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Analysis of nrDNA sequences and microsatellite allele frequencies reveals a cryptic chanterelle species *Cantharellus cascadensis* sp. nov. from the American Pacific Northwest

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In the Pacific Northwest, yellow chanterelles have long been referred to as *Cantharellus cibarius*, synonymous with the European yellow chanterelle. Broad scale genetic surveys of North American chanterelles with *C. cibarius*-like morphology have demonstrated that the nrDNA internal transcribed spacer exhibits length variability, suggesting that this common morphology masks a species complex. Recently researchers have used morphological and genetic data to identify the yellow chanterelle most frequently harvested from American Pacific Northwest forests as *C. formosus*, a species once thought to be rare in the region. We present three genetic data sets and one morphological data set that characterize a previously undescribed, species of yellow chanterelle from the central Cascade Mountains of Oregon. Phylogenetic analyses of the nrDNA large subunit and ITS regions show that *C. cascadensis* sp. nov., along with two other yellow chanterelle taxa (*C. cibarius* var. *roseocanus* and European *C. cibarius*), are more closely related to white chanterelles (*C. subalbidus*) than they are to *C. formosus*. Data from five microsatellite loci provide evidence that *C. formosus*, *C. subalbidus*, and *C. cascadensis* sp. nov. do not interbreed when they co-occur spatially and temporally in Douglas fir-western hemlock forests. This demonstrates that these three sympatric chanterelles are biological species with boundaries congruent with those delineated by nrDNA phylogenetic clades. Morphological data indicate that the colour of the pileus and shape of the stipe can be used to separate fresh collections of the two yellow species now known to co-occur in Douglas fir-western hemlock forests in Oregon.

INTRODUCTION

Chanterelles are popular edible fungi commercially and recreationally harvested from wild populations in Europe, Africa, Asia, and the western United States (Danell 1999). Species in the genus *Cantharellus* also are ectomycorrhizal with commercially harvested conifers and their economic and ecological importance has inspired research into the evolution and ecology of the genus (Danell 1999, Molina *et al*. 2001). Until recently, morphologically similar yellow chanterelles throughout North America were lumped under a single Latin binomen and considered synonymous with the European yellow chanterelle (*Cantharellus cibarius*). In contrast, throughout the twentieth century, mycologists have noted that yellow chanterelles found in North America not only are morphologically distinct from European species (Murrill 1912, Smith & Morse 1947, Corner 1966, Petersen 1979) but also exhibit intra-specific variation at regional scales across North America (Smith & Morse 1947, Feibelman *et al*. 1997, Redhead, Norvell & Danell 1997).

Genetic studies of North America chanterelles with *C. cibarius*-like morphology have demonstrated significant length variability in the nuclear ribosomal internal transcribed spacer (nrDNA ITS), which suggests that this common morphology masks a species complex (Feibelman, Bayman & Cibula 1994). Recently researchers have used morphological and genetic data to identify the yellow chanterelle most frequently harvested from American Pacific Northwest (PNW) forests as *C. formosus*, a species once thought to be rare in the region (Redhead *et al*. 1997). We have studied genetic variability within putative *C. formosus* populations using RFLP analysis of the ITS region (ITS 1, ITS 2, and the 5.8s gene) and co-dominant microsatellite markers (Dunham, Kretzer & Pfrender 2003). The *C. formosus* collections examined exhibited two

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genotypes that varied in the total length of the ITS region and in RFLP patterns. One ITS genotype from these collections matches that described for *C. formosus* (~1690 base pairs; Danell 1995, Redhead *et al.* 1997), the other, a ~1490 base pair (bp) ITS region, is more similar in length to the ITS from both white chanterelles (*C. subalbidus*; Dunham 2003) and European *C. cibarius* (Danell 1995).

Sequence and RFLP analysis of the ITS region is a popular method often used to characterize species in fungi (e.g. Shinohara, LoBuglio & Rogers 1999, Høiland & Holst-Jensen 2000). ITS length variability similar to that described for *C. formosus* has been observed in the ectomycorrhizal fungus *Cenococcum geophilum* and was thought to delineate several cryptic species (LoBuglio, Rogers & Wang 1991) until subsequent analyses showed that the length polymorphism is due to the presence of a family of group-I introns inserted near the 3′ end of the nrDNA small subunit. Hence, *C. geophilum* collections with ITS regions of different length actually represent a single species (Shinohara, LoBuglio & Rogers 1996, 1999). In contrast, studies of the ITS region in *Armillaria* (Anderson & Stasovski 1992) and *Hebeloma* (Aanen *et al.* 2000) have characterized situations where variability in ITS sequences is low (<1.0%) even among reproductively isolated species. Given that the evolutionary implications of ITS length and RFLP variability can be unclear, more precise molecular and morphological comparisons are needed to better understand species boundaries within *Cantharellus*. Chanterelles exhibit a limited number of conserved morphological characters (Smith & Morse 1947, Thiers 1985), thus application of a variety of molecular markers should prove useful in defining genetic lineages that can be closely examined for previously undetected phenotypic differences (e.g. Fisher *et al.* 2002).

Mapping species boundaries onto the hierarchical structure of a nrDNA phylogeny requires an objective method of recognizing the level at which dichotomous branching represents divergence of lineages on independent evolutionary paths (i.e. evolutionary species *sensu* Simpson 1951, 1961, Wiley 1978). Methods proposed for recognizing evolutionarily independent lineages include mapping morphological data onto a topology to identify minimum aggregations that share distinct, diagnosable phenotypic characters (Harrington & Rizzo 1999) or generating multiple phylogenies from independent loci and identifying concordant lineages (Taylor *et al.* 2000). These methods require either observable variability in morphology or sequencing multiple genes for each taxon. A third option exists when the taxa in question are sympatric in distribution. Variable co-dominant loci can be used to test for significant allele frequency differences among sympatric collections. This sampling strategy eliminates local adaptation or independent population histories as an alternate explanation for genetic differentiation (Fisher *et al.* 2000) while greatly reducing expense and allowing characterization of large samples prohibited by the expense of sequencing based studies.

This investigation describes evolutionary relationships among PNW chanterelle species and uses information from microsatellite loci to determine if observed ITS variability is indicative of biological species boundaries. Specific objectives of the present study are to: (1) analyse nrDNA sequences to determine the phylogenetic relationships among PNW chanterelle collections with alternate ITS genotypes and European *C. cibarius*; (2) analyse microsatellite allele frequencies from sympatric chanterelle collections that represent multiple ITS genotypes to test for gene flow among ITS genotypes; and (3) identify morphological characters useful in differentiating cryptic species within the *C. formosus* morphology. Finally, we describe a new species of *Cantharellus* based on these genetic and morphological characters.

MATERIALS AND METHODS

Material examined

To better characterize the total genetic diversity present in the ITS region within western chanterelles, samples for RFLP analysis were taken from several locations in Washington, Oregon, and California. A large proportion of the collecting effort was centered in the H. J. Andrews Experimental Forest (HJA, lat. 44° 15′ N, long. 122° 10′ W) and surrounding areas in the central Oregon Cascades during 1997–98 with additional collecting efforts made opportunistically in more broadly distributed localities during 1997–2000 (Fig. 1). The broad host range of *Cantharellus* allowed sample collection in forests containing a variety of host trees (i.e. *Pseudotsuga*, *Tsuga*, *Picea*, and *Quercus*) at elevations ranging from sea level to approximately 1700 m. Collections of European *C. cibarius*, *C. cibarius* var. *roseocanus*, *C. cinnabarinus* and *C. persicinus* were obtained from mycologists familiar with these species. nrDNA large subunit sequences for *Clavulina cristata*, *Hydnum umbilicatum*, *Craterellus odoratus*, and *Craterellus tubaeformis* used here as outgroup taxa were taken from Dahlman, Danell & Spatafora (2000). Dried voucher materials from all analysed collections are deposited in the Oregon State University herbarium (OSC, Table 1). More detailed information on the collection localities of voucher specimen can be found in Dunham (2003).

Chanterelle collections used to define phylogenetic lineages, test for gene flow among ITS variants, and to describe morphological variation were drawn primarily from the HJA. Elevations within the HJA range from 420 to 1630 m and the area is typical of the central portion of the western Cascade Mountains in Oregon (Franklin, Bledsoe & Callahan 1990). The primary overstory species of HJA study areas are Douglas fir (*Pseudotsuga menziesii*), western hemlock (*Tsuga heterophylla*), and Pacific silver fir (*Abies amabilis*) and

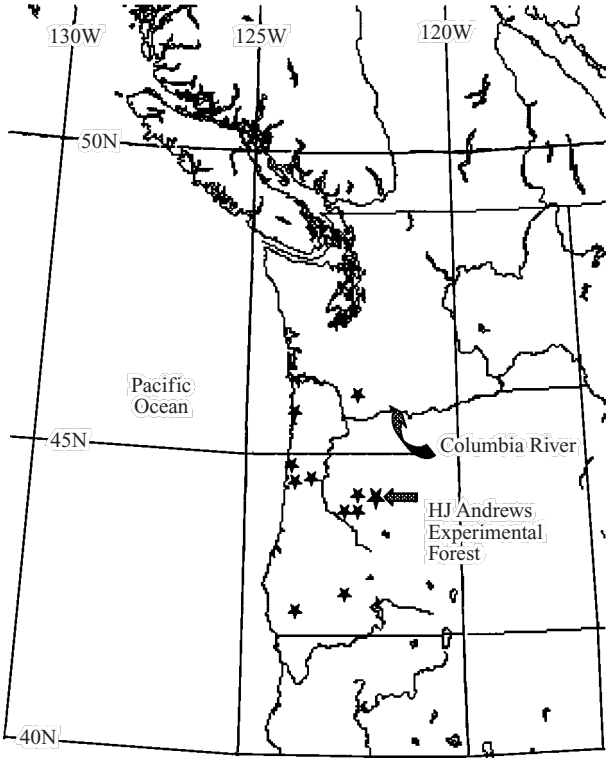

Fig. 1. A map of the Pacific Northwest showing the geographic distribution of chanterelle collections examined. The majority of collections were taken from the vicinity of the HJ Andrews Experimental Forest.

are in the western hemlock and Pacific silver fir zones of Franklin & Dyrness (1973). Collections made within the HJA came from 18 stands dominated by Douglas fir and western hemlock trees either 350+ yr old or stands that had been clear cut during 1950–61 and re-generated naturally. Second growth stands ranged from 10–15 ha and the area searched within old growth stands roughly approximated this range. Two collectors searched each stand for 1.5 h once in October and once in November and samples from these two time periods were pooled to derive data for microsatellite analyses. To avoid re-sampling individuals a minimum of 5 m (based on preliminary genet size estimates, Dunham *et al.* 1998) was kept between any chanterelle collections of the same colour (white or yellow) and collection locations were flagged to avoid re-sampling individuals encountered by collectors at different times. A minimum of five meters also was kept between collections made outside of the HJA but in these areas random search methods were not employed and most collecting took place along established hiking trails.

DNA extraction and amplification

After grinding a small amount of fresh or dried tissue in 1 ml of lysis buffer (100 mM Tris, 10 mM EDTA, 2% sodium dodecyl sulfate, 1 mg/ml proteinase-K, pH 8.0), each sample was incubated for 1.5 h at 55 °C and DNA was extracted using a standard phenol/chloroform emulsification and precipitation (Maniatis, Fritsch & Sambrook 1982). Resulting pellets were vacuum-dried and re-suspended in 50–200 μl of sterile TE buffer (10 mM Tris, 1 mM EDTA). Unquantified DNA samples were diluted 10–1000-fold prior to use and the polymerase chain reaction (PCR) was attempted on successive ten-fold dilutions until amplification of the desired nrDNA region was achieved. The entire ITS region spanning the 3′ end of the 18S, ITS-1, 5.8S, ITS-2, and 5′ end of the 28S was amplified with the fungal specific primer ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). The 5′ end of the nuclear large subunit was amplified with ITS4R (White *et al.* 1990) and LR5 (Vilgalys & Hester 1990). All amplifications of nrDNA regions were carried out in 40 μl volumes that contained 5 μl of diluted template. Final concentrations of PCR mix components were 200 μM of each dNTP, 0.2 μM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.75 units of DNA polymerase. Following an initial denaturation period at 95 ° for 3 min, reactions were subject to 35 PCR cycles with the following profile, denaturation at 95 ° for 1 min, primer annealing at 50 ° for 1 min, and primer extension at 72 ° for 90 s. Following the 35th cycle, samples were subjected to a final extension of 72 ° for 10 min.

Amplification of microsatellite loci were carried out using primer sequences developed for *Cantharellus formosus* described in Dunham *et al.* (2003). PCR reactions were carried out in 20 μl volumes and contained 2–3 μl diluted genomic DNA, 200 μM dNTPs, 0.25 μM of each locus specific primer, 1 U Taq DNA polymerase and 1X Assay Buffer A (Fisher Scientific). The PCR profile consisted of initial denaturation at 95 ° for 3 min followed by 35 cycles (95 °, 45 s; 55 °, 1 min; 72 °, 1 min) in a PTC-100 Programmable Thermal Controller (MJ Research, MA). Following the 35th cycle, samples were subjected to a final 72 ° extension of 60 min to maximize the proportion of complete PCR products prior to analysis. 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, Foster City, CA).

Restriction fragment length polymorphisms (RFLP's)

Unpurified PCR products amplified with ITS1-F and ITS4 were digested separately with four restriction enzymes, *Alu*, *HinfI*, *DPNII*, and *HaeIII*, according to the manufacturer's recommendations (GIBCO BRL, Grand Island, NY). Restriction fragments were electrophoretically separated on 3% agarose gels (2% Nu-Sieve agarose, 1% SeaKem LE agarose; FMC BioProducts, ME), stained with ethidium bromide, and scored against a 100 bp ladder using the Alpha Imager 2000 documentation and analysis system V 3.2 (Alpha Innotech Corp.). Each collection sampled in this study

Table 1. Collection numbers, geographical origins, habitat, and genbank accession numbers associated with samples used in molecular and morphological analyses.

Voucher specimen (OSC number)	Species	State/Country	County	Dominant overstory[a]	ITS type[b]	GenBank accession no.
76054	*Cantharellus formosus*	OR, USA	Lane	PT	A[1, 2, 3 & 4]	[1]AY041165, [2]AY041184
75930–31	*Ca. formosus*	OR, USA	Lane	PT	A[1, 3 & 4]	[1]AY041164, 66
75926, 81, 76024–25, 33, 37–41, 46, 48, 56, 59	*Ca. formosus*	OR, USA	Lane	PT	A[3 & 4]	
76021, 50, 58	*Ca. formosus*	OR, USA	Lane	PT	A[3]	
75972	*Ca. formosus*	OR, USA	Linn	PT	A[4]	
75906, 09–11, 41–42, 45–56, 73–74, 80, 87–89, 92–94, 96–99, 76000–01, 03–05, 08, 14, 16, 60	*Ca. formosus*	OR, USA	Lane	PT	A[4]	
75957–59	*Ca. formosus*	OR, USA	Benton	PT	A	
75960–67, 76065–70	*Ca. formosus*	OR, USA	Lane	PT	A	
67713–14, 76012–13	*Ca. formosus*	OR, USA	Lincoln	P	A	
76009–11, 15	*Ca. formosus*	OR, USA	Tillamook	P	A	
76057	*Cantharellus* sp.	CA, USA	Santa Barb.	Q	F[1]	[1]AY041167
75937, 76034	*Ca. subalbidus*	OR, USA	Lane	PT	C[1, 2 & 3]	[1]AY041149, 47; [2]AY041179, 78
75983, 76028, 32	*Ca. subalbidus*	OR, USA	Lane	PT	C[1 & 3]	[1]AY041146, 48, 50
75902–05, 27–29, 32–33, 35–36, 76020, 23, 26, 35–36, 42–43, 47, 49, 51, 53, 55	*Ca. subalbidus*	OR, USA	Lane	PT	C[3]	
75912, 68–71, 95, 76007	*Ca. subalbidus*	OR, USA	Lane	PT	C	
76061–64	*Ca. subalbidus*	OR, USA	Linn	PT	C	
75917	*Ca. cascadensis*	OR, USA	Lane	PT	B[1, 2, 3 & 4]	[1]AY041158, [2]AY041180
75908	*Ca. cascadensis*	OR, USA	Lane	PT	B[1, 2 & 3]	[1]AY041160, [2]AY041181
75975, 85	*Ca. cascadensis*	OR, USA	Lane	PT	B[1, 2 & 4]	[1]AY041163, 62; [2]AY041183, 82
75982	*Ca. cascadensis*	OR, USA	Lane	PT	B[1, 3 & 4]	[1]AY041161
75979	*Ca. cascadensis*	OR, USA	Lane	PT	B[1 & 3]	[1]AY041159
75907, 21, 23–25, 90, 76002, 18–19, 72	*Ca. cascadensis*	OR, USA	Lane	PT	B[3 & 4]	
75916, 18–20, 22, 84, 86, 91, 76006, 17	*Ca. cascadensis*	OR, USA	Lane	PT	B[3]	
75913–15, 34, 38, 76–78, 76073	*Ca. cascadensis*	OR, USA	Lane	PT	B[4]	
70397	*Ca. cascadensis*	OR, USA	Josephine	PL	B	
70396	*Ca. cascadensis*	OR, USA	Jackson	PT	B	
75940, 76027	*Ca. cibarius* var. *cibarius*	Sweden	–	B	E[1 & 2]	[1]AY041157, 55; [2]AY041177, 75
76031	*Ca. cibarius* var. *cibarius*	Sweden	–	Conifers	E[1 & 2]	[1]AY041156, [2]AY041176
67634	*Ca. cibarius* var. *roseocanus*	WA, USA	Skamania	P	D[1 & 2]	[1]AY041154, [2]AY041174
67712, 76045	*Ca. cibarius* var. *roseocanus*	OR, USA	Lincoln	P	D[1 & 2]	[1]AY041152, 53; [2]AY041172, 73
66326	*Ca. cibarius* var. *roseocanus*	OR, USA	Lincoln	P	D[1]	[1]AY041151
67711, 15, 75939, 43–44, 76052, 71	*Ca. cibarius* var. *roseocanus*	OR, USA	Lincoln	P	D	
76022, 29, 44	*Ca. cibarius* var. *roseocanus*	OR, USA	Linn	P	D	
76030	*Ca. cibarius* var. *roseocanus*	OR, USA	Tillamook	P	D	
69197	*Ca. cinnabarinus*	SC, USA	Oconee	MH	N/A[1]	[1]AY041168
69195	*Ca. persicinus*	SC, USA	Oconee	MH	N/A[1]	[1]AY041169
Outgroup Taxa						
–	*Craterellus odoratus*[c]	MS, USA	–	–	N/A[1]	[1]AF105306
–	*Cr./Ca. tubaeformis*[c]	Sweden	–	–	N/A[1]	[1]AF105307
49915	*Cr./Ca. tubaeformis*[c]	WA, USA	–	–	N/A[1]	[1]AF105309
–	*Hydnum umbilicatum*[c]	OR, USA	–	–	N/A[1]	[1]AY041170
–	*Clavulina cristata*[c]	OR, USA	–	–	N/A[1]	[1]AY041171

[a] Dominant overstory: PT, *Pseudotsuga menziesii* and *Tsuga heterophylla*; PL, *Pseudotsuga menziesii* and *Lithocarpus densifolia*; P, *Picea* sp.; B, *Betula* sp.; Q, *Quercus* sp.; and MH, mixed hardwoods.

[b] ITS type: RFLP types are compiled from data collected using four restriction enzymes (*Alu*, *DpnII*, *HinfI*, and *HaeIII*). Superscript codes: 1, included in sequence analysis of nrDNA large subunit; 2, included in sequence analysis of the ITS; 3, included in analysis of microsatellite data; and 4, included in study of pileus colour and other morphological features.

[c] Sequence received from Joey Spatafora from data set analysed in Dahlman *et al*. (2000).

was assigned to an ITS type that summarised information from the four restriction enzyme profiles. Thus, collections with the same ITS type designation share identical restriction fragment patterns across all four enzymes. Information including RFLP types, fruitbody morphology, and geographic location were used to identify the groups of interest in subsequent sequence and microsatellite comparisons.

DNA sequencing

Prior to sequencing PCR products were purified using QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and diluted 2-fold. Sequencing followed standard direct dye-terminator automated florescence methods. Both large subunit and ITS sequencing was performed using primers employed in PCR reactions. The length of the large subunit products permitted complementary strands to be compared for the 24 novel sequences produced in this study. Internal ITS primers (2 and 3; White *et al*. 1990) do not have high homology in *C. formosus* preventing their use as internal sequencing primers. The length of the ITS region from *Cantharellus* (1400–1600 bp) does not permit the production of overlapping sequences from opposite directions. Because complementary strands could not be obtained for either end of the ITS region, sequences were truncated at 500 bp from either direction. In our general experience with the ABI sequencing system 500 bp is within the range of maximum sequence accuracy, thus reducing the need for complementary sequences. Sequences were edited and manually aligned by eye using Sequence Navigator v. 1.0.1 (Applied Biosystems).

Sequence data analysis

nrDNA large subunit and ITS sequences were aligned to minimize the number of inferred gaps and portions were re-coded from the DNA format to the symbols format in order to use selected gaps as informative characters (*sensu* Bruns *et al*. 1992). Taxa used to root this analysis were selected based on phylogenetic relationships presented in Feibelman *et al*. (1997), Pine, Hibbett & Donoghue (1999), and Dahlman *et al*. (2000). Phylogenetic analyses on the resulting ITS alignment were conducted with the unweighted maximum parsimony algorithm and branch and bound search option available in PAUP* v 4.0b2 (Swofford 1999). The large subunit alignment was analysed using the heuristic search option with 1000 stepwise random sequence addition replicates and the tree bisection-reconnection (TBR) branch swapping option. Non-parametric bootstrapping (Felsenstein 1985, Sanderson 1989) was used to assess the reliability of individual nodes in the resulting trees. For the ITS bootstrap analysis, branch and bound search options were employed with 1000 bootstrap replicates, while for the large subunit analyses heuristic search parameters included ten replicates of random addition sequences for each of the 1000 bootstrap replicates.

Microsatellite data analysis

After RFLP analysis, samples for microsatellite analyses were selected from forest stands on the HJA where the two RFLP types found within yellow chanterelles were sympatric with each other and with *Cantharellus subalbidus*. Sympatric as used here means that fruit bodies with alternate ITS genotypes were found growing in close proximity in the same 10–15 ha stand. *C. subalbidus* was used as a reference to determine how microsatellite differentiation between the two yellow types compared to the differentiation between *C. formosus* and *C. subalbidus*. Prior to tests for genetic differentiation between the sympatric ITS genotypes, each pair of loci was tested for linkage disequilibrium and each locus was tested for departure from Hardy-Weinberg equilibrium (Weir 1990, Guo & Thompson 1992) within each ITS 'population' across all 18 stands. Allele frequency differences within and among ITS genotypes were analysed using Fisher exact tests (Raymond & Rousset 1995) and F-statistics (Weir & Cockerham 1984). All analyses were carried out using the web-based version of GENEPOP (v. 3.1c; Raymond & Rousset 1995).

Morphology

Morphological and colour data were taken from fresh collections representing the full range in chanterelle colour variation (hue and intensity) and fruit body shape. To avoid damaging valuable colour standards with fresh material, colours of fresh fruit bodies were compared to a set of Lucite paint chips. These chips were subsequently keyed out under the same lighting in colour standards commonly used in chanterelle species descriptions (Ridgway 1912, Munsell Color Company 1966) and given the nearest ISCC-NBS colour-name equivalent (Kelly & Judd 1976). For each fruit body, we determined the dominant colour of the pileus, hymenium, stipe, and immediate staining reactions in each of these locations. Prior to warm air drying voucher material, a sample of context was taken for later genetic analyses. Microscopic observations of spores and tissues were made from dried material mounted in 5% KOH at 1000× magnification using the methods of Smith & Smith (1973) and Largent, Johnson & Watling (1977). RFLP data were generated after collection of morphological data so that more subjective measurements (e.g. colour determination) were not biased by knowledge of the ITS genotypes.

RESULTS

Restriction enzyme analysis

PCR products amplified using ITS1-F and ITS4 were either ~1490 or ~1690 bp in length. The number of distinct RFLP profiles from the 180 collections examined (Table 1) differed between the four enzymes used. *HaeIII*, the most variable enzyme produced six

Table 2. Pooled restriction fragment profiles used to define ITS types assigned to 180 *Cantharellus* collections included in this study. Values identify restriction fragment profiles for each enzyme. In some cases the fragment sizes in an RFLP profile do not sum to the size of the original ITS product (1490 or 1690 bp) because the sizes of fragments below 100 bp were difficult to accurately estimate. Restriction fragment sizes (bp) for each profile are as follows: *Alu* (1) 540-490-340-200-120, (2) 490-380-250-220-150, (3) 490-350-250-220-150, (4) 740-490-340-120; *DPNII* (1) 1090-400-200, (2) 600-400-290-200, (3) 800-400-290-200; *HinfI* (1) 970-500-220, (2) 480-430-340-240, (3) 690-500-300, (4) 670-500-320, (5) 750-500-250; *HaeIII* (1) 780-330-180-170-140-90, (2) 600-220-200-170-160-140, (3) 690-170-160-140-100, (4) 690-310-220-180, (5) 690-180-150, (6) 690-300-180.

RFLP type	Restriction fragment profile				
	Alu	*DPNII*	*HinfI*	*HaeIII*	ITS size (bp)
A	1	1	1	1	~1690
B	2	2	2	2	~1490
C	2	2	3	3	~1490
D	2	2	3	4	~1490
E	3	2	4	5	~1490
F	4	3	5	6	~1690

distinct RFLP profiles followed by *HinfI* (five), *Alu* (four), and *DPNII* (three). In several profiles, particularly those generated with *HaeIII*, the lengths of individual restriction fragments (Table 2) sum to less than the 1490 or 1690 bp of the original ITS product. This is due to the high number of recognition sites for this enzyme in the ITS of *Cantharellus* and our inability to detect and accurately size RFLP fragments smaller than 90 bp. *HaeIII* digests typically exhibited several small fragments below this size cut off which were ignored during RFLP comparisons.

Information pooled from individual restriction fragment profiles was used to characterize six unique RFLP types assigned across the 180 collections analysed (Table 2). Eighty-eight yellow chanterelle collections possessed a ~1690 bp ITS region and this group was further differentiated into two RFLP types (A and F) that differed in profile for all four enzymes. The 87 fruit bodies that exhibited RFLP type A resemble both morphological and genetic descriptions given for *C. formosus* (Corner 1966, Danell 1995, Redhead *et al.* 1997) while the one collection exhibiting RFLP type F (taken from under *Quercus* in southern California) possibly represents an undescribed relative of *C. formosus*. The remaining 92 fruit bodies exhibited a total ITS length of ~1490 bp and were partitioned into four distinct RFLP types (B–E). Within this group *DPNII* RFLP profiles were monomorphic and consequently, RFLP types are based on differences found at only two or three enzymes out of the four analysed. White chanterelles (*C. subalbidus*; Smith & Morse 1947), also differentiated by their unique colour, were represented by 39 collections with a single unique RFLP type (C). European *C. cibarius* var. *cibarius* collections, characterized by a single RFLP type (E), were differentiated from 15 *C. cibarius* var. *roseocanus* (type D; Redhead *et al.* 1997) collections by three restriction enzymes. The remaining 35 yellow chanterelle collections characterized by RFLP type B represent a currently undescribed chanterelle species and have been given the Latin binomen *C. cascadensis* sp. nov. in all tables and figures based on genetic analyses described below.

Host associations of ITS RFLP types

Our sampling design was not specifically intended to define ectomycorrhizal host associations for chanterelles but we did detect some interesting associations between the dominant overstory and the distribution of RFLP types (species) throughout the study area that warrant future investigation. Two RFLP types (D and F) identified in the broader sampling effort were not found at the HJA possibly because they are ectomycorrhizal host specialists. *Cantharellus cibarius* var. *roseocanus* (D) collections were found only in coastal and high elevation forests containing spruce, a characteristic also mentioned by Redhead *et al.* (1997) and have recently been collected from pure stands of lodgepole pine (*Pinus contorta*; Tom Horton, pers. comm.) along the Oregon coast. RFLP type F (*Cantharellus* sp.) was not detected in any other forest type sampled other than oak. These forest types were not present in our sampling areas on the HJA. Similarly, *C. subalbidus* (C) and *C. cascadensis* (B) were collected only in forests dominated by Douglas fir despite efforts to locate these species in other forest types. Data from genet size research indicates that the occurrence of *C. cascadensis* may also be correlated with stand age within this forest type (Dunham *et al.* 1998). *C. subalbidus*, *C. cascadensis* sp. nov., and *C. cibarius* var. *roseocanus* also have the potential to extend into in stands dominated by *Abies* and the extent to which this occurs is unknown because stands of this type were not well represented in our sampling efforts. *C. formosus* (A) collections were present in forests containing Douglas fir and or spruce and probably extend into forests containing *Abies*, indicating that it may be more of a generalist than other chanterelle species.

DNA sequence characteristics

PCR amplification with the primers ITS4R and LR5 produced ~950 bp fragments from the 5′ end of the nrDNA large subunit gene. The 24 new sequences generated by this study were relatively free of length variation but proper alignment of the 29 analysed sequences required the introduction of gaps one to 32 nucleotides long resulting in a total of 982 recoded characters that were included in maximum parsimony analyses. With gaps included as a character state the large subunit contained 420 variable sites, of which 322 were parsimony informative and 103 of these parsimony informative sites included information from gaps. Large subunit sequences from *Cantharellus* species (ingroup) contained 27 parsimony informative sites,

ten of which included information from gaps. Levels of sequence divergence ranged from 0.0% within species represented by a single RFLP type, up to 33.2% between ingroup and outgroup taxa. The highest pair-wise divergence values observed within *Cantharellus* were between *C. cinnabarinus* and other taxa (~7.0%). Divergences among other *Cantharellus* species were below 3%. Sequence variability was extremely low (<1%) between the four species that possess ITS regions of ~1490 bp mirroring the lower RFLP variability seen within this group.

Combined sequences generated from the ITS contained 431 nucleotide bp from the 5′ end of the ITS 1 and 558 nucleotide residues from the 3′ end of the ITS 2 including the 5.8s nrDNA gene. These sequences also include 30 nucleotides each from the flanking nrDNA small (18s) and large (28s) subunits. Due to pronounced length variation in the ITS 1, alignment of the 13 analysed sequences required the introduction of gaps one to 74 nucleotides long resulting in a total of 989 recoded characters available for maximum parsimony analyses. With gaps included as a character state the alignment contained 95 variable sites, of which 32 were parsimony informative and 15 of these parsimony informative sites included information from gaps. Within these partial ITS sequences, pair-wise divergence values ranged from 0–0.5% within single RFLP types, up to 8.1% between *C. formosus* and other *Cantharellus* taxa. The lowest divergence values observed between two ingroup taxa with different RFLP types were between *C. cibarius* var. *cibarius* and *C. cibarius* var. *roseocanus* (0.7–0.8%). Sequence divergence values for all other ingroup comparisons were at least two times higher (1.4–3.0%).

Maximum parsimony analyses

Unweighted maximum parsimony analysis of the nrDNA large subunit resulted in 34 equally parsimonious trees, 479 steps in length. The relative amount of genetic difference (number of steps) between taxa are visually summarized in one of the equally parsimonious trees (Fig. 2A) selected based on its congruence with the strict consensus tree. Bootstrap consensus shows strong support for splits between *Cantharellus formosus*, the oak associated chanterelle (~1690 bp ITS region), and a poorly resolved clade containing European *C. cibarius* var. *cibarius*, *C. cascadensis*, *C. cibarius* var. *roseocanus*, and *C. subalbidus*. These splits represent on average 1.8% total sequence divergence in the large subunit between *C. formosus* collections and the oak associated chanterelle and an average of 2.3% between these two species and the collections that fall within the larger unresolved clade (Fig. 2A).

The analysis of ITS sequences used *C. formosus* to root the large poorly resolved clade identified in the large subunit tree. Unweighted analysis of the ITS alignment produced 3 equally parsimonious trees and a single tree that visually summarizes the relative amounts of genetic differentiation (Fig. 2B) is shown. Monophyletic groups with greater than 80% bootstrap support are consistent with those identified by RFLP analysis. Bootstrap analysis supports a close relationship between *C. cascadensis* and white chanterelles (*C. subalbidus*). Monophyly of *C. cibarius* var. *roseocanus* collections is strongly supported, as is its close association with *C. cibarius* var. *cibarius* (Redhead *et al.* 1997).

Microsatellite analysis

Multilocus genotypes for 72 sympatric collections (Table 1) of *Cantharellus formosus* (A), *C. cascadensis* (B), and *C. subalbidus* (C) were determined using five microsatellite loci recently developed for *C. formosus*. The number of alleles within each species ranged from one to five with an average number of 2.4 alleles per locus. Expected heterozygosities ranged from 0.05–0.61 in *C. formosus*, 0 (for two momomorphic loci) to 0.58 in *C. cascadensis*, and 0–0.67 in *C. subalbidus* which exhibited no amplification at locus Cf145 and was monomorphic at another locus. Across the five loci, each species had observed heterozygosities that matched those expected under the assumptions of Hardy-Weinberg equilibrium ($P>0.05$) and no convincing evidence of linkage between pairs of loci was observed.

Fisher's exact tests of population independence, performed for all pair-wise species comparisons at each locus indicated that allelic distributions differ significantly among the three species ($P\leqslant 0.05$; Table 3). Fixation indices are measures of differentiation that combine information on both allele frequency and identity. Assuming an infinite allele mutation model, F_{st} values calculated for pair-wise comparisons between *C. formosus*, *C. subalbidus*, and *C. cascadensis* sp. nov. at individual loci ranged from 0.22–1.0 (100% genetic differentiation) and mean F_{st} values calculated across all loci ranged from 0.66–0.68 (Table 3). Sympatric sampling excludes isolation by distance as an explanation for the extreme genetic differentiation. Results from these comparisons provide strong support for the hypothesis that *C. formosus*, *C. subalbidus*, and *C. cascadensis* are not interbreeding and represent biological species on independent evolutionary trajectories.

Morpholgical comparisons

Morphological comparisons made are primarily intended to differentiate *Cantharellus formosus* from *C. cascadensis* because the two co-occur extensively in Douglas fir-western hemlock forests. Of the microscopic characteristics examined only spore dimensions showed any potential for differentiating dried collections and were examined in more detail. Microscopic examination of spores from seven *C. formosus* and six *C. cascadensis* collections revealed little information

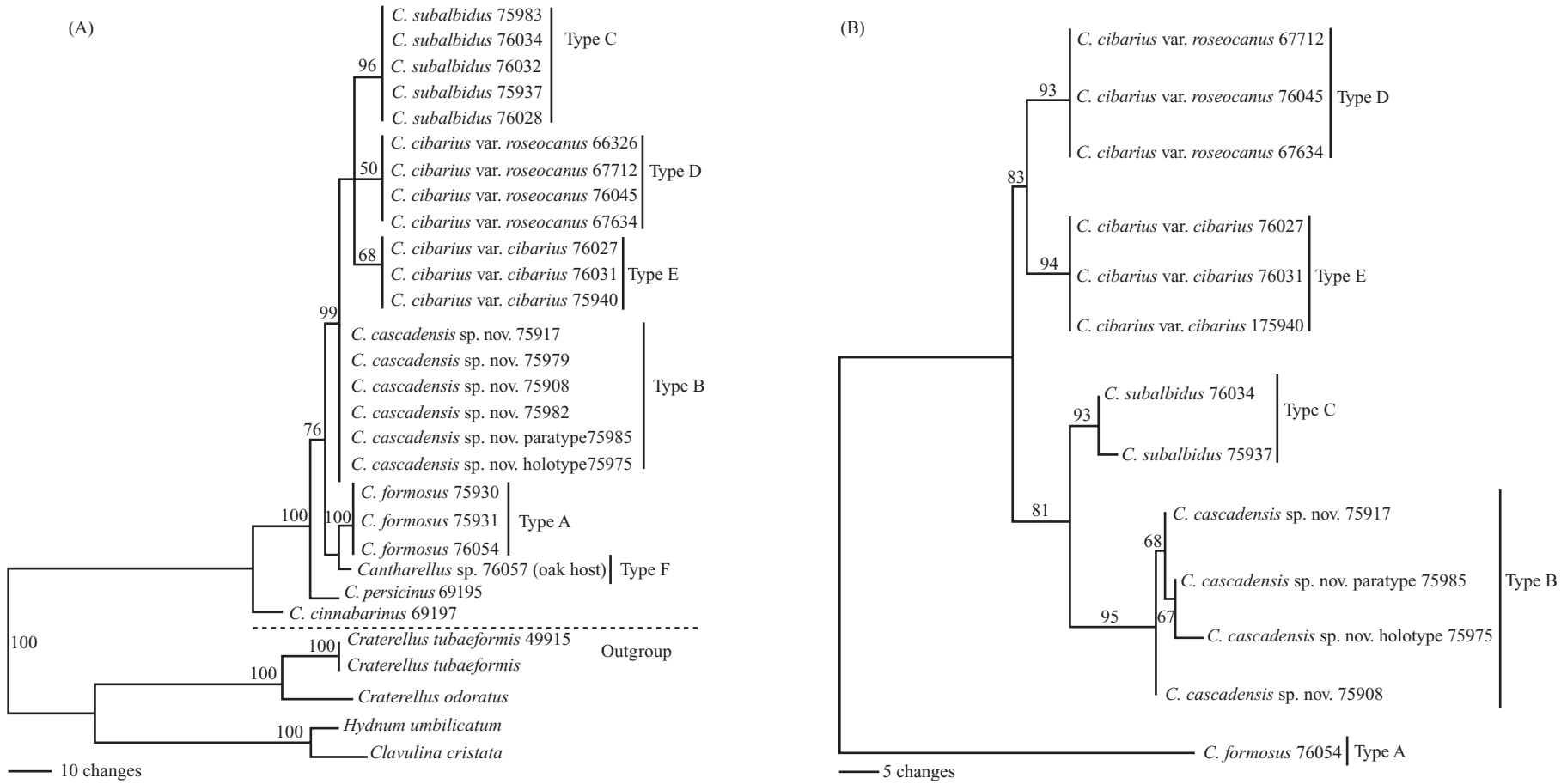

Fig. 2. (A) A phylogram of a single tree selected from the 34 equally parsimonious trees found in the analysis of the nrDNA large subunit alignment. This tree was selected based on its congruence with the majority rule concensus tree found using heuristic stepwise random addition analysis of the large subunit sequences (CI = 0.871 and HI = 0.129) and is intended to visually summarize the genetic distances between taxa. (B) A phylogram of a single tree selected from the 3 equally parsimonious trees found in the branch and bound analysis of the nrDNA ITS alignment. The majority rule consensus tree had consistency and homoplasy indices of 0.902 and 0.098 respectively. For both trees, above each branch are heuristic bootstrap values summarized from 1000 pseudo-replicates with 10 random stepwise addition searches per replicate. Numbers after each name correspond to OSC collection numbers presented in Table 1 and letters correspond to the RFLP type determined for each collection defined in Table 2.

Table 3. Summary of allele identities and frequencies at five microsatellite loci. Identification of species associated with each allele and sample sizes used to generate summaries: *C. cascadensis* ($n=24$)/*C. formosus* ($n=20$)/***C. subalbidus*** ($n=28$).

	Locus					
Allele	Cf642	Cf145	Cf339	Cf126	Cf113	
Allele 1	*0.95*	*0.27*	*0.15*	***0.02***	*0.97*	
Allele 2	*0.29*	*0.55*	*0.12*/*1.0*	***0.66***	***0.96***/*0.42*	
Allele 3	*0.71*	*0.18*	*0.73*/***1.0***	***0.32***/*1.0*	*0.03*/***0.04***/*0.58*	
Allele 4	*0.05*	*0.60*		*0.72*		
Allele 5	***0.46***	*0.02*		*0.28*		
Allele 6	***0.32***	*0.19*				
Allele 7	***0.11***	*0.17*				
Allele 8	***0.11***	*0.02*				
F_{st} and Fisher Exact test probabilities (Null hypothesis = random union of gametes)						
C. formosus vs C. cascadensis sp. nov.						
F_{st}	0.73	0.41	0.76	0.81	0.71	(Global = 0.68)
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
C. subalbidus vs C. cascadensis sp. nov.						
F_{st}	0.45	–	1.0	0.63	0.52	(Global = 0.67)
P	<0.0001	–	<0.0001	<0.0001	<0.0001	
C. subalbidus vs C. formosus						
F_{st}	0.59	–	0.22	0.56	0.94	(Global = 0.66)
P	<0.0001	–	<0.0001	<0.0001	<0.0001	

useful in differentiating the two species. Thirty spores were examined from each collection and mean spore length and width measurements (9.53 ± 0.19 µm (7–13 µm) × 6.55 ± 0.15 µm (5–8 µm) for *C. formosus*; and 9.3 ± 0.15 µm (7–13 µm) × 6.3 ± 0.23 µm (5–8 µm) for *C. cascadensis*) and spore length to width ratios (1.46 ± 0.04 (1.14–2) for *C. formosus*; and 1.49 ± 0.05 (1.13–2.2) for *C. cascadensis*) overlapped completely between the two species. In collections of both species, spores were subglobous to ellipsoid and smooth with large oil droplets, clamp connections were abundant and diverse in type, and hyphae from the context and trama appeared thin-walled and hyaline. These observations are comparable to those reported in other chanterelle species descriptions (Smith & Morse 1947, Corner 1966, Smith 1968, Petersen 1979, Redhead *et al.* 1997) indicating that molecular evolution within the genus is proceeding at a more rapid pace than changes in microscopic morphology.

Analysis of macroscopic morphology yielded a few characters more useful in separating field collections of the two species. Colours were most variable on the pileus and far less so on the hymenium, stipe, or in bruising reactions. A summary of colour notes (Table 4) taken from 81 fresh collections of yellow chanterelles (58 *C. formosus*; 23 *C. cascadensis*) shows that relatively consistent differences in fresh pileus colour can be used to differentiate these two species. In general, *C. cascadensis* tends toward a intensely bright pure yellow while *C. formosus* shows orange-yellow to brownish yellow hues. The maximum width of the pileus ranged from 2.5–15 cm in *C. formosus* and 4–12 cm in *C. cascadensis* but the distributions of these measurements were skewed in opposite directions with *C. formosus* (mean = 6.1 ± 2.45 cm) tending toward smaller pileus widths and *C. cascadensis* (mean = 8.6 ± 2.13 cm) tending toward larger pileus widths. A non-parametric analysis of maximum pileus width measurements shows that the means differ significantly (Mann–Whitney $Z = 3.85$; $P = 0.0001$).

TAXONOMY

Cantharellus cascadensis Dunham, O'Dell & R. Molina, **sp. nov.**

Etym.: *cascadensis*, from the Cascade Mountains, Oregon.

A *Cantharellus formosus* Corner ob conjunctione pilei vivide armeniaci vel pallidule armeniaci, in caelo humido a disco radialiter extrinsecus albescentis vel luteolescentis, in caelo sicco rimas profundas lateribus obscure brunneoaurantiis effectescentis, et stipitis plani, clavati, ventricosi, vel basi bulbosi differt.

Typus: **USA**: *Oregon*: Lane County, Mill Creek, southern boundary of H. J. Andrews Experimental Forest, south of intersection of forest roads 1501 and 2633, T 16S, R5E, section 5 south, alt. 730 m, 10 Oct. 1999, *S. Dunham & T. E. O'Dell* (OSC 75975 – holotypus).

Basidiomata solitary to gregarious or occasionally cespitose, 4–10 cm tall. *Pileus* 4–12 cm broad, plano-convex to slightly umbonate, becoming depressed to deeply depressed with age. Surface dry and finely tomentose, when developing in dry conditions, toward margin tomentum forming radial fibrils on some specimens. Surface bumpy or with occasional warts of hymenial tissue, often showing dark brownish orange discolouration along cracks with exposure to sun or very dry conditions. Most specimens are vivid orange

Table 4. Pileus and hymenium colours in mature basidiomata of *Cantharellus formosus* and *C. cascadensis*.

Colour name			Number of basidiomata with observed colour	
ISSCC – NBS colour/no.[a]	Munsell renotation[b]	Ridgway colour[c]	*C. formosus* ($n=58$)	*C. cascadensis* sp. nov. ($n=23$)
Dominant pileus Colours				
Vivid orange yellow/66	8.6YR 7.3/15.2	Cadmium yellow	1	19
Vivid yellow/82	3.3Y 8.0/14.3	Light cadmium yellow	1	1
Light orange yellow/70	9.4YR 8.3/6.8	Pale orange yellow	1	1
Brilliant orange yellow/67	0.1Y 8.1/10.5	Antimony yellow	7	2
Light yellowish brown/76	8.7YR 6.5/5.0	Yellow ochre	21	0
Mod. yellowish brown/77	9.5YR 4.4/3.9	Buckthorn brown	2	0
Deep orange yellow/69	8.6YR 6.0/12.1	Chamois	24	0
Mod. orange yellow/71	8.7YR 7.2/8.3	Cinnamon buff	3	0
Dominant hymenium colours				
Light orange yellow/70	9.4YR 8.3/6.8	Pale orange yellow	20	5
Pale orange yellow/73	9.2YR 8.7/4.4	Pale ochraceous salmon	37	12
Pale yellow/89	4.7Y 9.0/3.8	Massicot yellow	1	6

[a] Kelly & Judd (1976).
[b] Munsell Color Company (1966).
[c] Ridgway (1912).

yellow (8.6YR 7.3/15.2) to vivid yellow (3.3Y 8.0/14.3) or light orange yellow (9.4YR 8.3/6.8). Central disc occasionally fades to white or very pale yellow (5Y 9/4) when water soaked with fading progressing toward margin. *Margin* incurved at first (never inrolled) soon becoming uplifted with undulating or crisped margins. Tending to retain vivid yellow colouration during wet conditions. *Context* firm and yellowish white (2.5Y 9/2). Odor and taste not distinctive. *Hymenium* with ridges to 2 mm deep, close and narrow, long and strongly decurrent, variously forked or anastomosing. Colour ranging from light orange yellow (9.4YR 8.3/6.8) to pale yellow (4.7Y 9.0/3.8). *Stipe* occasionally even but more often clavate to ventricose or bulbose at base, flaring upward and indistinct from pileus. Solid, 2–4.5 cm tall × 1–2 cm wide at apex (below hymenium), 1.5–3 cm at widest spot near base. *Staining* reactions when bruised similar in all tissues with intensity increasing as fruit body dries. Colour ranging from deep orange yellow (10YR 7/10) to brownish red (10R 5/6). *Basidios spores* white to yellowish white (2.5Y 9/2) in deposits, 9.3 ± 0.15 μm (range 7–13 μm) long × 6.3 ± 0.23 μm (range 5–8 μm) wide, ellipsoid to subglobous and smooth with variable number of oil droplets. *Basidia* 85–100 × 7–9 μm with incurved sterigmata and 4–8 spores per basidia. *Hyphae* hyaline, thin-walled, wavy to interwoven in both context and trama, variable in width, 5–12 μ diam, regular and diverse clamp connections at cross walls.

Habitat: Douglas fir-western hemlock forests of variable age and elevation.

Distribution: Throughout the HJ Andrews Experimental Forest, forest stands along the eastern boundary of the HJA, along the west shore of Cougar reservoir, Willamette National Forest, Blue River ranger district, and the Powers Ranger District and Butte Falls Resource Area in southern Oregon.

Other specimen examined: **USA**: *Oregon*: Lane County, boundary of Mill Creek and Florence Creek drainage, south of forest road 700 one mile from intersection with forest road 1501, T 16S, R5E, section 9, south east, elevation 480 m, 10 Oct. 1999, *S. Dunham & T. E. O'Dell* (OSC 75985).

Observations: Carotenoid pigments produce the yellow colours observed in many chanterelle species, and the expression of colour produced by carotenoids can be altered by changes in environmental factors (e.g. carbon and nitrogen sources, pH, light, and temperature) that alter the physiology of fungal individuals. Alternatively, studies on *Neurospora* have shown that single genetic mutations also can significantly alter the expression of colour produced by carotenoids (Bramley & Mackenzie 1992). Our analysis has partitioned environmental and genetic sources of colour variability such that the range of colour variation can be used to differentiate two very similar species, *C. formosus* and *C. cascadensis*. These subtle differences in yellow hues are likely due to unique combinations of various carotenoid pigments, as has been demonstrated for other morphologically similar chanterelle species (Mui, Feibelman & Bennett 1998).

The centre of the pileus of *C. cascadensis* also tends to fade to white in wet weather giving the appearance of concentric circles of yellow increasing in intensity toward the outer edge. Particularly old or water-logged fruit bodies may only show yellow on the outer most edge and can resemble *C. subalbidus*. Both *C. subalbidus* and *C. cascadensis* develop large cracks in the pileus flanked by dark orange brown discolouration during dry weather. Discoloured cracking of this nature was not observed in *C. formosus*, which also is more likely to develop warts containing hymenial tissue regardless of environmental conditions. The stipe of *C. cascadensis* is only occasionally equal in width throughout its length and is more often clavate to ventricose or

bulbose. This contrasts with the stipe of *C. formosus* which is sometimes equal but more often tapered.

All of the characters discussed show a minor degree of overlap between *C. formosus* and *C. cascadensis* but, when taken in combination, are useful in differentiating field collections of the two species. Given that collections were made randomly under similar environmental conditions throughout the fall of 1998, it is reasonable to conclude that they present characters useful in differentiating *C. formosus* and *C. cascadensis*. Fruit body size can vary widely from year to year in the same location so pileus width is likely useful only when collections are made at the same time and place. It should also be noted that, within the HJA, the relative sample sizes used to make macroscopic morphological comparisons reflect the relative encounter rate with each species; that is *C. cascadensis* appears to occur in lower frequency relative to *C. formosus* in Douglas fir forests.

DISCUSSION

We use both phylogenetic species recognition criteria and biological species recognition criteria (Taylor *et al.* 2000) to diagnose a cryptic species within *Cantharellus* in the PNW. Molecular markers (e.g. RFLPs, RAPDs, AFLPs, SSCPs, microsatellites) have been used in other genera to distinguish fungal taxa difficult to characterize by traditional morphological means (Anderson, Petsche & Smith 1987, Bruns, White & Taylor 1991, Kohn 1992, Fukuda *et al.* 1994, Harrington & Wingfield 1995). The RFLP and phylogenetic analyses presented were used both to define genetically distinct lineages of chanterelles within the PNW and to identify multiple lineages within a single forest type to facilitate tests for interbreeding using microsatellites. These analyses are intended simply to differentiate species rather than attempt to determine their evolutionary relationships to other *C. cibarius* 'like' chanterelles. Our sampling was inadequate for this purpose as the relationships among the taxa analysed may change with the addition closely related taxa from several other regions in North America, particularly the southeast (Mui *et al.* 1998), which are proving to be very rich sources of new *Cantharellus* species. Until the species boundaries of eastern North American taxa are better clarified the construction of a comprehensive phylogeny of the genus is not practical. For example, our initial phylogenetic analyses included three eastern collections accessioned into OSC (nos 69198, 69199, and 69258) as *C. cibarius*. The sequences from these three collections were highly divergent from each other and from sequences reported here. To avoid problems with long-branch attraction in parsimony analyses (Felsenstein 1978, Huelsenbeck 1997) these collections were excluded from our analyses and we narrowed our focus to only collections from the PNW and Europe.

A suite of phenotypic characters that exhibit variability resulting from both evolutionary divergence and environmental plasticity confuse the morphological delineation of PNW chanterelle species. The lack of clear patterns in gross or microscopic morphological variation has led to a taxonomy that inadequately characterizes the species diversity within *Cantharellus*. Achieving a taxonomy based on fully differentiated chanterelle species boundaries requires characters that can demonstrate the genetically based discontinuities between evolutionarily independent groups. Our molecular and morphological data suggest there are at least four distinct clades within the genus *Cantharellus* inhabiting Douglas fir and spruce forests of Oregon (ITS RFLP types A, B, C, and D/E) and possibly a fifth distinct clade (ITS RFLP type F) inhabiting oak forests in California. We propose that these clades represent distinct evolutionary lineages that should be treated as biological species based on genetic, morphological, and ecological evidence.

The magnitudes of genetic divergence among clades supported by bootstrap analyses do not overlap with those within poorly resolved clades. In the nrDNA large subunit analysis (Fig. 2A), no sequence variability is observed within clades characterized by a single RFLP type. Divergence between collections in the clade containing *C. formosus* (RFLP type A) and *C.* sp. (oak host; RFLP type F) and taxa characterized by RFLP types B–E are on average ten times higher than divergence values among collections with RFLP types B–E. This disjunction in large subunit divergence indicates that length difference in the total ITS region is a good preliminary genetic character for detecting new species in *Cantharellus*. With the exception of differences observed between *C. cibarius* var. *cibarius* and *C. cibarius* var. *roseocanus*, genetic distances in the ITS sequence analysis (Fig. 2B) follows a pattern identical to the nrDNA large subunit analysis where divergences values among well supported clades are on average ten times higher than those within clades. This disjunction in sequence similarities indicates that ITS RFLP profiles generated using at least three restriction enzymes represent reliable characters for differentiating species in *Cantharellus* and that ITS length variability alone does not represent total species diversity. The close association and high sequence similarity between *C. cibarius* var. *cibarius* and *C. cibarius* var. *roseocanus* and lack of data from more variable markers make genetic delimitation of these two taxa as separate species more problematic. High bootstrap values generated in the ITS analysis result from seven fixed differences between these taxa and support full differentiation of *C. cibarius* var. *roseocanus* from *C. cibarius* var. *cibarius*. These fixed sequence differences provide weak support for raising *C. cibaris* var. *roseocanus* to full species status (a sixth lineage in the analyses presented here) as it likely represents an early but independent evolutionary lineage derived from *C. cibarius* var. *cibarius*. A larger sample of both European and North American

C. cibarius var. *cibarius* collections compared to *C. cibarius* var. *roseocanus* using more variable loci is required to completely understand the genetic boundaries between these taxa.

Of the five lineages defined using phylogenetic analysis of nrDNA sequences two show some evidence of restriction in ectomycorrhizal host associations. Collections of *C. cibarius* var. *roseocanus* have been found only in association with costal and high elevation spruce forests (Redhead *et al.* 1997) and in pure stands of lodgepole pine (*Pinus contorta*) along the Oregon coast (T. Horton, pers. comm.). In costal spruce habitats (at least) *C. cibarius* var. *roseocanus* overlaps in distribution with *C. formosus* and differentiating field collections of the two requires attention to details of hymenium and pileus colour as described by Redhead *et al.* (1997) particularly under dry conditions during the early fall (Dunham, unpubl.). Our analysis also includes a single chanterelle collection with a unique nrDNA characteristics, found growing in association with oak but not identified in other sampled forest types. This collection (RFLP type F) may represent the variety *C. cibarius* var. *pallidifolius* described from Michigan by Smith (1968) and described by Thiers (1985) as mycorrhizally associated with tanbark oak (*Lithocarpus densiflora*) in California. Unfortunately the identity of this collection could not be confirmed using genetics because DNA extracted from available *C. cibarius* var. *pallidifolius* type material was highly degraded, preventing PCR amplification of nrDNA regions. The distribution of our sampling locations is heavily biased toward forests dominated by Douglas fir, and while our limited sampling in other forest types detected *C. cibarius* var. *roseocanus* (RFLP type D) and *C.* sp. (RFLP type F), they were never observed in forests where spruce or pine (type D), or oak (type F) were absent. Three lineages (*C. formosus*, *C. subalbidus*, and *C. cascadensis*) have sympatric distributions in Douglas fir forests and provide an opportunity to extend our phylogenetic analysis from the identification of genetically isolated clades to the detection of interbreeding individuals and biological species boundaries.

A comparison of numerous sporocarps of *C. formosus* (RFLP type A), *C. cascadensis* (RFLP type B), and *C. subalbidus* (RFLP type C) revealed that most microsatellite loci show fixed allelic differences between at least two taxa (4 loci; Table 3) if not all three (2 loci). These qualitative differences at a large proportion of variable loci are characteristic of separate species, rather than conspecific populations (Avise & Ball 1990). Collections of *C. formosus*, *C. subalbidus*, and *C. cascadensis* used to compare microsatellite allele frequencies overlap completely in the time frame of collection and in their spatial distributions. We selected roughly equal numbers of each species from the random collections made in 18 separate stands at the HJA, and in many cases fruit bodies of each species were found growing within a few cms of each other. The maintenance of substantial genetic differentiation in the face of such close geographic proximity demonstrates that these three species have unique evolutionary histories that are not simply a result of isolation by distance. Sympatric, outcrossing species maintain species cohesion via pre or post-zytogic barriers to gene flow. If there is no gene flow between these three sympatric species then it is logical to assume that they have differing ecological adaptations (Harrington & Rizzo 1999) that either reduce hybrid fitness or inhibit sexual reproduction.

These data shed some light on the relationship between nrDNA sequence similarity and levels of gene flow. Global F_{st} values indicate that *C. cascadensis* is differentiated from *C. subalbidus* and *C. formosus* at similar levels even though nrDNA sequences divergence values differ drastically in these pairwise comparisons (Figs 2A–B, Table 3). *C. cascadensis* differs from *C. subalbidus* at only 1.5–2.2% of the 989 ITS nucleotide sites examined while it differs from *C. formosus* at 7.5–8.0% of nucleotide sites. The observed level of differentiation between *C. formosus* and the other two taxa may be limited by the constraints on microsatellite evolution discussed above. The substantial sorting of microsatellite alleles within *C. cascadensis* and *C. subalbidus* indicates that the 0.7–0.8% divergence observed between *C. cibarius* var. *roseocanus* and *C. cibarius* var. *cibarius* may be large enough to indicate species level differentiation. The 1.5–2.2% divergence observed between *C. cascadensis* and *C. subalbidus* is within the range of intraspecific ITS sequence variability reported for *Cenococcum geophilum* (0.0–4.0%; Shinohara *et al.* 1999), *Marasmius quercophilus* (1.11–1.25%; Farnet, Roux & Le Petit 1999), *Galerina marginata* (0.3–2.7%; Gulden, Dunham & Stockman 2001), and *Phialophora americana* (up to 2%; Yan, Rogers & Wang 1995), and similar to that observed between closely related species in *Hebeloma* (Aanen *et al.* 2000), *Dermocybe* (Liu, Ammirati & Rogers 1997), *Sarcodon* (Johannesson *et al.* 1999), and *Cortinarius* (Høiland & Holst-Jensen 2000). In addition, other studies have reported identical ITS sequences for morphologically distinguishable ascomycete species (Seifert, Wingfield & Wingfield 1995, Harrington & Potter 1997) including those demonstrated to differ in ecology and isozyme variability (Harrington *et al.* 1996, Harrington & McNew 1998, Witthuhn *et al.* 1998). While ITS sequences are often used to delineate relationships among fungal species within genera (e.g. Yan *et al.* 1995, Aanen *et al.* 2000, Høiland & Holst-Jensen 2000), expense often precludes these studies from including numbers of individuals adequate to characterize intraspecific sequence variability (Harrington & Rizzo 1999). These and our results demonstrate that, when working at the interface between population level variation and species boundaries, the meaning of sequence variability in these regions requires case specific interpretation.

The diagnosis of cryptic species in difficult groups of fungi currently is moving toward the use of multiple

gene genealogies where the transition from conflict to concordance among separate gene trees delimits species boundaries (Genealogical Concordance Phylogenetic Species Recognition, Taylor *et al*. 2000). When applicable, the use of microsatellite markers and sympatric sampling to test for gene flow among lineages defined by a single gene genealogy can greatly reduce the expense and effort of confidently defining species compared to generating multiple gene genealogies. Both techniques recently have been used in combination to define cryptic biological species within the pathogenic fungus *Coccidioides immitis* (Burt *et al*. 1996, 1997, Koufopanou, Burt & Taylor 1997, Fisher *et al*. 2000, 2002, Koufopanou *et al*. 2001). Concerns with using microsatellites to define species include limits on measures of genetic distance created by constraints on allele sizes (Garza, Slatkin & Freimer 1995, Lehmann, Hawley & Collins 1996) and high levels of homoplasy due to high mutation rates regenerating identical alleles in genetically isolated species. Both phenomena can create the appearance of gene flow when none exists. These issues are of minor concern in our comparisons because even though we did observe identical alleles in some pair-wise comparisons of species, their frequencies differed substantially and their presence did not cloud the obvious genetic differentiation among these three taxa.

In summary, phylogenetic analyses of the nrDNA large subunit and ITS regions show that *C. cascadensis*, along with two other yellow chanterelle taxa (*C. cibarius* var. *roseocanus* and European *C. cibarius* var. *cibarius*), are more closely related to white chanterelles (*C. subalbidus*) than they are to the most common yellow species in the PNW, *C. formosus*. Data from five microsatellite loci provide evidence that *C. formosus*, *C. subalbidus*, and *C. cascadensis* do not interbreed when they co-occur spatially and temporally in Douglas fir-western hemlock forests. This demonstrates that the three co-occurring species fit into biological species concepts congruent with nrDNA phylogenetic species concepts. Our findings have larger implications for management of fungi under the Northwest Forest Plan. If our knowledge of *Cantharellus* species concepts is similar to those for other major genera of ectomycorrhizal fungi in the PNW, these species-based conservation efforts will likely miss many rare, cryptic species of unknown ecological importance. There currently is no way to determine if the conservation of other well described but rare taxa will protect this unknown diversity in PNW forests.

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