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Genetic population structure of the ectomycorrhizal fungus *Pisolithus microcarpus* suggests high gene flow in south-eastern Australia

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Abstract *Pisolithus* are ectomycorrhizal fungi that associate with roots of numerous plant species in natural and plantation forests worldwide. Despite the fact that *Pisolithus* spp. are present in plantation forests in many countries, knowledge of the genetic population structure of *Pisolithus* spp. remains limited. In this study, we have tested the hypothesis that a propensity for long-distance spore dispersal in *Pisolithus microcarpus*, along with the widespread distribution of potential eucalypt and acacia plant hosts in south-eastern Australia facilitates gene flow that limits population differentiation. Five polymorphic simple sequence repeat markers were used to investigate the population structure of *P. microcarpus*. Isolates were grouped according to geographical origin and isolate genotypes were analysed among the geographical populations. Pairwise F_{ST} estimates indicated limited genetic differentiation among the geographical populations. Analysis of molecular variance revealed that most of the genetic variation present was within geographical populations, with only 1.3% of the genetic variation among *P. microcarpus* geographical populations. This was particularly pronounced for four geographical populations within a ca 7,000 km^2 area New South Wales, which were each separated by <100 km and appeared to be genetically homogeneous. The lack of population structure is suggested to be due to a high degree of gene flow, via basidiospores, between the New South Wales geographical populations.

Keywords *Pisolithus* · Ectomycorrhiza · SSR markers · Microsatellites · Genetic structure · Spore dispersal

Introduction

Aerial basidiospores are important in the dispersal of epigeous basidiomycetes, and their patterns of dispersal are a significant factor in shaping populations of these fungi (James and Vilgalys 2001). While most basidiospores are deposited within tens of metres of the parent basidiome, for certain wood-decay taxa at least, some may be dispersed over tens or even hundreds of kilometres (Rishbeth 1959; Kallio 1970; Stenlid and Gustafsson 2001). There is thus the potential for long-distance gene flow that may limit population differentiation on a large geographical scale. Despite this, there appears to be limited intercontinental gene flow in decomposer basidiomycetes, with populations of taxa such as *Phlebiopsis gigantea* or *Schizophyllum commune* displaying differentiation between continents (James et al. 1999; Vainio and Hantula 2000). At regional scales, however, the potential for long-distance gene flow appears to be realised for decomposers and pathogens such as *Armillaria ostoyae*, *Fomitopsis* spp., *Phlebia centrifuga* or *S. commune*, with little or no genetic differentiation being observed over scales of several hundred kilometres (Högberg et al. 1999; James et al. 1999; Kauserud and Schumacher 2003; Franzén et al. 2007; Prospero et al. 2008). For some taxa, this is dependent upon the availability of continuous suitable habitat, and there is evidence for local genetic differentiation in populations where habitat is fragmented, despite the ability for long-

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distance spore dispersal (Franzén et al. 2007; Högberg and Stenlid 1999; Parrent et al. 2004).

Ectomycorrhizal (ECM) fungi form mutualistic associations with forest trees and are important drivers of forest carbon and nutrient cycles (Smith and Read 1997). Although investigations of ECM fungal populations have focused largely on genet size and distribution in local populations (Fiore-Donno and Martin 2001; Kretzer et al. 2003), there is now increasing emphasis on understanding genetic differentiation and gene flow within and between populations of ECM fungi. Few genetic population structure investigations have been performed on ECM fungal species and most of these have been conducted over a relatively small geographical scale (<20 km). The structure of, for example, *Cantharellus formosus*, *Rhizopogon vinicolor*, *R. vesiculosis*, *R. occidentalis* and *R. vulgaris* populations (Dunham et al. 2006; Kretzer et al. 2005; Grubisha et al. 2007) has been examined using simple sequence repeat (SSR) markers, with evidence for genetic differentiation having been detected. In contrast, other studies at similar geographical scales have found little evidence for genetic differentiation between populations of some ECM taxa, such as *Suillus grevillei*, *Russula brevipes* and *Tricholoma matsutake* (Zhou et al. 2001; Bergemann et al. 2006; Lian et al. 2006). In these investigations, the absence of genetic differentiation was attributed to gene flow via basidiospore dispersal.

On a larger geographical scale, there is evidence of considerable genetic variation in some taxa, notably *Tuber melanosporum* and *Tricholoma scalpturatum* (Murat et al. 2004; Carriconde et al. 2008). Bergemann and Miller (2002) also identified genetic differentiation between populations of *R. brevipes* located 1,500 km apart in North America. Since the populations shared only two alleles, however, they suggested that the populations may, in fact, represent cryptic species. More recently, Roy et al. (2008) found little evidence of population structure in *Laccaria amethystina* over ca 500 km based on a combination of marker sets, including SSR loci. Similarly, single nucleotide polymorphisms revealed low, but significant, differentiation in populations of *T. matsutake* in south western China separated by ca 80–1,000 km (Xu et al. 2008). In both of these investigations, the low level of genetic differentiation was taken to indicate that long-distance gene flow, mediated by basidiospore dispersal, occurs between the populations (Roy et al. 2008; Xu et al. 2008).

Pisolithus spp. are ECM basidiomycetes that occur globally and have been used for decades in forestry inoculation programmes (Marx et al. 1977; Garbaye et al. 1988). Recent work has revealed the existence of multiple *Pisolithus* species worldwide, with strong relationships being identified between these and the native geographical origins of their plant hosts (Martin et al. 2002). Although now distributed with plantation eucalypts worldwide, *P. microcarpus* is thus considered to be a native Australian taxon (Martin et al. 2002; Hitchcock et al. 2003). The fungus is regarded as forming mycorrhizal associations with a broad range of eucalypts and acacias throughout Australia (Martin et al. 2002; Hitchcock et al. 2003) and, in common with other *Pisolithus* taxa, produces large, earthball type basidiomes that release large quantities of basidiospores over weeks or months (Fuhrer 1985). Based on rDNA internal-transcribed spacer (ITS) sequence analysis, three *Pisolithus* species that associate with *Kunzea ericoides* in New Zealand geothermal areas appear to be conspecific with Australian *P. albus*, *P. microcarpus* and *P. marmoratus*, and it seems likely that these were introduced to New Zealand by long-distance basidiospore dispersal across the Tasman Sea from Australia (Moyersoen et al. 2003).

Despite the likelihood of long-distance basidiospore dispersal, investigations of *Pisolithus* populations to date have considered only localised genet distribution and persistence based on inter-simple sequence repeat (ISSR) PCR (Anderson et al. 1998b, 2001a). In the present study, we used SSR markers to investigate the genetic structure of *P. microcarpus* populations in eastern Australia. Specifically, we have tested the hypothesis that a propensity for long-distance spore dispersal in these taxa, along with the widespread distribution of potential eucalypt and acacia plant hosts in eastern Australia, facilitates gene flow that limits population differentiation.

Materials and methods

Collection and maintenance of fungal isolates

Basidiomes of *P. microcarpus* were collected from various locations in south-eastern Australia (Table 1). Axenic cultures were established for 29 *Pisolithus* basidiomes collected in Ku-ring-gai Chase National Park during June–July 2003, along with four basidiomes from different locations in New South Wales (NSW) collected during 2002–2003. These isolates, together with 28 isolates from various locations in south-eastern Australia, which were collected and isolated as described in Anderson et al. (1998a, 2001a), were maintained on MMN agar at 20°C in the dark and sub-cultured every 6 to 8 weeks. A further 53 isolates, from which DNA had previously been extracted (Anderson 1996; Anderson et al. 1998a, b, 2001b) were also included in the study.

DNA extraction, SSR isolation and genotyping

DNA extraction was performed using the modified CTAB method of Gardes and Bruns (1993). ITS-PCR and RFLP

Table 1 Details of the *P. microcarpus* isolates included in the current study

Map location	Abbreviated names	Geographical location	Geocode	Year collected	Area sampled (km^2)	Number of isolates
1	BMNP	Blue Mountains National Park, NSW	33°43′S, 150°26′E	1996	5	6
2	KCNP	Ku-ring-gai Chase National Park, NSW	33°37′S, 151°16′E	1996/2003	50	37
3	RNP	Royal National Park, NSW	34°07′S, 151°03′E	1996	120	18
4	NWLB	Nth Wilberforce, NSW	33°33′S, 150°51′E	1996/2000	15	32
5	VIC	Warrandyte, VIC	37°44′S, 145°13′E	2000	150	8
–	–	Ballina, NSW	28°47′S, 153°34′E	2000		1
–	–	NSW	Unknown	Unknown		1
–	–	Brisbane Water National Park, NSW	33°27′S, 151°18′E	2002		1
–	–	Uraidla, SA	34°57′S, 138°44′E	2000		2
–	–	Ted Horwood Reserve, Baulkham Hills, NSW	33°45′S, 150°00′E	1996		5
–	–	Wollemi National Park, NSW	32°59′S, 150°20′E	2003		1

Geographical population 1 = Blue Mountains National Park (*BMNP*), 2 = Ku-ring-gai Chase National Park (*KCNP*), 3 = Royal National Park (*RNP*), 4 = North Wilberforce (*NWLB*) and 5 = Victoria (*VIC*). Abbreviations for Australian states: *NSW* New South Wales, *SA* South Australia and *VIC* Victoria

analysis using the *Taq* I restriction enzyme were conducted to confirm the species identity of the *P. microcarpus* isolates. This method has been show to be effective in separating *P. microcarpus* isolates from other Australian *Pisolithus* species (Hitchcock et al. 2003). Six SSR markers (PM1-6) described by Hitchcock et al. (2006), together with two markers (P2 and P7) described by Hitchcock et al. (2003), were used to amplify DNA from the *P. microcarpus* isolates. Identification and isolation of SSRs were performed as previously described (Hitchcock et al. 2003, 2006), with amplification achieved using the reagent concentrations and cycling parameters as previously outlined (Hitchcock et al. 2006).

Data analysis

Duplicate multilocus genotypes were excluded from the study, resulting in a clone-corrected dataset. In all cases, the duplicate multilocus genotypes were from basidiomes that were collected <1 m apart and were thus regarded as arising from a single genet. Genotyped isolates were grouped according to the geographical locations from which the basidiomes were collected (geographical populations numbered in Fig. 1). Geographical populations were defined as those comprising five or more isolates (with different genotypes). Thus, 89 *P. microcarpus* genotypes from five geographical populations (Table 1) were included in all data analysis. The isolates from locations that had less than five genotypes were only included in the Bayesian clustering (STRUCTURE) analysis, which was conducted using all *P. microcarpus* genotypes listed in Table 1 (excluding duplicate multilocus genotypes).

The genotypic data were summarised using GENEPOP version 3.4 (Raymond and Rousset 1995) for all geographical populations, including number of alleles and allele frequencies, genic differentiation using Fisher's exact test, the observed (H_O) and expected (H_E) heterozygosities and the inbreeding coefficient (f) values. Exact tests for departure from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci were also performed using GENEPOP and were corrected for multiple comparisons using the sequential Bonferonni procedure (initial α=0.05) (Holm 1979). The software Micro-Checker (van Oosterhout et al. 2004) was used to assess genotypic data for the presence of null alleles and scoring errors for each of the geographical populations. All tests were performed using the clone-corrected data sets, which were corrected for duplicate multilocus genotypes.

Genetic differentiation among the geographical populations was estimated using Weir and Cockerham's (1984) pairwise θ values, an unbiased estimate of F_{ST}, which were calculated between each of the geographical populations using FSTAT version 2.9.3.2. (Goudet 1995). The significance of pairwise F_{ST} values was calculated using 1,000 permutation tests and standard Bonferroni correction for multiple analyses was applied (Rice 1989). F_{ST} estimates were interpreted as follows: values that ranged from 0 to 0.05 indicated little genetic differentiation between geographical populations, values from 0.05 to 0.15 moderate genetic differentiation, values from 0.15 to 0.25 great genetic differentiation, and values above 0.25 indicated very great genetic differentiation (Hartl and Clark 1997).

To compare the genetic variation within and among geographical populations, the data were also subjected to a hierarchical analysis of molecular variance (AMOVA) using Arlequin Version 2.000 (Schneider et al. 2000) with 1,000 permutations to examine significance of the variance

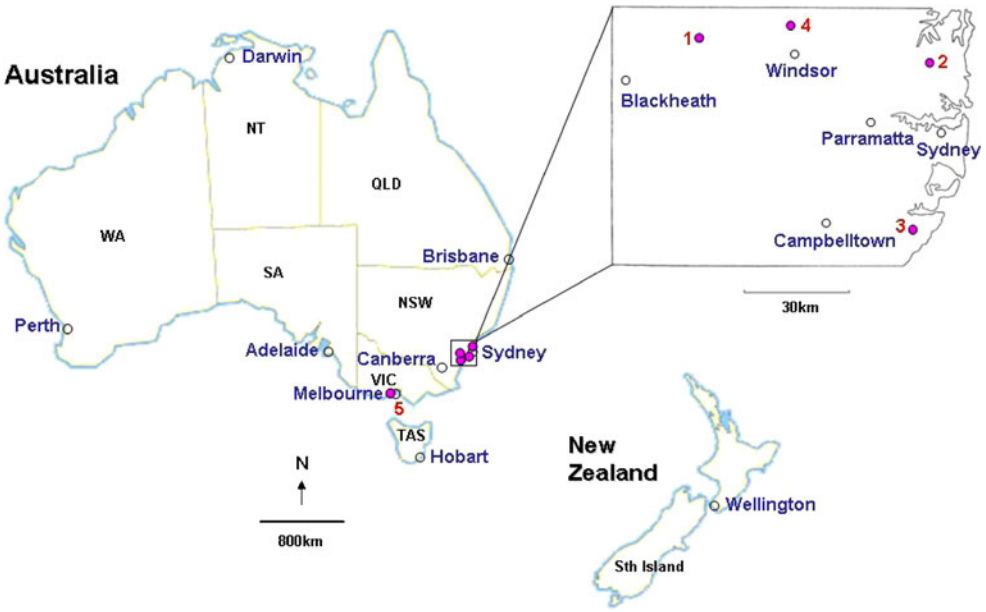

Fig. 1 Map of Australia and New Zealand and an enlarged section (85 km^2) of New South Wales showing the approximate locations of the *P. microcarpus* geographical populations used in the genetic population structure analysis (represented by *pink circles*). Geographical population 1 = Blue Mountains National Park (*BMNP*), 2 = Ku-ring-gai Chase National Park (*KCNP*), 3 = Royal National Park (*RNP*), 4 = North Wilberforce (*NWLB*) and 5 = Victoria (*VIC*). Abbreviations for Australian states: *WA* Western Australia, *NT* Northern Territory, *SA* South Australia, *QLD* Queensland, *NSW* New South Wales, *VIC* Victoria, *TAS* Tasmania

components. It was hypothesised that there would be spatial genetic structure between geographical populations based on geographical location. To investigate this, isolation by distance between geographical populations was tested using the Mantel test with 1,000 permutations implemented in the ISOLDE extension of GENEPOP. This programme was also used to compute the regression of $F_{ST}/(1-F_{ST})$ estimates to the natural logarithm of geographical distance.

To further examine the population structure, a Bayesian clustering analysis was performed using STRUCTURE Version 2.1 (Pritchard et al. 2000). Simulations were performed with an initial burn-in length of 200,000 followed by 200,000 iterations. The Markov Chain Monte Carlo simulations were performed for K=1 to K=10, and simulations were run in triplicate for each K. Analysis parameters included using no prior population information, the population admixture model and uncorrelated allele frequencies. All genotyped *P. microcarpus* (except duplicate multilocus genotypes) were included in this analysis.

Results

A total of 113 *P. microcapus* isolates from the eastern states of Australia were genotyped using eight SSR markers. The *P. microcarpus* geographical populations were polymorphic across all loci. After correcting for multiple comparisons, however, three loci showed significant deviations from HWE (PM2, PM3 and PM4) and were excluded from the analyses. Therefore, five loci (PM1, PM5, PM6, P2 and P7) were used to analyse the data. No linkage disequilibrium was detected between the loci after sequential Bonferonni correction. A heterozygote deficiency, possibly caused by null alleles, was detected by the Micro-Checker software for those loci that were found to deviate from HWE. The only exception to this was locus PM3 for the KCNP geographical population, which had a heterozygote excess.

The allele frequencies did not differ significantly for any of the geographical populations. Unique alleles were detected, with half of these being found in a single geographical population. Most of the alleles with the highest frequency were observed in all geographical populations (Table S1, Supplementary material). Mean heterozygosity values for all geographical populations were moderate to low (Table 2).

The pairwise estimates of F_{ST} among the five *P. microcarpus* geographical populations indicated that there was no significant genetic differentiation between most geographical populations. The only exception was between RNP and VIC

Table 2 Summary statistics for five *P. microcarpus* geographical populations including mean expected (H_E) and observed (H_O) heterozygosity and the inbreeding coefficient (f) calculated over all loci

Geographical populations	H_E	H_O	f
P. microcarpus			
BMNP	0.28	0.3	−0.37
KCNP	0.26	0.29	−0.14
RNP	0.28	0.28	0
VIC	0.23	0.26	−0.16
NWLB	0.26	0.25	0.02

BMNP Blue Mountains National Park, *KCNP* Ku-ring-gai Chase National Park, *RNP* Royal National Park, *VIC* Victoria and *NWLB* North Wilberforce

Table 3 F_{ST} estimates for all pairs of geographical populations (above the diagonal) and geographical distance (km) between populations (below the diagonal) for *P. microcarpus*

A

	BMNP	KCNP	RNP	VIC	NWLB
BMNP	0	0.009	0.0067	0.0256	0.0357
KCNP	72.5	0	−0.0003	0.0606	0.0044
RNP	89.0	64.5	0	**0.0808***	0.0107
VIC	659.0	735.0	676.0	0	0.0522
NWLB	32.0	48.0	81.0	670.5	0

Significant values are indicated in bold and underlined

*Significant differences reflect adjustment for multiple comparisons (P=0.005)

BMNP Blue Mountains National Park, *KCNP* Ku-ring-gai Chase National Park, *RNP* Royal National Park, *VIC* Victoria and *NWLB* North Wilberforce

geographical populations (F_{ST}=0.0808) which was significantly different from zero (P=0.005), suggesting a moderate level of genetic differentiation (Table 3). Genetic distance increased with geographical distance between the VIC geographical population and RNP, KCNP and NWLB geographical populations (P=0.015) (data not shown). The AMOVA results also indicated little genetic differentiation between *P. microcarpus* geographical populations, with genetic variation revealed to be 98.71% within geographical populations and only 1.29% attributed to between geographical populations (Table 4). In toto, these results suggest that extensive gene flow occurs between most *P. microcarpus* geographical populations.

To examine the population structure further, the Bayesian clustering programme STRUCTURE was used to investigate clustering within *P. microcarpus*. Based on the genotype data, *K* was equal to one, indicating no population structure.

Discussion

Analysis of the genotypic data from *P. microcarpus* isolates demonstrated a high level of genetic homogeneity between geographical populations. The majority of genetic variation was found within geographical populations, with little variation between geographical populations. A significant difference in pairwise F_{ST} estimates was, however, noted between the VIC and RNP geographical populations. While this may suggest moderate genetic differentiation, it should be noted that the VIC population comprised only eight isolates and further sampling would be required to confirm this. Similarly, significant isolation by distance was indicated between the VIC, RNP and KCNP geographical populations and the NWLB geographical population. Most of the NSW *P. microcarpus* isolates were collected from within a ca 7,000 km^2 area of NSW, with individual geographical populations separated by <100 km. In contrast, the VIC geographical population was located at a greater distance (>650 km) from the NSW geographical populations and this probably explains the genetic variation and reduced gene flow between them.

Overall, the results indicate extensive gene flow, mediated by basidiospore dispersal, between the NSW *P. microcarpus* geographical populations (BMNP, KCNP, RNP and NWLB) and that gene flow also occurs, but to a lesser extent, between the NSW and VIC geographical populations. Indeed, the NSW isolates may comprise one large population.

It is generally accepted that, for most basidiomycete taxa, a relatively small number of basidiospores are lifted into the wind and transported over long distances, and that the majority of basidiospores fall in the immediate vicinity of a basidiome (Stenlid 1994). Nevertheless, several studies suggest that long-distance dispersal of basidiospores can occur (Hirst and Hurst 1967; Stenlid 1994; Mitakakis and Guest 2001). Moyersoen et al. (2003) proposed that the *Pisolithus* spp. found in association with *K. ericoides* in New Zealand were established as a result of basidiospore movement from Australia by air flow across the Tasman Sea during recent times. *Pisolithus* basidiomes are long-lived earthballs containing large numbers of spores that are dispersed by wind over extended periods of weeks to months (Fuhrer 1985). Given these factors, it seems likely that basidiospore dispersal underlies the high gene flow observed between the *Pisolithus* geographical populations in the present study.

Some ECM fungi, such as *C. formosus* and *Rhizopogon* spp., have been found to have genetically differentiated

Table 4 Hierarchical analysis of molecular variance (AMOVA) showing distribution of genetic variation among and within five *P. microcarpus* geographical populations

Source of variation	*df*	Sum of squares	Variance component	Percentage of variance	*P* value
P. microcarpus					
Among populations	4	7.33	0.02	1.29	<0.05
Within populations	173	222.73	1.29	98.71	

populations (Kretzer et al. 2005; Dunham et al. 2006; Grubisha et al. 2007). Other studies have reported little or no genetic differentiation in populations of, for example, *S. grevillei*, *R. brevipes* and *T. matsutake* (Zhou et al. 2001, Bergemann et al. 2006; Lian et al. 2006). The lack of population structure in the latter studies was thought to be indicative of substantial gene flow via basidiospore dispersal. Compared to the current study, however, these studies focused on populations over relatively short distances (<18.5 km). In contrast, strong evidence for genetic differentiation at a regional scale has been obtained for some taxa such as *T. melanosporum* and *T. scalpturatum* (Murat et al. 2004; Carriconde et al. 2008).

To date, only a few ECM fungal population studies have been conducted at a geographical scale comparable to the current study. Little population structure was observed for *L. amethystina* over ca 500 km (Roy et al. 2008). Genetic differentiation was observed for *R. brevipes* populations separated by 1,500 km, but the authors considered that the populations probably represented cryptic species since they shared only two alleles (Bergemann and Miller 2002). Xu et al. (2008) investigated the genetic structure within and between populations of *T. matsutake* that were separated by ca 80–1,000 km situated in south western China. Their analyses revealed low, but significant, differentiation between populations, with a positive correlation between genetic and geographical distance detected between the populations. Xu et al. (2008) suggested that the low level of genetic differentiation indicated that long-distance gene flow was present due to the dispersal of basidiospores. Since we found evidence of only moderate differentiation between the VIC and NSW geographic populations, *P. microcarpus* appears broadly similar to *T. matsutake* in this regard.

Even in those fungi that can spread via long-distance spore dispersal and show little or no genetic variation over regional scales, local genetic differentiation is evident in populations of some taxa where habitat is fragmented, suggesting that continuous suitable habitat is required to facilitate gene flow (Franzén et al. 2007; Högberg and Stenlid 1999; Parrent et al. 2004). *P. microcarpus* is widely distributed in Australia and forms ectomycorrhizal symbiosis with a range of *Eucalyptus* and *Acacia* spp. (Chambers and Cairney 1999). Suitable natural and/or plantation forest habitat for *Pisolithus* spp. is widespread in the eastern states of Australia (Bougher and Syme 1998). Moreover, both taxa commonly fruit under amenity plantings of *Eucalyptus* spp. in parks and gardens throughout eastern Australia (unpublished personal observations), indicating that suitable habitat extends across urban areas. Coupled with the release of large numbers of basidiospores over extended periods and their apparent ability to spread over long distances by wind (Moyersoen et al. 2003), our data suggest that this enhances gene flow and so, limits population differentiation in *P. microcarpus* at the geographical scales we have investigated.

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