Class III endophytes, clandestine movement amongst hosts and habitats and their potential for disease; a focus on Neofusicoccum australe

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Abstract Neofusicoccum australe is a class III endophyte characterised by a quiescent passive life phase and an active pathogenic life phase as a latent pathogen. The latter life stage has been observed worldwide for numerous woody horticultural hosts. In this study, we have re-evaluated GenBank ITSrDNA sequence data to establish the current host and geographical range of N. australe. Additionally, we have interrogated the diversity of N. australe in Australia using microsatellite markers to ascertain if there are any host or site preference for different genotypes. N. australe has a widespread distribution across ten countries and colonises 46 hosts from 18 plant families; mainly angiosperms, some coniferous species and one monocot. Phylogenetic analysis of the ITSrDNA sequence indicates there is a single dominant ITS genotype present in most locations and there are another 12 rare or moderately rare genotypes. Populations of N. australe in Australia appear to be highly diverse, and there is no discernable host or habitat restriction. The dominance of N. australe in native forest throughout the southwest of Western Australia, and its rarity elsewhere in native vegetation, while being common as a pathogen of horticultural hosts, suggests that this species is endemic to Western Australia.

Keywords Botryosphaeriaceae · Population · Genetic diversity · Latent pathogens · Distribution

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Introduction

The life strategies of the Botryosphaeriaceae generally fit into the description of class III endophytes (Rodriguez et al. 2009). Many members have a broad host range (Punithalingam 1976, 1980), they fruit on senescing tissue (Slippers and Wingfield 2007), are found in above ground plant parts (Johnson et al. 1992) and multiple species are found often within localised infections in the host tissue (Sakalidis et al. 2011a, b; Spagnolo et al. 2011). However; unlike other Class III fungi they have occasionally been observed in host seeds (Johnson et al. 1998; Bihon et al. 2011) and there is also evidence of infections in horticultural crops occurring via pruning wounds (Brown and Hendrix 1981; Smith et al. 1994, 1996; Amponsah et al. 2011; Sakalidis et al. 2011c). Pathogenicity trials are successfully used to prove potential for disease development (Amponsah et al. 2011; Sakalidis et al. 2011c) but little is known about possible passive (endophytic) or delayed active (latent pathogen) life stages that may also initiate from the same infections.

Neofusicoccum australe (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, was originally described as Botryosphaeria australis from native Acacia spp. in Victoria and exotic Sequoiadendron giganteum in Canberra; both in eastern Australia (Slippers et al. 2004). N. australe had previously been identified as Botryosphaeria ribis from various hosts including Banksia coccinea (Shearer et al. 1995), Banksia caleyi and Eucalyptus marginata (Australia) and a Pistacia sp. (Italy) (Smith and Stanosz 2001) and as Botryosphaeria lutea on Fraxinus augustifolia (Portugal) (Phillips et al. 2002) and from Protea cynaroides (South Africa) (Denman et al. 2003). In 2006, an extensive phylogenetic evaluation of the Botryosphaeriaceae identified several lineages and Botryosphaeria was only retained for Botryosphaeria dothidea and Botryosphaeria

corticis (Crous et al. 2006). Taxa that were characterised by Fusicoccum-like anamorphs and Dichomera-like synanamorphs were placed into Neofusicoccum; which is where N. australe currently resides (Crous et al. 2006).

There have been many reports describing N. australe as a significant pathogen of grapevine; it is associated with a variety of symptoms affecting trunk, canes, buds and the fruit. Symptoms include stem cankers, dieback, wedge shaped lesions in grapevine stems and fruit rot. N. australe has been associated with symptomatic grapevine in Western Australia (Taylor et al. 2005), southern and eastern Australia (Cunnington et al. 2007; Pitt et al. 2010), New Zealand (Amponsah et al. 2009, 2011), South Africa (van Niekerk et al. 2004) and USA (California) (Úrbez-Torres et al. 2006; Úrbez-Torres and Gubler 2009). Other fruit crop declines have linked N. australe to olive "drupe rot" in Italy (Lazzizera et al. 2008) and dieback symptoms of peach, plum and apricots in South Africa (Damm et al. 2007; Slippers et al. 2007). Pavlic et al. (2007) established N. australe as a potential pathogen of Eucalyptus grandis x E. camaldulensis in which pathogenicity trials of young 2-year-old trees produced stem lesions significantly greater then control trees. In 2008, N. australe was isolated from symptomatic wood showing perennial cankers from Eucalyptus globulus and 20-year-old Pistachio vera in northern Spain (Armengol et al. 2008). Subsequent pathogenicity trials illustrated a rapid induction of symptoms including leaf wilting and canker formation in 8-month-old Eucalyptus seedlings, whilst it took 3 months for cankers to development in 2-year-old Pistachio seedlings.

Relatively few reports of N. australe have been recorded in eastern Australian native tree species; the only record of N. australe was linked to canker development on native Acacia spp. in the original description (Slippers et al. 2004). However, N. australe is the dominant Botryosphaeriaceae endophyte found amongst native tree species in the southwest of Western Australia (WA) (Shearer et al. 1995; Burgess et al. 2006; Taylor et al. 2009). Native hosts include Acacia rostellifera, Acacia cochlearis, Allocasuarina fraseriana, Agonis flexuosa, B. coccinea, Banksia grandis, Callitris preissii, Eucalyptus diversicolor, E. gomphocephala, E. marginata, Eucalyptus phylacis, Santulum acuminatum (Shearer et al. 1995; Burgess unpublished data; Burgess et al. 2005, 2006; Taylor et al. 2009). Slippers et al. (2004) suggested that N. australe is endemic to the southern hemisphere, and current evidence suggests N. australe is endemic to WA (Burgess et al. 2006; Taylor et al. 2009).

Interestingly, pathogenicity trials using more than one isolate of N. australe have, in some cases, found significant variation in the virulence between isolates (van Niekerk et al. 2004; Burgess et al. 2005; Taylor et al. 2005; Pavlic et al. 2007) suggesting there may be some level of genetic variation between isolates of this species. A one bp difference between N. australe isolates in the ITS gene region and up to three bp differences between isolates in the EF1-a gene region has been reported (Lazzizera et al. 2008).

In this study, we have re-evaluated GenBank sequence data to establish the current host and geographical range of N. australe worldwide. Additionally, we have then focussed on the Australian diversity of N. australe to ascertain if there is any host or site preference for different genotypes and to compare isolates from WA and eastern Australia.

Materials & methods

Collection of Australian material

Isolates from previous studies in WA were included (Burgess et al. 2006; Taylor et al. 2009). They were collected from four locations in the south-west of WA; Yalgorup National Park from E. gomphocephala, S. acuminatum, A. rostellifera, B. grandis, A. fraseriana, A. flexuosa and E. marginata; Woodman's Point from E. gomphocephala, S. acuminatum, A. rostellifera, A. cochlearis and C. preissii; and two plantations and adjacent native tree species close to Denmark on the south coast of WA, with samples taken from E. globulus, E. marginata and E. diversicolor. Samples, including type cultures, were also obtained from VPRI (Victorian Department of Primary Industries) and from CMW (collection of the Forestry and Agriculture Biotechnology Institute, University of Pretoria, South Africa) in Victoria, New South Wales and South Australia.

World-wide distribution

The majority of the N. australe isolates, including those used in the original descriptions (CMW9073, CMW9072, CMW6837 and CMW6853) can be separated from N. luteum (CMW9076/ICP7818 and CMW10309/CAP002) by five fixed polymorphisms in the ITSrDNA region. A smaller subset of N. australe isolates can only be separated from N. luteum by three to four of these polymorphisms. These polymorphisms were used to assist in identification of N. australe sequence from GenBank (Table 1).

Molecular identification

Molecular identification was used to confirm identification of isolates to species level. Cultures were grown on half-strength Potato dextrose agar plates for approximately 1 week at 20°C. The mycelial mass was harvested and

Table 1 Single nucleotide polymorphisms used to identify the individual haplotypes of Neofusicoccum australe haplotypes. The closely related species N. luteum is included for comparison

Haplotype	Single Nucleotide Polymorphisms																									No. of isolates	Location	Representative GenBank Number
	16	17	22	23	36	82	113	119	128	201	400	404	462	463	464	467	485	488	490	491	525	526	527					
H1	G	–	G	A	–	C	C	–	C	C	C	C	–	–	G	A	G	T	G	C	T	–	C	152	Australia, Chile, New Zealand, Portugal, South Africa, Spain, USA	AY339260
H2	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	USA	GQ857662
H3	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	USA	AF464949, AF464950
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	29	Australia, Italy, South Africa and Spain	EF638770
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	3	Australia	JF323951, JF323952 and JF323953
H6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	3	Australia	EF591891, EF591893 and EF591902
H7	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C		.	.	.	.	3	South Africa	EF445359, EF445356 and AY343387
H8	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	1	South Africa	FJ752745
H9	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	C	.	.	.	.	.	2	South Africa	FJ752744, FJ752743
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	3	China and Japan	AB454293, AB041245 and EU442284
H11	.	.	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	2	USA	DQ008323 and DQ233610
H12	.	.	.	.	.	.	T	.	T	.	T	.	.	.	.	.	.	C	.	.	.	.	.	3	China	FJ037758, FJ44165 and FJ441624
H13	.	.	.	.	.	.	.	.	.	.	.	.	T	G	A	C	A	T	A	A	T	.	.	2	Australia	EF591895 and EF591906
H14	–	G	.	.	.	.	.	G	.	.	T	T	.	.	.	.	.	C	.	.	.	.	.	2	New Zealand	N. luteum (AY236946)

placed into 1.5 mL sterile eppendorf tubes and freeze dried. A modified method from Graham et al. (1994) was used to extract DNA as described in Andjic et al. (2007), except DNA was suspended in 50 µL purified water.

A part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified for all isolates in 96-well plates using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The region was amplified using the following thermal cycling conditions 94°C for 2 min (initial denaturation) then 35 cycles of 94°C for 30 s (denaturation), 50–55°C for 45 s (primer annealing) and 72°C for 1 min (elongation) and 72°C for 5 min (extension). Purification of PCR reactions was undertaken according to the manufactures instructions using ExoSap. Except 96-well plates were initially incubated for 30 min on ice and then in the final step, to remove liquid from the wells, instead of spinning plates in a speed-vac they were left overnight in a laminar flow (lights off) or incubated at 60°C (without lid). They were sequenced as described by Sakalidis et al. (2011a). Identity was confirmed by phylogenetic analysis using the program PAUP 4.0b10 (Swofford 2003) as described previously (Sakalidis et al. 2011c).

Haplotype networks were constructed for the ITS data set using TCS version 1.13 (Clement et al. 2000). The program collapses DNA sequences into haplotypes and calculates the frequencies of haplotypes in the sample which are then used to estimate haplotype out-group probabilities, that correlate with haplotype age (Donnelly and Tavarè 1986; Castelloe and Templeton 1994) and then calculates an absolute distance matrix from which it estimates phylogenetic networks using a probability of parsimony, until the probability exceeds 0.95 (Templeton et al. 1992).

SSR primer development, amplification and visualisation

Four polymorphic markers (BotF11, BotF15b, BotF19 and BotF35) designed for Neofusicoccum parvum- N. ribis were also polymorphic among isolates of N. australe (Slippers et al. 2004). An additional polymorphic primer pair was developed from DNA of N. australe using the RAMS (random amplified microsatellites) sequencing, cloning and genome walking methods described by Burgess et al. (2001).

Five polymorphic loci that contain SSR (single sequence repeat) sequences were used to amplify DNA from N. australe isolates The reaction mixture contained on average 16.4 µL of Ultrapure PCR grade water, 2.5 µL of PCR buffer, 2.5 µL of dNTP's, 1 µL of Mg^{2+}, 0.5 µL of each forward primer and 0.5 µL of each reverse primer and containing 1.5 ng of the DNA template. 1 U of fast start Taq DNA polymerase (Roche Molecular Biochemicals, Almeda, California) was added to each tube individually. Reactions were run on a program of 95°C for 2 min then 10 cycles of: 95°C for 30 s, 50–54°C for 45 s, 72°C for 1 min, then 30 cycles of: 95°C for 30 s, 50–54°C for 45 s, 72°C for 1 min, with a final extension of 60°C for 40 min (this final extension reduces stutter in the microsatellite peaks) then held at 4°C. All primer pairs were run at these conditions with the following exceptions; Expand High™ Fidelity Taq polymerase (Roche Biochemicals) and Expand PCR buffer (2 mM Tris-HCl, pH 7.5; 1.5 mM $MgCl_2$; 10 mM KCl) were used in some cases.

Amplified products that exhibited non-overlapping base pair sizes were combined into one group. A dilution was made to an approximate concentration of 0.2 ng of product/µl of each amplified product. One µl of diluted product was added to 2 ml of formamide containing a LIZ-labelled GeneScan-500 size standard (Applied Biosystems) (14 µl LIZ ml^{-1} formamide). Products were diluted to approximately 0.2 ng of product/µl. These were then run on an ABI PRISM 3100 autosequencer (Perkin-Elmer Applied Biosystems). Data analyses were conducted with Genemarker software (SoftGenetics LCC).

Gene diversity

The frequency of each allele at each locus was calculated using the program Arlequin (Excoffier et al. 2005) using the data matrix generated in the distance analysis. For each isolate, a data matrix of multistate characters was compiled by assigning each allele at each of the five loci, a different letter (eg. EDBACLA) and gene diversity determined, using the program POPGENE (Yeh et al. 1999) and the equation $H = 1 - \sum x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973).

Distance analysis

The distance matrix between isolates of N. australe based upon total nucleotide length of each allele at each of the five loci was calculated based on absolute distance (D_{AD}) using ARLEQUIN (Excoffier et al. 2005). The total nucleotide length of alleles was used for these analyses, because the variation was due to both changes in micro-satellite repeats and the flanking sequence. The tree file was used to produce an unrooted tree in Treeview (Page 1996).

Population differentiation and gene flow

To assess whether isolates from different hosts and different areas could be combined Chi-square tests for differences in allele frequencies were calculated for each locus across clone corrected populations (Workman and Niswander 1970). Population differentiation, theta (θ) was calculated

for clone corrected populations in Multilocus (Agapow and Burt 2000) using an estimate of Wright's F_{ST}, as $q = Q - q/1 - q$ where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Burt et al. 1997; Weir 1997). Two populations with the same allele frequencies ($\theta = 0$) are considered to be identical whereas populations sharing no alleles ($\theta = 1$) are considered completely isolated. The statistical significance of θ was determined by comparing the observed value to that of 1,000 randomized datasets in which individuals were randomized among populations. A significant p value ($p < 0.05$) means that the null hypothesis of no population differentiation can be rejected. Theta can be determined even when there are few genotypes; however, the value will need to be higher to be statistically significant. Gene flow (M) or the number of migrants exchanged between populations in each generation will give the observed θ value, where $M = [(1/q) - 1)]/2$ (Cockerham and Weir 1993).

Mode of reproduction

The index of association (I_A) was used to measure multilocus linkage disequilibrium for each population and for all populations combined (Maynard Smith et al. 1993). I_A was calculated for the clone corrected population containing only one representative of each genotype. The tests were performed on a data matrix of five multistate characters using the program Multilocus (Agapow and Burt 2000). The expected data for 1,000 randomly recombining data sets were calculated and compared with the observed data. Where the observed data fall within the distribution range of the recombined data, then the hypothesis that the population was undergoing recombination cannot be rejected. If the observed data fall outside the distribution range with a significant p value ($p < 0.05$), the population is most likely clonal.

Results

Primer development

One primer pair was developed from the RAMS method; forward primer: MS1F GGA GAA ACA ATC ACG AAA AAG GG and reverse primer: MS1R GTG AAA GCC GGC ACC CCG AAC, repeat motif and length $(TCA)_{28}$.

Phylogenetic diversity and word-wide distribution

GenBank blast search of the ITS sequence of N. australe led to 135 sequence deposits identified as N. australe. Isolates were identified on GenBank as B. australis, B. dothidea, Botryospheria larincina, B. ribis, Guignardia larincina, F. luteum and N. australe. GenBank and literature searches indicated N. australe is present in six continents (Africa, Australia, Asia, Europe, North and South America) across ten countries (Australia, Chile, China, Japan, Italy, New Zealand, Portugal, South Africa, Spain and USA), 46 host species and 18 host families (Anacardiaceae, Araucariaceae, Arecaceae, Cupressaceae, Casuarinaceae, Ericaceae, Elaeocarpaceae, Fabaceae, Lauraceae, Myrtaceae, Oleaceae, Proteaceae, Pinaceae, Rhizophoraceae, Rosaceae, Salicaceae, Santalaceae and Vitaceae). Most reports of N. australe have come from isolations made from diseased material in horticultural crops, some hardwood plantation trees and diseased and asymptomatic native trees.

Of the 251 N. australe isolates (135 from GenBank and 116 from this study), 45 were excluded from the tree spanning network analysis as these isolates were missing informative fixed polymorphisms at the start or the end of the sequence. The final ITSrDNA dataset contained 208 isolates (including two N. luteum isolates). The alignment contained 545 characters of which 23 characters were informative. Intraspecific variation in ITS sequence data resulted in nine linked haplotypes (H1-9) and four haplotypes (H10-13) that could not be included in the network (H13 corresponded to N. luteum) (Fig. 1). H1 contained the majority of isolates (152) collected from Australia, Chile, New Zealand, Portugal, South Africa, Spain and USA. Most isolates fell within H1, including the type of N. australe (CMW9072). H4 contained 29 isolates; these included members from Australia, Italy, South Africa and Spain collected from 11 host species (one representative GenBank number is given for each host): Elaeocarpus holopetalus (VPRI32666A), Corymbia citriodora (FJ752740), Eucalyptus sp. (FJ752741), Olea europaea (EF638778), P. vera (EU375516), Pistacia sp. (AF293479), Prunus salicina (EF445354), Syzygium cordatum

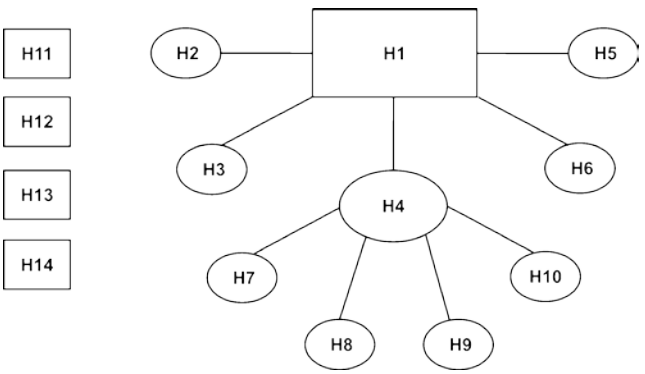

Fig. 1 Haplotype network depicting the relationship generated from sequence data of the ITSrDNA region of 206 Neofusicoccum australe isolates and two Neofusicoccum luteum isolates

(DQ316087), Vaccinium corymbosum (VPRI32789), Vitis vinifera (AY343407) and Wollemia nobilis (AY615165). No isolates in H4 came from Western Australia. All other haplotypes contained one to three isolates from one to two locations. Haplotypes 11–13 broke the network connection and may represent new species. H14 contained the two N. luteum isolates (CMW992 and CMW9076).

Within Australia, N. australe has been collected from 22 host species encompassing Haplotypes 1, 4, 5, 6 and 13; all these haplotypes except for H4 were found in Western Australia: Acacia cochlearis, Acacia mearnsii, Acacia rostellifera, Acacia sp., Agonis flexuosa, Allocasuarina fraseriana, B. coccinea, B. grandis, C. preissii, E. holopetalus, E. diversicolor, E. globulus, E. gomphocephala, E. marginata, E. phylacis, Phoenix canariensis, Salix sp., Santulum acuminatum, Sequoiadendron giganteum, Vaccinium corymbosum, Vitis vinifera, and W. nobilis (Shearer et al. 1995; Slippers et al. 2004, 2005; Burgess et al. 2005, 2006; Cunnington et al. 2007; Taylor et al. 2009; Pitt et al. 2010).

Within Asia, N. australe has been reported four times from China, from three host species; a Larix sp. (EU442284; H10), Bruguiera gymnorhiza (FJ441615 and FJ441624; H12) (these records are only linked to sequence data available from GenBank) and Sonneratia apetala (a mangrove sp.) (FJ037758; H12) (Xu et al. 2011). Within Japan, N. australe has been reported twice from Japan from Larix kaempferi (Motohashi et al. 2009) and an unknown host (Okane et al. 2001) (both H10).

Within Chile, N. australe (H1) has been reported twice on GenBank from V. corymbosum (Espinoza et al. 2009; Inderbitzin et al. 2010).

Within Europe, N. australe has been reported ten times on GenBank from three hosts from Italy (H4); Vitus sp. (HQ011406), O. europaea (Lazzizera et al. 2008) and Pistacia sp. (Smith and Stanosz 2001). N. australe has been reported twice in Portugal (H1) on Robinia pseudoacacia (van Niekerk et al. 2004) and a Rubus sp. (Phillips et al. 2006). N. australe has been reported four times from Spain from P. vera (H4) and E. globulus (H1) (Armengol et al. 2008) and P. cynaroides and a Protea sp. (both H1) (Marincowitz et al. 2008).

Within New Zealand N. australe has been reported six times on GenBank. From two hosts (H1); V. vinifera and Cytisus scoparius (Amponsah et al. 2009).

Within South Africa, N. australe has been reported 51 times on GenBank from 13 hosts: C. citriodora (H4: FJ752740 and H8: FJ752745), Eucalyptus dorrigoensis (H9: FJ752743), Eucalyptus saligna (H9: FJ752744), a Eucalyptus sp. (H4: FJ752741 and FJ752742), Malus domestica (H1) (Slippers et al. 2007), Prunus ameniaca (H1 and 7) (Damm et al. 2007), Prunus dulcis (H1) (Slippers et al. 2007), Prunus persica (H1) (Damm et al. 2007), P. salicina (H4) (Damm et al. 2007), (H1) (Slippers et al. 2007), Pyrus communis (H1) (Slippers et al. 2007), S. cordatum (H1 and 4) (Pavlic et al. 2007), V. vinifera (H1, 4 and 7) (van Niekerk et al. 2004) and Widdringtonia nodiflora (H1) (Slippers et al. 2005).

Within the USA, N. australe has been reported seven times; from Persea americana (H1 and 2) (McDonald et al. 2009), Sequoia sp. (H1 and 3) (Ma and Michailides 2002) and V. vinifera (H11) (Úrbez-Torres et al. 2006).

Gene diversity

The five primer pairs produced 54 alleles across five loci; amplifying fragments ranging from 202 to 468 bp. All loci were polymorphic. Of the 85 isolates collected there were a total of 72 multilocus haplotypes.

Distance analysis

Distance analysis separated isolates into two broad groups. Each group contained numerous clusters. Genotypes sampled from different hosts and different locations were grouped together in the distance analysis. There was no distinguishable pattern grouping together genotypes from the same host species or the same location. Genotypes from Western and eastern Australia were mixed throughout the distance tree (Fig. 2).

Population differentiation and gene flow

Chi squared tests comparing different hosts in each WA study site from Denmark (DEN), Woodman's Point (WP) and Yalgorup (YAL) indicated there were no significant differences in allele frequency between hosts within each site, therefore isolates from different hosts were pooled at each site (data not shown). Chi squared tests comparing the three WA sites indicated that samples from WP and YAL were not significantly different except at Botf19 ($\chi^2 = 4$, $p > 0.05$) and this dataset was pooled for future analyses. The DEN population was significantly different to the YAL and WP populations at three loci and five loci, respectively. Chi squared tests comparing isolates collected from NSW and Victoria (including one sample from South Australia) indicated that there were no significant differences in allele frequency between isolates in these regions. The pooled eastern Australian (EA) dataset was compared to the DEN population and the pooled YAL-WP dataset and was significantly different across all loci (Table 2).

The mean total gene diversity across all loci was 0.546. A higher gene diversity was exhibited by DEN population

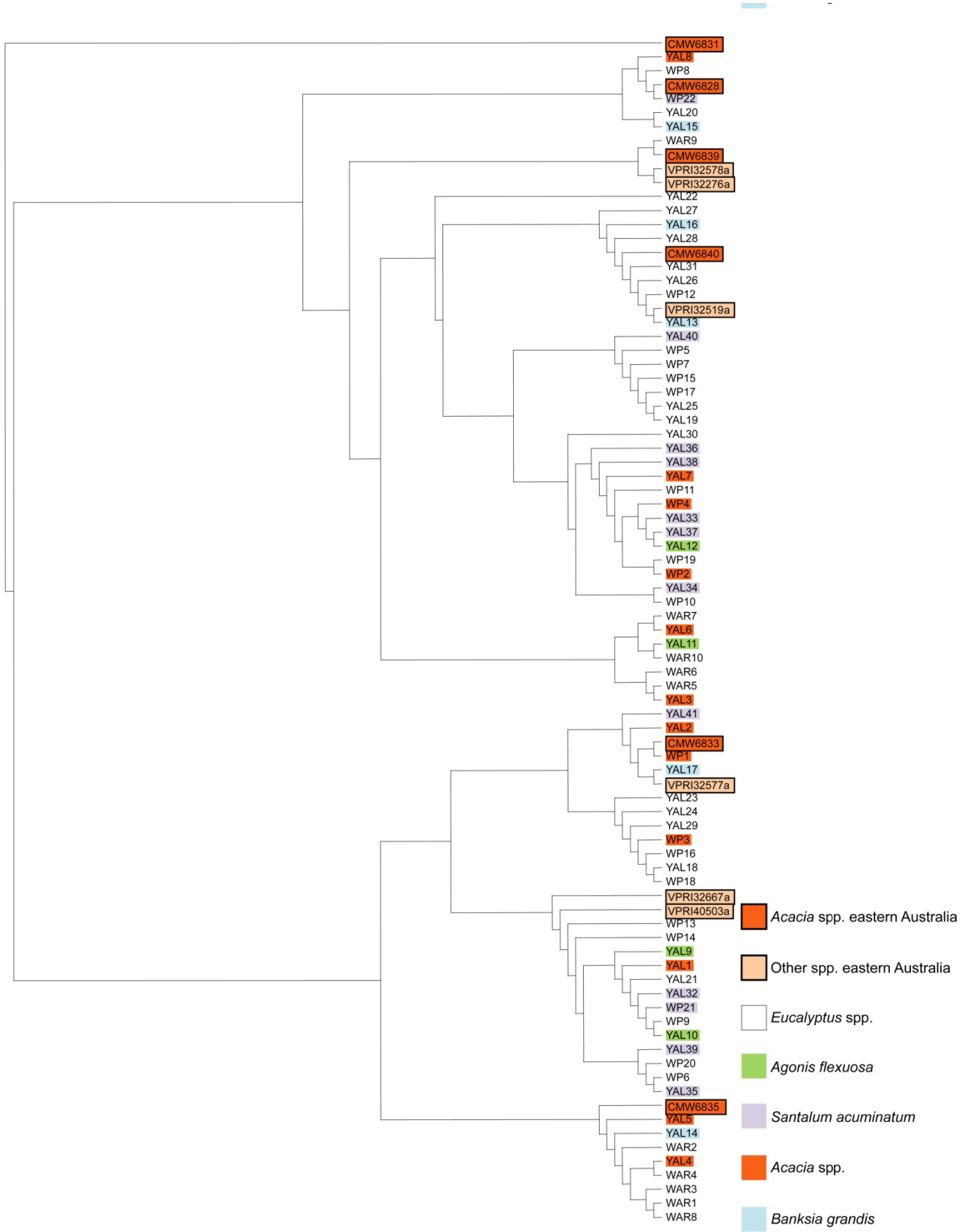

Fig. 2 Distance tree of *Neofusicoccum australe* isolates from eastem Australia (ClvfW or VPRI codes) and Wesrem Australia (WP-W>odman's Point, W\R.- Wame and YAL-Yalgorup)

Table 2 Contingency χ^2 tests for differences in allele frequencies for five polymorphic loci across clone corrected populations of Neofusicoccum australe from Yalgorup (YAL), Woodman's Point (WP), Denmark (DEN), Yalgorup and Woodman's Point combined (YALWP), New South Wales, Victoria and South Australia (NSW: VICSA) and eastern Australia combined (EA)

Locus	YAL:WP		YAL:DEN		WP:DEN		YALWP:DEN		NSW:VICSA		YALWA:DEN:EA	
	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2	df
BotF11	17.5^{NS}	15	33.4^{NS}	15	27*	10	43.5*	17	7.2^{NS}	6	82.2*	40
BotF15b	3.9^{NS}	3	14.1*	4	8.4*	3	18.7*	4	5.6^{NS}	2	23.2*	8
BotF19	4*	1	35*	3	23*	3	45*	3	0.8^{NS}	1	48*	6
BotF35	3.7^{NS}	4	20.1*	5	15.4*	4	27*	5	6.7^{NS}	4	44*	12
MS1	13.3^{NS}	16	26^{NS}	19	17.2*	8	32.5*	20	4.3^{NS}	4	53.4*	42

*=p<0.001, NS=p>0.05

(0.603). Lower gene diversities were exhibited by all other populations (Table 3). The small sample number of the populations means that gene diversity values must be treated with caution as they would probably be an underestimate of the true gene diversity present in these populations.

Theta values (θ) between all populations were significant ($p<0.05$- $p<0.01$) indicating high levels of population differentiation and low gene flow between all populations (Table 4). The level of significance ($p<0.001$) was higher comparing Warne to all other populations. The level of significance ($p<0.001$) was also higher comparing the combined YALWP to all other populations rather then individual YAL and WP populations to all others ($p<0.05$) (apart from comparisons to Warne which was always at a level of significance of $p<0.001$).

Mode of reproduction

The I_A of the observed data of individual populations fell within the values produced from the randomized data sets (p-value >0.05) (Table 5). Therefore, the null hypothesis that alleles are freely recombining could not be rejected, suggesting sexual reproduction in all populations.

Discussion

N. australe is commonly reported in association with its pathogenic and endophytic lifestage; with a widespread distribution found across ten countries and 46 hosts from 18 plant families; mainly angiosperms, some conifer species and one monocot. N. australe is widespread in the native forests in the southwest of WA; with limited reports of it occurring in native trees in eastern Australia, South Africa and China. N. australe is most commonly reported as a pathogen of various horticultural crops; its association with grapevine decline is particularly well documented. Phylogenetic analysis of the ITSrDNA sequence indicates there is a single dominant ITS haplotype and another 12 rare or moderately rare haplotypes. Based on microsatellite data, native populations of N. australe in Australia appear to be highly diverse, and there is no discernable host or habitat restriction.

Within Australia, reports of N. australe on native tree species were mainly restricted to the southwest of WA and these were mostly linked to studies attempting to determine the cause of specific native tree species decline (Shearer et al. 1995; Dakin et al. 2009; Taylor et al. 2009) or linking geneflow of N. australe between native trees and adjacent

Table 3 Gene diversity (H) frequencies for five polymorphic loci across clone corrected populations of Neofusicoccum australe from Yalgorup (YAL), Woodman's Point (WP), Denmark (DEN), Yalgorup and Woodman's Point combined (YALWP) and eastern Australia (EA). Numbers in superscript indicate standard deviation

Locus	Gene Diversity					No. of Alleles
	YAL	WP	DEN	YALWP	EA	
BotF11	0.864	0.761	0.370	0.872	0.800	20
BotF15b	0.218	0.408	0.469	0.310	0.180	4
BotF19	0.332	0.000	0.667	0.264	0.000	3
BotF35	0.304	0.000	0.719	0.245	0.694	6
MS1	0.820	0.636	0.790	0.795	0.722	21
N (alleles)	37	15	18	40	18	
MEAN	0.508	0.36	0.603	0.498	0.479	

Table 4 Pairwise comparisons of population differentiation (θ) (above the diagonal) and gene flow (below the diagonal) among Neofusicoccum australe populations from Yalgorup (YAL), Woodman's Point (WP), Denmark (DEN), Yalgorup and Woodman's Point combined (YALWP) and eastern Australia (EA). Values obtained are for clone corrected populations

	YAL	WP	DEN	YALWP	EA
YAL	–	0.032*	0.208***	NA	0.062*
WP	15.125	–	0.337***	NA	0.120*
DEN	1.904	0.984	–	0.234***	0.169***
YALWP	NA	NA	1.637	–	0.066***
EA	7.564	3.667	2.458	7.076	–

*=p<0.05, ***=p<0.001

plantation tree species (Burgess et al. 2005, 2006). In these studies, N. australe was the dominant species collected (up to 95% of isolates obtained). Other reports of N. australe in WA have been from Eucalyptus plantations (Burgess et al. 2005, 2006) and grapevines (Taylor et al. 2005). N. australe has been collected sporadically from hosts in eastern Australia; most reports were linked to ornamental or horticultural settings in Victoria (Slippers et al. 2004; Cunnington et al. 2007), Canberra (Slippers et al. 2004), New South Wales (Slippers et al. 2004; Pitt et al. 2010) and South Australia (Pitt et al. 2010), whilst N. australe has been collected from native hosts in only four instances, all from Acacia spp. (Slippers et al. 2004). Extensive sampling from native hosts in QLD, northern NSW (Burgess unpublished) and Tasmania (Burgess et al. 2006) did not uncover any additional isolates of N. australe. The dominance of N. australe in WA and the sporadic reports of this species in eastern Australia suggest that N. australe is endemic to Western Australia and introduced to eastern Australia.

N. australe has been reported on native hosts in South Africa and China. In these surveys N. australe was not the dominant Botryosphaeriaceae endophyte collected. N. australe accounted for 5% of Botryosphaeriaceae isolated from endemic S. cordatum populations in South Africa (Pavlic et al. 2007). In China, N. australe was found from two mangrove species (GenBank record, Xu et al. 2011). Other studies of Botryosphaeriaceae associated with native or naturalised hosts have been undertaken in Cameroon, Madagascar and South Africa (Begoude et al. 2010) and Uruguay (Pérez et al. 2010) but N. australe was not recorded in these studies. This suggests that N. australe is not part of the endemic microflora of native tree species in these locations.

Table 5 Index of association for individual Neofusicoccum australe data sets from Yalgorup (YAL), Woodman's Point (WP), Denmark (DEN), Yalgorup and Woodman's Point combined (YALWP) and eastern Australia (EA). Values obtained are for clone corrected populations

	Observed (original dataset)	Range (randomized datasets)	p-value
YAL	0.117	−0.310–0.460	0.153
WP	−0.164	−0.292–0.452	0.94
DEN	−0.287	−0.661–0.771	0.898
YALWP	0.036	−0.268–0.356	0.363
EA	0.107	−0.495–0.789	0.344

The wide variety of hosts available to N. australe is typical of many members of Botryosphaeriaceae, which, except for a few host specific species like Neofusicoccum pennatisporum (Taylor et al. 2009) and Fusicoccum ramosum (Pavlic et al. 2008) tend to be opportunistic endophytes/pathogens, which are not limited by the availability of specific hosts, instead colonising the plethora of plant species that may exist in a forest, garden, or horticultural setting. N. australe has been collected from 18 plant families across ten countries. Most isolations came from dicotyledonous angiosperm species, but in Australia, there was one record causing disease of a monocot palm (Cunnington et al. 2007), and the gymnosperms S. giganteum (Slippers et al. 2004) and W. nobilis (Slippers et al. 2005). N. australe was also isolated from W. nodiflora in South Africa (Slippers et al. 2005), and a Sequoia sp. in the USA (Ma and Michailides 2002). N. australe was commonly reported from horticultural crops, these included ten fruit (apple, apricot, avocado, blackberry, blueberry, grapevine, Japanese plum, peach and pear) and one nut (almond) hosts. The presence of N. australe in such a wide diversity of hosts confirms it as an opportunistic fungal species that can potentially colonise most woody plants species that it comes into contact with.

Most host species were recorded from a single location, with the exception of Vaccinium corymbosum and Vitis vinifera (grapevine). Collections were made from V. corymbosum in Australia (Cunnington et al. 2007) and Chile (Inderbitzin et al. 2010; Espinoza et al. 2009). Whilst, V. vinifera was recorded as a host of N. australe in Australia (Taylor et al. 2005; Cunnington et al. 2007; Pitt et al. 2010), Italy (HQ011406), New Zealand (Amponsah et al. 2009; 2011), South Africa (van Niekerk et al. 2004), Spain (Martos et al. 2011) and USA (Úrbez-Torres et al. 2006). There are also reports of N. australe occurring on grapevines in Mexico; however, sequence data are not currently publically available for this (Candolfi-Arballo et al. 2010). The presence of N. australe amongst grapevine in multiple locations suggests that this species has a significant endophytic presence and escapes detection in quarantine efforts during grapevine germplasm exchange.

Native and exotic populations of N. australe in Australia appear to be highly diverse. There was limited geneflow between sample sites in WA and between WA and eastern Australia. This may reflect the distance between the sites (between sites in WA and between WA and eastern Australian sites), but also it seems unlikely that the native plants from the isolated WA sites would come into contact with the disparate collection of eastern Australian exotic plants (collected from ornamental gardens and horticultural crops). The index of association indicated that all populations were sexually reproducing, this may account for the high genotypic diversity. Distance analysis found no detectable pattern between hosts. This supports the general consensus that N. australe is a host generalist. There was also a high genetic diversity within and between each site. The genetic diversity reflected within each site could be attributed to the lifestyle of endophytic Botryosphaeriaceae. As Class III endophytes they inhabit small localised pockets of their host (Müller et al. 2001; Rodriguez et al. 2009) and multiple genotypes can be found within a single host (Müller et al. 2001). Therefore amongst numerous hosts the overall genetic diversity would be expected to be high.

The variation among the ITS haplotypes within N. australe represent fixed mutations which most likely occurred post-introduction. This has been seen amongst other fungi such as Venturia inaequalis (scab disease of apple), populations of V. inaequalis approximately correspond to geographical location and differ from the endemic population range of China (Gladieux et al. 2008). Each population of V. inaequalis reflected a level of genetic diversity that is indicative of multiple introductions and post-introduction increases in population size (Gladieux et al. 2008). In comparison, amongst N. australe a single dominant haplotype was found in all countries except for China, Japan and Italy. One moderately rare haplotype was found in Australia, Italy, South Africa and Spain. This haplotype was the only haplotype identified in Italy. The 11 rare haplotypes (three or fewer isolates) were restricted in range to one or two countries. The existence of rare haplotypes in China, Japan, USA as well as South Africa and WA suggests that N. australe populations have been present in these locations for a long time. The variation seen amongst N. australe isolates suggests an expansion of introduced N. australe populations, a subsequent accumulation of fixed mutations within these populations and local environment as a significant driver of speciation, rather than a specific host.

The genetic variation apparent amongst the ITSrDNA region in N. australe suggests transfer of germplasm from WA to elsewhere in Australia and other countries on native plants (including Proteaceae species) and horticultural crops (possibly grape and fruit species). N. australe may then have moved from these exotic hosts into native hosts in China, eastern Australia and South Africa. The occurrence of N. australe in endemic S. cordatum populations may reflect gene flow between native and introduced plant species such as Eucalyptus spp. This has occurred between N. australe, native tree species and exotic eucalypt plantations within WA (Burgess et al. 2006). Overall, due to the low number of samples available from many countries it is possible that the membership of rare haplotypes is underestimated. More studies intensively sampling native hosts in other regions would be useful.

N. australe appears to be a genetically diverse species with a geographical distribution and life-strategy controlled by environmental factors. The occurrence of rare haplotypes within specific geographical regions provides support for the environment limiting rather than host(s) limiting the factors which drive speciation. N. australe may harbour multiple cryptic species; further sequencing of additional gene regions are required to confirm this. Microsatellite analysis indicates populations of N. australe within Australia appear to be sexually reproducing. N. australe poses a significant threat to horticultural, plantation, ornamental and native tree species. Management strategies reducing host stress should be utilised to combat N. australe becoming pathogenic and causing disease. Little is known about the interaction of multiple species of Botryosphaeriaceae within one host; further research should focus on multiple infections and the mechanism that triggers the switch from endophytic to pathogenic lifestyle.

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