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Evolutionary consequences of putative intra- and interspecific hybridization in agaric fungi

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Abstract: Agaric fungi of the southern Appalachian Mountains including Great Smoky Mountains National Park are often heterozygous for the rDNA internal transcribed spacer region (ITS) with >42% of collections showing some heterozygosity for indels and/or base-pair substitutions. For these collections, intra-individual haplotype divergence is typically less than 2%, but for 3% of these collections intra-individual haplotype divergence exceeds that figure. We hypothesize that high intra-individual haplotype divergence is due to hybridization between agaric fungi with divergent haplotypes, possibly migrants from geographically isolated glacial refugia. Four species with relatively high haplotype divergence were examined: *Armillaria mellea*, *Amanita citrina* f. *lavendula*, *Gymnopus dichrous* and the *Hygrocybe flavescens/ chlorophana* complex. The ITS region was sequenced, haplotypes of heterozygotes were resolved through cloning, and phylogenetic analyses were used to determine the outcome of hybridization events. Within *Armillaria mellea* and *Amanita citrina* f. *lavendula*, we found evidence of interbreeding and recombination. Within *G. dichrous* and *H. flavescens/ chlorophana*, hybrids were identified but there was no evidence for F_2 or higher progeny in natural populations suggesting that the hybrid fruitbodies might be an evolutionary dead end and that the genetically divergent Mendelian populations from which they were derived are, in fact, different species. The association between ITS haplotype divergence of less than 5% (*Armillaria mellea* = 2.6% excluding gaps; *Amanita citrina* f. *lavendula* = 3.3%) with the presence of putative recombinants and greater than 5% (*Gymnopus dichrous* = 5.7%; *Hygrocybe flavescens/ chlorophana* = 14.1%) with apparent failure of F_1 hybrids to produce F_2 or higher progeny in populations may suggest a correlation between genetic distance and reproductive isolation.

Key words: biodiversity, Dobzhansky-Muller incompatibility, hybridization, speciation

Introduction

Interspecific hybrids are common in higher plants and play a significant role in plant evolution (Abbott 1992), but reports of such hybrids for fungi are relatively rare (see Olson and Stenlid 2002, Schardl and Craven 2003, Le Gac and Giraud 2008 for summaries). Among ascomycetes, non-orthologous ITS2 sequences, suggestive of ancient hybridization, have been identified in populations of plant pathogenic *Fusarium* species (O'Donnell and Cigelnik 1997). In addition, Inderbitzin et al. (2011) reported that the plant pathogen *Verticillium longisporum* was a hybrid that originated at least three separate times.

Among basidiomycetes, hybridization as a mechanism to explain genetic variation was reported in several populations. For example, rare interspecific hybrids were reported in *Heterobasidion* (Garbelotto et al. 1998) and among cryptic species of *Coniophora puteana* in a region of geographical overlap (Kauserud et al. 2007, but see Skrede et al. 2012). Newcombe et al. (2000) reported rare hybrids in the rust genus *Melampspora*, while Morin et al. (2009) reported an apparent rust fungus hybrid between *Puccinia lagenophorae* and an unknown rust fungus on *Senecio* in Africa based on recovery of divergent ITS haplotypes. Le Gac et al. (2007) determined the

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rates of pre- and post-syngamy isolation in pairings of sympatric and allopatric sister species of anther smuts in the genus *Microbotryum*. Lindner and Banik (2011) reported recovery of divergent ITS genotypes in clones of the bracket fungus genus *Laetiporus* and the effect of these on clade circumscription in phylogenetic trees, but divergent sequences in that study could have represented hybridization with unresolved rRNA homogenization.

A probable hybrid, identified from a recombinant ITS sequence between *Flammulina rossica* and *F. velutipes*, was reported from Argentina where it might have been introduced (Hughes and Petersen 2001). Later, a European collection with an identical hybrid ITS sequence was identified suggesting that either this is an old hybridization that has survived and was propagated in distant locations or that this hybridization has occurred more than once on different continents (Ripková et al. 2010). Baumgartner et al. (2012) presented evidence from single-gene phylogenies of discordance between cytoplasmic and nuclear genes of Eurasian and an introduced African homothallic *Armillaria mellea* suggestive of intra-lineage hybridization. Similarly hybrids from heterothallic populations of *A. ostoyae* and *A. tabescens* have been noted (Schnabel et al. 2005, Hanna et al. 2007).

The southern Appalachians, including Great Smoky Mountains National Park (GSMNP), is a region of exceptional biodiversity within North America (Stein 2000). We sampled the fruit bodies of southern Appalachian agaric fungi over a 5 y period and obtained diagnostic nuclear ribosomal ITS sequences for 2172 collections. We found that a significant number (42%) were heterozygous for indels (data concerning base-pair heterozygosity in sequences without indels were not collected for the entire dataset). We evaluated both base-pair and indel heterozygosity for a random sampling of 100 heterozygous collections from this dataset and demonstrated that percent ITS sequence differences within an individual basidiome varied from one base pair (bp) to more than 3.3% bp divergence, but the great majority of these collections (97%) revealed less than 2% bp divergence among haplotypes within an individual and 99% of the heterozygotes had less than 3% divergence (Hughes et al. 2009). We suggested that for a geographically localized region such as the southern Appalachians biological species (individuals of an interbreeding population) could be defined for barcoding purposes by collections with a conservative 3% or less sequence divergence. We noted that this figure would miss recent speciation where barriers to interbreeding were established without significant concomitant sequence divergence, thus potentially underestimating species numbers suggested by ITS barcoding.

We hypothesize that observed heterozygosity (genetic distance between haplotypes) that is greater than 2% is the consequence of rare hybridization between divergent populations. To further examine the relationship between degree of heterozygosity and the fate of hybrid progeny, we examined four exemplars with >2% base-pair heterozygosity: *Amanita citrina* f. *lavendula* (ectomycorrhizal), *Armillaria mellea* (plant pathogen and wood decay saprobe), *Gymnopus dichrous* (saprobe) and the *Hygrocybe flavescens/chlorophana* complex (not ectomycorrhizal, possible rhizosphere or moss symbiont).

MATERIALS AND METHODS

Collections.—Collections were documented, photographed, given a Tennessee Field Book (TFB) number, dried and accessioned into TENN (available at http://tenn.bio.utk.edu/) (TABLE I). For long-term storage of tissue, a small piece of each basidiome (0.3 cm^3) was placed in a microfuge vial containing silica beads and stored at −80 C.

DNA extraction.—DNA was extracted by grinding a 2–3 mm^2 dried tissue sample with a mortar and pestle in a small volume of sterile sand at room temperature until powdered. The sample was added to 750 µL Carlson lysis buffer (Carlson et al. 1991), vortexed 5 s and heated at 74 C for 30 min with brief vortexing at 15 and 30 min. The sample was centrifuged to precipitate sand and cell debris, and the supernatant was mixed with an equal volume of 24:1 chloroform-isoamyl alcohol. The top layer containing DNA was removed and an equal volume of isopropanol added to precipitate DNA. The DNA pellet was washed with 80% ice-cold ethanol and resuspended in 100 µL sterile Tris-EDTA (TE) buffer. The ITS region of the ribosomal RNA repeat was amplified with primers ITS1F (Bruns and Gardes 1993) and ITS4 (White et al. 1990). PCR products were cloned (pGEM-T easy kit with M109 competent cells, Promega Corp., Madison, Wisconsin 53711). Between five and 10 clones were sequenced for each basidiome. Clone consensus sequences were not determined. Sequencing was performed with an automated ABI 3100 DNA sequencer (ABI Prism Dye Terminator cycle sequencing, Perkin-Elmer Inc.) using primers ITSIF and ITS4 (White et al. 1990). For *G. dichrous*, clade 1 and 2 reverse primers were designed from ITS sequences of both clades. These were used to test homogeneity of the ribosomal repeat in homozygotes.

Determination of heterozygosity.—Base-pair heterozygosity was inferred from the presence of double peaks on a chromatogram. Indels were inferred when peaks abruptly went out of phase. For a simple 1–2 bp indel, a comparison of forward and reverse sequences allowed determination of haplotypes. When heterozygosity due to overlapping indels was impossible to phase, the ITS region was cloned. Homozygotes were defined as sequences that had no double peaks in chromatograms and no apparent indels,

whereas heterozygotes were defined as sequences with double peaks and/or indels from chromatograms.

Distance estimates.—To calculate maximum and minimum base-pair differences among and within clades, a distance matrix was calculated using the ''distance'' program in GCG (GCG 2000) with an ''uncorrected distance'' setting. Highest and lowest distance estimates were identified from the distance matrix and percent base-pair differences were calculated manually from the aligned pairs of sequences. When comparing cloned sequences, an average error rate for Taq polymerase was determined by obtaining 10 reclones of two clones from different species (*Gymnopus dichrous* TENN67859c1 reclone error rate = 0.14%; *Russula* afn. *foetens* TENN67951c1 reclone error rate = 0.09%). Error rates were bp mutations/total base pairs. The former rate agrees well with estimates obtained by initial cloning of TENN67859c1–c10 (error rate = 0.14%). The larger estimate (0.14%) was subtracted from distance measurements of cloned sequences in this study and should provide a conservative estimate of distance. Each base pair in an indel was counted as a single nucleotide difference, except for *A. mellea*, the ITS region of which was characterized by a series of shared gaps. To evaluate relationships among isolates of *A. mellea* from eastern USA, parsimony and Bayesian analyses were performed on two datasets: (i) gap areas removed to keep large gaps from unduly influencing phylogenies and (ii) gaps treated as a fifth base.

Phylogenetic analyses.—Sequences were aligned with the Pileup program in GCG and adjusted manually. Phylogenetic relationships within a species were estimated by maximum parsimony and Bayesian analysis. For parsimony analysis implemented in PAUP* 4.0b10 (Swofford 2002), gaps were treated as a fifth base and characters were unordered and unweighted. Heuristic searches were conducted under these conditions: The starting tree was obtained via stepwise addition; the branch-swapping algorithm was tree-bisection-reconnection (TBR). Branch robustness was evaluated by 1000 bootstrap replicates (Felsenstein 1985) using the same conditions as above. ModelTest (Posada and Crandall 1998) was used to estimate the appropriate model of nucleotide substitution for Bayesian analysis using MrBayes 3.0 (Huelsenbeck and Ronquist 2000). If the selected model could not be implemented in MrBayes 3.0, the closest model with equivalent parameters or more relaxed parameters was selected. The MCMC search was run with four chains for 500 000 generations with sampling every 100 generations. The first 1000 trees were discarded based on preliminary analyses showing that likelihood values had reached stability with the first 1000 trees. Posterior probabilities were estimated by sampling trees generated after likelihood values diverged. A 50% majority rule consensus tree was generated. Aligned sequences were deposited in TreeBASE: *Amanita citrina* f. *lavendula* project number 14193, *Armillaria mellea* project number 14195, *Gymnopus dichrous* project number 14198, *Hygrocybe* project number 14199.

Determination of putative crossover regions.—Crossover regions were estimated visually for *Armillaria mellea* using shared gaps as markers. Tentative zones of recombination were identified between markers including the 5.8S gene region. Recombination points for four clones of a *Hygrocybe flavescens/chlorophana* hybrid were identified by DNA sequence. There were no recombinants in *Gymnopus dichrous* hybrids except for a single probable clone chimera. Variability within *Amanita citrina* f. *lavendula* complex was too extensive to allow identification of recombination points, and there were no identical clones in this dataset.

Tests for recombination.—Recombination was detected statistically with Φω (Bruen et al. 2006) as implemented in the program SplitsTree (Huson and Bryant 2006) using a window size of 100 bp. Recombination detection program (RDP) 3.44 (Martin et al. 2010) also was used to evaluate recombination with RDP (Martin and Rybicki 2000) that examines possible recombination and makes an attempt to identify breakpoints from the analyses of three sequences. We also used 3Seq (Boni et al. 2007), which is also a triplet-scanning procedure and the MaxChi method that identifies potential breakpoints (Smith 1992).

RESULTS

Armillaria mellea.—*Armillaria mellea* collections fell into two general clades distinguished primarily by sequence differences in the ITS2 region. Both clades were characterized by an extensive series of gaps that were present in all North American collections but absent from European collections. Gaps present in eastern North American collections were numbered. Gaps 1–3 were present and variable in both clades; gap 4 was present only in clade 1, gap 5 was present only in clade 2; gap 6 was present only in clade 1. Three sequences in clade 2 (AY213587, AF163587, AF163589) lacked gaps 5 and 6 (FIGS. 1, 2). Collections that were heterozygous for gaps (TENN063153, TENN60319, TENN61702 from GSMNP) were cloned to recover individual haplotypes, and in all three instances cloned haplotypes belonged to either clades 1 or 2, suggesting that the parental dikaryon was a hybrid between the clades (FIG. 2).

Gaps served as convenient markers to assay whether recombination was occurring among different eastern North American haplotypes. Examination of sequence data revealed that there were at least 10 different gap haplotypes and that ITS1 gaps were not well correlated with any gap haplotype pattern in the ITS2 region (FIG. 2), suggesting that recombination was occurring. To exclude the possibility that putative recombinants were artifacts generated by the PCR process, we examined sequences for homozygous uncloned collections in this study and found the same haplotypes as seen in sequences from cloned collections. A Φω test for recombination did not find significant evidence for recombination within clade 1 ($P = 0.09$), but statistically significant evidence for

TABLE I. Taxa used in the phylogenetic analyses, including information about the origin of the fungal material, collection numbers and GenBank accession numbers[a]

GenBank no.	Herbarium no.	Field no.	Name	Location
Armillaria mellea				
AF163578		B176	*Armillaria mellea*	UK
AF163579		B1247	*Armillaria mellea*	UK
AF163580		B1240	*Armillaria mellea*	UK
AF163581		B1212	*Armillaria mellea*	Hungary
AF163582		B186	*Armillaria mellea*	UK
AF163583		B1205	*Armillaria mellea*	Iran
AF163584		B527	*Armillaria mellea*	France
AF163586		B1245	*Armillaria mellea*	France
AF163587		B282	*Armillaria mellea*	USA, New Hampshire
AF163588		B497	*Armillaria mellea*	USA, New Hampshire
AF163589		B496	*Armillaria mellea*	USA, Massachusetts
AF163590		B497	*Armillaria mellea*	USA, Massachusetts
AJ250051		M1 (90260/1)	*Armillaria mellea*	Slovenia
AM269762		T4D	*Armillaria mellea*	Switzerland
AME250051			*Armillaria mellea*	Slovenia
AY163585		GD081	*Armillaria mellea*	France
AY213584		ST5-A; GB934	*Armillaria mellea*	USA, Virginia
AY213585		ST5-B; GB934	*Armillaria mellea*	USA, Virginia
AY213586		ST20; A3	*Armillaria mellea*	USA, Wisconsin
AY213587		ST21; TCH-2	*Armillaria mellea*	USA, New Hampshire
AY789081		PBM2470	*Armillaria mellea*	USA, Massachusetts
AY848938		UASWS0027	*Armillaria mellea*	Poland
JF313749	TENN50663	TFB4184	*Armillaria mellea*	Scotland
JF313750- JF313756 JF313770 JF313771 JF313778	TENN60319	TFB12071 c1-c10	*Armillaria mellea*	USA, Tennessee, GSMNP, Cades Cove
JF313757		Cif2005 340	*Armillaria mellea*	USA, Tennessee GSMNP
JF313758	TENN61407	TFB12942	*Armillaria mellea*	USA, Tennessee, GSMNP, Kephart Prong Trail
JF313759 JF313760 JF313765 JF313766	TENN61702	TFB13439 c1, c2, c3, c4	*Armillaria mellea*	USA, North Carolina, GSMNP, Big Fork Ridge Trail
JF313761- JF313764 JF313772- JF313777 JF313768 JF313769	TENN63053	TFB13617c1-c12	*Armillaria mellea*	USA, Tennessee. GSMNP, Cherokee Orchard
JF313767	TENN61396	TFB12931	*Armillaria mellea*	USA, Tennessee, GSMNP, Cades Cove
Amanita citrina f. *lavendula*				
AB015677		LEM960303	*Amanita porphyria*	Japan
AB015679		LEM960298	*A.* ''*citrina*''	Japan
AB015680		LEM970501	*Amanita* ''*citrina*'' var. *grisea*	Japan
AF085483		IFO-8261	*A.* ''*citrina*''	No data
AF085489		CBS441.91	*A.* ''*citrina*''	Austria
AJ633110			Environmental Sample	Finland
AY656916			Environmental Sample	USA, North Carolina
AY789079	TENN62305	AFTOL-ID 673 PBM 2429	*Amanita brunnescens*	USA, Massachusetts

TABLE I. Continued

GenBank no.	Herbarium no.	Field no.	Name	Location
DQ990869			Environmental Sample	Italy
EF619627			Environmental Sample	USA, North Carolina
EU597069		UBCOGTR0401s	*Amanita porphyria*	Canada, British Columbia
FJ210727			Environmental Sample	Italy
FJ596864-FJ596868	TENN61704	TFB13441 c1-c5	*Amanita citrina* f. *lavendula*	USA, North Carolina, GSMNP, Big Fork Ridge Trail
FJ715924			Environmental Sample	USA, Michigan
GU256206			Environmental Sample	Italy
JF313650	TENN61660	TFB13397	*Amanita flavorubens*	USA, Tennessee, GSMNP
JF313651-JF313652	LE241998	LE216-2004 c1, c3	*Amanita* cf. *rubescens*	Russia, Samara Region
JF313653	No specimen	LE259-2004	*Amanita* cf. *rubescens*	Russia, Priozersk district
JF313654	TENN62894	TFB13547	*Amanita* cf. *rubescens*	Sweden, Närke
JF313655	TENN61564	TFB12602	*Amanita flavoconia*	USA, North Carolina, Swain County, GSMNP
JF313656	LN2004-07-13-05a	LN2004-07-13-05a	*Amanita flavoconia*	USA, Tennessee, GSMNP
JF313657	RET 376-10	RT-7-13-04-B	*Amanita flavoconia*	USA, Tennessee, Sevier County, GSMNP
JF313658	TENN61382	TFB12917	*Amanita* "*citrina*"	USA, Tennessee, GSMNP
JF313659	TENN61557	TFB13296	*Amanita solaniolens*	USA, North Carolina, GSMNP
JF313660-JF313663	FCME Cifuentes 2005-311	CIF2005 311 c1-c4	*Amanita citrina* f. *lavendula*	USA, Tennessee, GSMNP
JF313664-JF313669	TENN61710	TFB13447 c1-c6	*Amanita citrina* f. *lavendula*	USA, North Carolina, GSMNP, Beech Grove School
Gymnopus dichrous				
AF241335		NA	Soil sample	USA, New Jersey
AY256702	TENN56726	TFB10014	*Gymnopus dichrous*	USA, Georgia, Macon County
DQ450007	TENN53792	TFB7920	*Gymnopus dichrous*	USA, Tennessee, GSMNP,
DQ450008	TENN48554	TFB2028ss5	*Gymnopus dichrous*	USA, North Carolina, Highlands
DQ450030	TENN50324	TFB4727	*Marasmius ramealis*	Sweden
DQ450031	SFSU DED4425		*Marasmius ramealis*	USA, North Carolina
DQ480115	Culture only	TFB10829ss6	*Gymnopus dichrous*	USA, North Carolina, Jackson County
FJ596781-FJ596782	TENN60673	TFB12506h1, h2	*Gymnopus dichrous*	USA, North Carolina, GSMNP, Brushy Mt. Trail
FJ596783	TENN61128	TFB12567	*Gymnopus dichrous*	USA, North Carolina, GSMNP, Baxter Creek Trail
JF313670	TENN62867	TFB13520	*Marasmius ramealis*	Sweden
JF313671-JF313672	TENN60014	TFB11785 c2, c5	*Gymnopus dichrous*	USA, Tennessee, GSMNP
JF313673-JF313677	TENN60027	TFB11814 c1-c5	*Gymnopus dichrous*	USA, Tennessee, GSMNP, Cherokee Orchard
JF313678-JF313693	TENN61624	TFB13361 c1a, c1b, c2a, c2b, c3a, c3h^2, c4a, c4b, c5-c12	*Gymnopus dichrous*	USA, North Carolina, GSMNP
JF313694	TENN48554	TFB2028ss4	*Gymnopus dichrous*	USA, North Carolina, Macon County
JF313695	TENN60308	TFB12060	*Gymnopus dichrous*	USA, Tennessee, GSMNP, Greenbrier
Hygrocybe flavescens/chlorophana complex				
AY969935			Soil sample, USA, NC	USA, New Jersey
EU435148	CFMR; CUW Boertmann 2002/9		*Hygrocybe chlorophana*	Denmark
EU435149	TENN61826	DJL05NC9	*Hygrocybe glutinipes* var. r*ubra*	USA, North Carolina GSMNP, Big Creek

TABLE I. Continued

GenBank no.	Herbarium no.	Field no.	Name	Location
EU784301	RBG Kew K (M) 61287		*Hygrocybe chlorophana* as *H. flavescens*	UK, Bedfordshire
EU784309	RBG Kew K (M) 126644		*Hygrocybe glutinipes*	UK, East Sussex
EU784312	RBG Kew K (M) 128171		*Hygrocybe glutinipes* as *H. vitellina*	UK, North Somerset
EU784329	RBG Kew K (M) 139410		*Hygrocybe chlorophana* as *H. mollis*	UK, Shetland Islands
EU784354	RBG Kew K (M) 126650		*Hygrocybe vitellina*	UK, East Sussex
FJ313704	TENN60268	TFB12008	*Hygrocybe flavescens*	USA, Tennessee, GSMNP, Tremont
FJ313705	TENN59945	TFB11987	*Hygrocybe flavescens*	USA, Tennessee, GSMNP, Crib Gap
FJ596922	TENN68015a	DJL06TN16.1 (TN139)	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Greenbriar
FM208855		H4	*Hygrocybe chlorophana*	Hungary
FM208856		H5	*Hygrocybe chlorophana*	Hungary
FM208858		H7	*Hygrocybe chlorophana*	Hungary
JF313699-JF313702	TENN60864	TFB12267 c1-c3, c5	*Hygrocybe flavescens*	USA, Tennessee, GSMNP, Cosby
JF313703	TENN68010	DJL08TN26 (TN285)	*Hygrocybe flavescens*	USA, Tennessee, GSMNP, Cosby
JF313706	TENN61923	DJL05NC102	*Hygrocybe* sp. afn. *flavescens*	USA, North Carolina, GSMNP, Beech Gap Trail
JF313707	TENN61897	DJL05NC68	*Hygrocybe* sp. afn. *flavescens*	USA, North Carolina, GSMNP, Cataloochee Cove
JF313708-JF313710	TENN68016	DJL06 TN25 c3-c5 (TN148)	*Hygrocybe flavescens*	USA, Tennessee, GSMNP, Greenbriar
JF313711	Vince P. Hustad #71	VPH71	*Hygrocybe flavescens*	USA, Tennessee, GSMNP,
JF313712-JF313715	CFMR DJL VT2-11	DJL-VT2 c2-c5	*Hygrocybe flavescens*	USA, Vermont
JF313716-JF313719	TENN61898	DJL05NC69	Interspecific hybrid as *H. glutinipes*	USA, North Carolina, GSMNP, Cataloochee
JF313720-JF313724	TENN60277	TFB12018 c1-c5	*Hygrocybe chlorophana*	USA, North Carolina, GSMNP, Waterville
JF313725-JF313728	TENN61457	TFB12993c1-c3, c5	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cades Cove
JF313729	TENN68013	DJL06TN45 (TN171)	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cades Cove
JF313730-JF313733	TENN68018	DJL06NC113 c1, c3-c5 (NC245)	*Hygrocybe chlorophana*	USA, North Carolina, GSMNP, Cataloochee
JF313734	TENN68012	DJL06TN46 (TN172)	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cataloochee
JF313735	TENN68017	DJL06NC114 (NC246)	*Hygrocybe chlorophana*	USA, North Carolina, GSMNP, Cataloochee
JF313736-JF313737	TENN68014	DJL06TN34 c1, c2 (TN157)	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cades Cove
JF313738	TENN60572	TFB12498	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Husky Gap Trail
JF313739	TENN68015b	DJL06TN16.2 (TN-139)	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Greenbriar
JF313740	TENN60682	TFB12515	*Hygrocybe flavescens*	USA, North Carolina, GSMNP, Big Fork Ridge
JF313741-JF313742	TENN68011	DJL06TN47 h1, h2	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cades Cove
JF313743-JF313744	TENN60942	TFB13105 h1, h2	*Hygrocybe chlorophana*	USA, Tennessee, GSMNP, Cades Cove
JF313745-JF313748	CFMR NY-46	DJLNY11b c1-c3, c5	*Hygrocybe chlorophana*	USA, New York, Albany County, Joralem Street Park

[a] NA = not applicable, c = clone number, h = haplotype number, ss = single spore isolate, TFB = Tennessee Field Book, GSMNP = Great Smoky Mountains National Park.

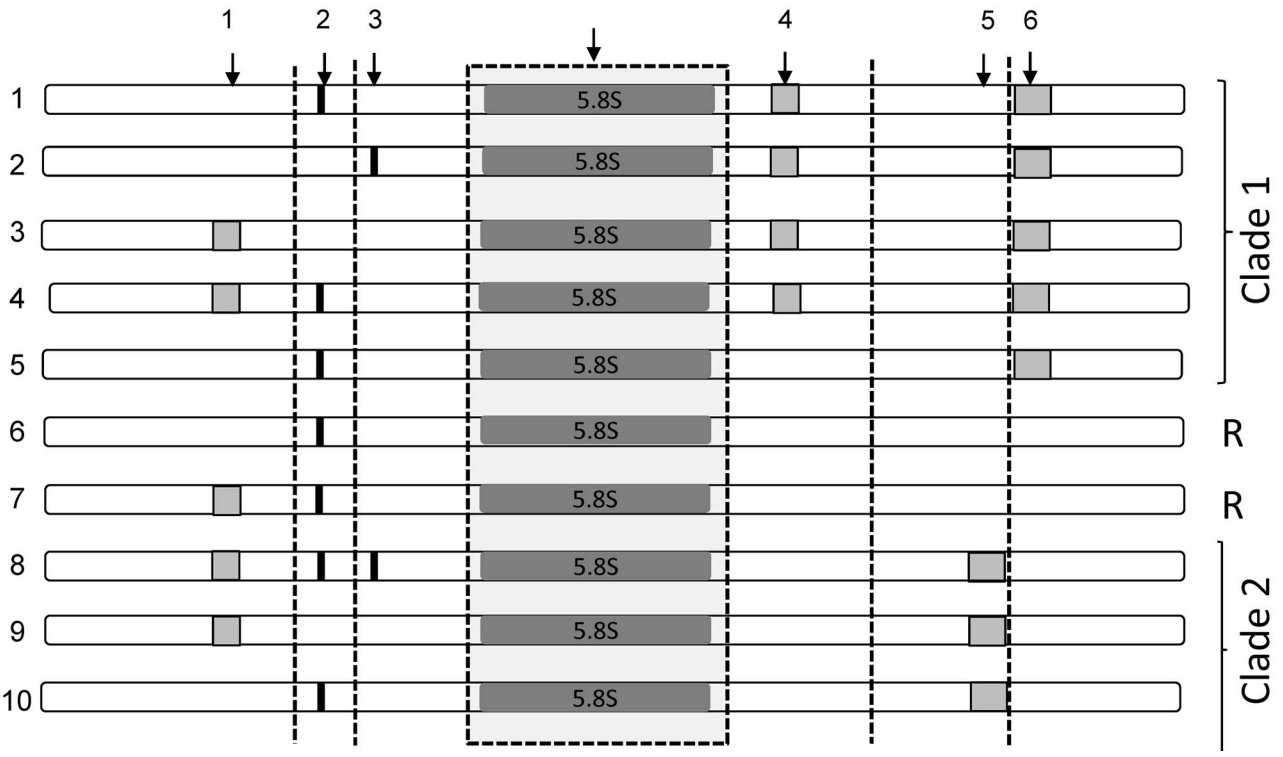

FIG. 1. Position of gaps in the ribosomal ITS1-5.8S-ITS2 region in collections of *Armillaria mellea* from the eastern United States. Deletions 1–6 are indicated by arrows. Gap positions in the aligned dataset of eastern North American and European collections starting with the first base of ITS1 were: gap 1 bases 133–144 (12 bp); gap 2 bases 180–182 (3 bp); gap 3 bases 205–206 (2 bp); 5.8S region bases 264–423 (159 bp); gap 4 bases 605–618 (14 bp); gap 5 bases 658–687 (30 bp); gap 6 bases 696–717 (22 bp). Potential regions of recombination are shown as dotted lines or boxes. R = putative recombinants.

recombination was found within clade 2 ($P < 0.01$) and within the combined dataset ($P < 0.01$). RDP, 3SEQ and MaxChi methods did not detect recombination in clade 1 or in clade 2, but 3Seq detected six possible recombinants in the entire dataset including those sequences (AY213587, AF163587, AF163589) in clade 2 that lacked gap 6.

A distance matrix was calculated for datasets 1 and 2 to identify the minimum and maximum genetic distance between collections. Within clade 1 percent base-pair divergence with gaps included was 0.0–5.3% bp and with gaps excluded was 0.0–0.8% bp. Within clade 2, percent base-pair divergence with gaps included was 0.1–2.2% bp and with gaps excluded was 0.1–2.6% bp. Between clades 1 and 2, percent base-pair divergence with gaps included was 9.3–9.6% bp and with gaps excluded was 1.2–2.6% bp (FIG. 2).

Amanita citrina *f.* lavendula.—Four collections were made in Great Smoky Mountains National Park. Of these, three were heterozygous for multiple indels in the ITS region. Cloning revealed several haplotypes that differed by as much as 3.5% (TENN61704c2 and c4 = 3.1%; TENN61710c1 and c3 = 3.5%; Cif2005-311c1 and c3 = 3.3%). After subtracting estimated cloning errors (i.e. 0.14%), the maximum ITS divergence observed within individual fruitbodies was 3.3–4.6%, only slightly exceeding the 3% bp divergence suggested as a benchmark for intraspecies variation within a defined geographical area (Hughes et al. 2009). Haplotypes from the three fruit bodies were interspersed in the phylogeny as would be expected in an interbreeding population (FIG. 3). A Φω test for recombination found highly significant evidence for recombination ($P = 0.001$). 3Seq detected three putative recombinants in the dataset but RDP3 was unable to identify the start or end of the breakpoints.

Gymnopus dichrous.—Two distinct ITS haplotypes for *G. dichrous* collections from the southern Appalachians differed primarily in the ITS2 region (FIG. 4). Variation within clade 1 was 0.3–1.0% (minimum/maximum divergence between paired sequences). Variation within clade 2 was 0.0–1.1%. Divergence between clades 1 and 2, however, was much greater, 5.5–5.7%. There were a number of consistent within-clade synapomorphies (23 bp) that separated haplotypes from the two clades. Three collections (TENN60014, TENN60027, TENN61624)

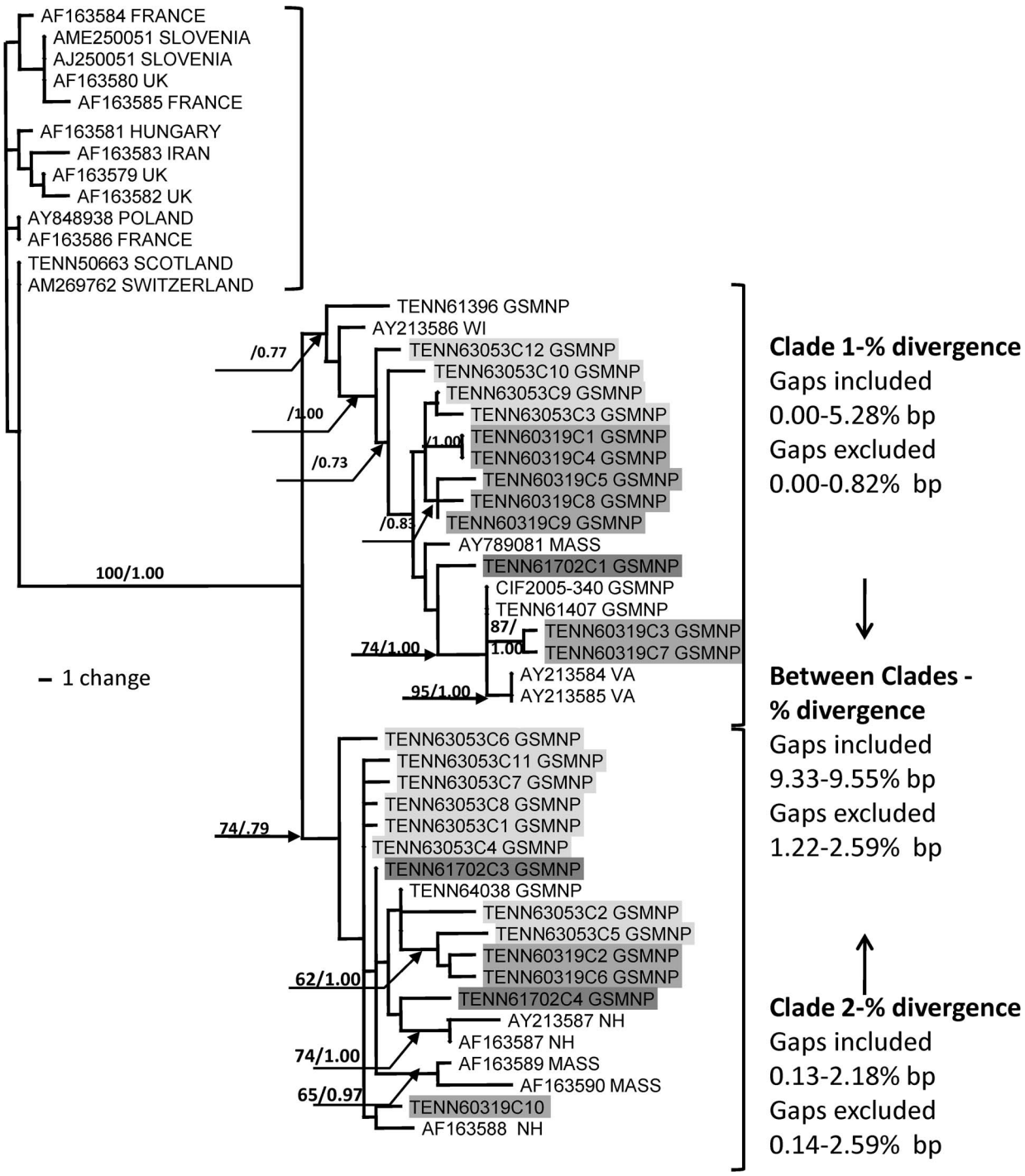

FIG. 2. Parsimony analysis of *Armillaria mellea* collections from the eastern United States. Sequence data in gaps was removed for this analysis. Tree illustrates 1 of 1000 most parsimonious trees, 156 steps long. Search was heuristic with gaps treated as a fifth base. Characters were unordered and equally weighted. Bootstrap values and Bayesian posterior probabilities greater than 70% are to the left of the supported node. c = clone number, Env = environmental sample. Shades of gray distinguish clones from the same basidiome.

were heterozygous and their cloned haplotypes segregated into either clade 1 or 2 (FIG. 4). A single clone of TENN61624 contained intermixed sequence elements of both clades. Whether this represents a cloning-generated artifact between sequences of clades 1 and 2 or a recombination event is unknown. In that this recombinant haplotype is the only instance in the dataset, the chimeric sequence is

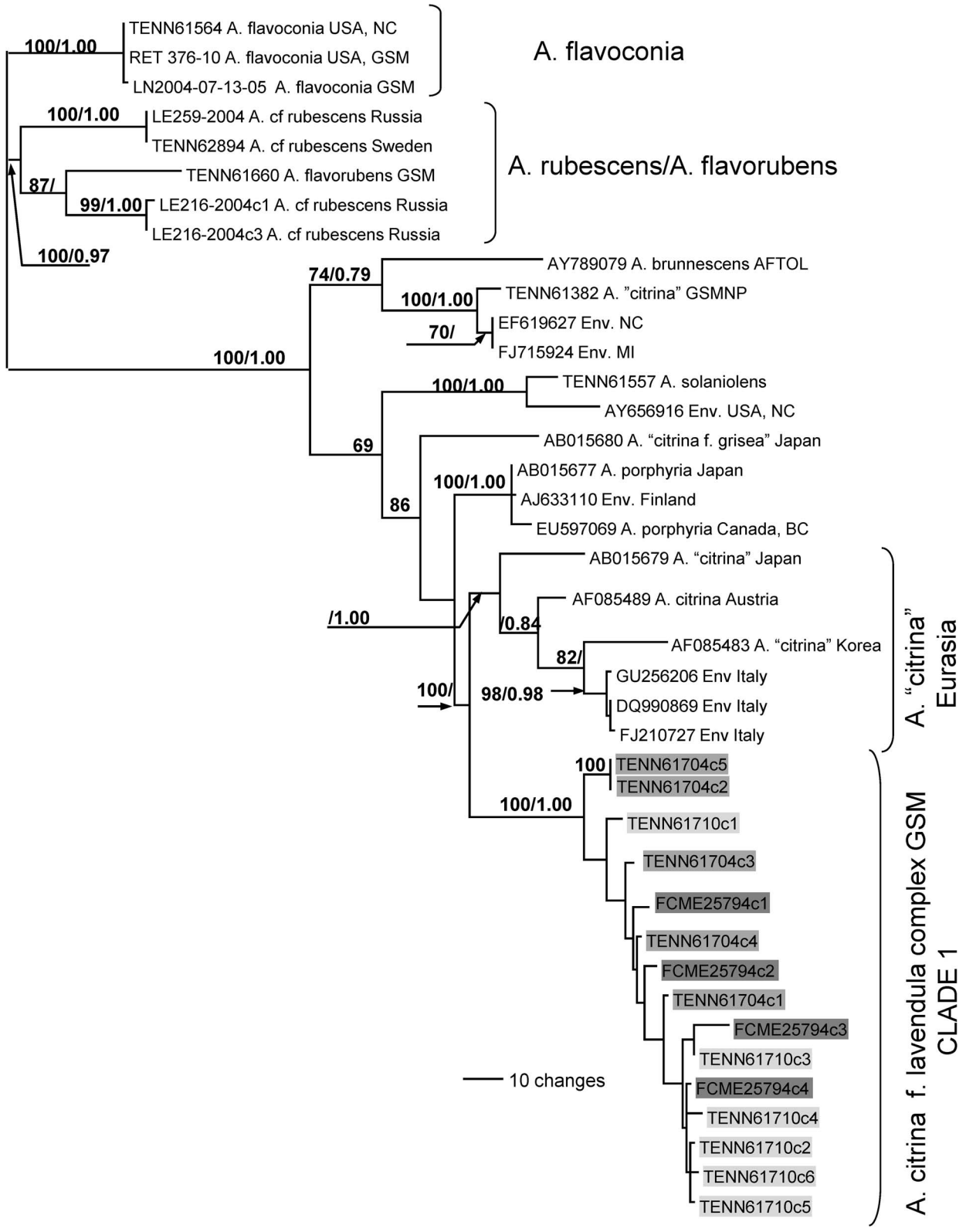

FIG. 3. Parsimony analysis of *Amanita citrina* f. *lavendula* and related taxa. This is the single most parsimonious tree, 653 steps long. Search was heuristic with gaps treated as a fifth base. Characters were unordered and equally weighted. Bootstrap values and Bayesian posterior probabilities greater than 70% are to the left of the supported node. c = clone number, Env = environmental sample. Shades of gray distinguish clones from the same basidiome.

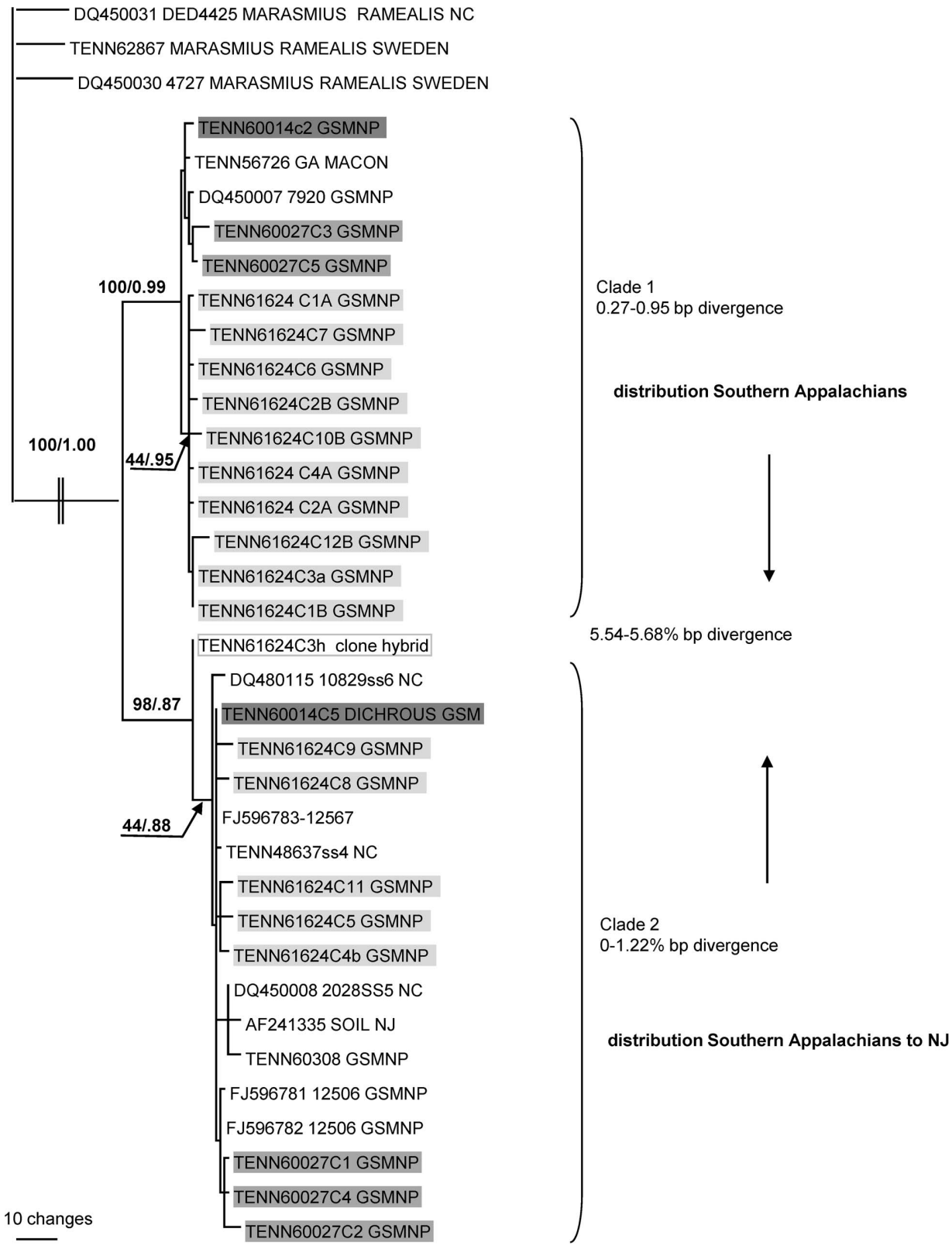

FIG. 4. Parsimony analysis of *Gymnopus dichrous* collections from the eastern United States. Illustration is 1 of 20 most parsimonious trees, 290 steps long. Search was heuristic with gaps treated as a fifth base. Characters were unordered and equally weighted. Bootstrap values and Bayesian posterior probabilities greater than 70% are to the left of the supported node. c = clone number. Shades of gray distinguish clones from the same basidiome.

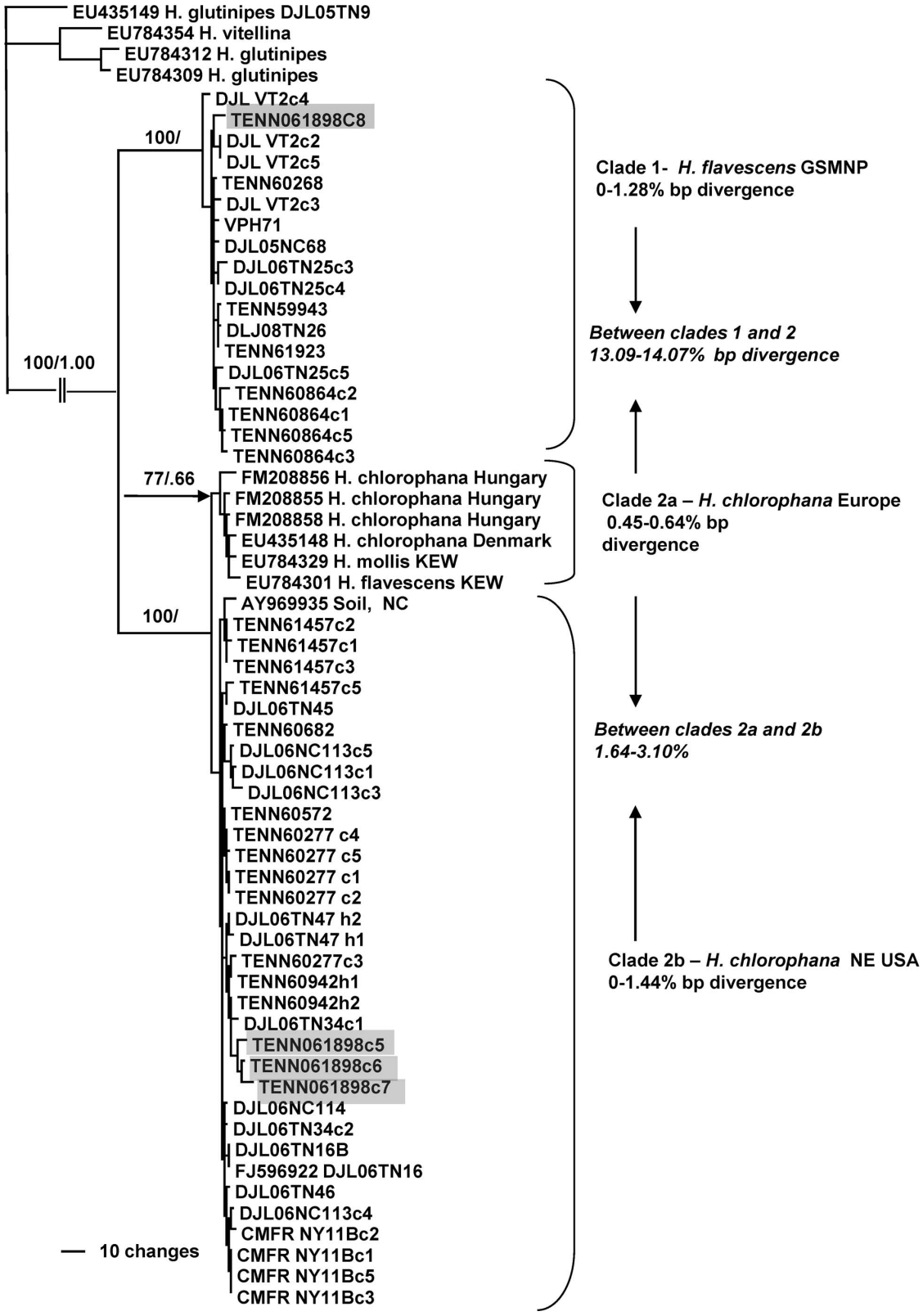

FIG. 5. Parsimony analysis of the *Hygrocybe flavescens/H. chlorophana* complex. Tree illustrates 1 of 1000 most parsimonious tree, 631 steps long. Search was heuristic with gaps treated as a fifth base. Characters were unordered and equally weighted. Bootstrap values and Bayesian posterior probabilities greater than 70% are to the left of the supported node. Arrows indicate clones from a single basidiome. Recombinant ITS sequences were not included in the analysis. c = clone number. Shades of gray distinguish clones from the same basidiome.

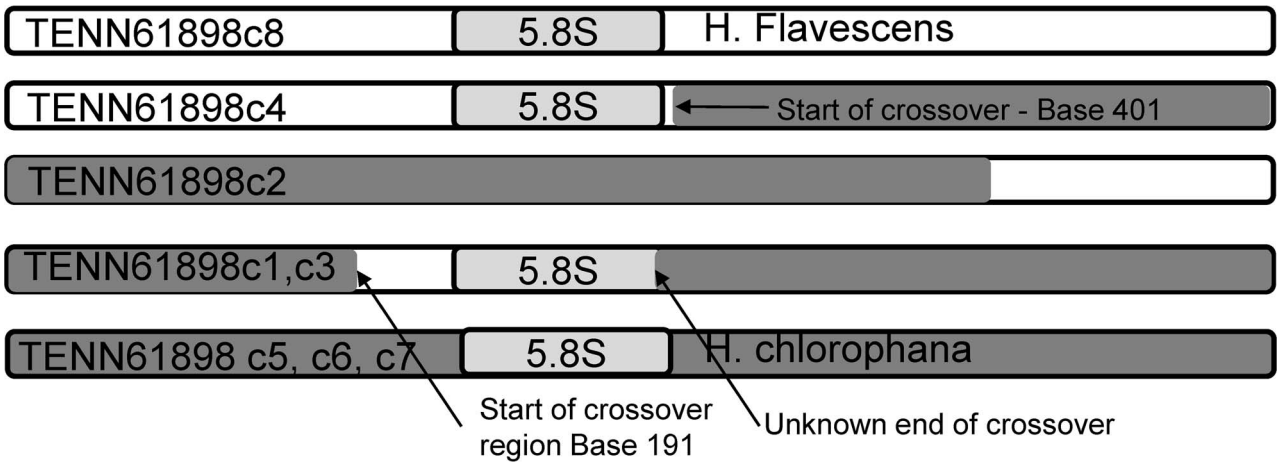

FIG. 6. Clones derived from a putative hybrid (TENN61898) between *Hygrocybe* afn *flavescens* and *H. chlorophana*. Clone 8 was *H.* afn *flavescens*; clones 5, 6 and 7 were *H. chlorophana*. The recombination point for clone 4 was at base 401. The recombination point for clones 1 and 3 was at base position 191. The end point of this recombinant is unknown.

likely an artifact of cloning. The possibility of apparent hybrids representing intragenomic variability instead of a true hybrid was tested by amplifying *G. dichrous* homozygotes with clade-specific primers and finding only one PCR product, whereas apparent hybrids showed both PCR products. A Φω test for recombination did not produce significant evidence for recombination within clade 1 ($P = 0.7$) or within clade II ($P = 0.8$) but did find statistical evidence for recombination when sequence data for both clades were combined ($P < 0.001$ with 36 informative sites), but RDP, MaxChi and 3Seq did not identify recombinants. In this case, results from Φω disagree with our direct observations and with other tests for recombination. The reason for this failure is unknown but might be due to shared variable sites within the two sister clades.

Hygrocybe flavescens/chlorophana *group.*—Results of the phylogenetic analysis are illustrated (FIG. 5). Percent ITS sequence divergence within clade 1 (*H.* afn. *flavescens*) was 0–1.3%. Within clade 2a (European *H. chlorophana*), percent sequence divergence was 0.5–0.6% and clade 2b (North American *H. chlorophana*) was 0–1.4%. In contrast, percent ITS sequence difference between clades 1 and 2 was much greater, 13.1–14.1%. This high variation in ITS region clearly indicates that these most likely are distinct species. In addition, 22 collections from GSMNP belonged unambiguously to one of the two clades and there was no evidence of hybrids between the two clades. Therefore it was surprising to find that cloned ITS haplotypes from a collection under the field name *H. glutinipes* (DJL05NC69 = TENN61898) were identical to *H.* afn *flavescens*, *H. chlorophana* or were complex hybrids between both clades. Clone 8 was a complete sequence of clade *H. flavescens*; Clones 5, 6 and 7 were complete sequences of clade *H. chlorophana*; The remaining clones were complex hybrids between clades 1 and 2. (FIG. 6). Two additional collections of *H.* afn. *flavescens* were cloned and no sequences of *H. chlorophana* were identified in the cloned sequences. Six additional collections of *H. chlorophana* were cloned and no sequences of *H.* afn. *flavescens* were identified in the cloned sequences. A Φω test for recombination did not find evidence for recombination within *H.* afn. *flavescens* ($P = 0.89$) or within North American *H. chlorophana* ($P = 0.06$). The Φω test did find statistically significant evidence for recombination in the combined *H.* afn. *flavescens* + *H. chlorophana* + putative recombinants TENN61898c1–c4 dataset ($P = 0.01$), but with the putative crossovers removed, it did not find evidence for recombination ($P = 0.22$). Similar results were found with 3Seq with the same set of clones of TENN61898c1–c4 identified as recombinant, but crossovers were not detected by RDP and MaxChi.

DISCUSSION

Allopatric speciation typically require geographical separation, followed by the accumulation of independent genetic differences between populations and ultimately leading to establishment of new species. When geographic isolation breaks down and previously differentiated allopatric populations come back into contact, several outcomes are possible: Populations may hybridize and if genetic divergence is sufficient to cause reduced fitness and/or partial reproductive prezygotic/postzygotic barriers (Dobzhansky-Muller model; Muller 1942, Dobzhansky 1951), inhibition of

gene flow may be reinforced and speciation processes would continue. When genetic divergence is sufficient to block gene exchange, this is equivalent to establishment of biological species (Dobzhansky 1951, Mayr 1970). If genetic divergence is an insufficient barrier to gene exchange, previously isolated populations can interbreed with resulting introgression and/or formation of a genetically variable hybrid swarm with new adaptive combinations (Herder et al. 2006). In theory, greater genetic distances between parental genotypes are correlated with higher probabilities of transgressive variation and novel genetic combinations (Stelkens and Seehausen 2008, Donovan et al. 2010).

In this study, four cases of putative hybridization between divergent haplotypes were examined. In two cases, hybridization has resulted in apparent recombination and persistence of hybrid progeny (*A. mellea*, *A. citrina* f. *lavendula*). In the remaining two cases, *G. dichrous* and *H. chlorophana/flavescens*, although hybrid progeny were identified, no evidence was found for persistence of recombinant F_2 or backcrossed progeny in natural populations.

Armillaria mellea.—ITS sequences of eastern North American collections are characterized by a series of indel regions, and using these as markers we identified reasonable evidence of hybridization and recombination. An indel in an aligned dataset can result from either a deletion or a duplication, but sequences present in indel regions of eastern North American collections are not duplication of adjacent ITS DNA sequence and for the most part are homologous to sequences from European *A. mellea* collections. For these reasons it is likely that most haplotypes in eastern North American populations evolved by a series of deletions resulting in an unusually high number of small (2-bp) to large (51-bp) gaps. It is possible that an original deletion might have destabilized the ITS region, resulting in further deletions through mispairing. Some gap combinations do not occur, suggesting that there may be structural constraints on the total number and/or positions of gaps that can be accumulated without damaging ITS secondary structure sufficiently to cause lethality. If this is the case, there should be an evolutionary cost for hybridization and recombination and selection gradually should favor alternate scenarios (barriers to hybridization, elimination of some haplotypes from the population etc.). If gaps are derived, then European and North American populations diverged before evolution of gap recombinants.

In the absence of interbreeding among populations of *A. mellea* in eastern North America, we should expect to see within the population a few dominant haplotypes that segregate into distinct clades. Instead, using indel sites as informative sites, multiple haplotypes are distributed throughout the population (as has been shown in eastern and western North American populations of *A. mellea*; Baumgartner et al. 2010, Travadon et al. 2012), suggesting that extensive recombination has taken place. At this time, we cannot distinguish whether intra-lineage hybridization is limited to the southern Appalachians but the repeated recovery of two major clades suggests that there were two (or more) distinct parent populations in eastern North America that are interbreeding. Evidence for population subdivision leading to divergence among populations was provided by Baumgartner et al. (2010) who sampled fruit bodies of *A. mellea* from eastern North America and recovered genotypes restricted to the Ozark and southern Appalachian subpopulations, in addition to the genotypes that were relatively unique to more northern populations. They hypothesized that southern populations might have inherited alleles from divergent source populations. A plausible scenario is the northward expansion by post-glacial migration of *A. mellea* populations from Mexico (or other southern refugia), where the species also is found.

Amanita citrina *f.* lavendula.—*Amanita citrina* is a Persoon name with a probable topotype in western Germany (Persoon 1797). *Amanita citrina* f. *lavendula* (Coker) Veselý 1933 Ann Mycol 31:239 originally was described by Coker from oak/pine woods as a variety of *A. mappa* Batsch 1783 in North Carolina differing from *A. citrina* (= *A. mappa* = *A. bulbosa* f. *citrina*) in that the cap of the basidiome is paler than in the European *A. citrina* and both cap and stalk can be tinged with lavender or brown. In addition, the flesh of the eastern North American taxon is nearly white, sometimes turning brown or lavender when cut. Lavender is most likely to appear after exposure of the basidiome to near freezing temperatures (see // www.amanitaceae.org?Amanita+citrina+f.+lavendula). *Amanita citrina* is sister to *A. citrina* f. *lavendula* but likely is a different species based on ITS sequence divergence, toxin profiles, culture characteristics and morphology (Petersen 1977; also see http://www.amanitaceae.org?Amanita+citrina+f.+lavendula).

Three collections of A. citrina *f.* lavendula were heterozygous, and cloning of the ITS region produced multiple haplotypes. The three collections were from geographically disparate locales within the southern Appalachians suggesting that hybridization of different haplotypes is not a localized phenomenon and occurred by either multiple hybridization or the range expansion of putative hybrids. Further, haplotypes did not separate into

discrete clades. Instead, as with *A. mellea*, variable sequence motifs occurring within ITS1 and ITS2 were unlinked and all haplotypes from GSMNP collapsed into a single clade, suggesting that a single, large interbreeding population undergoing recombination might be a likely explanation for the lack of separation of haplotypes. The high divergence among haplotypes within GSMNP suggests interbreeding between two or more divergent parent populations that has progressed to the extent that signatures of the original parental populations are no longer evident and have produced a single, highly heterogeneous population. An alternate and plausible hypothesis is incomplete lineage sorting as a result of recent divergence.

Gymnopus dichrous.—*Gymnopus dichrous* (Berk. & M.A. Curtis) Halling originally was described from South Carolina but apparently extends as far west as Indiana and northward into New England (Halling 1983). Of 13 collections of *G. dichrous*, sequences of 10 collapsed into one of two clades that may represent two historically allopatric populations that have come into contact. Populations of *G. dichrous* with clades 1 and 2 haplotypes are currently sympatric in the low elevation cove-hardwood forests of the southern Appalachians where they fruit on oak bark and woody debris. Of the three collections that are hybrids of clades 1 and 2, cloned haplotypes sorted into either clade 1 or 2 suggesting that these fruit bodies represent an F_1 hybrid. Collection TENN61624 (TFB13361), however, produced a single clone (out of 12 cloned sequences) that had sequence elements from clades 1 and 2 and is likely a cloning chimera. The lack of apparent recombination between the two clades observed in populations from the southern Appalachians raises the question as to whether hybrids between clades 1 and 2 are inter-fertile and/or viable in nature. No recombinant fruit bodies suggestive of F_1 meiotic recombination and second generation hybrid fruit bodies have been collected. Preliminary studies by RHP (unpubl) provide evidence that single-spore isolates of clades 1 and 2 will dikaryotize in vitro, but mycelia germinated from spores of a natural F_1 hybrid (TENN53972 = TFB7920) produced mycelia with a reduced ability to dikaryotize mycelia germinated from clade 1 spores. This is preliminary evidence of a reduction in hybrid fitness that limits the viability of hybrid offspring.

Hygrocybe flavescens/chlorophana *group.*—The *H. flavescens*/*H. chlorophana* complex in GSMNP comprises two variable, small, bright yellow *Hygrocybe* species that are not reliably distinguishable macro- or micromorphologically, but they do have different ITS sequences. Both fruit in mixed cove-hardwood areas of GSMNP. Within both taxa there was sufficient sequence variation (usually 1 bp indels) such that cloning was necessary to recover haplotypes.

Hygrocybe chlorophana was described from Sweden and has a presumed amphi-Atlantic distribution, whereas *H. flavescens* (Kauffman) Singer was described from Michigan, USA. Although the stipe surface is described as dry, moist or subviscid in *H. chlorophana* but viscid in *H. flavescens*, this character was highly variable within both taxa (Lodge and Hughes 2009). It is uncertain therefore whether the name *H. flavescens* can be applied to clade 1 until the holotype or an epitype from the topotype locality is sequenced, although an environmental clone sequence from Michigan matches those in our putative *H. flavescens* clade 1 (GenBank GU174284). Clade 2b is congruent with *H. chlorophana* collections from Hungary and Denmark as deposited by knowledgeable collectors (FIG. 5, clade 2a). The large percent base-pair difference (13.1–14.1%) between ITS sequences of clades 1 (*H.* afn *flavescens*) and 2 (*H. chlorophana*) suggests that they are two distinct species and ITS sequences of 22 collections separated unambiguously into one of the two distinct clades. The finding of a collection with sequence motifs characteristic of both clades (TENN61898 = DJL05NC69) was therefore a surprise and is not easily explained. This collection might represent a rare hybrid between genetically divergent sister species. Also not easily explained is the mixture of complex chimeras, which were not recovered from any other cloned basidiome. Chimeric sequences might result from the cloning process per se or might result from meiotic or mitotic in vivo recombination or repair processes (Hamady et al. 2008).

In this paper examples of hybridization between genetically divergent taxa are described. It is feasible that hybrids between genetically divergent organisms may form more readily in tetrapolar fungi than in other organisms because of their unique mating system. Most agaric fungi have a tetrapolar mating system (i.e. two mating genes with multiple alleles). To dikaryotize, two monokaryotic hyphae must be different for both alleles. This mating system favors retention of rare mating type alleles in the population while there may be selection against common alleles (identical mating alleles prevent dikaryotization). This balancing selection for mating type genes would have the effect of retaining the full spectrum of mating alleles in any population of agarics and would act to retard drift with respect to mating alleles. A number of studies that paired monokaryons from Europe, Asia and North America demonstrated successful dikaryotization in vitro despite apparent

genetic and/or morphological differentiation (Petersen 1991, 1992; Petersen and Bermudes 1992a, b; Petersen and Hughes 1993; Petersen 1995; Gordon and Petersen 1997, 1998; Johnson and Petersen 1997; Petersen and McCleneghan 1997; James et al. 1999; Le Gac and Giraud 2008). These studies and data from *H. flavescens/H. chlorophana* and *G. dichrous* are compatible with modeling observations of basidiomycetes by Giraud and Gourbier (2012) revealing that loss of ability to dikaryotize in *Microbotryum* initially declined at short genetic distances then leveled off. The retention of mating ability in *Microbotryum* allowed for remarkably high interspecific gamete fusion between divergent sister species. At greater genetic distances, however, postsyngamic hybrid sterility and inviability continued to increase. If these studies are more broadly applicable, allopatric speciation in basidiomycetes only secondarily might involve mating-type genes and the ability for divergent lineages to dikaryotize might be conserved. Thus, when previously allopatric and genetically divergent populations/species come back into contact, hybridization between genetically divergent individuals may be feasible.

In this paper we have examined four exemplars of putative intra- and/or interspecific hybrids of agaric fungi with ITS haplotype divergence greater than 2% bp. Two examples, *A. mellea* and *A. citrina* f. *lavendula*, retain the ability to interbreed in the face of significant ITS sequence divergence and both reveal evidence of recombination and reassortment leading to a hybrid swarm. In these species, hybrids apparently are viable and reproduce. Originating haplotypes are not evident in *A. mellea* or *A. citrina* f. *lavendula* GSMNP collections. Maximum ITS divergence between haplotypes for *A. citrina* f. *lavendula* was a little over 3%. Haplotype divergence for *A. mellea* was difficult to access because of extensive gaps.

Two additional examples (*G. dichrous* and *H. flavescens/ chlorophana*) suggest that the ability to dikaryotize and form F_1 fruit bodies has not been eliminated completely by genetic divergence. *Gymnopus dichrous* and *H. flavescens/ chlorophana* form hybrid fruit bodies but there were no F_2 recombinant progeny recovered from natural populations despite extensive collecting. This might be due to establishment of pre- or post-zygotic barriers. For *G. dichrous*, the ITS genetic distance between the two clades is 5.5–5.7% bp and these clades may represent two biological species with no gene flow between them. For *H. flavescens/ chlorophana*, the ITS divergence is 13.1–14.1%, so *H. flavescens* and *H. chlorophana* also might represent distinct biological species.

The identification of hybrid fruit bodies with divergent haplotypes raises some interesting questions: Is hybridization evident in the southern Appalachians unusual or is this phenomenon geographically widespread and what is the origin of such hybrids? Twenty thousand years ago, the southern Appalachians contained a far different assemblage of plants than are present today. Known refugia for the cove-hardwood forests that currently are present in the southern Appalachians were the US Gulf Coast region, along the lower Mississippi River bluffs and in Mexico and Central America. As glaciers retreated, cove-hardwood forests migrated north to their current positions thereby creating new assemblages of plants, and by inference, their associated fungi (Delcourt 1975, 1985, 2002; Delcourt and Delcourt 1984, 1994; Lickey et al. 2002). The repeated finding of usually two or on occasion three distinct and often divergent haplotypes within a number of different southern Appalachians agaric ''species'' could be explained by re-contact between fungal populations previously residing in glacial refugia. We speculate that hybridization between such populations could explain the high heterozygosity observed in many southern Appalachian agaric taxa. Vercken et al. (2010) have reported similar evidence of divergent genotypes originating from separate glacial refugia in a *Microbotryum* species that causes anther smut on *Silene latifolia* in Europe. A number of European taxa show evidence of isolation in southern European refugia followed by post-glacial re-contact (Demesure et al. 1996, Hewitt 1999). Willis (2000) noted that full glacial refugia patterns might have had more influence on patterns of biodiversity in temperate regions than in tropical zones. Refugia for western North America and the consequences of migration and re-contact are discussed in Shafer et al. (2010). Thus, one origin of the unusual biodiversity for fungi and other organisms in the southern Appalachians (Stein et al. 2000) might be migration of differentiated populations from isolated glacial refugia, followed by hybridization with generation of new genetic variability in some cases and reinforcement of reproductive barriers in others. Further, altitude, soil and water gradients may maintain biodiversity through niche diversity with hybridization occurring at the periphery. A question remains as to whether agaric fungi can undergo sympatric speciation, given a predominantly outcrossing mating system and the retention of many mating-type alleles in a population. It is difficult to see how this might occur without some kind of geographical or ecological isolation.

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