Characterization of Pacific golden chanterelle (*Cantharellus formosus*) genet size using co-dominant microsatellite markers

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Abstract

We characterized five co-dominant microsatellite markers and used them to study Pacific golden chanterelle (*Cantharellus formosus*) genet size and its relation to forest age and disturbance. Fruit-bodies were mapped in and collected from nine replicate study plots in old-growth, recently thinned, and unthinned 40–60-year-old second-growth stands dominated by Douglas fir (*Pseudotsuga menziesii*). Information from microsatellite loci, combined with random fragment length polymorphism analysis of the nuclear DNA internal transcribed spacer indicates that putative 'C. formosus' fruit-body collections may include a cryptic chanterelle species. Small genets were characterized for both genetic types with mean maximum widths of 3.2 ± 3.6 m for *C. formosus* and 1.5 ± 1.7 m for the alternative genetic group. Variance in genet size was high and some multilocus genotypes were observed on multiple plots separated by 0.3 km or more, indicating that genets were not fully resolved by the loci described here. There was no evidence that genet size differed across the three disturbance treatments.

Introduction

Concern for the population viability of ectomycorrhizal forest fungi has drawn attention to the need to consider fungi in Pacific Northwest forest management plans (Molina *et al*. 2001). Achieving this goal requires an understanding of how evolutionary processes act within fungal populations at different spatial and temporal scales. At a more basic level, public land managers require knowledge of spatial patterns in intraspecific genetic diversity. These patterns can reveal important clues about the evolutionary significance of past and present ecological and demographic events (Avise 1994), particularly for species not easily observed during most of their life cycle.

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Proper analysis and interpretation of genetic spatial patterns requires study areas that are large enough to encompass populations governed by independent evolutionary processes and samples dispersed such that the variance of process effects can be quantified (Wiens 1989). Several characteristics of fungi impede our ability to select study areas of appropriate size and geographical orientation for population genetic studies. The design of landscape-scale sampling strategies requires an understanding of how individuals are distributed. This presents a difficult obstacle with fungi because the mycelia that give rise to fruit-bodies are hidden in soil or wood and cannot easily be observed. For fungi, individuals possessing unique genotypes are usually referred to as genets. Ectomycorrhizal basidiomycete fungi establish new genets by fusion of two monokaryotic mycelia produced by germination of sexual basidiospores. Newly formed dikaryotic mycelia undergo vegetative growth and produce fruit-bodies that continue the reproductive cycle (Todd & Rayner 1980).

Studies characterizing population-level variation in ectomycorrhizal fungi typically rely on fruit-body sampling.

Understanding the mean and variance in genet size for different ectomycorrhizal species is critical to avoid re-sampling genets and associated problems with lack of sample independence. Past research on ectomycorrhizal fungi has identified basidiomycete species capable of producing persistent genets (Dahlberg & Stenlid 1990; De La Bastide *et al.* 1994; Selosse *et al.* 1998, 1999; Sawyer *et al.* 1999; Gryta *et al.* 2000) that can grow to 20–40 m in diameter (Dahlberg & Stenlid 1994; Dahlberg 1997; Bonello *et al.* 1998). Similar studies have also identified species that form much smaller genets, probably because individuals persist for a shorter time (Baar *et al.* 1994; Gryta *et al.* 1997; Gherbi *et al.* 1999; Zhou *et al.* 1999; Redecker *et al.* 2001). To understand better the relative roles of basidiospore dispersal and vegetative growth in the life histories of ectomycorrhizal fungi, many studies have characterized the genet size of ectomycorrhizal species across different habitats. The results from these studies demonstrate that environmental factors such as forest age and disturbance are associated with variation in genet lifespan and size and that larger genets typically occur in older or less disturbed forests (Dahlberg & Stenlid 1990, 1995; Dahlberg 1997; Guidot *et al.* 2002). Exceptions to this pattern have also been identified, indicating that genetics may play a larger role in longevity and genet size for other ectomycorrhizal species (Fiore-Donno & Martin 2001; Redecker *et al.* 2001). Knowledge of genet size unique to each ectomycorrhizal species and environment will facilitate our understanding of the scale at which population boundaries should be encountered, and help determine minimum study area size.

A practical approach to expanding our understanding of ectomycorrhizal fungal population genetics to landscape spatial scales involves research on species that fruit reliably and are broadly distributed. Pacific golden chanterelles (*Cantharellus formosus*, Redhead *et al.* 1997) are commercially harvested basidiomycetes that form ectomycorrhizal associations with a wide range of economically important host trees (Molina *et al.* 1993; Redhead *et al.* 1997). This species fruits abundantly in several different habitats over a broad geographical range and is a good candidate for landscape-level population genetic studies. Population genetic research also requires genetic markers that exhibit levels of variability appropriate for use in studies ranging from parentage determination to characterization of landscape-level genetic patterns. This paper describes the development of five co-dominant microsatellite markers for *C. formosus* and characterizes the variability of these loci using a sample of chanterelles from an area of 6400 ha. To test the utility of these microsatellite markers, we applied them in a study aimed at determining the size of chanterelle genets in a Douglas fir (*Pseudotsuga menziesii*)/western hemlock (*Tsuga heterophylla*) habitat in central Oregon. Three forest types were sampled including old-growth, naturally regenerated 40–60-year-old second-growth, and second-growth recently disturbed by forest harvest. Characterization of genet sizes under these stand conditions is intended to determine whether propagation strategies (sexual or vegetative) of chanterelles are altered by forest age or harvesting disturbance.

Materials and methods

Study area and sampling

Samples used to assess microsatellite variability. Fruit-bodies were collected from 18 forest stands distributed over 6400 ha in and around the H.J. Andrews experimental forest (HJA; 44.2° latitude, 122.2° longitude). Once in October 1998 and once in November 1998 fixed length searches of each stand were conducted and data from these two months were pooled for analysis. To avoid re-sampling genets all collection sites were marked and a minimum of 5 m was kept between any two fruit-body collections. This distance is based on a preliminary study of genet size using Inter Simple Sequence Repeat (ISSR) markers and the fine-scale samples described below (data not shown).

Samples used to characterize genet size. Samples used to characterize genet size were taken from nine plots located in 40–60-year-old second-growth (SG) and 350 + old-growth (OG) stands along the southeast boundary of the HJA. Plots located in SG stands are part the Young Stand Thinning and Diversity Study (YSTDS), a joint effort between the Cascade Center for Ecosystem Management, Oregon State University, USDA Forest Service Pacific Northwest Research Station, and the Willamette National Forest McKenzie, Blue River and Middle Fork Ranger Districts. The goal of the YSTDS is to investigate the effects of SG thinning techniques on several ecosystem variables including chanterelle production. To characterize genets we selected seven chanterelle productivity plots from two YSTDS sites (18 km apart) closest to the southeast boundary of the HJA. The two YSTDS treatments sampled in this study included unthinned control stands (250 trees per acre) and lightly thinned stands (~100–120 residual trees per acre). In addition to the YSTDS plots we installed plots in OG stands adjacent to each replicate site. This sampling encompasses three disturbance treatments including undisturbed OG, SG control stands disturbed once by clear-cut harvest 40–60 years ago, and thinned SG stands similarly disturbed by clear-cut harvest 40–60 years ago, and recently by a light thin harvest.

In total, we sampled two plots in OG stands, three plots in two YSTDS control stands, and four plots in two YSTDS light thin stands with a minimum distance of 0.3 km between any two plots. Plot locations were selected non-randomly by searching stands for areas where chanterelle fruit-bodies occurred in high abundance. When such an

area was located, a 16-m diameter plot encompassing the greatest number of fruit-bodies was installed. Fine-scale samples used to characterize genets were collected 2 weeks after the first autumn rain of 1997. Within each plot the spatial coordinates of all chanterelle fruit-bodies were mapped to the nearest decimeter and azimuth (0–360°) direction from plot centre. Each mapped fruit-body was collected and samples were taken for subsequent genetic analyses.

DNA extraction and amplification of the internal transcribed spacer (ITS) region

To aid in the detection of possible multiple taxa within our chanterelle sample we analysed all fruit-bodies using restriction fragment length polymorphism analysis of the internal transcribed spacer region of the nuclear ribosomal repeat (ITS-RFLP), a technique commonly used to characterize community level species diversity (Gardes & Bruns 1996; Horton & Bruns 2001). After grinding approximately 5 mm³ of fresh tissue in lysis buffer (100 mm Tris–HCl, 10 mm ethylenediaminetetraacetic acid, 2% sodium dodecyl sulphate, pH 8.0, 1 mg/mL proteinase-K), each sample was incubated for 1.5 h at 55 °C and DNA was extracted using phenol/chloroform emulsification and precipitation (Sambrook *et al*. 1989). Unquantified DNA samples were diluted 10- to 100-fold, and the polymerase chain reaction (PCR) was attempted on successive 10-fold dilutions until amplification of ITS region was achieved. The region spanning the 3′ end of the 18S rDNA, ITS-1 spacer, 5.8S, ITS-2 spacer, and 5′ end of the 28S rDNA was amplified with the fungus-specific primer ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). PCR mix components were 1× Assay Buffer A (Fisher Scientific), 200 μm dNTPs each, 0.2 μm of each primer, 0.75 units *Taq* DNA polymerase (Fisher Scientific). Reactions were subject to initial denaturation at 95 °C for 3 min followed by 35 PCR cycles (95 °C for 45 s, 52 °C for 55 s, 72 °C for 90 s) in a PTC-100 Programmable Thermal Controller (MJ Research Inc.). Following the 35th cycle, samples were subjected to a 10-min 72 °C extension.

RFLP analysis of the ITS region

Unpurified PCR products were digested separately with four restriction enzymes, *Alu*I, *Hin*fI, *Dpn*II and *Hae*III, according to the manufacturer's recommendations (GIBCO BRL). These enzymes were chosen because they cut at four base-pair recognition sites and have demonstrated utility in the molecular typing of ectomycorrhizal root tips (Gardes & Bruns 1996). Restriction fragments were electrophoretically separated on 3% agarose gels (2% Nu-Sieve agarose, 1% SeaKem LE agarose; FMC BioProducts), and scored against a 100-base-pair (bp) molecular weight

standard using the Alpha Imager 2000 documentation and analysis system (v. 3.2, Alpha Innotech Corp.). Each collection was assigned an ITS type that summarized information from the four restriction enzyme profiles. Collections with the same ITS type designation share identical restriction fragment patterns across all four enzymes.

Microsatellite enrichment protocols and primer development

The enrichment largely followed the protocol of Fleischer & Loew (1995) with minor modifications. Genomic DNA was extracted from a dried *Cantharellus formosus* fruit-body and remaining voucher material was placed in the Oregon State University herbarium under accession number OSC 76037. Approximately 10 μg of DNA were digested with *Taq*I and size-fractionated by agarose gel electrophoresis. Fragments in the 500–1000 bp size range were recovered from the gel and ligated to SAULA (5′-GCG GTA CCC GGG AAG CTT GG-3′)/*Taq*B (5′-CGC CAA GCT TCC CGG GTA CCG C-3′) oligonucleotide linkers. The SAULA linker was used as a primer to amplify double-stranded ligation products. Ligation PCR products were hybridized to three biotinylated probes (GGAT)$_7$ and (GACA)$_7$ (mixed in a single reaction) and (CAC)$_{10}$ (used separately) and the products were immobilized onto Dynabeads M-280 Streptavidin (DYNAL France). Immobilized hybridization reactions were washed repeatedly at increasing temperatures with final washes at 95 °C to remove the single-stranded fraction enriched for microsatellite repeats. Retained single-stranded fragments were amplified using the SAULA primer and the double-stranded products were digested with *Taq*I to partially remove linkers and prepare fragments for ligation into the pUC19 *Acc*I restriction site. Plasmids were transformed into *Escherichia coli* DH5α and transformants were detected on LB$_{Amp+X-Gal}$ plates. Transformants containing microsatellite repeats were detected by hybridization to ^{32}P end-labelled oligonucleotide probes. Nucleotide sequences for positives were generated from the M13 forward primer using the BigDye Terminator sequencing kit and an ABI 377 automated sequencer (PE Applied Biosystems). Sequences flanking microsatellites five repeats or longer were used to design locus-specific PCR primers with annealing temperatures at 55 °C.

Microsatellite screening

Allelic diversity of co-dominant microsatellite loci was characterized using a sample that included both collections from the HJA and selected samples representing unique multilocus genotypes on circular plots. PCR reaction conditions were as described for ITS amplification. The PCR profile consisted of initial denaturation at 95 °C for 3 min followed by 35 cycles (95 °C for 45 s; 55 °C for 60 s;

72 °C for 60 s). Following the 35th cycle, samples were subjected to a final 72 °C extension of 60 min to maximize the proportion of complete PCR products. PCR products were analysed on an ABI 377 automated sequencer using the GS500 Tamra internal size standard. Band sizes were determined using GENESCAN software (PE Applied Biosystems).

Data analysis

The web-based version of GENEPOP (v. 3.1c; Raymond & Rousset 1995) was used to calculate expected and observed heterozygosities, exact tests for departure from Hardy–Weinberg, and tests for linkage. Variance in the size of genets was examined graphically. Fruit-body locations from circular plots were converted to x/y coordinates using algebraic transformations and SIGMAPLOT (v. 2.01; Jandel Scientific 1993) was used to generate a map for each plot. A spatial grid with a resolution of 10 dm was superimposed onto each plot map and used to estimate the maximum distance between fruit-bodies with identical multilocus genotypes. Statistical comparisons of genet length were made using STATMOST (Dataxiom Software In*c*. 2001).

Results

ITS-RFLP variability

During 1997, 355 chanterelle fruit-bodies were collected from the nine circular plots used to study genet size. Random searches of forest stands in the HJA during 1998 yielded a sample of 348 yellow chanterelle fruit-bodies. Amplification of the ITS region yielded PCR products of either 1690 bp or 1490 bp. Later sequence analysis showed that the two variants differ in length as a result of multiple insertion/deletion events in the ITS 1 region (see GenBank Accessions AY041164–1690 bp, AY041160–1490 bp for representative sequences) rather than a single insertion event as has been shown for other species of ectomycorrhizal fungi (Shinohara *et al*. 1996). RFLP analysis of the ITS region consistently produced two RFLP types that correspond to the two ITS PCR product lengths (Table 1). ITS-RFLP type A matches the genetic description given for *Cantharellus formosus* by Danell (1995) and Redhead

et al. (1997) and we refer to these samples as *C. formosus* throughout the remainder of the manuscript. The length of the other ITS-RFLP type (B, Table 1) is similar to that given for Swedish *C. cibarius* (Danell 1995) but confirmation of identity requires further investigation.

Microsatellite enrichment

Attempts to isolate microsatellites from the *C. formosus* genome resulted in the screening of two partial libraries containing a total of 3300 clones. The longest microsatellite identified contained 13 repeats with most less than 10 repeats. The first enrichment yielded 15 positive clones containing eight microsatellites long enough to warrant primer development. Of the eight primer sets screened, two loci (Cf642; Cf145; Table 2) showed variability. In the second partial library, 97 positive clones were identified. DNA from 37 clones was amplified and sequenced and primers were designed for 15 clones. Three primer sets (Cf339; Cf126; Cf113; Table 2) amplified polymorphic repeats.

Variable microsatellite loci

To characterize microsatellite variability, 44 samples representing both ITS-RFLP types were selected from fruit-bodies collected throughout the HJA (Table 3). Genetic information from these 44 samples was pooled with information from 45 samples representing unique multilocus genotypes on circular plots. Each primer set consistently amplified a single product (locus) from all collections screened. In the combined sample, polymorphic loci had three to eight alleles with expected heterozygosities ranging from 0.54 to 0.83. Observed heterozygosities were 23–70% lower than the heterozygosities expected under Hardy–Weinberg and all departures were significant ($P = 0.001$; Table 2). The number of alleles per locus, expected heterozygosities and observed heterozygosities changed substantially when the sample was partitioned by ITS-RFLP type and no significant departures ($P = 0.05$) from Hardy–Weinberg equilibrium were detected. The 45 collections that represent *C. formosus* possess two to four alleles per locus, with expected heterozygosities that ranged from 0.09 to 0.64. The 44 collections that possess ITS-RFLP type B show one to five alleles per locus with expected

	Type A	Type B
Total ITS length	1690	1490
*Alu*I fragments	540, 490, 340, 200, 120	490, 380, 250, 220, 150
*Hin*fI fragments	970, 500, 220	480, 430, 340, 240
*Dpn*II fragments	1090, 400, 200	600, 400, 290, 200
*Hae*III fragments	780, 330, 180, 170, 140, 90	600, 220, 200, 170, 160, 140

Table 1 Characterization of variability in the internal transcribed spacer (ITS) region of yellow chanterelles collected in this study using four restriction enzymes. Fragment sizes are reported in approximate number of base pairs

Table 2 Loci, primer sequences, and additional characteristics of five *Cantharellus formosus* microsatellites

Locus	Primer sequence (5′–3′)	Genbank accession no.	Repeat motif	No. of alleles	Allele size range	H_E	H_O
Cf642	F: 6-FAM-CTA TGC AAA TCC GCC AGC	AY077690	$(gaca)_9$	4	275–279	0.64	0.30*
	R: GGG ACA AGT GCC ACA C			ITS-A: 2	275, 279	0.09	0.09
				ITS-B: 2	276, 277	0.49	0.52
Cf145	F: 6-FAM-GTA CGA AAC TGA TTG GAT GG	AY077691	$(gaca)_2(ggta)_4N_{120}$	8	342–473	0.83	0.64*
	R: GTG TTT GCA TGT GGC TGG		$(gcgc)_4N_{29}(gct)_5$	ITS-A: 3	342–350	0.64	0.69
				ITS-B: 5	465–473	0.67	0.59
Cf339	F: 6-FAM-GAA TCG GGT TCG CAG AAG	AY077694	$(ggt)_6$	4	173–182	0.54	0.16*
	R: GAA CTT CTT CAT CCG ACT CC			ITS-A: 4	173–182	0.31	0.31
				ITS-B: 1	176	0.0	0.0
Cf126	F: 6-FAM-GCT TGT GCG GGA TGA CGG	AY077692	$(ggt)_6$	3	221–231	0.62	0.23*
	R: CCA CGT TCA TTT CAT TCT ACC C			ITS-A: 2	228, 231	0.45	0.44
				ITS-B: 1	221	0.0	0.0
Cf113	F: HEX-CGA TCT CGC TGT TAT TGG AG	AY077693	$(ggt)_5$	4	96–120	0.69	0.32*
	R: TGC GAC CAA CTC ATC TAA TCC			ITS-A: 4	96–120	0.29	0.29
				ITS-B: 2	117, 120	0.50	0.34

Summaries are based on a sample of 89 collections, 45 combined from nine circular plots used to study genet size and 44 collected from the HJ Andrews Experimental forest. For each locus characteristics are given for all samples combined and for samples from each ITS-RFLP type (A = 45 and B = 44) separately.
F, forward primer; R, reverse primer; H_E, expected heterozygosity; H_O, observed heterozygosity.
*Significant departure from Hardy–Weinberg equilibrium ($P = 0.001$).

Table 3 Oregon State University (OSC) collection numbers for the 44 yellow chanterelle vouchers taken from areas in and around the HJ Andrews Experimental forest and screened for variability using five microsatellite loci

OSC	ITS RFLP type	OSC	ITS RFLP type
75926	A	75907–08	B
75930–31	A	75916–25	B
75981	A	76072	B
76021	A	75979	B
76024–25	A	75982	B
76033	A	75984	B
76037–41	A	75986	B
76046	A	75990–91	B
76048	A	76002	B
76050	A	76006	B
76054	A	76017–19	B
76056	A		
76058–59	A		

ITS-RFLP types are compiled from patterns generated using four restriction enzymes (*Alu*I, *Dpn*II, *Hinf*I and *Hae*III), see Table 1 for details.

heterozygosities ranging from 0 (for two monomorphic loci) to 0.67. For three of the loci (Cf642, Cf145, Cf126) the samples representing the two ITS-RFLP types share no alleles. At a fourth locus (Cf339) the ITS-RFLP type B sample is fixed at an allele present at low frequency in *C. formosus* and at the fifth locus (Cf113) the two ITS-RFLP types share one allele that occurs at different frequencies in

the two samples (120 bp allele frequencies; *C. formosus*–0.02; ITS-RFLP type B–0.56).

All pairwise comparisons of loci were tested for linkage disequilibrium using the 44 fruit-bodies (20 *C. formosus* and 24 type B) drawn from the HJA. When the two ITS types are combined, all pairs of loci were found to have significantly associated combinations of alleles ($P < 0.001$ for all loci pairs). When samples from each ITS type were analysed independently no significant linkage was found between pairs of loci within *C. formosus*. In ITS-RFLP type B, two pairs of loci appeared to be linked namely Cf642 with Cf145 ($P = 0.02$) and Cf642 with Cf113 ($P = 0.08$).

Resolution and size of chanterelle genets

To study the size of chanterelle genets, 355 fruit-bodies were collected from nine circular plots placed in YSTDS and adjacent OG stands. The majority (285) of fruit-bodies were *C. formosus*. Screening the total sample with the microsatellite loci yielded 36 unique multilocus genotypes (19 *C. formosus* and 17 type B). Eight multilocus genotypes were found on more than one plot with seven genotypes occurring on two plots each and one genotype occurring on three plots. Plots were separated by substantial distances so identical multilocus genotypes were thought to represent distinct genets when they were found on different plots. The total number of genets resolved was thus 45 (25 *C. formosus* and 20 type B; Table 4).

Maximum genet width was estimated visually by superimposing a 10-dm grid over each plot map and measuring

Table 4 Number of chanterelle fruit-bodies and number of unique genotypes found within nine circular plots used to study genet size of yellow chanterelles

ITS pattern	Old-growth		YSTDS control			YSTDS light thin			
	Site 1 Plot 1	Site 2 Plot 1	Site 1 Plot 1	Site 2 Plot 1	Site 2 Plot 2	Site 1 Plot 1	Site 1 Plot 2	Site 2 Plot 1	Site 2 Plot 2
Number of fruit-bodies									
A	0	0	81	61	97	4	0	23	19
B	19	8	0	36	0	0	5	2	0
Number of genets resolved									
A	0	0	4	4	10	1	0	3	3
B	5	5	0	6	0	0	3	1	0
Total	5	5	4	10	10	1	3	4	3

Totals are partitioned by their ITS-RFLP types (A and B).
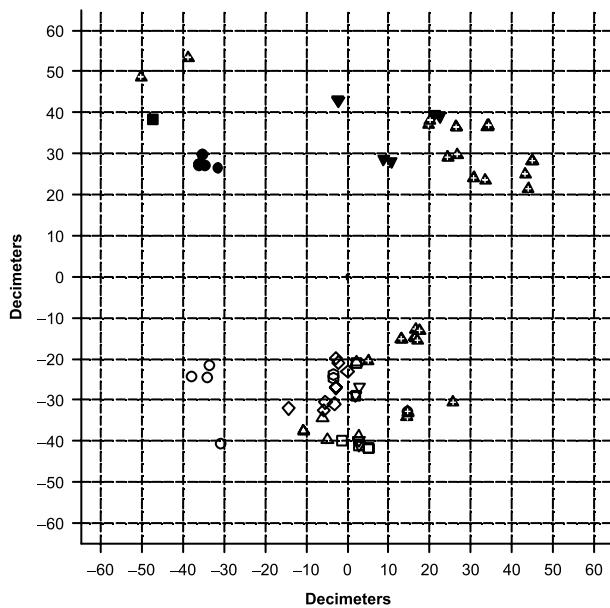
Fig. 1 A map of plot number one in the YSTDS control SG site number two. Each symbol marks the location of a single chanterelle fruit-body. Filled symbols represent *Cantharellus formosus* fruit-bodies and open symbols represent ITS-RFLP type B fruit-bodies. Multiple similar symbols delineate the extent of a putative genet measured against a 10-dm grid superimposed on the plot map. Plot sampling boundaries are not shown to improve resolution.

the distance between the two furthest fruit-bodies possessing like multilocus genotypes (Figs 1 and 2, maps and data for plots not shown can be obtained from the primary author). There was no evidence that the mean maximum width of chanterelle genets differed between the three forest types for the pooled sample or for either ITS-RFLP type. There was statistical evidence that the mean maximum genet width for *C. formosus* (3.2 ± 3.6 m) was larger than the mean width for ITS-RFLP type B genets (1.5 ± 1.7 m) (*t*-
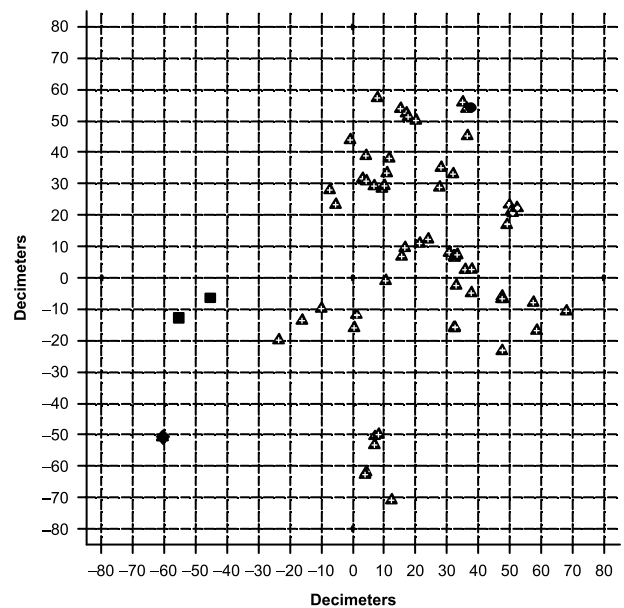

Fig. 2 A map of plot number one in the YSTDS control SG site number one. Filled symbols represent 81 *Cantharellus formosus* fruit-bodies, 77 of which possess identical multilocus genotypes. Plot sampling boundaries are 80 dm from the 0/0 mark that indicates the plot centre.

value 2.02, $P = 0.05$, 43 d.f.), but the variance within both groups was high (Fig. 3).

The presence of multilocus genotypes repeated on separate plots indicates that marker variability is insufficient to resolve all the genets sampled. A plot of the mean number of genotypes resolved against number of loci scored (Fig. 4) shows that the curves for the total sample and for each ITS-RFLP type are approaching, but have not reached, saturation. Allele frequencies (Table 2) were used to calculate expected frequencies for all multilocus genotypes encountered on our plots. All eight genotypes found
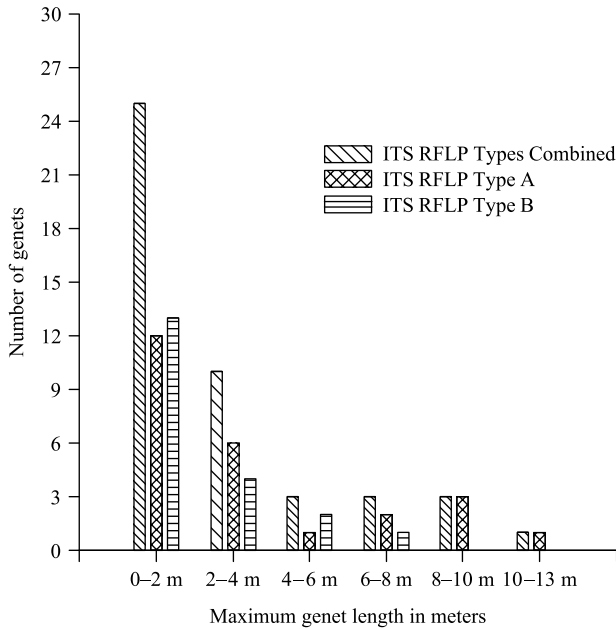
Fig. 3 Frequency distribution of maximum genet width measured across all plots in three disturbance treatments. Groups of fruit-bodies larger than 8 m (*Cantharellus formosus*) or 4 m (ITS-RFLP type B) may encompass several unresolved genets.
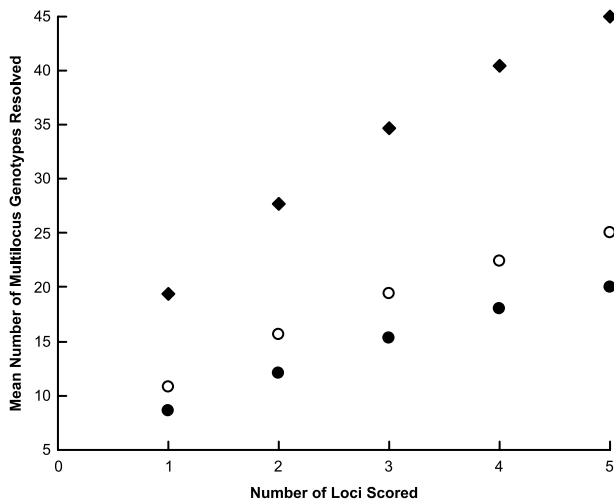

Fig. 4 Mean number of multilocus genotypes resolved by all possible combinations of one, two, three, four and five microsatellite loci. ◆ represent the number of genotypes identified in the combined sample (ITS-RFLP types A and B), ● represent the genotypes identified in *Cantharellus formosus* only, ○ indicate ITS-RFLP type B only.

on more than one plot had a greater than 1% chance (range 1.2–6.8%) that two fruit-bodies drawn from the same plot might share that genotype as a result of chance rather than of vegetative propagation. In contrast, genotypes not repeated on different plots had expected frequencies < 0.1%.

Comparison of the maximum genet width between genets with expected genotype frequencies greater than and less than 1% showed that the mean genet width variability was two to three times higher in the group with expected frequencies > 1% (expected frequency > 1%: standard deviation for *C. formosus* = 4.1 m, type B = 2.0 m; expected frequency < 1%: standard deviation for *C. formosus* = 2.3 m, type B = 0.7 m). The increased maximum genet width variability in the group with expected multilocus genotype frequencies > 1% indicates that these genotypes encompass unresolved genets on some plots. The largest genet identified provides an example of a single genotype with a high-expected frequency (5.9%) that likely includes several unresolved genets. This genet is 13 m wide (3 + standard deviations from the mean *C. formosus* width) and is represented by 77 fruit-bodies (Fig. 2).

Chi-square analysis of genet counts (Table 4) demonstrated that the two ITS-RFLP types were not evenly distributed across the three disturbance treatments (χ^2 = 152.25, $P < 0.001$, 5 d.f.). Most notably *C. formosus* was absent from both OG plots but only absent from 1 of seven SG plots. Alternatively ITS-RFLP type B was present in both OG plots but absent from four of seven SG plots.

Discussion

Implications of intraspecific variability in the ITS region in Cantharellus

Throughout the 1900s, mycologists have noted that North American yellow chanterelles are morphologically distinct from European species and exhibit intraspecific variation (Smith & Morse 1947) of unknown evolutionary importance. Delineation of most fungal species relies on the morphological characteristics of fruiting structures. Genetic analyses and mating experiments have shown that exclusive use of morphology to determine species boundaries is inadequate for many groups (Petersen & Hughes 1999) because commonly used characters, such as pileus colour, can vary at the population level or mask cryptic species when they are conserved (see Redecker *et al.* 2001 for examples from *Russula*).

Genetic studies of the ITS from North American yellow chanterelles have demonstrated length variability in the ITS 1 spacer (Feibelman *et al.* 1994), suggesting that either the ITS 1 varies at the population level or this group is a species complex. Recently researchers have used morphological and genetic data to identify the yellow chanterelle most frequently harvested from Pacific Northwest forests as *Cantharellus formosus*, a species once thought to be rare in the region (Redhead *et al.* 1997). A second yellow chanterelle species (*C. cibarius* var. *roseocanus*) apparently restricted to spruce (Redhead *et al.* 1997) and pine forests (T. Horton personal communication) also has been recognized

from the region. We have examined several collections of *C. cibarius* var. *roseocanus* that have ITS regions of ~1490 bp and ITS-RFLP patterns that differ from those produced by the fruit-bodies collected from our plots under Douglas fir (S. M. Dunham, unpublished data). Our data indicate the possibility of a third undescribed yellow chanterelle variety or species but it is unclear whether *C. formosus* and ITS-RFLP type B collections are different taxa or locally adapted populations. These two ITS-RFLP types are significantly segregated by habitat and genetic differences may reflect local adaptation within populations of *C. formosus*. Any phenotypic differences between the two yellow chanterelle ITS-RFLP types are subtle and were regarded as environmental variation during collecting for this study. If ITS-RFLP type B does represent an undescribed species it is difficult to distinguish from *C. formosus* in the field.

Species boundaries cannot be discerned using ITS-RFLP data alone, but the heterozygote deficiencies and significant linkage tests present in the combined microsatellite analysis (Table 2), both of which are reversed when the two groups are analysed separately, indicate that these ITS-RFLP groups may not be interbreeding. However, Chakraborty & Zhong (1994) have shown that the power of exact tests to detect significant departures from Hardy–Weinberg equilibrium is dependent on both the number of segregating alleles and allele frequencies. Partitioning our sample by ITS-RFLP type reduced both the sample size and the number of segregating alleles present in each group. As a result of likely reductions in statistical power, caution must be used in interpreting what these tests mean because we may simply have lost the ability to detect departures from Hardy–Weinberg when the sample was split.

Overall there were several problems with application of microsatellite primers in ITS-RFLP type B that were not observed in *C. formosus*. Patterns of linkage were not consistent between the two ITS-RFLP types, indicating that ITS-RFLP type B may have scoring problems, possibly because of the presence of null alleles, in loci Cf145 and Cf113 (Pemberton *et al.* 1995). The presence of null alleles may have resulted in nonrandom associations between the amplifiable alleles at these loci and the alleles at locus Cf642. This hypothesis is supported by low observed heterozygosities calculated for ITS-RFLP type B at these two loci (Table 2). Nonrandom associations between these loci were not detected in *C. formosus*, indicating that linkage in ITS-RFLP type B is the result either of scoring problems, or of the distance between these loci being reduced by chromosomal rearrangements. Enrichment protocols were carried out on DNA from a *C. formosus* specimen. If the variation observed is indicative of species level differences then these primers do not cross-amplify well between the two species.

Results from our research reveal the need for more molecular data to define better the species boundaries of

Cantharellus species in the Pacific Northwest. The evolutionary relationships between these taxa will only be resolved with an analysis of DNA sequence data coupled with close scrutiny of morphology. Questions regarding biological species boundaries (*sensu* Mayr 1963) should be addressed with microsatellite analysis of sympatric broad-scale collections of relevant taxa. We are not able to address this question fully with the collections presented here because they represent a limited number of genets and are confounded by differences in habitat.

Abundance of microsatellite repeats in the Cantharellus genome

The overall efficiency of attempts to isolate microsatellites from the *C. formosus* genome was low relative to that observed for nonfungal taxa. The frequencies of different types of microsatellite motifs are known to vary widely between taxa (Hancock 1999; Tóth *et al.* 2000). ISSR primers based on $(CAC)_n$ repeats have been used to generate polymorphic fingerprints in other species of fungi (Longato & Bonfante 1997; Anderson *et al.* 1998; Liu *et al.* 1998; Gherbi *et al.* 1999; Sawyer *et al.* 1999; Zhou *et al.* 1999; Kanchanaprayudh *et al.* 2002). Prior to our enrichment attempts we screened several *C. formosus* collections with ISSR primers based on various microsatellite motifs. ISSR fingerprint profiles indicated that the repeat types used in our enrichments were fairly common in our target species. Use of these markers to guide our motif selection may have resulted in poor choices if the primers were able to anneal to imperfect repeats or if ISSR profiles exhibiting discrete bands (rather than smears) are actually indicative of low repeat abundance. Tóth *et al.* (2000) have shown that in the intron and intergenic regions of some fungal genomes, tetranucleotide repeats constitute the least frequent class of microsatellites. How this relates to the abundance of these motifs in other regions of the fungal genome is unknown.

The low efficiency of our enrichments may also reflect the true abundance of microsatellite motifs in the *C. formosus* genome. Several studies have reported a positive correlation between the frequency of microsatellite regions and total genome size (e.g. Primmer *et al.* 1997; Field & Wills 1998; Kubis *et al.* 1998; Hancock 1999). For example the avian genome is roughly one-third the size of the human genome (~1200 Mb for the domestic chicken *Gallus gallus*; Bloom *et al.* 1993; Wachtel & Tiersch 1993) and recent research has demonstrated that microsatellite repeats occur less frequently in birds relative to other vertebrates (Primmer *et al.* 1997). The few fungal genomes that have been well characterized are modest in size with high gene density (Goffeau *et al.* 1996); average basidiomycete genome size ranging between 35 and 40 Mb (e.g. Ramírez *et al.* 2000), roughly 30 times smaller than the typical avian genome. Microsatellites predominantly occur in noncoding

regions, and if the proportion of noncoding DNA in the genome acts as the main constraint for the evolution of simple repeats, both the relative and absolute numbers of repeats in fungal genomes may naturally be low. Further research on the abundance of microsatellites in fungi is needed to clarify if the low number of loci characterized in this study result from factors involved in the generation of simple repeats, selection against 'superfluous' repetitive DNA, or low abundance of the repeat motifs we enriched for in the *C. formosus* genome.

Genet size and resolution of fungal genets

The largest ectomycorrhizal fungal genets measured to date cover areas of approximately 300 m^2 (*Suillus* sp., Dahlberg & Stenlid 1994; Dahlberg 1997; Bonello *et al.* 1998). Small genets measured for species thought to proliferate by repetitive establishment via basidiospores rather than vegetative growth range from < 1 m^2 for *Laccaria amethystina* (Gherbi *et al.* 1999) and *Russula cremoricolor* (Redecker *et al.* 2001) to 12.5 m^2 for *Laccaria bicolor* (Baar *et al.* 1994). The widths for most of the chanterelle genets we measured are small compared to other ectomycorrhizal species, with < 4 m between most fruit-bodies with identical multilocus genotypes (Fig. 3). Genets of *C. formosus* were significantly larger than those of ITS-RFLP type B but a few large genets interrupted by fruit-bodies with unlike genotypes drive this difference (Fig. 1). This indicates that the statistical difference between genet sizes of the two ITS-RFLP types may be the result of unresolved or fragmented genets.

Estimating genet size using fruit-bodies introduces error from several sources. Fruit-bodies are connected to mycelia that, to acquire nutrients, grow through soil and colonize ectomycorrhizal root tips (Smith & Read 1997). Fruit-body production occurs during a limited season dependent on environmental conditions and species-specific biological cycles. Guidot *et al.* (2001) demonstrated that the spatial and temporal correspondence between the presence of fruit-bodies and ectomycorrhizal root tips was high for *Hebeloma cylindrosporum* but how well these results extend to other ectomycorrhizal taxa is unknown. Even if spatial locations of fruit-bodies are correlated with ectomycorrhizal root tip locations, fruit-body sampling may not reflect the distribution of mycelia involved in the capture of soil nutrients. In addition, some genets may continuously produce fruit-bodies in different locations over the course of a fruiting season while others fruit inconsistently (e.g. *Laccaria* sp. Selosse *et al.* 2001). This makes it difficult to estimate genet size without taking replicate samples throughout the entire fruiting season. Finally, mycelia can persist throughout the year and genet size and shape probably vary between years. When fruit-bodies are used to represent the distribution of mycelia, these sources of error

probably result in underestimates of genet size . Fruit-bodies, however, are frequently sampled in ecological studies so having an estimate of the mean distance between fruit-bodies produced by individual genets is extremely useful prior to initiating research on ectomycorrhizal species.

The maximum width for *C. formosus* genets ranged from < 1 m up to 13 m and this high variance may have occurred for several reasons. An important source of error in this study was that the five microsatellite loci characterized were not variable enough to resolve all the genets on our study plots (Fig. 4) and this probably led to overestimates of genet size. Ectomycorrhizal fungi do not produce mitotic (asexual) spores (Hutchinson 1989) that could allow long-distance dispersal of multilocus genotypes so the presence of multilocus genotypes repeated on plots separated by kilometres supports this conclusion. However, because chanterelles are frequently eaten and collected by various animals, an alternative explanation for the existence of identical multilocus genotypes several kilometres apart is the long-distance transport of mycelium fragments. To our knowledge, this mode of dispersal has not been identified in ectomycorrhizal fungi. A related explanation for the high size variance within plots is genet fragmentation. Long- or short-distance dispersal of ectomycorrhizal fungi is assumed to occur via movement of sexually produced basidiospores, but short-distance dispersal could occur by vegetative growth of mycelia that subsequently become disjointed. On our study plots fruit-bodies of larger genets were dispersed and interrupted by different multilocus genotypes (Fig. 1), indicating that fragmentation might occur in chanterelles.

Some larger genets may have been more difficult to resolve because of inbreeding among established genets (Gryta *et al.* 2000). Every fruit-body produces millions of basidiospores, most of which fall in the immediate vicinity of their source. Although ectomycorrhizal fungi possess mating-type genes that reduce inbreeding, basidiospores from the same genet can germinate and fuse to form a dikaryotic mycelium capable of fruit-body formation. The mating system for *C. formosus* is unknown but even small amounts of inbreeding coupled with the low numbers of alleles present at our loci (Table 2) would exacerbate the difficulty in resolving some genets. For example, the multilocus genotype of the largest genet observed (13 m in diameter) was heterozygous at only one of five loci scored. Genets established by basidiospores from a genet with this genotype could only be detected with a 50% probability. Also, our study plot locations were biased towards patches with high fruit-body abundance and this may have contributed to the difficulty in resolving genets because close proximity might facilitate inbreeding. On the other hand, frequent inbreeding or selfing should convert heterozygous loci to a homoallelic state, but no significant heterozygote deficit was detected in samples subdivided by ITS-RFLP

type (but see the discussion on power of test in subdivided samples).

How ectomycorrhizal fungi propagate under different forest stand conditions has been the focus of several previous studies characterizing fungal genets (Dahlberg & Stenlid 1990; De La Bastide *et al.* 1994; Gryta *et al.* 1997, 2000; Bonello *et al.* 1998; Selosse *et al.* 1998, 1999; Gherbi *et al.* 1999; Sawyer *et al.* 1999; Zhou *et al.* 1999; Fiore-Donno & Martin 2001; Guidot *et al.* 2001, 2002; Redecker *et al.* 2001). Uniformly small genets are generally thought to be indicative of continual establishment via sexual basidiospores (Dahlberg 2001; Guidot *et al.* 2002). The numerous, small genets of *C. formosus* and ITS-RFLP type B in all forest types indicate that genets may be frequently established by basidiospore dispersal and that sexual reproduction is a more prominent component in the life history of chanterelles compared to vegetative growth. In OG forests this might be facilitated by high turnover rates of fine roots (Vogt *et al.* 1986) continually providing new opportunities for genet colonization. Our study design lacks the temporal sampling needed to address the persistence of genotypes, so we cannot eliminate the possibility that *C. formosus* genets are slow growing or simply do not produce fruit-bodies across the entire spatial distribution of their mycelium each year.

The broader-scale life history strategy of *C. formosus* is still in question because it is not clear that we actually sampled genets from this species on our OG plots. ITS-RFLP type B dominated the OG sites and because there is a strong possibility that this sample represents an undescribed species, comparisons of genet size among all three treatments are suspect. If ITS type B represents an OG-adapted population of *C. formosus* then genet size may actually be smaller in OG than in SG. If ITS type B represents a different species, then we cannot address how *C. formosus* genet establishment and growth patterns are affected by environmental differences associated with stand age. This will require another genet study aided by better chanterelle species concepts.

Conclusions

Our knowledge of patterns in fungal genet propagation has increased substantially over the past 10 years and with this knowledge of pattern has come some understanding of the processes that shape genetic diversity in fungal populations (Dahlberg 2001). Each study of genet size has produced unique and sometimes unexpected estimates for different species. These results indicate that investigating genet size is a prerequisite for subsequent studies of genetic diversity at larger spatial scales. Here we describe five co-dominant markers that demonstrate a high degree of utility in the characterization of chanterelle genets. Our investigation of genet size in *C. formosus* in Douglas fir

forests has pointed to the possible existence of a cryptic and undescribed species of yellow chanterelle in the Pacific Northwest that may be associated with OG habitats. Regardless of the evolutionary status of these genetic entities, our data shows that mean maximum width of chanterelle genets is < 4 m, indicating that genet propagation is more likely the result of basidiospore dispersal than vegetative spread.

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