a 0.8wagarose gel in 0.5 r TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85and isualised under U light in a transilluinator TF-35C, ilber Louratfollowing ethidiu broide staining.

PCR products were puried by using a IAuick PCR Purication Kit iagen GbH, Gerany. The puried PCR products were seuenced using the AI Pris377 DNA Seuencer Perkin-Eler, Norwalk, CN. The cycle seuencing reaction with 2040 ng of DNA teplate and 3.2 pol prier in a total olue of 10 l was carried out with a Dye Terinator Cycle Seuencing Ready Reaction Kit containing ApliTa DNA Polyerase Perkin-Eler. The reaction was set up as 25 cycles of 96 for 30 s, 50 for 15 s, and 60 for 4 in. The resulting fragents were nally puried using Centri-Sep Spin coluns Princeton Separations, Adelphia, NJand loaded onto the seuencing gel.

Phylogenetic analysis

Nucleotide seuences of the histone and ITS rDNA of this study Genank accession nos AF404591 AF404641 and AF404642AF404667and those of the outgroup, Fusariunygaai Genank nos 94174 and U34426were assebled using Te-Edit Plus ender, Toaol.co. The alignent of the seuence les were conducted using the CLUSTAL W software Thopson, Higgins Gibson 1994. Adustents for iproeent were ade by eye where

Table 1. Isolates of Alternaria spp. sapled froapple core rot and citrus black rot syptos in South Africa.

Accession no.	Other no.	Morpho-species[a]	Phylospecies	Genank accession no. ITS, Histone	ariety or cultiar [b]	Location[c]
CR 1	STE-U 4242	A. arborescens	Alternaria group, clade 1	AF397218, AF404591	A, Top Red	WC, Grabouw
CR 2	STE-U 4243	A. arborescens	A. arborescens, clade 3	AF397219, AF404592	A, Top Red	WC, Grabouw
CR 3	STE-U 4244	A. arborescens	A. arborescens, clade 3	AF397220, AF404593	A, Top Red	WC, Grabouw
CR 5	STE-U 4246	A. arborescens	A. arborescens, clade 3	AF397222, AF404594	A, Top Red	WC, Grabouw
CR 6	STE-U 4247	A. arborescens	Alternaria sp., clade 2	AF397223, AF404595	A, Top Red	WC, Grabouw
CR 7	STE-U 4248	A. tenuissia	Alternaria group, clade 1	AF397224, AF404596	A, Top Red	WC, Grabouw
CR 8	STE-U 4249	A. arborescens	Alternaria group, clade 1	AF397225, AF404597	A, Top Red	WC, Grabouw
CR 12	STE-U 4253	A. infectoria	A. infectoria, clade 5	AF397229, AF404598	A, Top Red	WC, Grabouw
CR 14	STE-U 4255	A. arborescens	Alternaria sp., clade 2	AF397231, AF404599	A, Top Red	WC, Grabouw
CR 15	STE-U 4256	A. infectoria	A. infectoria clade 5	AF397232, AF404600	A, Top Red	WC, Grabouw
CR 16	STE-U 4257	A. tenuissia	Alternaria group, clade 1	AF397233, AF404601	A, Top Red	WC, Grabouw
CR 17	STE-U 4258	A. tenuissia	Alternaria group, clade 1	AF397234, AF404602	A, Top Red	WC, Grabouw
CR 18	STE-U 4259	A. arborescens	Alternaria group, clade 1	AF397235, AF404603	A, Top Red	WC, Grabouw
CR 19	STE-U 4260	A. tenuissia	Alternaria group, clade 1	AF397236, AF404604	A, Top Red	WC, Grabouw
CR 20	STE-U 4261	A. tenuissia	Alternaria group, clade 1	AF397237, AF404605	A, Top Red	WC, Grabouw
CR 21	STE-U 4262	A. tenuissia	Alternaria group, clade 1	AF397238, AF404606	A, Top Red	WC, Grabouw
CR 22	STE-U 4263	A. tenuissia	Alternaria group, clade 1	AF397239, AF404607	A, Top Red	WC, Grabouw
CR 23	STE-U 4264	A. arborescens	Alternaria sp., clade 2	AF397240, AF404608	A, Top Red	WC, Grabouw
CR 24	STE-U 4265	A. tenuissia	Alternaria group, clade 1	AF397241, AF404609	A, Top Red	WC, Grabouw
CR 25	STE-U 4266	A. tenuissia	Alternaria group, clade 1	AF397242, AF404610	A, Top Red	WC, Grabouw
CR 26	STE-U 4267	A. tenuissia	Alternaria group, clade 1	AF397243, AF404611	A, Top Red	WC, Grabouw
CR 27	STE-U 4268	A. tenuissia	A. tenuissia , clade 4	AF397244, AF404612	A, Top Red	WC, Grabouw
CR 28	STE-U 4269	A. infectoria	A. infectoria, clade 5	AF397245, AF404613	A, Top Red	WC, Grabouw
CR 30	STE-U 4271	A. infectoria	A. infectoria, clade 5	AF397247, AF404614	A, Top Red	WC, Grabouw
CR 31	STE-U 4272 EGS 27-193[d]	A. infectoria	A. infectoria, clade 5	AF397248, AF404615	Triticu sp.	U.K.
MA 1	STE-U 4324	A. tenuissia	A. tenuissia , clade 4	AF404642, AF404616	C, Minneola tangelo	MP, Ngonini
MA 2	STE-U 4325	A. tenuissia	Alternaria group, clade 1	AF404643, AF404617	C, Minneola tangelo	MP, Ngonini
MA 3	STE-U 4326	A. pellucida	Alternaria group, clade 1	AF404644, AF404618	C, Minneola tangelo	MP, Ngonini
MA 4	STE-U 4327	A. tenuissia	Alternaria group, clade 1	AF404645, AF404619	C, Minneola tangelo	MP, Ngonini
MA 5	STE-U 4328	A. citri	Alternaria group, clade 1	AF404646, AF404620	C, Minneola tangelo	MP, Ngonini
MA 6	STE-U 4329	A. tenuissia	A. tenuissia , clade 4	AF404647, AF404621	C, Minneola tangelo	MP, Ngonini
MA 7	STE-U 4330	A. alternata	Alternaria group, clade 1	AF404648, AF404622	C, Minneola tangelo	MP, Ngonini
MA 8	STE-U 4331	A. pellucida	Alternaria group, clade 1	AF404649, AF404623	C, Minneola tangelo	MP, Ngonini

Table 1 cont.

Accession no.	Other no.	Morpho-species[a]	Phylospecies	Genank accession no. ITS, Histone	ariety or cultiar [b]	Location[c]
MA 9	STE-U 4332	A. pellucida	Alternaria group, clade 1	AF404650, AF404624	C, Minneola tangelo	MP, Ngonini
MA 10	STE-U 4333	A. alternata	Alternaria group, clade 1	AF404651, AF404625	C, Minneola tangelo	MP, Ngonini
MA 11	STE-U 4334	A. alternata	Alternaria group, clade 1	AF404652, AF404626	C, Minneola tangelo	WC, Citrusdal
MA 12	STE-U 4335	A. pellucida	Alternaria group, clade 1	AF404653, AF404627	C, Minneola tangelo	NP, Letaba
MA 13	STE-U 4336	A. pellucida	Alternaria group, clade 1	AF404654, AF404628	C, Minneola tangelo	NP, Letaba
MA 14	STE-U 4337	A. alternata	Alternaria group, clade 1	AF404655, AF404629	C, Nael orange	WC, Citrusdal
MA 15	STE-U 4338	A. alternata	Alternaria group, clade 1	AF404656, AF404630	C, Nael orange	WC, Citrusdal
MA 16	STE-U 4339	A. pellucida	Alternaria group, clade 1	AF404657, AF404631	C, Nael orange	WC, Citrusdal
MA 17	STE-U 4340	A. pellucida	Alternaria group, clade 1	AF404658, AF404632	C, Nael orange	WC, Citrusdal
MA 18	STE-U 4341	A. alternata	Alternaria group, clade 1	AF404659, AF404633	C, Cleentine	WC, Citrusdal
MA 19	STE-U 4342	A. pellucida	Alternaria group, clade 1	AF404660, AF404634	C, Cleentine	WC, Citrusdal
MA 20	STE-U 4343	A. pellucida	Alternaria group, clade 1	AF404661, AF404635	C, Cleentine	WC, Citrusdal
MA 21	STE-U 4344	A. pellucida	Alternaria group, clade 1	AF404662, AF404636	C, Cleentine	WC, Citrusdal
MA 22	STE-U 4345	A. arborescens	A. arborescens, clade 3	AF404663, AF404637	C, Cleentine	WC, Citrusdal
MA 23	STE-U 4346	A. alternata	Alternaria group, clade 1	AF404664, AF404638	C, Cleentine	WC, Citrusdal
MA 24	STE-U 4347	A. pellucida	Alternaria group, clade 1	AF404665, AF404639	C, Cleentine	WC, Citrusdal
MA 25	STE-U 4348	A. pellucida	Alternaria group, clade 1	AF404666, AF404640	C, Cleentine	WC, Citrusdal
MA 26	STE-U 4349	sterile	Alternaria group, clade 1	AF404667, AF404641	C, Minneola tangelo	MP, Ngonini

[a] ased on cultural characteristics Serdani et al. 2001, and sporulation groups Sions Roberts 1993.
[b] A, AppleC, citrus.
[c] ProinceMP, MpualangaNP, Northern ProinceWC, Western Cape.
[d] Reference isolate of A. infectoria.

necessary. Alignent gaps were coded as fth base in the analysis. Phylogenetic analyses were perfored with PAUP version 4.0b6 (Swofford 2001). A partition hoogeneity test in PAUP was conducted for the ITS and histone seuence alignent to eaine the possibility of a oint analysis of the two data sets. The ost parsionious trees MPTwere deterined frothe indiidual and cobined data sets using the heuristic search options with 1000 randoseuence input orders with MULPARS on and TR branch swapping for the eact solution. The unconstrained topologies of the eually parsionious trees were copared using the KishinoHasegawa test of PAUP. The best topology was selected as the ost parsionious tree topology. Parsiony bootstrap with 1000 replicates in PAUP was applied to the tree to ealuate the stability. Other easures including tree length, consistency, retention, rescaled consistency and hooplasy indees TL, CI, RI, RC and HIwere also calculated. The aiu likelihood analyses with 100 seuence randoisations were also perfored for the data sets to test the parsionious tree topology. Substitution odel was selected for the likelihood setting with transitiontransersion ratio estiation. The distinct key seuence insertions eist in arious regions in both ITS and histone data sets. In order to avoid the effect of these insertions to their local regions in the alignent, analyses ecluding the insertions were conducted. As nucleotide coposition of these key seuence insertions distinguished the species inoled, aiulikelihood analysis with 1000 seuence randoisations and substitution odel of transitiontransersion ratio estiation on the subdataset of only the key seuence insertion regions and the releant taa were also perfored. Maiu likelihood bootstrap with 1000 replicates in PAUP was applied to the tree to ealuate the stability. Maiu parsiony analysis with ehaustie and branch and bound search options and neighbour-oining analysis with uncorrected poption were also perfored for the key seuence data set. The decay indices were also

calculated using AutoDecay Eriksson 1998to further test the robustness of the branches of all the trees generated.

RESULTS

Phylogenetic analysis

The alignent Treease accession no. SN 1195of the oint data sets of histone and ITS spans 1116 sites in total ITS 1585, histone 5861116including 472 constant, 428 ariable but parsiony-uninforatie and 216 parsiony-inforatie characters. Other than for inor changes, the aor transitiontransersion substitutions are depicted in Fig. 1 for Alternaria arborescens, A. infectoria and A. tenuissia .

The aiuparsiony analysis of the indiidual ITS and histone data sets with 1000 randoseuence input orders in heuristic search produced 2 and 288 eually ost parsionious trees MPTrespectiely. The aority-rule consensus trees were generated with 1000 randoseuence input orders and 1000 bootstrap replications in heuristic search for both data sets. The ITS gene tree distinguished two clades Fig. 2. Isolates of A. infectoria clustered together with strong bootstrap support 100, while a large clade assebled all the other isolates with strong bootstrap support 100. In the large clade, two isolates of A. tenuissia frocitrus, MA1 and MA6 fored a subclade with high bootstrap support 87. The topology of the histone gene tree data not shownis identical to the oint gene tree Fig. 3of the ITS and histone data sets, and also has siilar bootstrap alues.

The result of the partition hoogeneity test P 0.20, where Pf 0.05 was signicantly incongruentshowing the haronious phylogenetic eolution of ITS and histone genes in Alternaria spp. and suggesting a oint analysis of the two data sets. The aiuparsiony analysis of the alignent using the heuristic search option in PAUP with 1000 randoseuence input orders generated 252 MPTs and a shortest tree of 805 steps. The best tree topology of the 252 MPTs was selected as the oint gene tree Fig. 3through the KishinoHasegawa likelihood test in PAUP data not shownand ealuated with 1000 parsiony bootstrap replications in a heuristic search for clade stability. The neighbour-oining analysis of the alignent with uncorrected p and 1000 bootstrap replications in PAUP produced a aority-rule consensus tree which was topologically identical to the oint gene tree Fig. 3and receied stronger neighbour-oining bootstrap supports data not shownto the clades of the Alternaria group clade 1, Alternaria sp. clade 2, A. arborescens clade 3, A. tenuissia clade 4and A. infectoria clade 5. Maiu-likelihood analysis in heuristic search with 100 randoseuence input orders and was also perfored in PAUP for the data set, which produced a tree topology data not shownidentical to that of the oint gene tree Fig. 3. The oint gene tree Fig. 3based on the alignent of histone and ITS data sets fored two aor clades coprising 5 saller clades. The aor clade coprising clades 1 and 2 receied strong bootstrap and decay indices support 98d8. The other aor clade coprising clades 35 also receied strong bootstrap and decay indices support 100d66. Clade 1 consisted of a large nuber of isolates orphologically identied as A. alternata, A. tenuissia , A. citri, A. arborescens and A. pellucida froapple and citrus with siilar branch lengths and receied strong parsiony bootstrap and decay indices supports 86d2. The separation of clade 2 CR6, CR14 and CR23froclade 1 was strongly supported by bootstrap and decay indices 95d3. Clades 35 represented A. arborescens CR2, CR3, CR5 and MA22, A. tenuissia CR27, MA1 and MA6and A. infectoria CR12, CR15, CR28, CR30 and CR31. Each of these three clades were strongly supported by bootstrap and decay indices, naely 99 d4, 99d3 and 100d7, respectiely. To aoid their possible effect on local regions in the alignment, maximum parsiony, aiu-likelihood and neighbour-oining analyses with the sae setting were conducted on the saller data set ecluding the key seuence insertion regions. The tree topologies data not shown generated were concordant with the oint gene tree Fig. 3. The parsiony bootstrap alues 95, 93, 97, 98, 100for the e respectie clades were siilar to those in the oint gene tree Fig. 3.

Maiuparsiony analysis with ehaustie search option and idpoint rooting on the saller data set of the key seuences of the 12 releant taa resulted in 4 MPTs. The aiuparsiony gene tree of the key seuences Fig. 4was selected using the Kishino Hasegawa test in PAUP and ealuated with 1000 bootstrap replications in branch and bound search. The clades of A. arborescens, A. tenuissia and A. infectoria in the tree Fig. 4receied strong parsiony bootstrap and decay indice supports of 99d4, 100d9 and 100 d38, respectiely. In the A. infectoria clade isolates CR30 and CR31 were strongly supported by bootstrap and decay indices 99d3, indicating a ore intiate genealogical tie between the. The subclade of isolates CR15 and CR28 receied a bootstrap support of 81 but failed to receie decay indices. The aiulikelihood analysis with 1000 seuence randoisations and substitution odel of transitiontransersion ratio estiation and the neighbour-oining analysis with uncorrected poption and 1000 bootstrap replicates were also perfored for the saller data set of the key seuences and generated tree topologies data not shown alost identical to the aiuparsiony gene tree of the key seuences Fig. 4.

DISCUSSION

The histone and ITS nucleotide seuence gene trees obtained in the present study separated the 51 Alternaria isolates inestigated into e clades, naely a large clade containing orphologically ariable isolates,

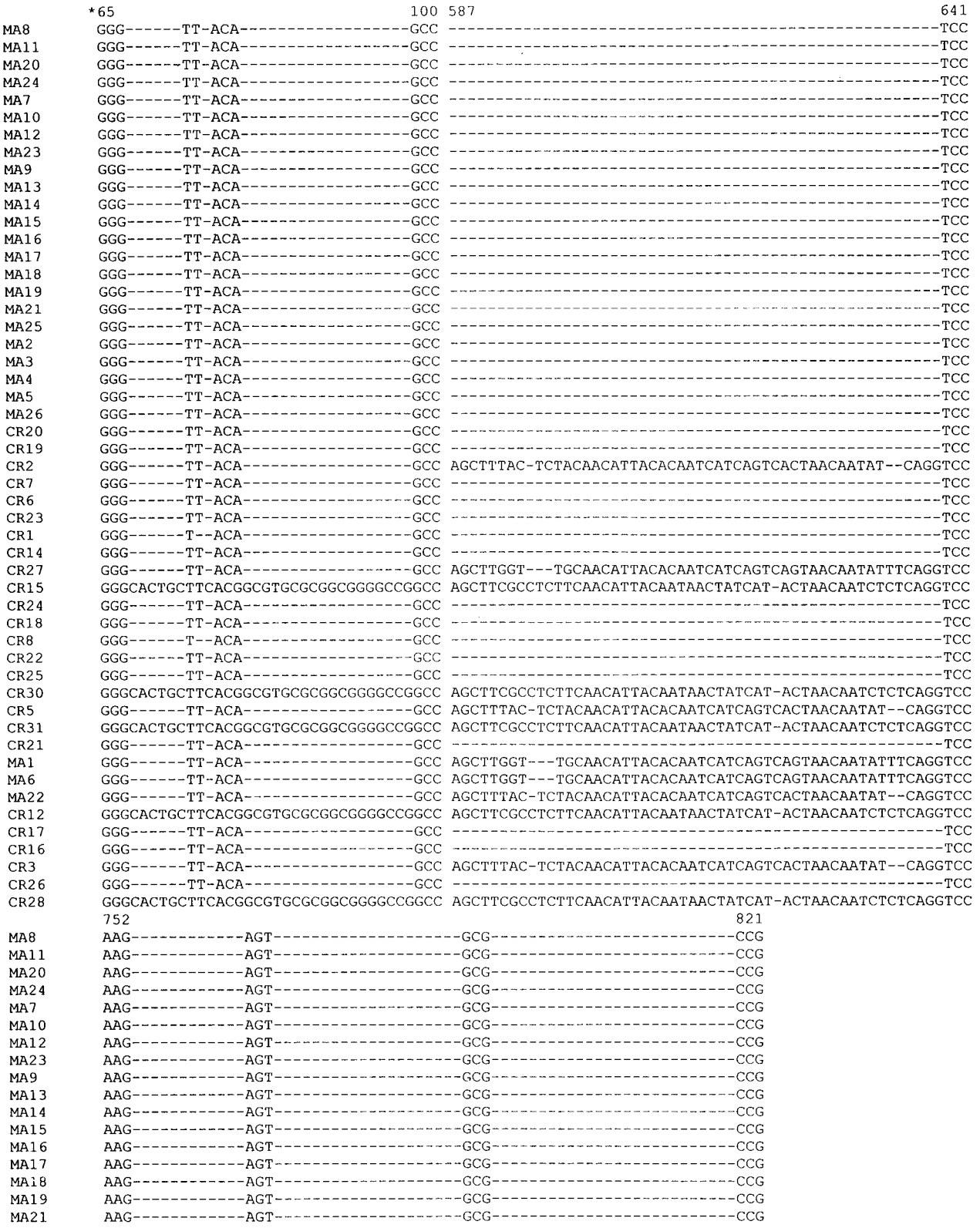

Fig. 1. For legend see opposite page.

the Alternaria group clade 1, Alternaria sp. clade 2, A. arborescens clade 3, A. tenuissia clade 4and A. infectoria clade 5.

Alternaria black rot of citrus has traditionally been ascribed to A. citri rown Eckert 1988. Contrary to this belief, howeer, our ndings show that Sions 1999awas correct in stating that seeral sall-spored species of Alternaria are inoled in causing this disease. Although the ITS data set only distinguished a large Alternaria clade and A. infectoria Fig. 2, the cobined ITS and histone data set separated ore taa frothe large Alternaria group clade 1, naely Alternaria sp.

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MA25    AAG-----------AGT--------------------GCG-------------------------CCG
MA2     AAG-----------AGT--------------------GCG-------------------------CCG
MA3     AAG-----------AGT--------------------GCG-------------------------CCG
MA4     AAG-----------AGT--------------------GCG-------------------------CCG
MA5     AAG-----------AGT--------------------GCG-------------------------CCG
MA26    AAG-----------AGT--------------------GCG-------------------------CCG
CR20    AAG-----------AGT--------------------GCG-------------------------CCG
CR19    AAG-----------AGT--------------------GCG-------------------------CCG
CR2     AAGTCCGCACCGGTTAGT-ACCATCTTCATTTCATCTTGCGATGCATC-TTTCTAAC----ATGCTCCAG
CR7     AAG-----------AGT--------------------GCG-------------------------CCG
CR6     AAG-----------AGT--------------------GCG-------------------------CCG
CR23    AAG-----------AGT--------------------GCG-------------------------CCG
CR1     AAG-----------AGT--------------------GCG-------------------------CCG
CR14    AAG-----------AGT--------------------GCG-------------------------CCG
CR27    AAGTCCGCACCGGTTAGT-ACCATCTCCACACTATCTTGCGATCCATCC-TTCTAAC----ATGCTCCAG
CR15    AAGTCCGCACCGGTTAGTTACTATCCTCACCTCATCATGCGATGCATC---TCTAACGTTAATCTTCCAG
CR24    AAG-----------AGT--------------------GCG-------------------------CCG
CR18    AAG-----------AGT--------------------GCG-------------------------CCG
CR8     AAG-----------AGT--------------------GCG-------------------------CCG
CR22    AAG-----------AGT--------------------GCG-------------------------CCG
CR25    AAG-----------AGT--------------------GCG-------------------------CCG
CR30    AAGTCCGCACCGGTTGGT-ACTATC---ACTTCATCATGCGATGCACGC--TCTAAC----ATATTCCAG
CR5     AAGTCCGCACCGGTTAGT-ACCATCTTCATTTCATCTTGCGATGCATC-TTTCTAAC----ATGCTCCAG
CR31    AAGTCCGCACCGGTTGGT-ACTATC---ACTTCATCATGCGATGCACGC--TCTAAC----ATATTCCAG
CR21    AAG-----------AGT--------------------GCG-------------------------CCG
MA1     AAGTCCGCACCGGTTAGT-ACCATCTCCACACTATCTTGCGATCCATCC-TTCTAAC----ATGCTCCAG
MA6     AAGTCCGCACCGGTTAGT-ACCATCTCCACACTATCTTGCGATCCATCC-TTCTAAC----ATGCTCCAG
MA22    AAGTCCGCACCGGTTAGT-ACCATCTTCATTTCATCTTGCGATGCATC-TTTCTAAC----ATGCTCCAG
CR12    AAGTCCGCACCGGTAAG--ACTATCCTCACCTGATCATGCGATGCATG---TCT--CTAACATCCTTCAG
CR17    AAG-----------AGT--------------------GCG-------------------------CCG
CR16    AAG-----------AGT--------------------GCG-------------------------CCG
CR3     AAGTCCGCACCGGTTAGT-ACCATCTTCATTTCATCTTGCGATGCATC-TTTCTAAC----ATGCTCCAG
CR26    AAG-----------AGT--------------------GCG-------------------------CCG
CR28    AAGTCCGCACCGGTTAGTTACTATCCTCACCTCATCATGCGATGCATC---TCTAACGTTAATCTTCCAG
* positions in the alignment Mxxx
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Fig. 1. Key seuences in histone and ITS rRNA genes which can be used to design species specic probes and priers to distinguish Alternaria arborescens, A. tenuissia and A. infectoria.

clade 2, A. arborescens clade 3, A. tenuissia clade 4 and A. infectoria clade 5. The sae phenoenon was also recently obsered in a study on DCR of apple, where two separate data sets ITS seuence data, as well as secondary etabolite, pathogenicity and sporulation group datadistinguished the A. arborescens, A. infectoria and A. tenuissia species-groups Serdani et al. 2002. In contrast to the low resolution power of the ITS region for differentiating Alternaria spp., the histone data set was able to distinguish ore taa Figs 23. Roberts, Reyond Andersen 2000also concluded that the ITS region of the rDNA gene was too consered to be used as sole easure of the ariation present aong the sall-spored species of Alternaria. They furtherore concluded that although any of the sall-spored species of Alternaria were closely related, it was unwise to accept the liited ariation depicted in the ITS region as intraspecic ariation within A. alternata.

The Alternaria species inoled in the DCR cople of apple and the AR copleof citrus hae not yet been fully characteried, and presently ost papers still refer to A. alternata as the cause of DCR, and A. citri as the cause of AR Cobrink et al. 1985a, b, rown Eckert 1988. Serdani et al. 2002reported the ost coon species-group isolated froDCR syptos to be A. arborescens, while the A. tenuissia species-group proed to be the ore iportant pathogen. Howeer, gien that a reference isolate of A. alternata EGS 34-016clustered within the A. arborescens clade Serdani et al. 2002, we hae opted to siply refer to this large clade of isolates as the Alternaria group in the present study, as isolates that we regard orphologically siilar to A. arborescens Sions 1999b grouped in cluster 3 based on the histone seuence data Fig. 3. Furtherore, although the present olecular data could not distinguish additional taa in the larger Alternaria group clade 1, we still obserued morphological and cultural differences among seeral of these isolates, but were unable to relate this to presently recognied species. Whether the isolates in clade 1 really represent additional species, or siply the range of ariation within one species, will only be reealed once ore genes are studied. ased on secondary etabolite, cultural and sporulation group data Serdani et al. 2002, it sees that this clade still contains several different species. It is clear, therefore, that although the histone gene represents ore inforatie data than the ITS region, we still cannot eplain all the orphological and cultural ariation obsered aong the isolates in clade 1 Fig. 3.

With related species that are constantly eoling, it is usually accepted that the ones containing the least changes eoled frothe ore ariable ones Aise et al. 1987, Aise 1989, Harrison 1991. The large Alternaria group clade 1, which includes orphologically assorted isolates corresponding with the descriptions

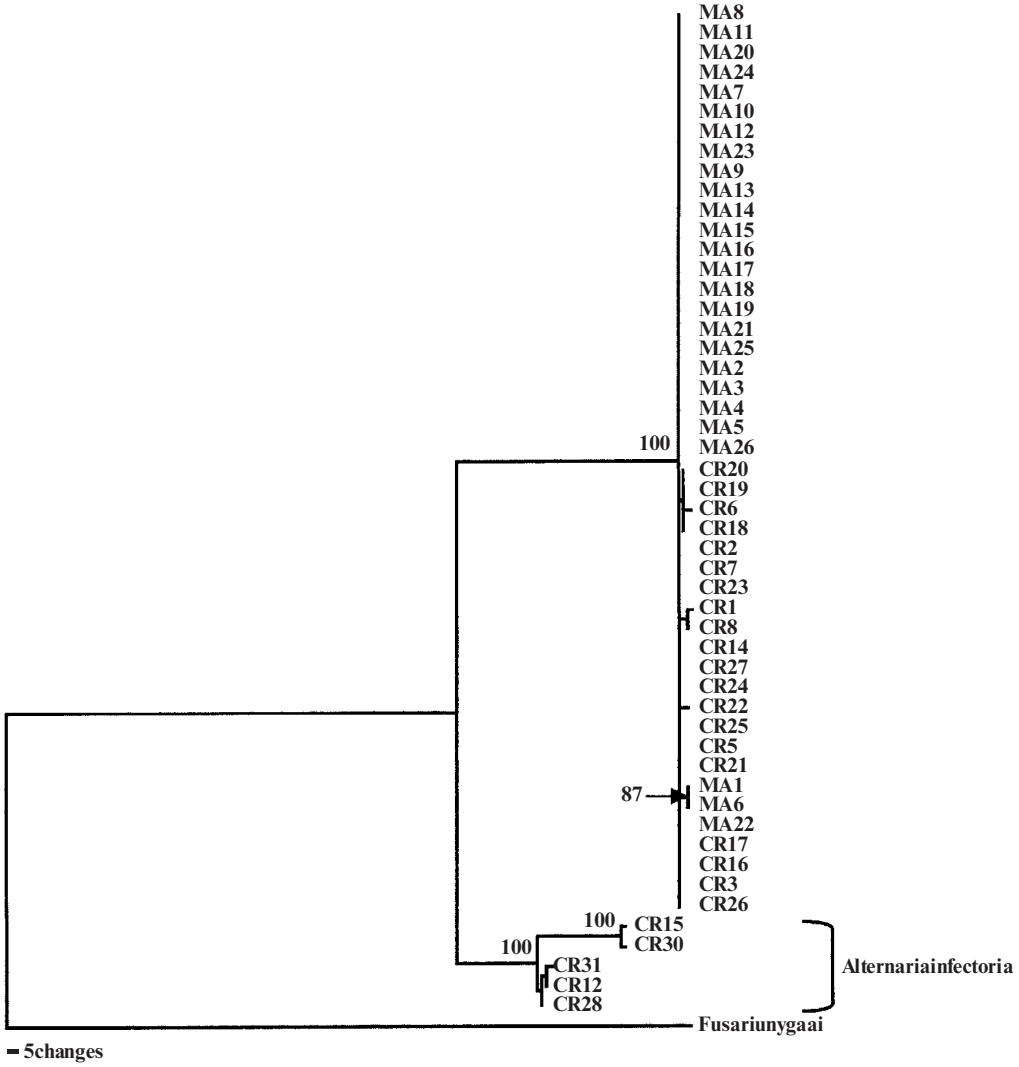

Fig. 2. One of 2 MPTs obtained froparsiony analysis of ITS using a heuristic search in the aiuparsiony analysis with 1000 randoseuence input orders. The tree is rooted with the outgroup Fusariunygaai TL 86 steps, CI 0.953, RI 0.982, RC 0.937, HI 0.047. Parsiony bootstrap alues of 1000 replicates are indicated aboe the branches.

of A. alternata, A. citri, A. pellucida and A. tenuissia , in fact has the least substitutions in alignent. ased on ITS and histone seuence data, therefore, it can be concluded that clade 1 represents a newly eoled taon. To the contrary, howeer, is the arguent that the ITS region Roberts et al. 2000as well as histone, are too consered to distinguish all these taa.

In the present study isolates of the Alternaria sp. clade 2CR6, CR14 and CR23ehibited a certain nuber of transitiontransersion substitutions. Isolates in clade 1 Alternaria groupare closely related to this species clade 2. A. arborescens clade 3ehibits the typical sporulation group ascribed to A. aborescens Sions Roberts 1993, Sions 1999b, whereas the Alternaria sp. (clade 2) differs in this regard. In a preious study using RAPDs, Roberts et al. 2000 delineated two groups of isolates ehibiting the A. arborescens sporulation pattern, thereby supporting the hypothesis that there could be ore species in this cople.

The key seuences Figs 1, 4presented here for A. arborescens clade 3, A. tenuissia clade 4and A. infectoria clade 5proide aluable references that can be used in further diagnostic studies of these pathogens. The key seuence insertion at positions 587638 for A. arborescens, A. infectoria and A. tenuissia proed to be uniue for these taa. A aiuparsiony analysis based on a saller data set of the key seuences of the 12 releant taa representing A. arborescens, A. infectoria and A. tenuissia Fig. 4separated the three clades with strong bootstrap supports of 99, 100 and 100, respectiely. Furtherore, it also separated subclades within A. infectoria, which again suggests that additional groups eist within this species, thereby supporting the ariability obsered within the A. tenuissia species copleby Sions Roberts 1993.

A further point of interest was that soe species recognied to be inoled with AR of citrus were also isolated froapples with DCR, naely the Alternaria group clade 1, A. arborescens clade 3and

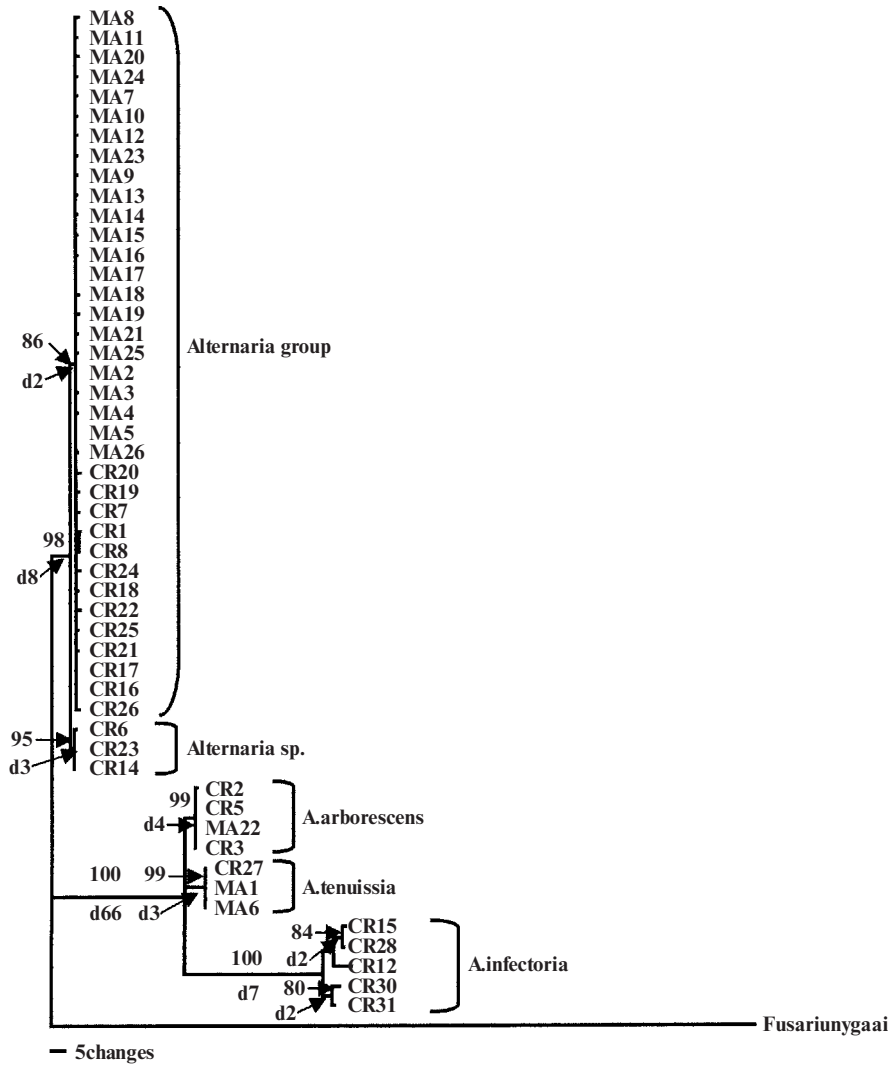

Fig. 3. One of 252 MPTs obtained frothe oint alignent of ITS, and histone data sets using the heuristic search in aiuparsiony with a 1000 randoseuence input orders TL 805 steps, CI 0.916, RI 0.957, RC 0.876, HI 0.084. ootstrap alues obtained fro1000 replicates in heuristic search and decay indices are indicated aboe and below the tree branches, respectiely.

A. tenuissia clade 4. Using a tooth-pick inoculation ethod, Serdani et al. 2002were able to proe that A. tenuissia was the ore iportant pathogen inoled with DCR of apples in South Africa. Although it has not yet been deterined which species plays the aor role in AR of citrus, inoculation studies with representatives of the different groups identified in DCR of apple showed that the apple isolates were also pathogenic to citrus Serdani, unpubl. data. This cross-pathogenicity is further supported, therefore, by the olecular data sets obtained in the present study, where apple and citrus isolates clustered together.

Further research is still reuired to resole the identity of A. citri, and to deterine which of the CR species identied here represent the ain pathogens in this disease cople. It is apparent, howeer, that, contrary to other reports about specicity in Alternaria Kusaba Tsuge 1995, Peeer et al. 1999, 2000, host specicity sees to play a lesser role in post-harest diseases such as citrus black rot and dry core rot of apple. This is in contrast to the situation in the Alternaria spp. causing brown spot of citrus, where Alternaria isolates frorough leon C. abhiri and Eperor andarin C. reticulatacaused disease on their original hosts, but not on other hosts Whiteside 1976, which in turn suggested host-specic toins to be inoled Whiteside 1976, Kohoto et al. 1979. Sions 1999bconfronted the host-specic toin producing concept eployed in pathotypes of A. alternata Kusaba Tsuge 1994, 1995and found that eaples of these groups were actually representatie of different species of Alternaria. In a further study, Roberts et al. 2000concluded that, based on their pathological, orphological, biocheical and genetic data, the sall-spored species of Alternaria represented a copleof taa rather than a collectie species. They therefore recoended that the pathotype systebe abandoned. Furtherore, it was also concluded that

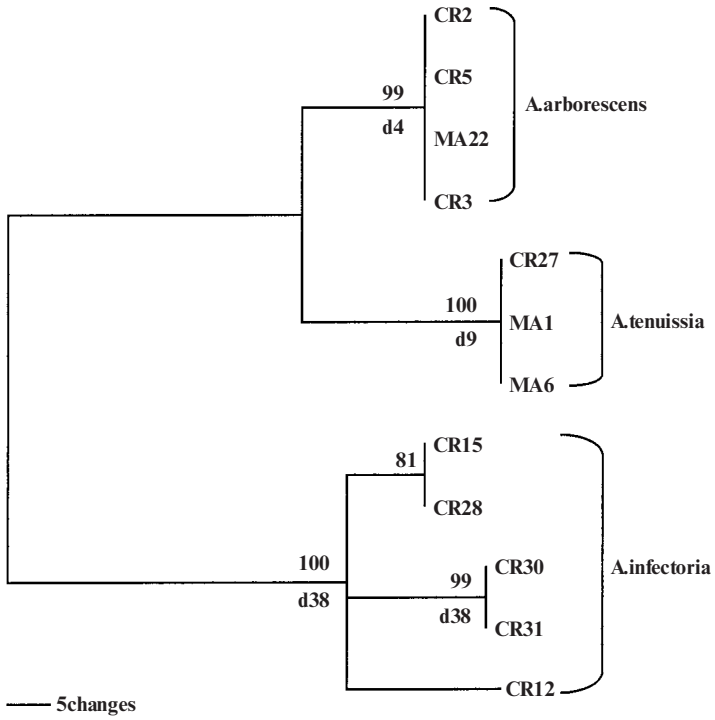

Fig. 4. One of four MPTs obtained frothe key seuences of 12 selected taa using an ehaustie search option and idpoint rooting in aiuparsiony analysis TL 86 steps, CI 0.953, RI 0.982, RC 0.937, HI 0.047. ootstrap alues ealuated with 1000 bootstrap replications in branch and bound search and decay indices are shown aboe and below the tree branches, respectiely.

A. alternata, the nae coonly eployed to describe a sall-spored species of Alternaria, is rarely encountered in nature.

Masunaka et al. 2000stated that the pathogen causing brown spot of citrus could be distinguished frothe AR species based on its pathogenicity and the production of an ACT-toin. Sions 1999a, howeer, segregated 135 isolates causing leaf spots on citrus into ten newly described species. All of these were orphologically distinct froboth A. alternata and A. citri. A certain correlation was also seen between citrus cultiars and geographic distribution. An analysis of RAPD fragent patterns of 260 sall-spored Alternaria isolates by Roberts et al. 2000deonstrated that isolates from different substrates and geographic origins group together based on their three-diensional sporulation patterns. Furtherore, their analyses also reealed that soe species consisted of ore than one clade or subgroup, indicating additional ariation within the presently acknowledged taa, and hence t-ting with their concept of species groups containing seeral as yet unnaed taa. Peeer et al. 1999also eployed RAPDs to elucidate the population structure of Alternaria spp. causing brown spot of citrus. An interpretation of the species concepts as proposed by Sions 1999aled theto conclude that they could be dealing with four distinct species. Howeer, they also found seeral points where their data did not agree with the concepts proposed by Sions 1999a. An isolate of A. lioniasperae proed to hae a RAPD prole identical to that of two reference isolates of A. alternata. An isolate of A. citriarbusti proed to be identical to two isolates of A. citriacularis , etc. Furtherore, siiso-lates associated with CR were found to cluster aong isolates causing citrus brown spot, thus also uestioning the differentiation between these two groups of isolates. In conclusion, Peeer et al. 1999reported seeral con-icts between the orphological concepts proposed by Sions 1999aand their olecular data sets. Roberts et al. 2000were of the opinion that when studying sall-spored Alternaria spp., RAPD data had to be suppleented by orphological data sets in the sub-seuent cluster analysis to obtain a ore realistic group-ing of isolates. Neertheless, it appears that a olecular conration of the species associated with citrus brown spot reains lacking and this aspect will hae to be resoled.

In a study focussing on the host specicity of the Alternaria spp. causing citrus brown spot, Peeer et al. 2000reported Alternaria spp. to eist as discrete, in-dependently eoling lineages. They further reported that the specicity obsered ay be due to host selec-tion rather than tissue specicity. The only eplanation for the lack of specicity obsered in the present study between citrus and apple isolates is possibly due to the isolates already occurring endophytically within healthy apple and citrus tissue Wager 1939, Serdani et al. 1998before harest, and that aderse cliate or

cold-storage conditions stiulate isolates occurring in the ower parts and calyby giing theaccess to the succulent fruit tissue, resulting in rot.

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