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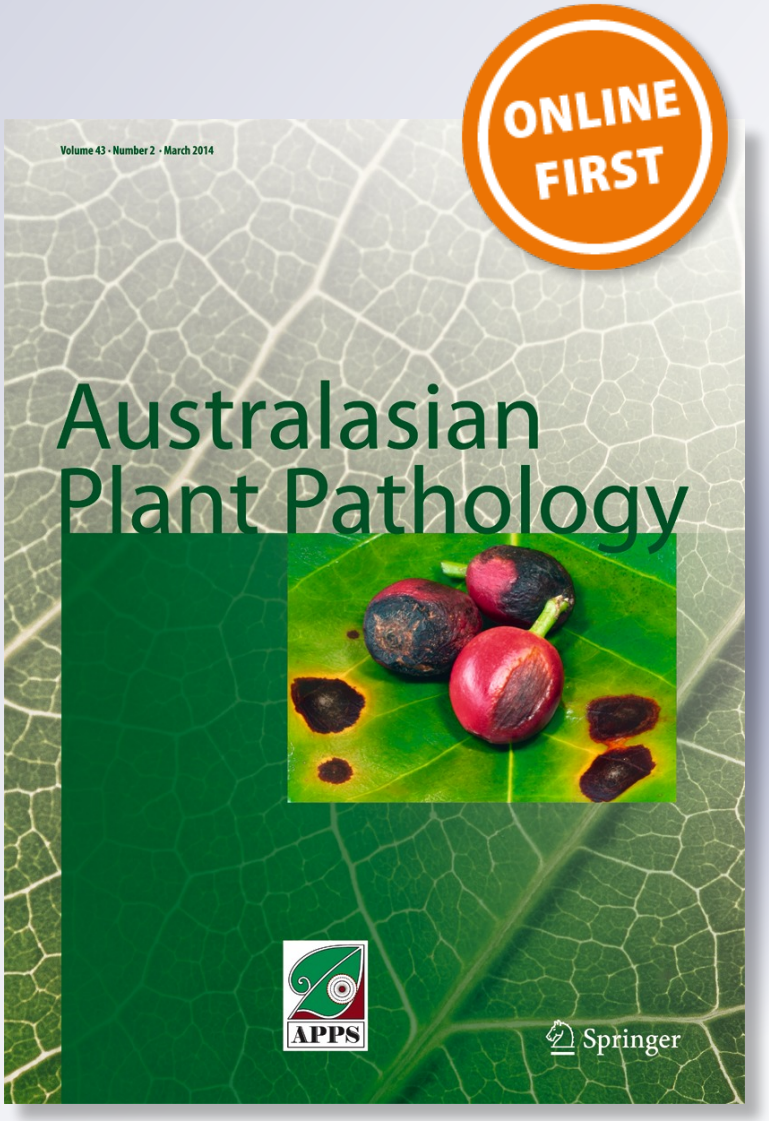

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Clonal structure of *Ceratocystis manginecans* populations from mango wilt disease in Oman and Pakistan

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Abstract *Ceratocystis manginecans* has recently been described from Oman and Pakistan where the fungus causes a serious wilt disease of mango. In both countries, the disease has moved rapidly throughout mango producing areas leading to the mortality of thousands of mango trees. The disease is associated with the infestation of the wood-boring beetle *Hypocryphalus mangiferae* that consistently carries *C. manginecans*. The aim of this study was to consider the population structure of *C. manginecans* isolated from Oman and Pakistan using microsatellite markers and amplified fragment length polymorphisms (AFLPs). Population genetic analysis of *C. manginecans* isolates from diseased mango tissue and bark beetles associated with the disease in Oman and Pakistan, showed no genetic diversity. The apparently clonal nature of the population suggests strongly that *C. manginecans* was introduced into these countries as a single event or from another clonal source.

Keywords *Ceratocystis acaciivora* · Clonal populations · Insect transmission · Mango wilt

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Introduction

Ceratocystis manginecans M. Van Wyk, A Al Adawi, and M.J. Wingf is a serious and relatively recently recognised pathogen causing wilt of mango trees (*Mangifera indica* L.) in Oman and Pakistan (Al Adawi et al. 2006, 2013b; Van Wyk et al. 2007). Although mango has been planted in Oman and Pakistan for several centuries, the disease was first observed in 1998 in both countries. In Oman, mango wilt disease was first reported on a small number of trees in Barka, southern Al Batinah. The disease subsequently spread to all mango growing areas killing thousands of trees (Al Adawi et al. 2003, 2006). Although the mango industry in Oman is relatively small (8600 t per annum), it accounts for 30 % of mango consumption in the country (Ministry of Agriculture and Fisheries [MAF] 2009b; Royal Oman Police [ROP] 2002, 2008). In contrast, Pakistan is a major mango producer and exporter, with production exceeding 1.7 million tons per annum and net profits from mango exports exceeding 20 million $US (FAOSTAT 2007). The establishment and spread of mango wilt disease has resulted in substantial loss and threatens mango production in both countries (Kazmi et al. 2005; Al Adawi et al. 2006).

Disease symptoms in Oman and Pakistan include vascular discolouration, gummosis and wilt of part, or the whole of the infected trees. The disease is usually accompanied by visible infestation by the wood boring beetle *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) (Al Adawi et al. 2006; Van Wyk et al. 2007). The first record of *H. mangiferae* attacking mango trees in Oman and Pakistan was concurrent with the first report of mango wilt disease. Furthermore, the pathogen responsible for the disease, *C. manginecans* (Al Adawi et al. 2006), was recovered from *H. mangiferae* isolated from diseased mango trees in both countries (Al Adawi et al. 2006; Van Wyk et al. 2007). In Oman, the high levels of recovery (13–83 %) of *C. manginecans* from *H. mangiferae*

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associated with the disease and the random distribution and rapid progress of mango wilt across northern Oman, has suggested the involvement of the bark beetle as vector for the pathogen (Al Adawi et al. 2006, 2013a).

Ceratocystis manginecans was described as a species in the *C. fimbriata sensu lato* complex (Van Wyk et al. 2007). This complex includes several newly described and numerous other unresolved species isolated from a wide range of hosts in many parts of the world (Webster and Butler 1967a, b; Kile 1993; Roux et al. 2000; Barnes et al. 2001; Baker et al. 2003; Van Wyk et al. 2013). Such species include *C. albifundus* M.J. Wingf., De Beer and Morris, the cause of a serious wilt disease of *Acacia mearnsii* (Wingfield et al. 1996); *C. pirilliformis* Barnes and M.J. Wingf. from *Eucalyptus* (Barnes et al. 2003); *C. cacaofunsta* Engelbrecht and Harrington from *Theobroma cacao* and *C. platani* Engelbrecht and Harrington from *Platanus* sp. (Engelbrecht and Harrington 2005).

Mango wilt disease has been known in Brazil since the 1930s (Viegas 1960) and has similar symptoms to those observed in Oman and Pakistan. The causal agent of the disease in Brazil resides in the *C. fimbriata* complex that is believed to include a number of distinct taxa (Van Wyk et al. 2007, 2011, 2013; Harrington et al. 2011) but the relatedness of these to *C. manginecans* remains to be fully resolved. The purported vector of *C. manginecans* in Oman, *H. mangiferae* is also associated with mango wilt in Brazil (Castro 1960; Rossetto et al. 1980; Al Adawi et al. 2013a) where it is considered an invasive alien species.

In Sao Paulo State, Brazil, two strains of *C. fimbriata s.l* were found to be predominant. Mango varieties evaluated for resistance showed differential responses. The varieties Coração Boi, Espada, Ubá and Vitória were resistant to one strain but susceptible to the second (Ribeiro 1993; Ribeiro et al. 1995). Given the fact that the pathogen/s in Brazil may encompass a number of discrete taxa or is one having considerable genetic variability (Harrington et al. 2011; Van Wyk et al. 2011), information on population genetic structure will be important for the development of effective management strategies for mango wilt disease in Oman, Pakistan and elsewhere (McDonald 1997; McDonald and Linde 2002; Milgroom and Peever 2003).

The origin of *C. manginecans* in Pakistan and Oman is unknown but field evidence suggests that it is an introduced pathogen. It is known that there have been large consignments of mango fruit imported into Oman from Pakistan (ROP 2002, 2008) and the proximity of the two countries encourages regular unauthorised introductions of mango planting material into Oman from Pakistan (Al Adawi, *personal observation*). The health status of this material is difficult to determine but illicit trade represents a clear means by which pathogens could enter Oman.

The aim of this study was to gain an understanding of the population structure of *C. manginecans* isolates from both diseased mango trees and bark beetles associated with mango wilt in Oman and Pakistan by using two sets of microsatellite markers previously developed for *C. fimbriata* (Barnes et al. 2001; Steimel et al. 2004) and an AFLP (Vos et al. 1995) protocol. In this way, it was anticipated that it might be possible to better understand the origin of the pathogen in Oman and to inform programmes focussed on developing disease-tolerant planting stock.

Materials and methods

Sampling and fungal isolations

Isolates of *C. manginecans* were collected from stems of diseased mango trees and *H. mangiferae* beetles in Oman and Pakistan. Samples from Oman were collected between November 2003 and June 2004 from five areas (Shinas, Liwa, Sohar, Rustaq and Quriayat) where disease incidence was high. Liwa and Shinas are within 50 km of Sohar while Rustaq and Quriayat are 110 and 250 km distant from each other respectively (Fig. 1). Between them, these areas represent 41.8 % of the total mango production in Oman (Ministry of Agriculture and Fisheries [MAF] 2009a). Diseased mango samples from Pakistan were collected during May 2006 from Faisalabad and Multan (230 Km apart) in the province of Punjab (Fig. 2). In addition, thirty adult *H. mangiferae* were collected from mango trees from Faisalabad and Multan using an aspirator. Punjab represents 52 % of the total area under mango cultivation in Pakistan with an annual production of over 700,000 t (67 %) (Collins et al. 2006).

Samples were made by cutting pieces of woody tissue from the leading edges of lesions on the stems of trees. These samples were washed with tap water; surface disinfested in 1 % NaOCl for 1 min, rinsed in sterile distilled water and blotted dry on sterile filter paper. To bait for, and induce sporulation of *C. manginecans*, infected wood pieces were incubated in moist chambers and/or placed between two slices of carrot pre-treated with streptomycin (100 mg/l) (Moller and DeVay 1968).

Single adult *H. mangiferae* beetles were crushed and placed in a cavity made on the inner surface of a pair of carrot discs pre-treated by soaking in a streptomycin solution. Carrot discs inoculated with either diseased wood pieces or beetles were incubated for 7–10 days at room temperature (22–25 °C) under conditions of high humidity. Once ascomata had developed on the carrot discs, single ascospore masses were transferred onto 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin (100 mg/l).

To confirm pathogen identity, fungal structures from randomly selected 10-day-old cultures of isolates were transferred to glass slides, mounted in lactic acid and examined under a compound microscope. *C. manginecans* isolates were

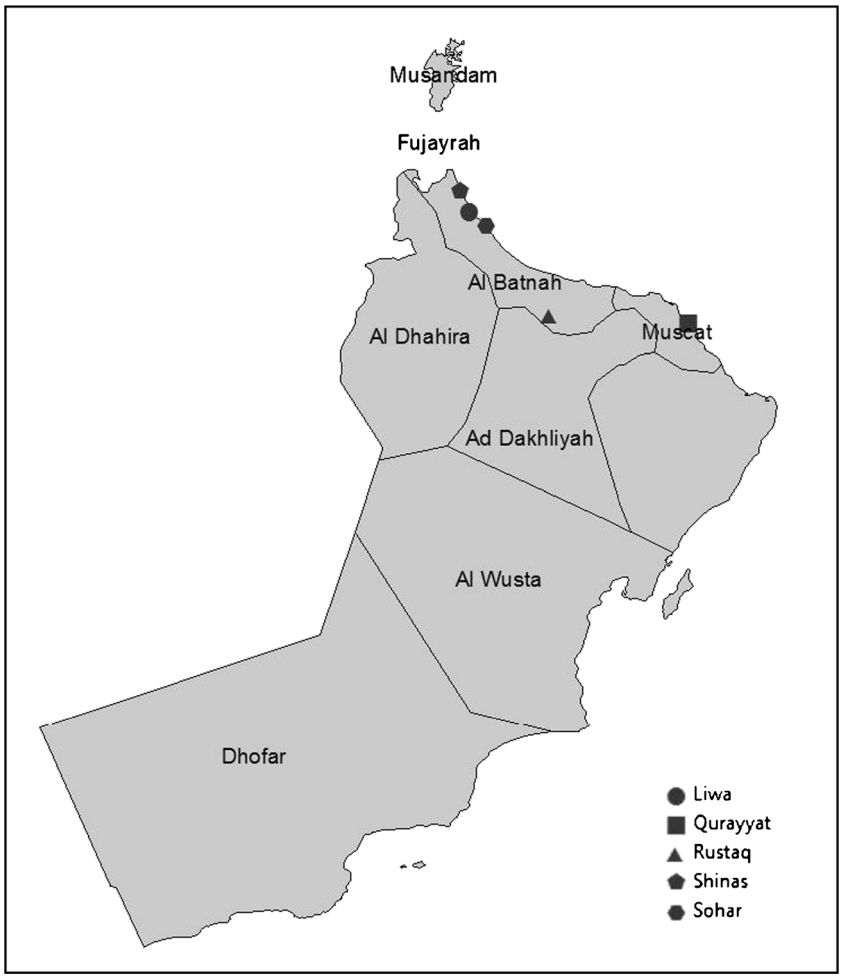

Fig. 1 Map of Oman showing the collection sites for *C. manginecans*

identified based on morphological characteristics such as colour of colonies, ascomatal shape, presence of hat shaped ascospores and production of cylindrical conidia (Van Wyk et al. 2007). All isolates used were preserved in the culture collection (CMW) at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

DNA extraction

All isolates were grown on 2 % MEA and incubated for 2 weeks at 25 °C. Mycelium, including ascomata and ascospores was scraped from the agar surface using a sterile spatula and transferred to 1.5 ml Eppendorf tubes. These were freeze dried, the mycelium crushed to a fine powder using a glass rod and the DNA was extracted following the method described by Barnes et al. (2001). Extracted DNA was run on 1.5 % agarose gels stained with ethidium bromide. The presence and intensity of the extracted DNA for each isolate was examined under ultraviolet (UV) illumination. DNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, Delaware) and DNA dilutions (30–60 ng/μl) made with sterile distilled water.

SSR-PCR and genescan analysis

Cross-species amplification of the available 27 microsatellite markers developed for *C. fimbriata* (Barnes et al. 2001; Steimel et al. 2004) were tested on three isolates of *C. manginecans*. PCRs were performed using 25 μl reaction mixtures consisting of 2 ng DNA, 2.5 μl of Expand HF buffer containing 1.5 mM $MgCl_2$, 300 nM of the forward and reverse microsatellite primers, 200 μM of dNTP and 0.35 U of *Taq* polymerase. Amplification of microsatellite primers was carried out using an Eppendorf thermocycler programmed as described previously (Barnes et al. 2001; Steimel et al. 2004). PCR products were separated by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide and visualized under UV light.

Primers that successfully amplified *C. manginecans* were used on the isolates obtained from Oman and Pakistan following the same methods described above. Primers were fluorescently labelled with PET, VIC, FAM or NED dyes

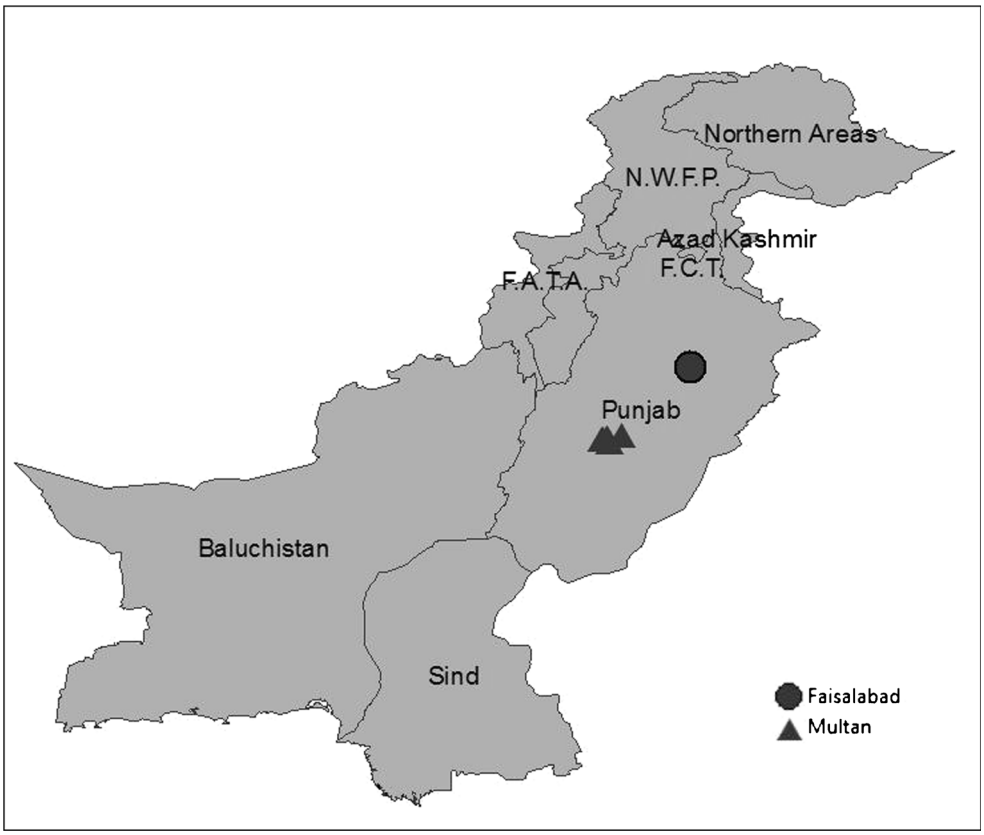

Fig. 2 Map of Pakistan showing the locations where *C. manginecans* was collected

using the G5 labelling kit (Applied Biosystems, Warrington, UK).

Microsatellite products were multiplexed according to the fluorescent dye and the amplicon size. This was to enable the simultaneous analysis of the maximum possible loci in a single gel run. The fluorescently labelled SSR-PCR amplicons (0.5 μl containing approx. 1.5 ng DNA) were mixed with 0.4 μl GeneScan-500 Liz internal size standard (Applied Biosystems Inc., Foster City, California) and 10 μl formamide. The majority of samples were run on an ABI Prism 377 DNA sequencer (Applied Biosystems Inc., Foster City, California) and the remaining samples were separated using an ABI Prism 3100 DNA sequencer. Due to allele shifts between instruments, three reference samples were analyzed on both instruments. GeneMapper version 3.0 (Applied Biosystems Inc., Foster City, California) was used to analyze gels and determine allele sizes. All gel runs contained the same reference samples to ensure reproducibility.

All alleles that were only one base pair different from each other at a locus were sequenced to confirm the allele scoring. The PCR reactions for three isolates (CMW15337, 23629, 23637) of *C. manginecans* were repeated using the same conditions as described above but with non-fluorescent primers. The PCR products were purified using 6 % Sephadex G-50 columns (1 g sephadex in 15 ml sterile water, Sigma-Aldrich, Steinheim, Germany). Sequencing reactions were prepared using 5–10 ng of cleaned PCR product, 1 μl primer, 2 μl 5× sequencing buffer, 2 μl of ABI Prism Big Dye Terminator mix, v. 3. 1 (Applied Biosystems Inc., Foster City, California) in a total volume of 10 μl. Sequencing PCR consisted of 25 cycles at 96 °C for 10 s; 50 °C for 4 s; 60 °C for 4 min. Sequencing reactions were cleaned using Sephadex G-50. Sequences were determined using an ABI PRISM 3100 Autosequencer (Applied Biosystems Inc., Foster City, California) and aligned using MEGA 5.0 (Tamura et al. 2011).

Selected isolates from the *C. fimbriata s.l* complex from Brazil (including *C. mangicola* and *C. mangivora*) and *C. platani* were included in this study (Table 1) to test the robustness of the microsatellite locus amplification and for comparison with *C. manginecans* isolates.

AFLP analyses

Twenty two isolates of *C. manginecans* (ten from Oman and 12 from Pakistan) and three isolates of *C. platani* were screened using the AFLP protocol (Vos et al. 1995) modified by (Myburg et al. 2001) (Table 1). DNA was digested using restriction endonucleases *Eco*RI and *Mse*I and the restricted fragments were ligated to the corresponding adapters. The pre-amplification reaction was performed with two pre-selective primers (M E00 and M M00) using the following

Table 1 Isolates of *Ceratocystis manginecans* used in this study. Isolates of *C. fimbriata s.l, C. mangicola, C. mangivora* and *C. platani* were used as controls

Ceratocystis spp.	CMW isolates[a]	Source	Area	Country	Sample size
C. manginecans	**15337**[b]–15353	*Mangifera indica*	Shinas	Oman	16(2)[c]
	15366, 15369, 15370, 15385–15389, 15391	*M. indica*	Liwa	”	9(3)
	15313, **15314,** 15315–**15316**, 15367, 15390	*M. indica*	Sohar	”	6(3)
	15371–15377	*M. indica*	Quariyat	”	7(1)
				Total	**38(9)**
C. manginecans	**15317**–15336	*Hypocryphalus mangiferae*	Sohar	Oman	20(1)
	15354–15365, **15384**	*H. mangiferae*	Liwa	”	13(3)
	15368	*H. mangiferae*	Rustaq	”	1(1)
	15378–**15381, 15382, 15383**, 15392	*H. mangiferae*	Quariyat	”	7(1)
				Total	**41(6)**
C. manginecans	17567	*M. indica*	Faisalabad	Pakistan	1(1)
	23637–23642, 23643	*M. indica*	Multan	”	7(3)
				Total	**8(4)**
	23628, 23629, **23630–23634,** 23635, 23636	*H. mangiferae*	Faisalabad	Pakistan	**9(1)**
C. mangicola	14797	*M. indica*	–	Brazil	1
C. mangivora	15052	*M. indica*	–	Brazil	1
C. fimbriata s.l	14806	*Ficus* sp.	–	Brazil	1
C. fimbriata s.l	4903	*Eucalyptus* sp.	–	Brazil	1
C. platani	**1894**	*Platanus* sp.	–	Switzerland	1
	2219	*Platanus* sp.	–	France	1
	2242	*Platanus* sp.	–	Italy	1

[a] All the *C. manginecans* isolates were collected by A. O. Al Adawi. All isolates are maintained in the culture collection (CMW) of the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, South Africa
[b] Isolate number in bold were used in the AFLP analysis
[c] Number in parenthesis indicates the number of sites where the samples were collected

PCR mixture: 5 μl Restriction/Ligation (R/L) mixtures; 1× PCR buffer; 0.2 mM dNTPs and 1.5 units *Taq* polymerase. The PCR conditions for pre-amplification of the R/L DNA fragments involved: 30 s at 72 °C, 25 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C with an increment of 1 s per cycle, followed by 2 min at 72 °C. The PCR mixture for selective amplification was carried out as above using one primer combinations (Mo4 + E-AC) and the *Eco*RI primer was labelled with the infrared dye, IRDye 700 (LI-COR, Lincoln, NE). The final PCR amplification consisted of 13 cycles of 10 s at 94 °C, 30 s at 65 °C with a decrement of 0.7 °C per cycle, 1 min at 72 °C; followed by 23 cycles of 10 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C with an increment of 1 s per cycle; and a final extension step at 72 °C for 1 min. Formamide loading buffer was added to the products resulting from the final amplification and these were run on a polyacrylamide gel using automated DNA sequencer 4200 LI-COR (Myburg et al. 2001). AFLP gel images generated from LI-COR automated sequences were visually evaluated and banding patterns of *C. manginecans* isolated from mango and *H. mangiferae* from Oman and Pakistan were compared to the *C. platani* isolates.

Results

Sampling and isolations

Seventy nine isolates of *C. manginecans* were collected from diseased mango trees and from bark beetles (*H. mangiferae*) from five areas of Oman (Table 1; Fig. 1). Isolations from Faisalabad, Pakistan, yielded 17 *C. manginecans* isolates, nine of which were from bark beetles (Table 1; Fig. 2). In total, 96 isolates of *C. manginecans*, three isolates of *C. platani,* one isolate each of *C. mangicola* and *C. mangivora* from mango in Brazil and two isolates from the *C. fimbriata s.l* clade representing those from different hosts were used for the SSR studies (Table 1).

SSR-PCR and genescan analysis

From the 27 loci amplified with the microsatellite markers, five loci (AG15/16, AG17/18, CF21/22, CAG900 and GACA60) were excluded from the analysis as they produced monomorphic alleles in all isolates, including those from the *C. fimbriata s.l* group. Loci AG1/2, AG7/8, CF11/12,

CF15/16, CF17/18, CF23/24 and CAT1200 amplified alleles with a one base pair polymorphism. After sequencing, 100 % sequence similarity was found between the alleles that had one base pair difference between them. They were thus scored as a single allele with the following sizes; 264, 283, 219, 474, 270, 158 and 373.

Sizes of the SSR-PCR products in the remaining 22 loci ranged from 123 to 480 bp. In the *C. manginecans* populations from Oman and Pakistan, 79 alleles were obtained from the amplification of twenty two polymorphic SSR loci (Table 2). Two alleles amplified in loci CF11/12 and AAG9 but in both cases, one of the alleles had a low allele frequency value of 0.03 and 0.01 respectively (Table 3). The remaining loci yielded monomorphic alleles in all isolates of *C. manginecans* from Oman and Pakistan. Therefore, in almost all loci examined, the allelic frequency values were one and gene diversity values were zero for the *C. manginecans* isolates from Oman and Pakistan (Table 3). All isolates of *C. manginecans* from either diseased mango or bark beetles represented a single multilocus genotype for the above mentioned loci. Furthermore, all the *C. manginecans* isolates had the same alleles as *C. mangicola* and *C. mangivora* from mango in Brazil at nine of the loci (40.1 %) and they shared the same allele with the Brazilian fig and Eucalyptus isolates at six loci (27.3 %). The *C. mangicola* and *C. mangivora* isolates varied from each other at 6 loci. *C. platani* isolates shared the same alleles with the *C. manginecans* populations in two microsatellite loci (9 %) (Table 2).

Three loci (AAG9, CAT3K and CAT9X) amplified more than one allele per locus within the Oman and Pakistan *C. manginecans* population. Locus AAG9, amplified multiple alleles in one isolate of *C. manginecans* from Oman. One of these alleles was the same as that in the Brazilian mango (*C. mangicola* and *C. mangivora*) and *Eucalyptus* isolates of *C. fimbriata s.l.* In addition, locus CAT3K amplified double alleles (309/321) for *C. manginecans* isolated from Oman and Pakistan and shared one of the alleles (309) with all the Brazilian isolates, irrespective of their host origin (Table 2).

AFLP profiles

The AFLP profiles of 18 loci for all isolates of *C. manginecans* isolated from diseased mango trees and bark beetles associated with mango wilt disease in Oman and Pakistan were identical. The three isolates of *C. platani* from *Platanus,* that were used as controls, shared 14/18 (78 %) bands with the *C. manginecans* isolates in the Mo4 + E-AC primer combination. In addition, 10 unique AFLP bands were present in the profiles of the *C. platani* isolates and these bands were not present in the *C. manginecans* profiles.

Discussion

Microsatellite and AFLP analyses in this study showed a lack of variation amongst isolates of *C. manginecans*. Furthermore, there were no intra or inter geographical differences between *C. manginecans* isolates from Oman and Pakistan or between isolates from mango trees or bark beetles. The results, therefore, suggest a recent founder event with *C. manginecans* having been recently introduced and established in the region.

Results of this study affirm the efficacy of the microsatellite markers developed previously for *C. fimbriata s.l.* for studying populations of isolates of *C. manginecans, C. mangivora, C. mangicola* as well as *C. albifundus*, *C. platani*, *C. cacaofunesta* and *C. pirilliformis* (Engelbrecht et al. 2004, 2007; Barnes et al. 2005; Kamgan et al. 2009). This might indicate that flanking regions surrounding these markers are highly conserved across taxa, allowing cross species amplification of those markers (Selkoe and Toonen 2006).

The overall genetic analysis of *C. manginecans* isolates from Oman and Pakistan has shown that they represent a single clone of the pathogen. This supports the view that mango wilt disease in Oman and Pakistan emerged from single introduction of a single haplotype and the population has expanded clonally to infect mango trees in many parts of both countries. However, the source of *C. manginecans* in Oman and Pakistan is unknown. Tracking records of customs and plant quarantine offices in Oman has revealed that there have been regular importations of mango fruit and germplasm from India and Pakistan (Royal Oman Police [ROP] 2002, 2008). In addition, there is evidence of illegal introductions of mango seedlings into Oman from Pakistan that is facilitated by the proximity of the two countries (Al Adawi, *personal observation*). It is, therefore, possible that Pakistan represents the source of infection in Oman.

There are possible origins of *C. manginecans* in Oman other than Pakistan. For example, there are recent but inconclusive observations that mango wilt disease is in India and Bangladesh (Ploetz and Freeman 2009; Mosharraf Hossain, *personal communication*) and this might be the source of the infections in both Pakistan and Oman. Results of this study, however, provide no clues as to the origin of the pathogen. The fungus has been found on *Acacia mangium* in Indonesia (Tarigan et al. 2011), a country where mango is also widely planted. Alternatively *C. manginecans* could originate from South America, which appears to be an area of substantial genetic variation for *C. fimbriata s.l.,* including the many cryptic species encompassed by this group (Barnes et al. 2001; Baker et al. 2003; Engelbrecht and Harrington 2005; Rodas et al. 2008; Van Wyk et al. 2009, 2010, 2011).

Prevention of further introductions of *C. manginecans* into Oman and Pakistan will be important as this will preclude an

Table 2 Alleles sizes for *C. manginecans* populations collected from Oman and Pakistan revealed through the amplification of 22 microsatellite loci. Allele sizes for *C. mangicola*, *C. mangivora*, *C. fimbriata s.l* and *C. platani* are also included for comparison purposes

Species	Oman		Pakistan		Brazil			France	Italy	Switzerland	No of alleles
	C. manginecans		*C. manginecans*		*C. mangicola/C. mangivora*	*C. fimbriata s.l*		*C. platani*	*C. platani*	*C. platani*	
Locus/Host	*M. indica* (38)[A]	*H. mangiferae* (41)	*M. indica* (8)	*H. mangiferae* (9)	*M. indica* (2)	*Ficus* sp. (1)	*Eucalyptus* sp. (1)	*Platanus* sp. (1)	*Platanus* sp. (1)	*Platanus* sp. (1)	
AG1/2	264	264	264	264	**264**[D]	**264**	262	266	266	266	3
AG7/8	283	283	283	283	**283**	286	289	286	286	286	3
CF5/6	365	365	365	365	**365**	**365**	**365**	376	376	376	2
CF11/12	217 (2) [B], 219 (36)	219	219	219	**219**	214	**219**	230	230	230	4
CF13/14	406	406	406	406	**406**	417	**406**	415	415	415	3
CF15/16	474	474	474	474	472	477	477	480	480	480	4
CF17/18	270	270	270	270	**270**, 278	**270**	268	**270**	**270**	**270**	3
CF23/24	158	158	158	158	152, 160	**158**	155	160	160	160	4
AAG8	176	176	176	176	174	179	179	174	174	174	3
AAG9	404	404 (40), 404/399 (1)[C]	404	404	**399**	402	**399**	407	407	407	5
CAA9	224	224	224	224	174, 204	200	174	–	204	–	4
CAA10	123	123	123	123	129, 132	132	132	126	126	126	4
CAA15	324	324	324	324	**324**	330	321	315	315	315	4
CAA38	338	338	338	338	–	330	**338**	**338**	–	–	2
CAA80	304	304	304	304	323, 328	323	311	–	308	–	5
CAT1	252	252	252	252	255	**252**	262	255	255	255	3
CAT3K	309/321	309/321	309/321	309/321	**309**	**309**	**309**	–	**309**	**309**	3
CAT9X	274/280	274/280	274/280	274/280	268	271	280/286	279/282	279/282	279/282	5
CAT1200	373	373	373	373	**373**	382	377	390	–	–	4
CAG5	319	319	319	319	**319**	337	**319**	328	–	–	3
CAG15	270	270	270	270	260, 283	274	255	–	–	–	5
GACA650	214	214	214	214	220	208	**214**	–	–	–	3

[A] Number of isolates used in the study
[B] Number of isolates in population with specific allele
[C] Two alleles were produced during amplification in a single individual
[D] Alleles that are shared with those found in Oman/Pakistan are indicated in bold

Table 3 Summary statistics for *Ceratocystis manginecans* isolates from Pakistan and Oman using 22 microsatellite markers

Loci	No. of isolates		No. of alleles		Allele frequency				H[a]	
	Oman	Pakistan	Oman	Pakistan	Oman		Pakistan		Oman	Pakistan
					A	B	A	B		
AG1/2	79	7	1	1	1.00		1.00		0.00	0.00
AG7/8	79	17	1	1	1.00		1.00		0.00	0.00
CF5/6	79	17	1	1	1.00		1.00		0.00	0.00
CF11/12	79	17	2	1	0.97	0.03	1.00		0.05	0.00
CF13/14	79	17	1	1	1.00		1.00		0.00	0.00
CF15/16	79	16	1	1	1.00		1.00		0.00	0.00
CF17/18	79	17	1	1	1.00		1.00		0.00	0.00
CF23/24	79	17	1	1	1.00		1.00		0.00	0.00
AAG8	79	13	1	1	1.00		1.00		0.00	0.00
AAG9	79	17	2	1	0.99	0.01	1.00		0.03	0.00
CAA9	78	17	1	1	1.00		1.00		0.00	0.00
CAA10	77	17	1	1	1.00		1.00		0.00	0.00
CAA15	78	17	1	1	1.00		1.00		0.00	0.00
CAA38	73	16	1	1	1.00		1.00		0.00	0.00
CAA80	78	17	1	1	1.00		1.00		0.00	0.00
CAT1	76	17	1	1	1.00		1.00		0.00	0.00
CAT3K	79	17	1	1	1.00		1.00		0.00	0.00
CAT9X	79	17	1	1	1.00		1.00		0.00	0.00
CAT1200	79	17	1	1	1.00		1.00		0.00	0.00
CAG5	77	17	1	1	1.00		1.00		0.00	0.00
CAG15	79	16	1	1	1.00		1.00		0.00	0.00
GACA650	79	17	1	1	1.00		1.00		0.00	0.00

[a] H = Nei's (1973) gene diversity

expansion of the genetic diversity of the pathogen in these countries. *C. manginecans* reproduces asexually via conidia and by ascospores arising from unidirectional mating type switching (Webster and Butler 1967a, b; Harrington and McNew 1997; Witthuhn et al. 2000). The fungus is able to outcross and there is emerging evidence of hybrids between species in the *C. fimbriata s.l.* complex (Naidoo et al. 2013; Fourie et al. *unpublished*). A greater genetic diversity in *C. manginecans*, emerging from sexual outcrossing or hybridisation will complicate efforts to manage mango wilt disease in Oman and neighbouring countries. Clearly, every effort should be made to prevent the introduction of strains of *C. manginecans* or other species in the *C. fimbriata s.l.* complex.

The lack of genetic variation between isolates of *C. manginecans* from *H. mangiferae* and mango in Oman and Pakistan suggests the direct involvement of *H. mangiferae* as a vector for the spread of the fungus in these countries (Rossetto et al. 1980; Al Adawi et al. 2006, 2013a; Van Wyk et al. 2007; Masood et al. 2008). *H. mangiferae* is host specific on mango and is indigenous to southern Asia where mango originated. Other than in Brazil, Oman and Pakistan where the insect is found in association with *C. manginecans*, *H. mangiferae* also occurs in many parts of the world including India, Malaysia, Indonesia and southern Florida where mango wilt disease is not known (Wood 1982; Butani 1993; Atkinson and Peck 1994). However, *C. manginecans* was recently reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011) and *C. fimbriata s.l* has been reported to cause disease on various crops including coffee (*Coffea arabica* L.), taro (*Colocasia esculenta*), pomegranate (*Punica granatum*) and rubber (*Hevea brasiliensis*) in the same area (Ploetz and Prakash 1997; Somasekhara 1999). Yet there has been no connection between these diseases and *H. mangiferae*, suggesting that the *C. manginecans* has not co-evolved with the insect (Ploetz and Freeman 2009).

The management of mango wilt disease involves using systematic fungicides and resistance mango cultivars (Rossetto et al. 1996; Ploetz and Freeman 2009; Al Adawi et al. 2013c). The development of genetic material resistant to the mango wilt pathogen, *C. manginecans*, is considered an important element in disease management. The Ministry of Agriculture in Oman, in collaboration with FAO, has begun a programme to introduce new mango germplasm from Brazil and Australia and to evaluate this for to infection by

C. manginecans under local conditions. The results of the present study showing clonality of the pathogen in Oman suggests that selection and breeding for resistance will be simpler than it would have been if *C. manginecans* were represented a diverse population of isolates.

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