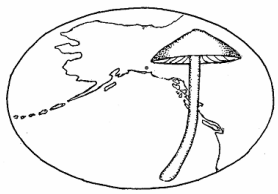
Pacific Northwest Fungi



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Genetic Structure of *Cantharellus formosus* Populations in a Second-Growth Temperate Rain Forest of the Pacific Northwest

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Abstract: *Cantharellus formosus* growing on the Olympic Peninsula of the Pacific Northwest was sampled from September – November 1995 for genetic analysis. A total of ninety-six basidiomes from five clusters separated from one another by 3 - 25 meters were genetically characterized by PCR analysis of 13 arbitrary loci and rDNA sequences. The number of basidiomes in each cluster varied from 15 to 25 and genetic analysis delineated 15 genets among the clusters. Analysis of variance utilizing thirteen apPCR generated genetic molecular markers and PCR amplification of the ribosomal ITS regions indicated that 81.41% of the genetic variation occurred between clusters and 18.59% within clusters. Proximity of the basidiomes within a cluster was not an indicator of genotypic similarity. The molecular profiles of each cluster were distinct and defined

as unique populations containing 2 - 6 genets. The monitoring and analysis of this species through non-lethal sampling and future applications is discussed.

Key Words: Pacific golden chanterelle, ectomychorrhizal fungi, molecular analysis, PCR

Introduction: Every year, thousands of tons of basidiomes representing several fungal species are commercially harvested from forests throughout the world for human consumption. The Pacific golden chanterelle (*Cantharellus formosus*), is a popular commercially harvested ectomycorrhizal fungi for domestic and foreign markets (Love et al., 1998). *C. formosus* occurs frequently throughout the Pacific Northwest and is often associated with spruce (*Picea sitchensis*), hemlock (*Tsuga heterophylla*) and Douglas fir (*Pseudotsuga menziesii*). Ectomycorrhizal symbiosis is a plant-fungal association that plays an important role in the biology, ecology and health of the forest ecosystems. Mutualistic benefits in the form of growth enhancement, water and nutrient acquisition, and protection from root disease have been reported through symbiotic interactions (Smith& Read 1997).

In recent years, concerns for the health and sustainability of forest ectomycorrhizal fungal populations exposed to frequent, heavy harvesting practices have arisen. Incorporation of fungal harvesting guidelines in the Pacific Northwest forest management plans has been suggested (Molina et al., 2001). These concerns do not appear to be unfounded. Earlier studies on harvesting practices of *Cantharellus cibarius (Fr.)* showed a decrease in fruiting body abundance due to human impacts; e.g. walking and disrupting the mycelia mats present in the forest floor (Egli & Ayer 1997). Management of natural ecosystems is complex and baseline data must be established for effective management guidelines to be designed. Foremost, a better understanding of spatial and temporal dynamics of mushroom populations influenced by harvesting must be established. In recent years, there have been several molecular genetic studies of several ectomycorrhizal species (Gherbi et al., 1999; Huai et al., 2003) including the Pacific golden chanterelle (Dunham et al., 2003). However, there have been no studies conducted in which a non-lethal sampling technique was incorporated. In so doing, our studies will allow us to revisit existing fungal populations in which individual basidiomes have been analyzed and the genet profiles (individuals possessing unique genotypes) established. Monitoring and comparing the genet profiles will provide baseline data to assess the possible impacts on the structure, health and sustainability of fungal populations in the presence and absence of harvesting. This information will allow resource managers to design management strategies to ensure ectomycorrhizal sustainability.

The goal of this study was to non-lethally genetically characterize *C. formosus* basidiomes within and between geographically distinct clusters in the absence of harvesting. This was determined by the presence or absence of polymorphic PCR markers, rDNA ITS restriction enzyme digestions, and ITS sequence analysis. The distribution of genets prior to imposing treatments for studying long term effects of harvesting on genotype expression is discussed.

Materials and Methods

Sample site and collection A mixed coniferous forest site of spruce and hemlock was identified on the Olympic Peninsula of Washington State that supported five spatially distinct clusters of *C. formosus* basidiomes and defined as "site 5". The large, mature trees were harvested approximately 55 years ago and presently, site 5 consists of second growth spruce and hemlock trees. Five *C. formosus* clusters (Figure 1 & 2A) were separated from one another by 3 to 25 meters, and a bench mark centrally established between and within the five clusters. Compass azimuth and distance measurements of each individual basidiome were mapped relative to one another. Each cluster contained between 15 and 25 basidiomes that were distributed over a 0.5 to 5 meter area (Figure 1, A - E). Approximately 20 mg of tissue from the pileal context was harvested for DNA extraction from 96 basidiomes between September and November of 1995 (Figure 2B). Samples were placed in microcentrifuge tubes containing 500 ul of DNA preservation buffer (150 mM EDTA, 50 mM Tri-HCl pH 8.0). Samples were collected without displacement of the fruiting body structure and disruption of underground mycelia complex. Samples were immersed in DNA preservation buffer and stored at room temperature (RT) or processed for total DNA extraction and stored at –70C.

DNA isolation: Total DNA was extracted from tissue samples immersed in DNA preservation buffer based on a previously published protocol (Rodriguez 1993). Samples were homogenized by hand with small pestles and 0.1 volume of 20% (w/v) sarkosyl added, samples mixed and incubated at 65 C for 30 min, and centrifuged 5 min at 14K rpm. The supernatant (S/N) was transferred to a new tube containing 300 µl of a solution of 20% polyethylene glycol (PEG, Mr 8000) and 2.5 M NaCl, mixed, and incubated 5 min at RT. The precipitated DNA was centrifuged for 5 min at 14K rpm, the DNA pellet re-suspended in 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). For the removal of RNA, polyphosphates and protein from samples, 100 µl of 7.5 M ammonium acetate was added, samples mixed, placed on ice for 5 min, and centrifuged for 5 min at 14K rpm. The S/N was transferred to a new tube containing 300 µl of n-propanol, mixed gently, and centrifuged for 5 min at 14K rpm. The DNA pellet was re-suspended in 200 µl TE buffer, NaCl to 0.1M and 400 µl of 95% ethanol added, samples mixed and centrifuged for 5 min at 14K rpm. The purified DNA pellet was re-suspended in 500 µl of TE buffer and placed at –70C for long term storage.

PCR and electrophoresis: PCR's (Saiki et al., 1985) were carried out in 20 µL volumes containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2% Triton X-100, 200 µM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.2 units Taq DNA polymerase, 500 ng of each oligonucleotide primer and 0.4 to 400 ng of fungal DNA. Amplification reactions were carried out in Barnstead/Thermolyne thermocyclers. Arbitrarily primed PCR (apPCR) involved amplification with single primers and 35 cycles of a temperature regime consisting of denaturation at 93C for 15 seconds, primer annealing (Table 1) for 1.5 min following a 30 s ramp from 93C, and synthesis at 72C for 1.5 min following a 1 min ramp from annealing temperatures. Prior to the initiation of the cycles, the reactions were incubated at 93C for 2 min. The temperature regime was the same for dual primer PCR (dpPCR) except the ramp times were eliminated and two primers were added to each reaction. Electrophoresis of the amplified products was performed for 1.5 hr at 12 to 16 V/cm in 2%

agarose and products visualized by staining with ethidium bromide and visualized using 305 nm UV light (Sambrook et al., 1989).

Generating marker-specific primer sets for dpPCR: Five basidiomes from five clusters in site 5 (Figure 1, A-E) were analyzed by apPCR with 2 single primers. Polymorphic bands were isolated, cloned, and sequenced using standard protocols (Sambrook et al., 1989) and marker-specific primer sets were generated based on sequences near the terminal regions of the cloned apPCR products (Ostberg & Rodriguez 2002; Rodriguez et al., 2004). The primer sets were used for dpPCR using the conditions described above. Sequences of the dpPCR primers and the annealing temperatures used for amplification are listed in Table 1. Eight primer sets were derived from polymorphic apPCR products which amplified 13 genetic markers that were used to analyze all 96 individuals representing the 5 basidiome clusters (Table 2).

Ribosomal DNA analysis: The rDNA ITS-1 region of *C. formosus* DNA was amplified with primers p1233 and p1234 (White et al., 1990) using conditions described above for dpPCR. The amplified products were precipitated with 0.1M NaCl and 2 volumes of 95% ethanol, incubated at RT for 60 min, and centrifuged at 14K rpm for 5 min. The amplified ITS regions were re-suspended in 10 mM Tris buffer (pH 8.5) and analyzed by restriction enzyme analysis using standard protocols (Sambrook et al., 1989). PCR products were sequenced by Northwoods DNA Inc. (http://www.nwdna.com).

Data Analyses: The genetic distance between all pairs of individuals was calculated as a Euclidean metric using the AMOVA-PREP program (http://herb.bio.nau.edu/~miller). The resulting distance matrix was subjected to AMOVA analysis (Excoffier et al., 1992) using WINAMOVA (http://anthropologie.unige.ch/ftp/comp) and the following variance component calculated: within and between populations in site 5. Genotypic Diversity was calculated using a normalized Shannon's diversity index (Goodwin et al., 1992):

$$[1] \qquad Hs = \frac{-Pi \ lnPi}{lnN}$$

Where Pi is the frequency of the ith multilocus genotype and N is the sample size. The values for Hs range from 0 to 1 where 1 reflects a population comprising genetically unique individuals and 0 indicates a clonal population (Table 2).

Results and Discussion

Sampling site identification and strategy: Site 5, was specifically chosen to determine the genetic structure of *C. formosus* due to the presence of a large number of basidiomes (a total of 96 sampled throughout the season) in five spatially distinct clusters, with each cluster containing 15-25 basidiomes (Figure 1 & 2A). This site presented an ideal setting because: 1) spatially distinct clusters may aid in the identification of genetic polymorphisms; 2) several different harvesting regimes could be imposed among the five clusters; and 3) the large number of basidiomes per cluster would lend fidelity to our statistical analysis. To perform this study it was necessary to: 1) design a non-lethal sampling technique, 2) use PCR analysis to verify species, 3) develop high fidelity, population discriminating markers for genetic structure analysis, and 4) address concerns regarding the presence of contaminants in original samples collected.

Sample collection: A non-lethal, efficient, and inexpensive sampling technique was developed for the effective long-term storage of tissue samples. The estimated costs for the sample storage and processing was less than $1 per sample and over 100 samples easily processed within a few hours. Due to the high EDTA and Tris buffer content, the preservation buffer was effective for the long term storage of fungal tissue at either 4C or RT. Samples stored in such a manner for 10+ years were processed and no significant degradation of DNA observed (data not shown). As such, this preservation buffer allows one to inexpensively collect a large number of samples from numerous seasons which can then, at some later date, be processed and analyzed at leisure, without fear of sample ruin. In addition, a minimal amount of tissue (approximately 20 mg) was taken from basidiomes to avoid genetic concerns about basidiome removal (Figure 2B). It was possible to collected samples without displacing the fruiting body structures and careful footing within site 5 minimized the disruption of the underground mycelial complex (personal observations). Tissue samples were collected near the outer rim of the pileal context resulting in a tell-tale triangular-shaped mark on the basidiome. In so doing, previously sampled basidiomes were easily identified and sampling of only the newly emerged basidiomes throughout the season (September – November) was possible. We chose to sample only firm, fresh basidiomes to decrease the chance of sampling tissues contaminated with microorganisms (bacteria and/or fungal).

Species Identification: The rDNA ITS-1&2 regions of all 96 basidiome samples were analyzed by PCR/RFLP analysis and sequenced from 10 individuals from each cluster to confirm their identity as *C. formosus* (data not shown). To determine if cluster specific differences could be identified in the ITS-1 region, PCR amplified products were treated with restriction enzymes having 4 base pair (bp) recognition sequences (Cfo1, Csp61, Hae111, Hsp9211, and Msp1). The amplified products were approximately 1200 bp in length as anticipated (Feibelman et al., 1994) and were digested by Csp61 and Hsp9211 but not by the other three enzymes (data not shown). Although there was no indication of cluster specific digestion patterns with these enzymes, two ITS products (approx. 1000 bp and 1100 bp) were amplified from all of individuals in cluster A and a single ITS product was amplified from all of the individuals representing clusters B - E (data not shown). To increase the resolution of analyses, the ITS-1 region of four individuals representing each of the clusters (B - E) that produced single rDNA products by PCR amplification was sequenced. Although minor differences were observed between the individuals, there was one small region of the ITS sequence that was polymorphic between clusters. Approximately 500 bp from primer 1233 (Table 1), there was a series of adenosines that varied in number from 7 - 13 between the five clusters. The four individuals representing clusters B - E had 10, 8, 7, and 13 adenosines, respectively, indicating cluster-specific differences in number of adenosines. The length of time required for such cluster specific polymorphisms to be acquired is hard to determine. However, these results do indicate that the 5 clusters represent unique populations. In the remainder of the text, the term cluster and population will be used inter-changeably.

Population analysis: Single primer PCR was performed on all 96 basidiomes to identify polymorphic markers that could be used to discern

genets within and between populations. Two simple sequence repeat (ssr) primers identified several polymorphic and monomorphic markers (data not shown). The polymorphic markers were isolated and sequenced to design marker-specific primers for dpPCR (Table 1). The seven generated dpPCR primer sets either amplified single products (cf31, cf34), multiple polymorphic products (cf28, cf30, cf33), or both monomorphic and polymorphic markers (cf13, cf36). The amplified regions were designated ssr-loci since dpPCR primer sets were derived from ssr primers p89 & p135 (Table 1).

Analyses of 13 ssr loci and the rDNA ITS regions revealed that the five clusters in site 5 represented distinct polymorphic populations. Each cluster contained 2 to 6 genotypes and a total of 15 unique genets delineated among the five clusters (Table 2). In addition to identifying the clusters as distinct populations, several of the markers were polymorphic among individuals within a population. In fact, all five populations showed polymorphic differences among the individuals with one or more markers indicating that each population were comprised with multiple individuals. There appeared to be no obvious spatial relationships between polymorphic individuals within a population. Only two genotypes (genotype 2 and 4) were shared between two clusters (A and C) , however, rDNA analysis indicated that these populations were genetically distinct. Although there were individuals within each population that had identical genotypes, there appeared to be little to no correlation between proximity of individuals with clonality (Figure 1). A summary of genotype diversity and distribution is presented in Table 2 and Figure 1, respectively. All of the populations represented unique genotypes although the composition and distribution frequency varied significantly. The highest Hs (0.51) was observed in Population A which comprised 6 genotypes and the lowest Hs (0.05) was found in population D which was dominated by a single genotype (Table 2). Several markers analyzed were population-specific either by their absence or presence in all of the individuals from a cluster, and some intra-population monomorphic markers had population-specific patterns (Table 2). The uniqueness of each population was also confirmed by AMOVA which indicated that 81.41% of the observed genetic variation was attributed to inter-population differences and 18.59% of the variation occurred within populations. Random permutation tests indicated that the variance components were significant ($P < 0.001$; Table 3). Collectively, the genotype distribution patterns and AMOVA results suggest that individuals within populations are more closely related than individuals between populations.

Addressing contamination concerns:
It was important to ensure that the genetic markers used in these studies represented *C. formosus* genomes and not other fungal or bacterial residents/parasites of basidiome tissues (Danell et al., 1993). We addressed these concerns by: 1) performing dpPCR on all *C. formosus* samples with eubacterial-specific rDNA PCR primers (p208 and p209, Table 1; DeLong, 1992). Bacterial DNA was detected in less than 5% of the samples and there was no correlation between the presence of bacterial DNA and specific *C. formosus* genotype patterns and; 2) NCBI blast analysis of monomorphic and polymorphic marker sequences revealed no similarity to any bacterial (including several Pseudomonads) or other fungal genomes .

Our genetic analysis showed unique +/- polymorphisms occurring within each of these five *C. formosus* populations.

This baseline data allows us to infer on aspects of life histories, dispersal, and establishment of these populations. For instance, these populations may have started from one or more clonal individuals and the level of polymorphism detected are a result of the accumulation of mutations over time since establishment. If so, this would imply that population D represents a recent introduction at this site and that the other populations were established at different times with population A being the oldest. It is also possible that *C. formosus* may mutate at relatively high rates in response to certain environmental conditions. This would indicate that population D had not experienced conditions conducive to mutation. The other populations may reflect either the magnitude of mutation inducing conditions, the number of mutation events, or a combination of the two over time. Alternatively, the populations may have been established by groups of related individuals that were genetically polymorphic and had little to no genetic exchange with adjacent populations.

The information from this single sampling season study has allowed us to better define the genetic basis of site 5, *C. formosus* populations in the Olympic Peninsula. To better understand the dispersal, establishment, and impacts of various harvesting regimes on the health and maintenance of these fungal populations, a long-term study was designed. Since individual clusters represent distinct genetic populations, it is important to determine potential impacts of commercial harvesting practices and environmental conditions on the health and sustainability of *C. formosus* populations.

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Table 1. Primers and annealing temperatures. [a]primers with the same number were used as forward (f) and reverse (r) primers in dpPCR, p89 and p135 were used for apPCR, and cf33 produced different products at the indicated temperatures. cf primers were derived from polymorphic products amplified with p89 and p135. [b]direction of nucleotides.

Primer[a]	**Sequence (5'>3')**[b]	**Annealing (°C)**
p89	tggtggtggtggtgg	56
p135	ctgctgctgctgagct	56
p208(27f)	agagtttgatcctggctcag	64
p209(1492r)	ggttaccttgttacgactt	64
p1233(ITS1)	tccgtaggtgaacctgcgg	54
p1234(ITS2)	gctgcgttcttcatcgatgc	54
cf13f	caatgatggggaaagcgtag	64
cf13r	gtaaactgggatcatgggac	64
cf14f	tccaacgatgggtactcttg	64
cf14r	gcgaaatgaagatcgttccc	64
cf28f	aaccacccaactcttcttgg	62
cf28r	ccatctgcgcacatctctaa	62
cf30f	ccatccgcattcatactcta	60
cf30r	acgaaagctgagaagcactg	60
cf31f	atcaagggcagttaccaagg	66
cf31r	ttggcccacttttggacatga	66
cf33f	aagtcctgcaggtctctgat	50/68
cf33r	cgtctctaacacattgggtg	50/68
cf34f	ggggactttcagagcacatt	64
cf34r	catcaagggcagttaccaag	64
cf36f	tcaatccgtgtggcttcaag	56
cf36r	acaagcaggttctggatgcg	56

Table 2. Site 5 genotype composition of 96 *C. formosus* basidiomes from five populations (A-E) using 13 PCR gnerated markers. Genotypes were based on the presence (1) or absence (0) of 13 dpPCR markers. Ninety-six basidiomes from 5 populations (A-E) were analyzed and fifteen genets identified. The number of basidiomes falling into each of the genet profiles (1-15) are summarized on the right. Basidiomes were categorized as the total number of individuals of each genotype profile (genets 1-15) from each population (A-E). Genotype composition numbers in large bold font represent marker profiles that are unique to a cluster and defined as population discriminating markers. The genotype diversity (see text) for each population is indicated on the lower right in bold.

	Genotype Composition														#Individuals/ Genotype/ Population				
PCR primers	cf13	cf13	cf28	cf28	cf28	cf30	cf30	cf31	cf33	cf33	cf34	cf36	cf36						
Marker sizes in base pairs	1400	700	375	625	650	400	1200	980	400	800	1050	550	800						
Marker #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**						
Genets															**A**	**B**	**C**	**D**	**E**
1	1	1	0	1	1	1	0	1	1	1	1	1	1	=	5				
2	1	1	0	1	1	1	0	1	0	1	1	1	1	=	6		5		
3	0	1	0	1	1	1	0	1	1	1	1	1	0	=	1				
4	1	1	0	1	1	1	0	1	0	1	1	1	0	=	6		13		
5	1	1	0	1	1	1	0	1	1	1	1	1	0	=	1				
6	1	1	0	1	1	1	0	1	0	1	0	1	0	=	1				
7	1	1	**1**	1	1	1	0	1	1	0	1	1	0	=		11			
8	1	1	**1**	1	1	1	0	1	0	0	1	1	0	=		4			
9	1	1	0	1	1	1	1	1	0	1	1	1	0	=		1			
10	**0**	1	0	1	1	1	0	0	0	0	1	1	0	=			1		
11	**0**	1	0	1	1	1	0	1	0	1	1	1	0	=			1		
12	1	1	0	0	**0**	1	0	1	0	1	1	1	1	=				1	
13	1	1	0	0	**0**	1	0	1	0	1	1	1	0	=				24	
14	1	1	0	0	1	**0**	**1**	1	1	0	1	1	0	=					6
15	1	1	0	0	1	**0**	**1**	1	0	0	1	1	0	=					9
													Genotype Diversity (Hs)	=	**0.51**	**0.28**	**0.31**	**0.05**	**0.25**

Table 3. Analysis of molecular variance (AMOVA) for five populations of *C. formosus.* df = degrees of freedom, SSD = sum of squared deviation, MSD = mean squared deviation, P = probability after 1000 random permutations

Source of variation	df	SSD	MSD	Variance Component	%Total Variance	P
Among populations	4	109.45	27.36	1.42	81.41	<0.001
Within populations	91	29.51	0.32	0.32	18.59	<0.001

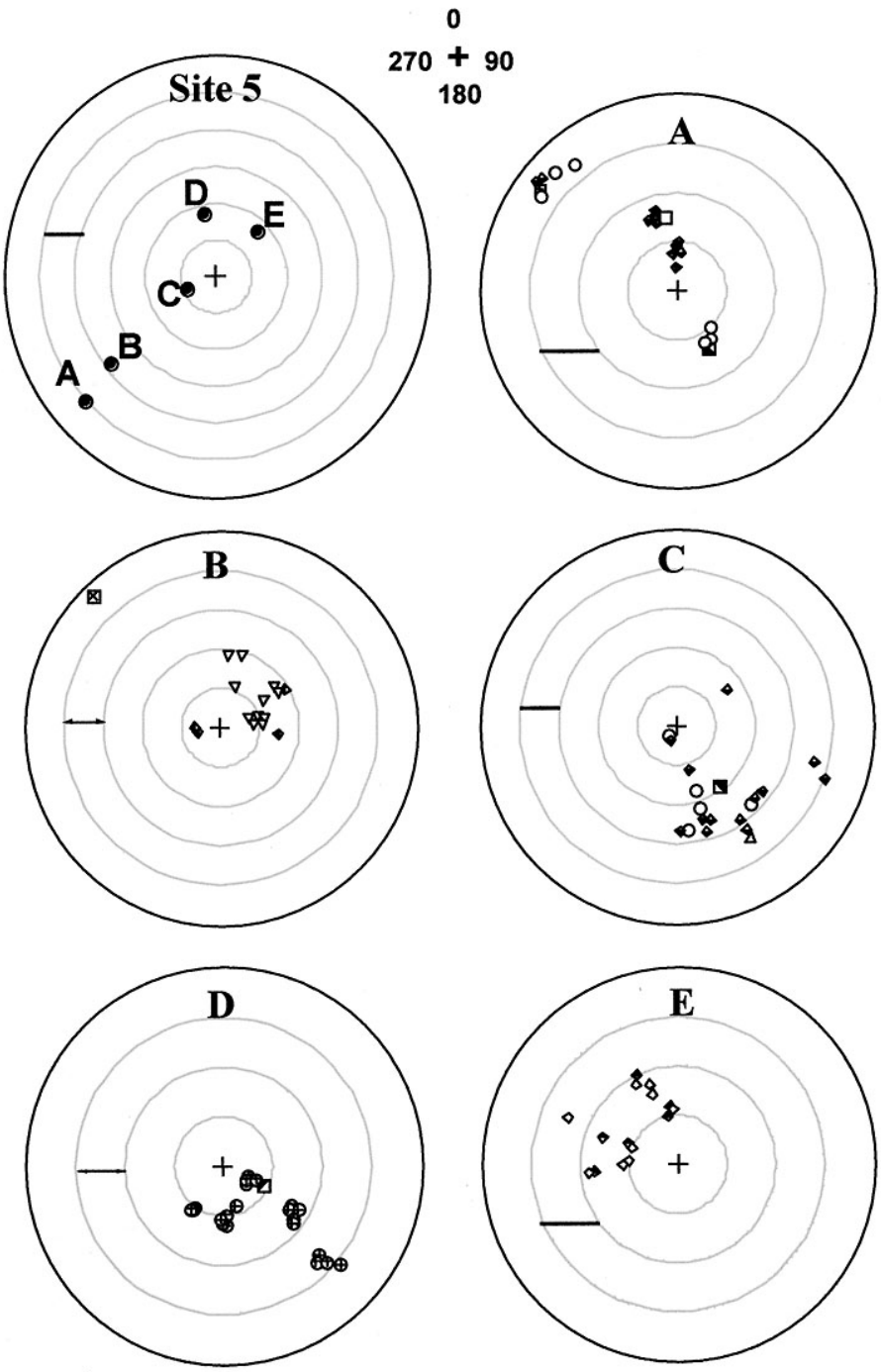

Figure 1. Spatial distribution patterns between 5 clusters of *C. formosus* population in site 5 and individual basidiomes within each cluster (A-E). Fifteen unique genets are indicated by different symbols. The relationship between clusters and individuals within clusters is based on compass azimuth and distance in meters (m) with the dark horizontal line drawn in each circle representing: Site 5 = 5 m; clusters A-D = 1 m; and cluster E = 0.25 m.

Figure 2A. Representative photo of a *C. formosus* population in site 5.

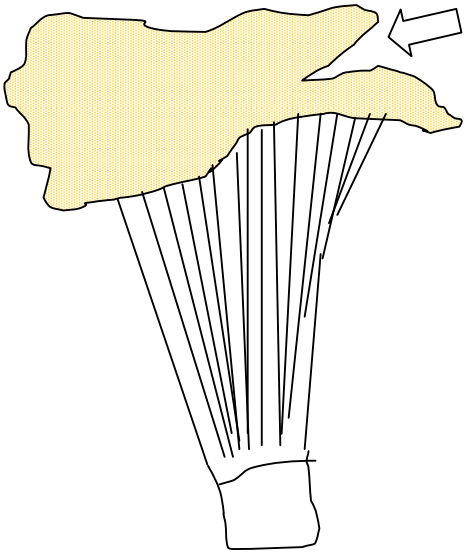

Figure 2B: Illustration of a representative basidiome left with a 'tell-tale" triangular-shaped (indicated by the arrow) marking after non-lethal sampling.