Molecular Ecology (2009) **18**, 817–833 doi: 10.1111/j.1365-294X.2008.04030.x

The ectomycorrhizal fungus *Amanita phalloides* was introduced and is expanding its range on the west coast of North America

ANNE PRINGLE,* RACHEL I. ADAMS,† HUGH B. CROSS* and THOMAS D. BRUNS‡
*Department of Organismic and Evolutionary Biology, Biological Laboratories, 16 Divinity Avenue, Harvard University, Cambridge, MA 02138, USA, †Department of Biological Sciences, Gilbert Hall, Stanford University, Stanford, CA 94305-5020, USA, ‡Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720, USA

Abstract

The deadly poisonous *Amanita phalloides* is common along the west coast of North America. Death cap mushrooms are especially abundant in habitats around the San Francisco Bay, California, but the species grows as far south as Los Angeles County and north to Vancouver Island, Canada. At different times, various authors have considered the species as either native or introduced, and the question of whether *A. phalloides* is an invasive species remains unanswered. We developed four novel loci and used these in combination with the EF1α and IGS loci to explore the phylogeography of the species. The data provide strong evidence for a European origin of North American populations. Genetic diversity is generally greater in European *vs*. North American populations, suggestive of a genetic bottleneck; polymorphic sites of at least two loci are only polymorphic within Europe although the number of individuals sampled from Europe was half the number sampled from North America. Endemic alleles are not a feature of North American populations, although alleles unique to different parts of Europe were common and were discovered in Scandinavian, mainland French, and Corsican individuals. Many of these endemic European haplotypes were found together at single sites in California. Early collections of *A. phalloides* dated prior to 1963 and annotated using sequences of the ITS locus proved to be different species of *Amanita*. The first Californian collections that we confirmed as *A. phalloides* were made from the Del Monte Hotel (now the Naval Postgraduate School) in Monterey, and on the campus of the University of California, Berkeley, in 1938 and in 1945. These historical data are used in combination with data on *A. phalloides*' current distribution to estimate a rate of spread for *A. phalloides* in California. Many species of ectomycorrhizal (EM) fungi have been introduced across and among continents, but with this evidence, the death cap becomes the only known invasive EM fungus in North America.

Keywords: conservation biology, death cap mushroom, exotic, fungal or microbial biogeography, invasive, native, range expansion

Received 31 July 2008; revision received 20 October 2008; accepted 24 October 2008

Introduction

The twin forces of habitat loss and invasion by non-indigenous species imperil the endemic biodiversity of the globe, and perhaps especially within the biodiversity hotspots of California (Bossard *et al*. 2000; Chaplin *et al*. 2000; Wilcove & Master 2005; but see Gurevitch & Padilla 2004). The diversity of North American fungi (37 800 species) is estimated to be twice that of plants (18 400 species) and yet their conservation has attracted little attention (Wilcove & Master 2005). Only 226 fungal species (0.6%) are classified as imperilled or critically imperilled, in contrast, 2917 plant species (16%) are listed as imperilled or critically imperilled (NatureServe 2008). Either fungal species are less likely to be endangered than plant species, or we are lacking a great deal of information. A limited understanding

Correspondence: Anne Pringle. E-mail: pringle@oeb.harvard.edu

of fungal diversity compounds the problem: approximately 5% of the world's fungi are described, perhaps 74 000 of approximately 1.5 million species (Hawksworth 1991, 2001).

Although there is a great deal of scattered information on introduced fungi, for example truffles and truffle-like species (Dennis 1975; Sogg 2000; Trappe & Cázares 2000; Fogel & States 2001; Yun & Hall 2004; Murat *et al*. 2008; Vellinga *et al*. in review), the only well studied fungal invasions involve plant pathogens (e.g. Chestnut Blight, Dutch Elm Disease). Ectomycorrhizal (EM) species are obligate symbionts and grow in association with a tree's roots to supply various benefits in exchange for carbon. EM fungi form species-rich communities in which an individual fungus may colonize multiple trees (Horton & Bruns 1998; Kennedy *et al*. 2003), and an individual tree associates with multiple fungi (Bruns 1995). Fungal networks may enhance or limit plants' competition with other plants (Horton & Bruns 1998; Horton *et al*. 1999; Bruns *et al*. 2002; Kennedy *et al*. 2003), as well as shape the movement of carbon through biogeochemical cycles (Hobbie & Hobbie 2006; Hobbie & Horton 2007). Basic data on EM fungal invasions and the concomitant potentials for environmental change are lacking, and biologists do not know the number of species that may have been moved across or between continents, the number or kinds of species that have become invasive, and the origins or spread rates of those EM species which are invasive (Schwartz *et al*. 2006).

Amanita phalloides (Vaill. Ex Fr.Fr.) Link is a notorious and deadly EM fungus that was first described from France (Vaillant 1727); although the death cap is native to Europe, it has been introduced to Australasia, Southern Africa, South America, and the east coast of North America (Pringle & Vellinga 2006). The fungus does not appear to be spreading from any of these points of introduction (Pringle & Vellinga 2006). In Australia, the mushroom is restricted to urban and suburban sites in Canberra and Melbourne. On the east coast of North America, *A. phalloides* is restricted to parks or plantations, although plantations may be of trees native to the East Coast (especially *Pinus strobus*, B. Wolfe and A. Pringle, personal observations).

In California, *A. phalloides* is rumoured to have been introduced from Europe (Saylor 1984a, b), and in contrast to its biology on the East Coast, the fungus fruits abundantly in the undisturbed habitats of especially coastal live oak woodlands, including the Point Reyes National Seashore. The appearance of *A. phalloides* mushrooms in local forests has been carefully described by the amateur mycological community (Arora 1975; D. Arora personal communications). Cases of *A. phalloides* poisonings at California hospitals are on the rise (Freedman 1996a, b, 2003), and California has been described as the 'epicentre of amatoxin poisoning' (Benjamin 1995). However, the anecdotal nature of early literature from California does not establish *A. phalloides* as an introduction to California (Pringle & Vellinga 2006) and because early collections of other *Amanita* species were often misidentified as *A. phalloides*, these herbaria accessions are not proof of the early presence of the fungus in California (Pringle & Vellinga 2006).

In this work, we used genetic data to test if *A. phalloides* is an introduced and currently invasive species. An older view of microbial species as globally distributed is changing as phylogeographies reveal previously unsuspected levels of endemism associated with cryptic genetic species (Koufopanou *et al*. 1997; James *et al*. 1999; Dettman *et al*. 2003; Pringle *et al*. 2005; Taylor *et al*. 2006). The population genetics of two other *Amanita* species provide clear evidence for the native status of both *A. pantherina* and *A. muscaria* in North America (Oda *et al*. 2004; Geml *et al*. 2006). Using a range of loci, we developed haplotype networks to test whether North American populations appear to be genetically diverged from European populations, as would be expected of a native species with a disjunct distribution, or whether European and North American populations appear panmictic, as would be expected if the species was recently introduced to California by humans. We also used a molecular marker diagnostic of *A. phalloides* to annotate herbaria accessions collected between 1911 and 1962, and used these to verify the earliest date at which *A. phalloides* was collected in California.

Materials and methods

Collecting European and American Amanita phalloides

Individual mushrooms of *A. phalloides* were collected from across Europe and North America (Table 1). Populations were sampled across a rough latitudinal gradient on both continents, focused on Norway, the South of France and the island of Corsica in Europe, and from British Columbia (Canada) south to Los Angeles on the west coast of North America. In California, sampling focused on the counties surrounding the San Francisco Bay. Although *A. phalloides* has been introduced to the East Coast, because it is not spreading only samples from Pennsylvania and New Jersey were included in this study. We collected a total of 24 European, 41 West Coast, and 7 East Coast individuals.

Confirming the identity of mushrooms collected as A. phalloides

The nuclear ribosomal internal transcribed spacer (ITS) is diagnostic of *A. phalloides* (Table 2). With the exception of one indel common to two mushrooms collected from Seattle, Washington, and Kragerø, Norway, the ITS1 and ITS2 regions of an initial survey of 30 European and American mushrooms were invariant. Homologous

Table 1 The identity and provenance of *Amanita phalloides* specimens used in this study

No. of mushrooms (Accession ID)	Population	City and state or region	County or equivalent	Country	Date collected
Northern Europe					
1 (HbO65378)	Rottåsberga	Tingvoll, Møre og Romsdal		Norway	September 2001
1 (HbO53858)	Sømsøy i Hafrsfjord	Sola, Rogaland		Norway	September 1982
1 (HbO53842)	Lovisenberg	Kragerø, Telemark		Norway	September 1986
2 (HbO53852, HbO53849)	Sjåen-Storkollen	Kragerø, Telemark		Norway	September1985/ September 1992
1 (HbO53851)	Barlandskilen	Kragerø, Telemark		Norway	September 1985
1 (HbO53853)	Jomfruland	Kragerø, Telemark		Norway	September 1985
1 (HbO53828)	Søndre Sandøy	Hvaler, Østfold		Norway	September 1971
1 (HbO70024)	Vigeland	Arendal, Aust-Agder		Norway	September 1994
1 (Dmark 1)	Nørreskov Forest	Farum, Sjœlland Island		Denmark	September 2003
France (southern mainland)					
1 (RdeM 97)	Région de Montpellier	Near Montpellier, Languedoc-Roussillon	Herault	France	1997
1 (BdeF 81)	Bois de Fabregues	near Montpellier, Languedoc-Roussillon	Herault	France	1981
2 (Av 02 1, Av 02 2)	—	Auvergne	—	France	2002
3 (Mtl 02 1, Mtl 02 2, Mtl 02 3)	—	Montlaur	—	France	2002
3 (CESAC 1, CESAC 30, CESAC 70)	Centre d'Ecologie des Systemes Aquatiques Continentaux (CESAC)	Toulouse, Midi-Pyrénées	Haute-Garonne	France	2002
France (Corsica)					
1 (Am.pha.Cor02.1)	St Jean	near Corte, Provence Alpes Côte d'Azur	Haute-Corse	France	November 2002
1 (Ap.Qs6)	Perticato	near Galeria, Provence Alpes Côte d'Azur	Haute-Corse	France	September 2002
2 (Am.pha.PV02.1, Am.pha.Arca02.3)	Arca Pianelli	near Porto Vecchio, Provence Alpes Côte d'Azur	Corse du Sud	France	October 2002
West Coast North America					
1 (DAVFP26775)	Uplands	Victoria, Vancouver Island	British Columbia	Canada	October 2002
1 (DAVFP26773)	downtown Victoria, by Budget Rent-a-Car/Crystal Gardens	Victoria, Vancouver Island	British Columbia	Canada	September 2002
3 (ST99-276, ST00-252, ST02-281)	2700 block of Mount St Helens Place	Seattle, Washington	Kings	USA	September–October 1999, 2000, 2002
1 (RT SOMA)	off Highway 128	between Navarro and Philo, California	Mendocino	USA	January 2003
2 (LR1, LL1)	Leona Canyon	Oakland, California	Alameda	USA	December 2001
2 (DR1, DR2)	Oakland Hills	Oakland, California	Alameda	USA	January 2003
2 (AH3, AH20)	Albany Hill	Albany, California	Alameda	USA	December 2001

Table 1 *Continued*

No. of mushrooms (Accession ID)	Population	City and state or region	County or equivalent	Country	Date collected
1 (ecv2984)	Sterling Avenue, Berkeley Hills	Berkeley, California	Alameda	USA	January 2003
7 (ecv2860, ecv2861, ecv2862, ecv2978, ecv2979, THA UCB, THSE 1)	University of California Campus	Berkeley, California	Alameda	USA	January–August–October 2002/January 2003
4 (ecv2977, LFP1S1, LFP2S1, LFP3S1)	Tilden Regional Park	E of Berkeley, California	Contra Costa	USA	January 2002/ January 2003
3 (ecv2864, ecv2865, ecv2866)	intersection of Ravenswood and Laurel	Menlo Park, California	San Mateo	USA	September 2002
2 (ecv 2959, ecv2960)	Upper Crystal Springs Reservoir	San Francisco Watershed, California	San Mateo	USA	December 2002
4 (ecv2980, ecv2981, ecv2982, ecv2983)	China Camp State Park	NE of San Rafael, California	Marin	USA	January 2003
1 (EHTBSP4)	Indian Beach, Tomales Bay State Park	NW of San Rafael, California	Marin	USA	December 2002
1 (HH3)	Lucas Valley	N of San Rafael, California	Marin	USA	January 2003
2 (JH1 Scruz, JH4 Scruz)	along Highway 1	SE of Aptos, California	Santa Cruz	USA	January 2003
1 (Pisto House)	—	Monterey, California	Monterey	USA	December 2006
1 (Pebble Beach)	conrner of Congress and Colton	Pebble Beach, California	Monterey	USA	December 2006
1 (LA Victim)	Altadena	Los Angeles, California	Los Angeles	USA	April 2003
1 (NJW 055)	Descanso Gardens	La Crescenta, California	Los Angeles	USA	November 2004
East Coast North America					
3 (MMK 487 Nos. MMK1, MMK2, MMK3)	Nockamixon State Park	near Quakertown, Pennsylvania	Bucks	USA	November 2002
3 (RET 328-3 Nos. RT1, RT8, RT11)	Jakes Landing Road	Belleplain State Forest, New Jersey	Cape May	USA	October 2002
1 (JL 53)	Jakes Landing Road	Belleplain State Forest, New Jersey	Cape May	USA	October 2006

Table 2 The internal transcribed spacer (ITS) locus is diagnostic of *Amanita phalloides*. A partial sequence of the ITS1 is shown (base pairs 110–146); positions 111 and 139 are autapomorphies for *A. phalloides*. The alignment includes other species of *Amanita* section Phalloideae. Position 1 is the beginning of ITS1. Additional informative SNPs and indels are found elsewhere in ITS1, as well as within ITS2. Sequences of *Amanita* species outside Phalloideae, including *A. brunnescens*, *A. citrina*, *A. calyptrata*, *A. muscaria*, *A. pantherina*, and *A. velosa* are greatly diverged and could not be aligned to *A. phalloides*; these taxa are not included in the table. Sequences of *A. phalloides* were generally invariant (see text). Sequences of other species were often variable, especially within the morphological species *A. virosa*. Novel sequences generated for this table are deposited in GenBank (EU909441–EU909453), and a file of all sequences as well as details on collections are available from the first author

	(*n*)	110									120										130										140					145
A. phalloides	30	C	**C**	T	T	G	A	C	C	**A**	G	T		C	T	C	T	T	**G**	A	G	A	**-**		**A**	**G**	T	T	**G**	A	A	**A**	**A**	T		C	**-**	T	**G**	G		G
A. ocreata type 1	3	.	**T**	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	**G**		.	**A**	.	.	.	.	.	.	**-**	.		.	.	.	.	.		.
A. ocreata type 2	2	.	**T**	.	.	.	.	.	.	**G**	.	.		.	.	.	.	.	**A**	.	.	.	.		.	.	.	.	.	.	.	.	**-**	.		.	.	.	**A**	.		.
A. bisporigera	4	.	**T**	.	.	.	.	.	.	**G**	.	.		.	.	.	.	.	.	.	.	**G**	**G**		.	**A**	.	.	.	.	.	**C**	**-**	.		.	.	.	.	.		.
A. verna	2	.	**T**	.	.	.	.	.	.	**G**	.	.		.	.	.	.	.	.	.	.	.	.		**T**	.	.	.	.	.	.	.	**-**	.		.	.	.	.	.		.
A. virosa type 1	2	.	**T**	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	**G**		.	**T**	.	.	**C**	.	**T**	.	**-**	.		.	.	.	.	.		.
A. virosa type 2	1	.	**T**	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	**G**		.	**A**	.	.	.	.	.	.	**-**	.		.	**C**	.	.	.		.
A. virosa type 3	2	.	**T**	.	.	.	.	.	.	**G**	.	.		.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	**-**	.		.	.	.	**A**	.		.

sequences of closely related species, and distantly related species that appear to be morphologically similar, are highly diverged from the standard sequence of *A. phalloides*. Therefore, we consider this locus as diagnostic of *A. phalloides* and used standard fungal primers (ITS1-F and ITS4-B, Gardes & Bruns 1993) to sequence the ITS locus of all of the mushrooms included in our study, and confirm their identity as *A. phalloides*. One mushroom that proved to be a species other than *A. phalloides* was discarded. No additional variability of the ITS region was discovered. We also used this locus to confirm the identity of herbarium accessions named as *A. phalloides* (see below).

Locating herbarium accessions for molecular annotation

Because a modern description of western North American *A. phalloides* was not available until 1977 (Ammirati *et al*. 1977), early collections of mushrooms named as '*A. phalloides*' may have been misidentified (Pringle & Vellinga 2006). Because *A. phalloides* is a well-known and even notorious species, it is unlikely that the species would have been collected and given a different name. To determine the authenticity of accessions named as *A. phalloides* and collected prior to 1963, the date of the earliest collection used by Ammirati *et al*. (1977), all accessions made from California before 1963 were taken from herbaria at the New York Botanical Garden, the San Francisco State University, the University of California, Berkeley, and the University of Oregon, Corvallis, and annotated using a molecular marker. A total of 11 accessions were gathered. To our knowledge, there are no other collections of Californian mushrooms named as *A. phalloides* and collected prior to 1963. Moreover, to confirm the identity of mushrooms originally described as *A. phalloides* by Ammirati *et al*. (1977), an additional four accessions from this publication were chosen at random and sampled from San Francisco State University.

Molecular markers: four loci (TC9, GT9, GGT8, CA11) developed from the genome of Amanita bisporigera

The incomplete genome of a species closely related to *A. phalloides* was used to develop four novel and variable molecular markers; markers were used to collect phylogeographical data. Complete protocols are described in detail by Adams *et al*. (2005). Briefly, the *A. bisporigera* genome was used to identify microsatellite loci. Primers were designed around these microsatellites and used to cross-amplify DNA from *A. phalloides*. No useful microsatellites were recovered from *A. phalloides*, but a total of four loci containing from one to six single nucleotide polymorphisms (SNPs) were identified. The loci are named TC9 (190 bp, 1 SNP), GT9 (213 bp, 1 SNP), GGT8 (288 bp, 6 SNPs) and CA11 (277 bp, 5 SNPs) (Adams *et al*. 2005).

Additional molecular markers: loci EF1α and IGS 2

Standard fungal primers were used to amplify one additional locus, the translation elongation factor (EF-1α, 577 bp, 3 SNPs), using primers EF1-983F and EF1-1567R (Rehner 2001).

Finally, novel primers were developed for the intergenic spacer (IGS) 2 region (see Appendix S1, Supporting Information). The organization of this region with an *A. phalloides* genome is complex and a single individual can house up to four IGS 2 variants, or 'types'. A total of 11 types were found among all *A. phalloides* sampled. Although each

Table 3 Numbers and kinds of IGS 2 types recovered from different *Amanita phalloides*. A representative sequence of each of the 11 types is available from GenBank (EU909430-EU909440)

Accession ID	Name as used in IGS2 research	Population and collection site; date collected	No. of clones sequenced	No. of IGS 2 types recovered (which types)*	Additional IGS2 types recovered from direct sequencing (which types)*	Total no. of IGS 2 types
LFP1S1	Berkeley 1	See Table 1	11	1 (1)	2 (2, 8)	3
ecv2980	Marin	"	10	1 (1)	2 (2, 3)	3
NJW 055	Los Angeles	"	14	2 (1, 3)	2 (2, 8)	4
Pisto House	Monterey	"	11	2 (1, 9)	3 (2, 3, 8)	5
Pebble Beach	Pebble Beach	"	13	2 (1, 2)	2 (3, 8)	4
JL 53	New Jersey	"	10	2 (1, 2)	2 (3, 8)	4
Mtl 02 3	France 1	"	16	1 (1)	3 (2, 3, 8)	4
CESAC 1	France 2	"	12	1 (1)	1 (2)	2
Av 02 1	France 3	"	14	1 (1)	1 (2)	2
Cor02.1	Corsica	"	31	2 (4, 5)	2 (1, 2)	4
Dmark 1	Denmark	"	14	2 (7, 10)	2 (1, 2)	4
HbO53851	Norway 1	"	12	1 (7)	1 (2)	2
HbO53852	Norway 2	"	13	3 (6, 9, 11)	2 (1, 2)	5

*The numbers in parentheses are lists of which types were recovered from either cloning or direct sequencing. See Supporting Information, Appendix S1, Table S1, Table S2, and Table S3, and Fig. 3 for information on types.

of these types is attached to a relatively less invariant 5S allele, the types themselves cannot be aligned to each other. To explore the phylogeography of this locus, clone libraries were created for a subset of *A. phalloides* individuals (Table 3) and sequencing of clones continued until variability appeared to be exhausted. Several of the IGS 2 types identified from the cloned sequence data were low in number and suggested a possible polymerase chain reaction (PCR) bias for one type over another (see Appendix S1), or alternatively, a difference in copy number between types within an individual. To confirm the presence or absence of a given type in all target individuals, primers were designed within each IGS 2 type to directly amplify and sequence only that type (see Table S1, Supporting Information) and these primers were used to probe target individuals for the presence or absence of particular types (Table 3).

DNA extraction, PCR amplification and sequencing: loci TC9, GT9, GGT8, CA11, EF1α

DNA extraction followed standard Bruns laboratory protocols (Peay *et al*. 2007). PCR amplification and sequencing are described in detail by Adams *et al*. (2005). Initial data confirmed that *A. phalloides* is a dikaryotic (functionally diploid) fungus (data not shown). The haplotypes of individuals heterozygous for multiple SNPs of a single locus were cloned and sequenced. Sequences of one European individual were deposited in GenBank (DQ1333971–DQ1333978, see Adams *et al*. 2005; also EU886739 for the locus EF-1α).

DNA extraction, PCR amplification and sequencing: the IGS locus

These data were collected after the first author established an independent laboratory. Because they are not described elsewhere, exact protocols are described in detail in Appendix S2, Supporting Information.

DNA extraction, PCR amplification and sequencing: molecular identification of herbarium accessions

DNA of herbarium specimens was extracted separately from the DNA of recently collected mushrooms, and protocols used in ancient DNA work were developed for use with this herbarium material (H.B. Cross and A. Pringle unpublished). Protocols are described in Appendix S3. Only very short fragments can be reliably amplified from the extracted DNA of older herbaria accessions. For this reason, we did not amplify any of our newly identified loci (TC9, GT9, GGT8, CA11) or the EF1α or IGS 2 regions from older tissue.

Phylogeographical analyses: loci TC9, GT9, GGT8, CA11, EF1α

To determine if Californian populations of *A. phalloides* manifest any signal of endemicity, we used a variety of approaches. Summary statistics for each of the five loci were used to explore whether the data follow expectations of neutrality, and if there is evidence for recombination within any locus. In every analysis, the

entire array of haplotypes was included, i.e. each individual was represented as two haplotypes. The method of Tajima (1989) compares two estimates of genetic diversity and uses the different sensitivities of these estimates to detect departures from neutrality (Hartl 2000). Fu and Li's methods (Fu & Li 1993) use the pattern of mutations along a genealogy to test for neutrality. Both Tajima's *D* and Fu and Li's *D**, as well as Fu and Li's *F** were used to explore the neutrality of the five loci. Loci were also pruned to include only haplotypes from the west coast of North America, and tests of neutrality repeated. The possibility for recombination within each locus was tested using a four-gamete test of internal recombination. Analyses used either the program SITES (available from lifesci.rutgers.edu/~heylab/HeylabSoftware.htm), or DnaSP version 4.10.4 (Rozas & Rozas 1999; Rozas *et al*. 2003), or both.

A nonparametric test of panmixia was used to explore whether the haplotypes of each locus are geographically structured, or whether the haplotypes conform to a null hypothesis of no genetic differentiation (Hudson *et al*. 1992). Tests were implemented using the program available from G. Achaz (wwwabi.snv.jussieu.fr/~achaz/hudsontest.html). The technique creates artificial data sets drawn from the original pool of haplotypes and compares the mean pairwise difference (Ks) and mean pairwise log(1 + difference) (K*s) of the original data to the distribution of Ks and K*s of artificial data sets. In these analyses, haplotypes of Europe were compared to haplotypes of the west coast of North America (these included Californian sequences and haplotypes from Washington State and British Columbia). Additional tests used locus CA11 to compare the genetic structure of groups from the Bay Area of California (defined as Alameda, Contra Costa, Santa Clara, San Mateo and Marin counties) and the different regions of Europe.

To understand whether West Coast populations have experienced a genetic bottleneck, as may be expected for populations that grow from a few founding individuals and has sometimes been demonstrated for introduced species (but see Wares *et al*. 2005), the program DnaSP version 4.10.4 (Rozas & Rozas 1999; Rozas *et al*. 2003) was used to estimate both pi (π) and Watterson's theta (Θ) for Europe and the west coast of North America. The genetic diversity at each locus was summarized using three statistics, calculated as pi, the average number of nucleotide differences per site between two sequences (Nei 1987), or calculated as estimates of theta for each site or sequence where $\Theta = 4N\mu$ and is based on either Watterson (1975) or Tajima (1993) (see http://www.ub.edu/dnasp/DnaSPHelp.pdf).

Biogeographical structure is readily detected from haplotype networks, and these networks have been used to demonstrate a clear disjunct in the genetic structure of native populations of a variety of fungal species, including at least two other *Amanita* species that also grow in both North America and Europe (Oda *et al*. 2004; Geml *et al*. 2006). To draw haplotype networks using these data, exploratory phylogenies were made using maximum-parsimony criteria in PAUP* version 4.0b10 (Swofford 2004). All data were treated as nonadditive, equally weighted characters and gaps were treated as missing data. The maximum-parsimony analyses initially used heuristic searches with 100 random addition sequence replicates, holding no more than 10 trees per replicate, and tree-bisection–reconnection (TBR) branch swapping. The trees resulting from this initial search were used as starting trees in additional rounds of TBR branch swapping, holding up to 10 000 most parsimonious trees (MPT) at each round. To ascertain support for the MP trees, bootstrap support values were calculated using 500 replicates with five random addition sequence replicates each. Parsimony trees were used to draw the haplotype networks by hand.

IGS analyses

Because different IGS types could not be aligned to each other, an alignment of the adjacent 5S sequences was made using MacClade version 4 (Maddison & Maddison 2002). Each allele was verified as a 5S sequence by comparing sequence data to the 5S sequences of other groups of fungi and the canonical 5S sequence (see Table S2, Supporting Information). Three additional 5S sequences from an *Amanita vaginata* (PH 82906-11 collected by B. Wolfe at the Harvard Forest, Petersham, MA) were aligned with the *A. phalloides* sequences and used as outgroups. The data matrix was imported into PAUP* 4.0b10 (Swofford 2004) for phylogenetic analyses using neighbour-joining (NJ) optimality criteria. Bootstrap support values were calculated using 1000 replicates.

Results

Summary statistics of neutrality, internal consistency, panmixia and genetic diversity

Loci appear to be neutral (test statistics not shown), although internal recombination (or double mutation) appears likely within locus GGT8. Loci appear to be neutral even when the data of any individual locus are pruned to include only haplotypes from the west coast of North America. The four gametic types possible for sites 78 and 196 of locus GGT8 are found within the data; the majority of haplotypes are TA, but 10 are CG, 9 are CA, and 3 are TG. Subsequent analyses of GGT8 used only base pairs 79 to 288 as necessary to avoid violating the assumptions of any given analysis, e.g. in phylogenetic analyses using maximum parsimony.

In tests for panmixia that used loci with a single SNP, global populations appear panmictic (locus TC9: probability of

panmixia using Ks = 0.813, using K*s = 0.813; locus GT9: Ks = 1.0, K*s = 1.0), but when more information is available (i.e. when using the three loci with more than one SNP) populations of Europe and the west coast of North America appear genetically different (locus EF1α: Ks = 0.00, K*s = 0.00, locus GGT8: Ks = 0.00, K*s = 0.00, locus CA11: Ks = 0.00, K*s = 0.00). Hudson *et al.* (1992) test for geographical subdivision is known to be sensitive to the addition of nucleotide differences. Greater genetic diversity is concentrated within the haplotypes collected from Europe (see below), and it is not surprising that artificial data sets that mix European and American haplotypes and distribute genetic diversity evenly between the American and European populations look very different from the original data. These differences in amounts of diversity give the result of genetically different and geographically subdivided groups of European and American *A. phalloides*.

To understand these results more clearly, additional tests for panmixia used the locus with the most number of SNPs and no evidence of internal recombination, CA11, and focused on comparisons between single regions of Europe and the Bay Area of California, or different parts of Europe. Populations from the Bay Area of California (Alameda, Contra Costa, Marin, Santa Clara and San Mateo counties) and mainland France are panmictic (Ks = 0.59, K*s = 0.22), but populations from the Bay Area and Corsica are not (Ks = 0.00, K*s = 0.00), and neither are populations from the Bay Area and north Europe (Ks = 0.00, K*s = 0.00). The two populations from north Europe and Corsica are themselves only marginally panmictic (Ks = 0.07, K*s = 0.056).

The European group of *A. phalloides* is more diverse than the group taken from the west coast of North America (Table 4), despite the smaller sample size of European mushrooms. Although not significant, the trend is clear and consistent in every locus and with every measure of genetic diversity. The result is especially dramatic when estimates are taken from loci possessing more than one SNP (e.g. CA11) or when genetic diversity is estimated using pi. Diversity is often confined to the European population; variable SNPs of two loci are only polymorphic within the smaller population of haplotypes from Europe.

Phylogeny and haplotype networks

None of the loci divided into strictly European or North American populations (Fig. 1). Instead, most haplotypes were found in both Europe and North America, with each haplotype existing in variable proportions on either continent. The jumbling of haplotypes contrasts with data collected for dozens of other species of fungi (James *et al*. 1999; Dettman *et al*. 2003; Pringle *et al*. 2005; Taylor *et al*. 2006), including *Amanita* species (Oda *et al*. 2004; Geml *et al*. 2006), and is strong evidence against a geographically disjunct population of endemic *A. phalloides* in North America.

Only one of these loci possesses a clear biogeographical structure (CA11, Fig. 2). The natural history of *A. phalloides* within Europe is only partially understood, and there are no published data to suggest that the fungus is divided into northern or southern European types. But in this locus, there is one haplotype that appears to be restricted to southern France, and is the most common haplotype of that region, and another that is restricted to Norway and Denmark. Both of these apparently endemic haplotypes are also found on the west coast of North America. Other common haplotypes are found only in Europe, and not in North America. Although one haplotype is represented by a single sequence collected from California, it is in the middle of the haplotype network and was found only once. The simplest explanation for this pattern is that haplotypes endemic to different parts of Europe were independently introduced and subsequently combined within North America; in these data, single American mushrooms may possess either or both of the haplotypes endemic to north and south Europe.

Table 4 Comparisons of genetic diversity between Europe and the west coast of North America, including Vancouver Island and Seattle, Washington. The greater diversity in each comparison is bolded. There is a trend to more diversity in the European populations

Locus	TC9	GT9	EF1	GGT8	CA11
			pi		
Europe	**0.00333**	**0.00294**	**0.00131**	**0.00746**	**0.00965**
West coast North America	0.00305	0.00291	0.00094	0.00208	0.00212
			Watterson's ∪/site (SD)		
Europe	**0.00187 (0.00187)**	**0.00131 (0.00131)**	**0.00133 (0.00083)**	**0.00474 (0.00194)**	**0.00527 (0.00272)**
West coast North America	0.00159 (0.00159)	0.00117 (0.00117)	0.00106 (0.00065)	0.00388 (0.00173)	0.00275 (0.00169)
			Watterson's ∪/sequence (SD)		
Europe	**0.248 (0.248)**	**0.228 (0.228)**	**0.723 (0.451)**	**1.365 (0.557)**	**1.175 (0.605**
West coast North America	0.21 (0.21)	0.204 (0.204)	0.612 (0.375)	1.02 (0.456)	0.615 (0.376)

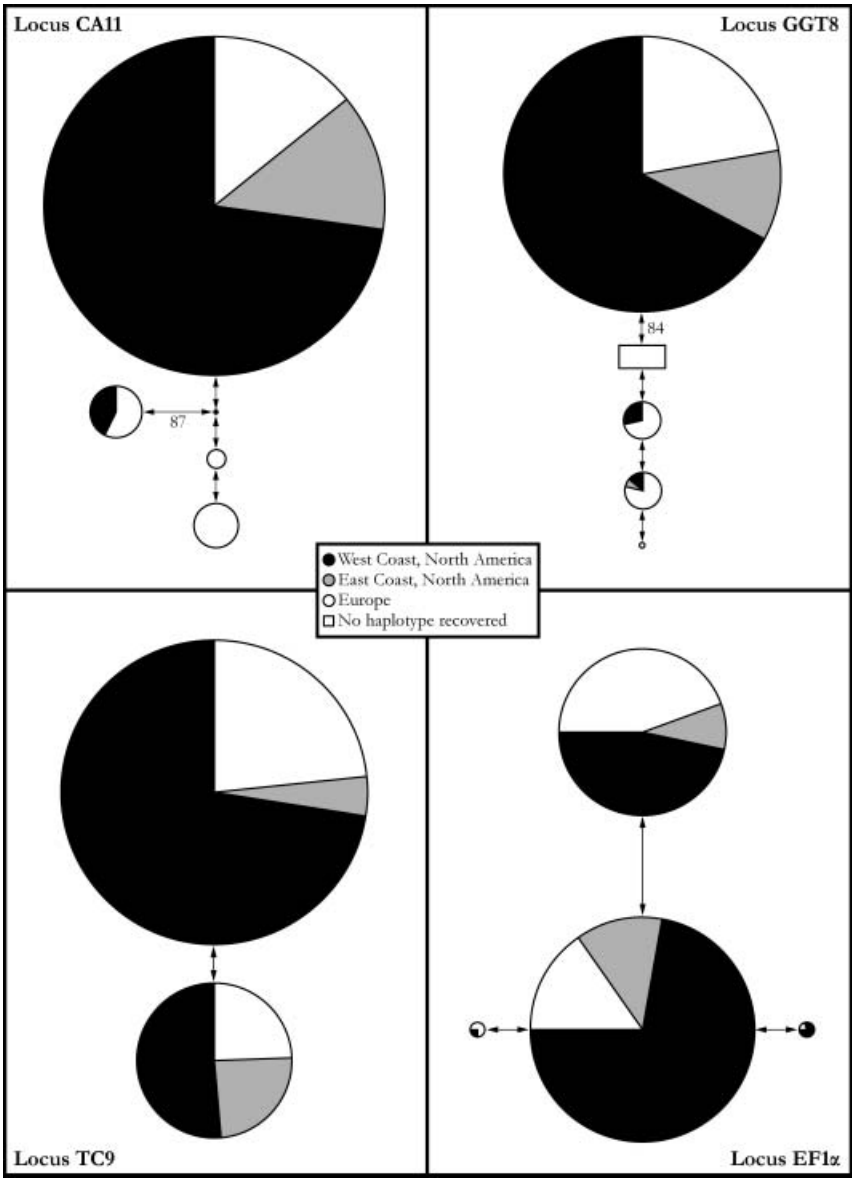


Fig. 1 Haplotype networks of loci CA11, GGT8, TC9, and EF1α. Circles are proportionate to the number of samples belonging to any particular haplotype and the different shades mark different geographical regions. Numbers are bootstrap supports above 75% and are taken from maximum parsimony analyses; there was a single most parsimonious tree for each locus. The haplotype network of locus TC9 has a pattern similar to the network of locus GT9. None of the haplotype networks divide into separate North American vs. European clades.

The IGS 2 locus

Data from the IGS 2 locus are analogous to the data collected from locus CA11. No endemic types were recovered from North America (Fig. 3, Table 3, Table 5), although endemics were common to Europe, and types that appear endemic to different parts of Europe are found together in California. Types 1 and 2 were found in nearly every North American and European individual. Types 4 and 5 appear to be endemic to Corsica, and types 6, 7, 10 and 11 appear to be endemic to Scandinavia (Norway and Denmark). In Europe, type 3 is only found on mainland France, but it is also found in New Jersey and California. The mean number of types found in a single individual was 3.5, the maximum number was 4, and the minimum number of types found in a single mushroom was 2.

Because the different IGS 2 types could not be aligned to each other, it was impossible to build a single phylogeny using the IGS 2 sequence data. However, a distance tree based on an analysis of the adjacent 5S alleles serves as a proxy for the relationships among the types (Fig. 3). Most of the IGS 2 types were linked to a single 5S allele; the exception is the type 1 IGS 2 type, which was linked to two somewhat different 5S alleles. Because the genome of each individual fungus housed multiple IGS types, each individual also possessed more than one 5S allele. Many of the endemic types had 5S alleles that formed strongly supported clades, and the types endemic to Corsica also

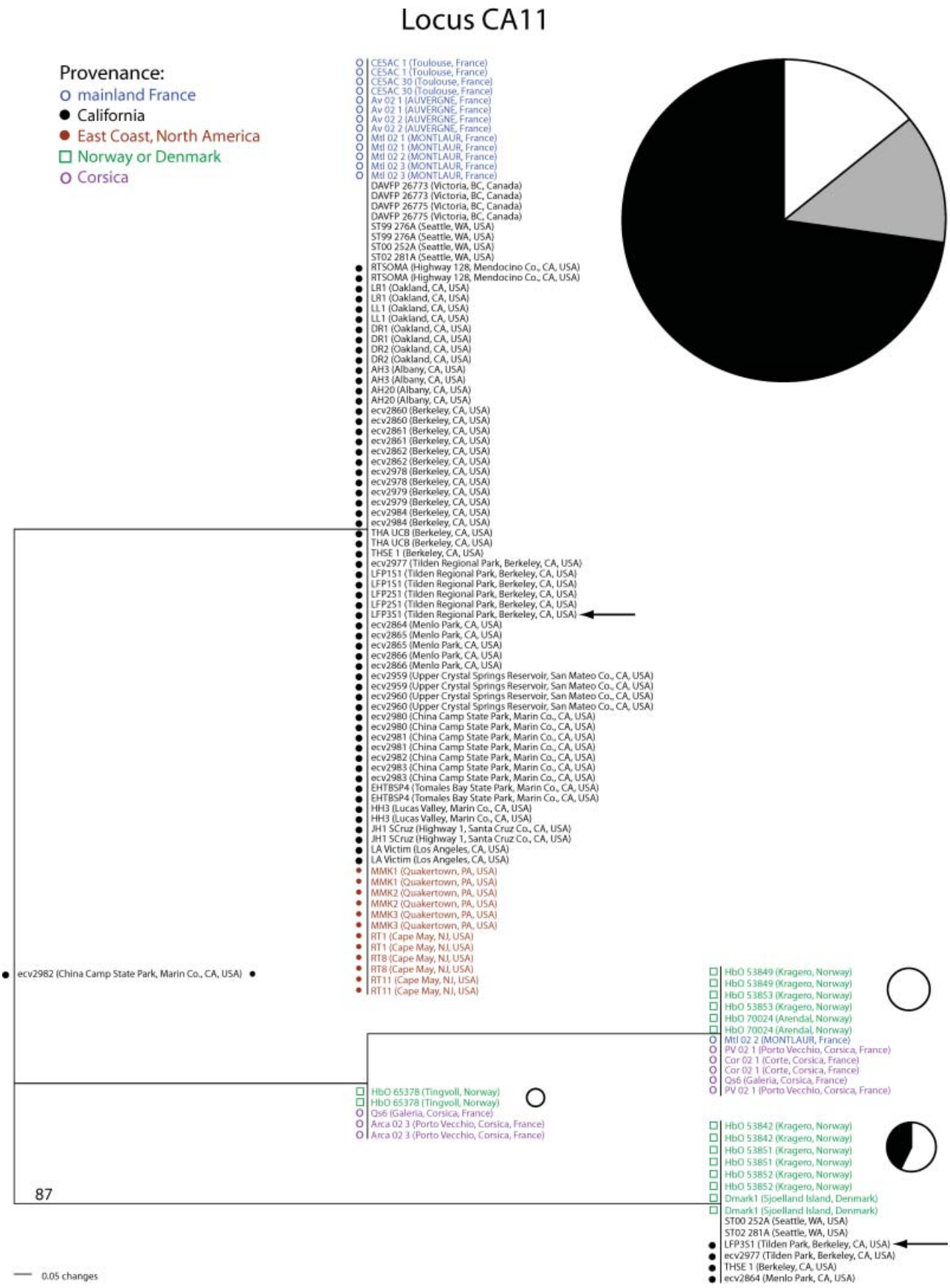


Table 5 The presence (1) or absence (0) of different IGS 2 types in *Amanita phalloides* individuals

IGS 2 type	1	2	3	4	5	6	7	8	9	10	11
Individual											
Berkeley	1	1	1	0	0	0	0	1	0	0	0
Marin	1	1	1	0	0	0	0	0	0	0	0
Los Angeles	1	1	1	0	0	0	0	1	0	0	0
Monterey	1	1	1	0	0	0	0	1	1	0	0
Pebble Beach	1	1	1	0	0	0	0	1	0	0	0
New Jersey	1	1	1	0	0	0	0	1	0	0	0
France 1	1	1	1	0	0	0	0	1	0	0	0
France 2	1	1	1	0	0	0	0	0	0	0	0
France 3	1	1	0	0	0	0	0	0	0	0	0
Corsica	1	1	0	1	1	0	0	0	0	0	0
Denmark	1	1	1	0	0	0	1	0	0	1	0
Norway 1	0	1	0	0	0	0	0	0	0	0	0
Norway 2	1	1	0	0	0	1	0	0	1	0	1

Table 6 Molecular annotation of mushrooms collected as *Amanita phalloides* before 1963

Herbarium and collection no.	Month collected	Year collected	Site	County	Sequence identity
NYBG 46009	March	1911	—	Santa Cruz	*A. ocreata*
NYBG 66087	February	1912	Madera Creek, near Stanford University	Santa Clara	*A. pantherina**
NYGB 46226	January–April	1913	Mission Canõn [sic]	Santa Barbara	*A. ocreata*
NYBG 66086	April	1916	San Francisquito Creek, above Stanford U	Santa Clara	—*
NYBG 45985	February	1934	Merced High School, Merced	Merced	aff. *A. virosa*†
OSU 9020	February	1935	Pacific Grove	Monterey	*A. ocreata*
UCB 605324	November	1938	Del Monte Hotel grounds	Monterey	*A. phalloides*
UCB 699589	December	1945	U.C. Berkeley	Alameda	*A. phalloides*
UCB 733960	January	1947	U.C. Berkeley	Alameda	*A. phalloides*
UCB 759917	April	1948	—	Marin	*A. pantherina**
UCB M269926	November	1962	U.C. Berkeley	Alameda	*A. phalloides*

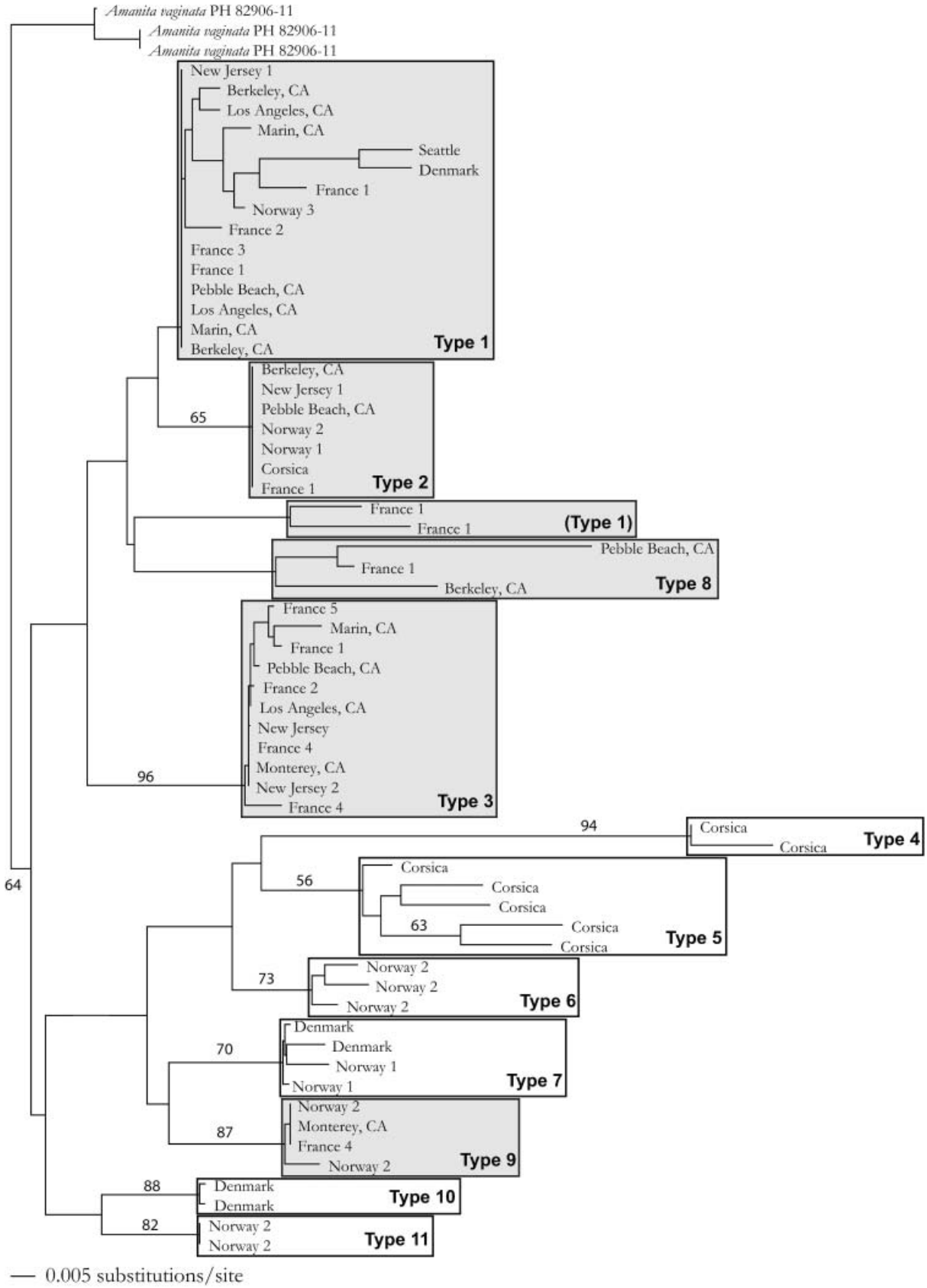
*Specimens gave a faint (NYBG 66086) or no amyloid reaction.
†Collections named as *A. virosa* or *A. bisporigera* possess a complex grade of ITS1 sequences.
U.C. Berkeley, University of California in Berkeley.

formed a single although unsupported clade. Among the widespread types, only types 3 and 9 formed strongly supported clades. Multiple 5S alleles and IGS 2 types were also recovered from the outgroup fungus, *Amanita vaginata*, as well as other species of *Amanita* (H.B. Cross and A. Pringle unpublished data), suggesting that the escape from concerted evolution is a phenomenon common to this group of species. And as was true for the other loci used in this study, the genetic diversity of IGS 2 in Europe is greater than it is in North America.

The history of A. phalloides *in California*

The first collections of *A. phalloides* in California date back to 1938 and 1945 (Table 6). None of the collections made prior to 1938 and named as *A. phalloides* were correctly identified, and these mushrooms are a mix of species including *A. ocreata*, *A. pantherina*, and others. We do not consider the 1916 collection to be *A. phalloides* because its spores are inamyloid. Amyloidy is a morphological hallmark of *A. phalloides*. This 1916 mushroom was also

Fig. 2 The maximum parsimony phylogeny of locus CA11 provides a more nuanced view of the CA11 haplotype network; compare circles at right to Fig. 1. Geographic information is given by colours, symbols at left, and text. More details on accessions are given in Table 1. Arrows point to a single Californian mushroom with haplotypes characteristic of different regions of Europe. Unique haplotypes isolated from different parts of Europe were commonly found at single sites within California.



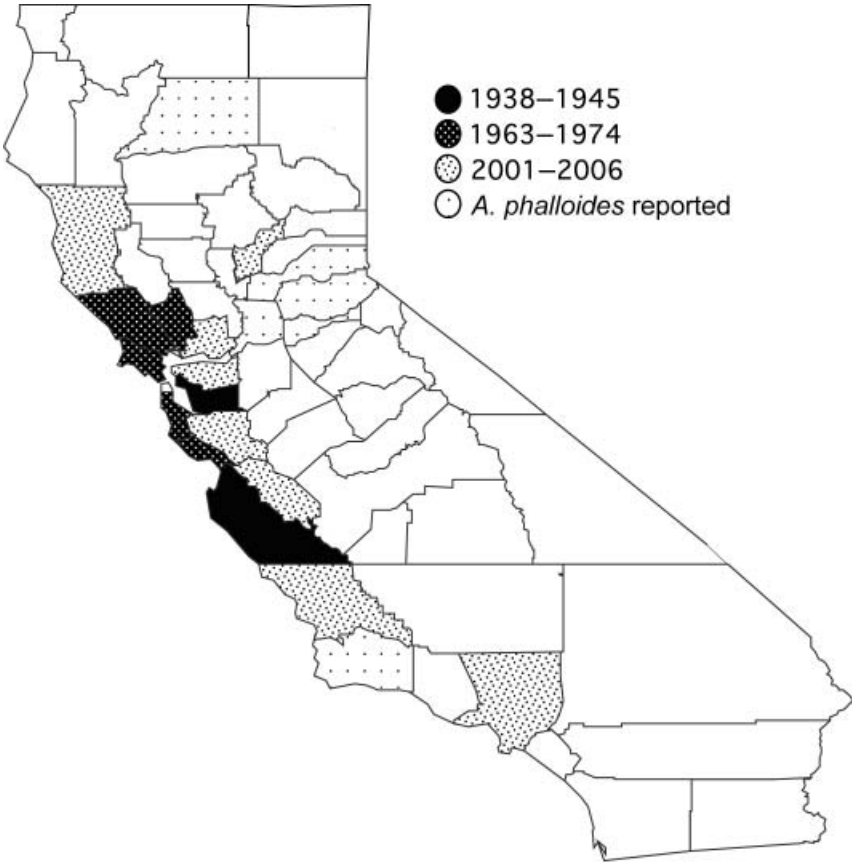


Fig. 4 An hypothesis of *Amanita phalloides'* history in California. Records for 1938–1945 were confirmed using the molecular annotation of herbarium specimens, see text; records from 1963 to 1974 were taken from Ammirati *et al*. (1977); records from 2001 to 2006 are from collections made or sent to the authors (see Pringle & Vellinga 2006). These data are used to estimate a crude spread rate.

collected in April, an unusual time for *A. phalloides* to fruit in an otherwise undisturbed habitat. Although *Amanita ocreata* was formally described in 1909 (Peck 1909), it was rarely mentioned in the mycological literature until Ammirati *et al*. (1977) provided a modern description and it is not surprising that collections of this fungus made in the early part of the 20th century were misidentified as *A. phalloides*.

The molecular annotation of mushrooms described by Ammirati *et al*. (1977) as *A. phalloides* confirm that these specimens are in fact *A. phalloides*; the ITS1 is an exact match. This annotation confirms Ammirati *et al*. (1977) as the earliest accurate record of *A. phalloides*' presence around the Bay Area of California.

In combination with data on *A. phalloides*' current distribution in California (Pringle & Vellinga 2006), these data can be used to estimate *A. phalloides*' spread within California (Fig. 4). Using either the 1938 Monterey County or 1945 Alameda County records as starting points, and calculating distances to farthest points north (near Navarro, Mendocino County), south (in Altadena, Los Angeles County) or east (near Spenceville, Yuba County), we find that estimated rates of spread range between 2.68 and 9.39 km/year, and average 5.13 km/year.

Discussion

Amanita phalloides was clearly introduced to the west coast of North America; the combination of data on genetic diversities, haplotype networks, and herbaria accessions provide evidence for *A. phalloides'* introduced status. European populations are genetically more diverse than American populations and the polymorphic sites of at least two loci are only polymorphic within Europe, even though

Fig. 3 Neighbour joining tree of 5S alleles from multiple sequences of 19 individuals of *Amanita phalloides* obtained by direct sequencing as well as cloning and sequencing, with three sequences of an *Amanita vaginata* individual used as an outgroup. Numbers are bootstrap values above 50%. Every 5S allele is linked to an IGS 2 allele and the IGS 2 types associated with the different 5S alleles are marked with boxes and text. Geographically widespread types are shaded. None of the 5S alleles or IGS 2 types is endemic to North America. This figure includes sequences from samples (e.g. Seattle) which were incompletely characterized and are not included in other IGS 2 analyses; they are included to provide additional geographical information (e.g. Type 9 is not endemic to Norway).

the number of individuals sampled from Europe was half the number sampled from North America. Estimates of both pi and Watterson's theta suggest that European *A. phalloides* either have greater effective population sizes, or have experienced greater mutation rates, or both. Although exact counts of fungal individuals are difficult to make, it may be reasonable to assume that these data reflect larger numbers of *A. phalloides* in Europe, and a longer history of this species on that continent.

The haplotype networks of multiple loci do not provide evidence for genetically isolated North American populations. Instead, multiple haplotypes that are unique to either Scandinavia, mainland France, or Corsica grow together at single sites in California. The locus CA11 is an example of this pattern; genetic variants otherwise unique to either the mainland of France or Scandinavia were isolated from single Californian populations and even from a single mushroom growing at Tilden Regional Park above Berkeley, California (see arrows, Fig. 2). A similar pattern is given by the data from the IGS 2 locus; a total of five IGS 2 types were present in North America, and none of these were endemic there. By contrast, one individual from Corsica possessed four IGS 2 types (and multiple 5S alleles), two of which were endemic. Collectively, three individuals from Norway had six IGS 2 types, two of which were endemic. Some but not all of these Corsican and Norwegian types were also found in California (Tables 3 and 5). The data of these loci suggest multiple introductions of *A. phalloides* to California.

The data also suggest a phylogeographical structure of *A. phalloides* in Europe; populations from northern Europe, mainland France and Corsica appear genetically distinct from each other. As Corsica is an island isolated from mainland France, it seems reasonable to find a subset of alleles endemic to Corsica (Grubisha *et al*. 2007; Peay *et al*. 2007). But the number of European samples included in this work precludes firm conclusions about the genetic structure of *A. phalloides* in Europe.

However, the divergences from panmixia suggested by Hudson *et al*.'s (1992) test for geographical subdivision suggest that as a group, the *A. phalloides* of the Bay Area of California are more closely related to one of the subsets of European mushrooms included in our study. Sampling in Europe focused on three regions. Bay Area *A. phalloides* are panmictic with the group of mainland French *A. phalloides*, but not with either the northern European or Corsican group of mushrooms.

The introduction of *A. phalloides* to California may have been mediated by the trade of plants in soil (Vellinga *et al*. in press); when imported forestry stocks are planted, the symbiotic fungi and bacteria growing in and around the roots of the trees will also be planted. Although *Amanita phalloides* is native to Europe, it has been introduced to other parts of the world as well, e.g. South America and Australia (Pringle & Vellinga 2006), and the source of California's *A. phalloides* is unknown.

The earliest known collections of Californian *A. phalloides* date to 1938 and 1945, and both collections were taken from sites known to harbour imported plants. The 1938 mushrooms of Monterey County were collected from the grounds of the Del Monte Hotel, a site famous for its unusual and exotic gardens (Cain 2004). The 1945 mushrooms were collected from the University of California, Berkeley, a campus that had been planted with trees collected from across the world (Cockrell 1976). Although *A. phalloides* is likely to have been introduced with imported plants, the mushroom is now abundant in otherwise undisturbed habitats (Arora 1975, 1986; Saylor 1984a, b; Fig. 4).

Migration data for nonpathogenic fungi are rare; *A. phalloides* appears to be moving across the landscape at a rate less than 10 km/year. As far as we know, this is the first estimate of the aerial spread rate of an ectomycorrhizal fungus. The data can be compared to estimates from a saprobic species. Although *Clathrus archeri* is dispersed by insects (not wind), it has invaded Europe from Australasia and year-by-year distribution maps are published (Parent *et al*. 2000). The fungus appeared in the Alsace region of France in 1920 but by 1999 (and perhaps earlier), *C. archeri* had travelled to and established in the Galicia region of Spain, a distance of at least 1400 km in approximately 70 years, or 20 km/year. That estimate is twice our fastest estimate for *A. phalloides*. Saprophytes may establish more easily than symbiotic fungi because they do not require a host, although *C. archeri* may also have been introduced to Europe more than once.

These simple calculations for *A. phalloides*' spread assume a homogeneous habitat and a normal (not leptokurtic) dispersal kernel (Skellam 1951; Kot *et al*. 1996; Hastings *et al*. 2005); moreover, the Monterey and Berkeley populations may not be the only or original sources of *A. phalloides* in California. Nonetheless, the numbers serve as a reasonable first approximation. Recent analyses of plant invasions within Europe conclude that many follow a Fisher–Skellam diffusion model (Williamson *et al*. 2005), and the limited data on spore dispersal within the genus *Amanita* suggest that the vast majority of spores settle within 100 m of the mushroom (Lacey 1996; Li 2005). In comparison to *A. phalloides*, data from plants suggest an average spread of 2 km/year, with a great deal of variation around that mean (Williamson *et al*. 2005); fungal invasions may be more rapid than plant invasions.

Different uses of the word 'invasive' are discussed in the plant and animal literature (Richardson *et al*. 2000; Rejmánek *et al*. 2002; Colautti & MacIsaac 2004). We follow Richardson *et al*. (2000) and conclude by defining *A. phalloides* as invasive because it has recently migrated from one continent to another, presumably carried by humans on the roots of trees, and because *A. phalloides* has established and expanded its range on the west coast of North America.

However, because the fungus is an obligate symbiont, another and perhaps more experimentally tractable definition is possible: *A. phalloides* appears to be associating with a plant endemic to California, the coast live oak *Quercus agrifolia* (Arora 1986; B. Wolfe and A. Pringle. unpublished). An invasive symbiont could be defined as a symbiont that has shifted associations and is spreading to geographically novel and previously unexploited hosts.

Acknowledgements

We are very grateful to the many people and institutions who lent or donated collections of *Amanita phalloides*, including Gro Gulden (Botanisk Museum/Universitas Osloensis, Norway); Thomas Laessøe (Denmark); Marie Bianchi, Monique Gardes, Monique Moutet, and Franck Richard (France); Brenda Callan and John Dennis (Forest Pathology Herbarium/Pacific Forestry Centre, Canada); Dennis Desjardin (San Francisco State University Harry D. Thiers Herbarium), Roy Halling (New York Botanical Garden), Earl Hazelton, Jason Hoeksema, Marion M. Kyde, David Rust, Chef John Pisto, Joey Spatafora and Richard R. Halse (Oregon State University Herbarium), Isabelle Tavares (University Herbarium, University of California at Berkeley), Steve Trudell, Rod Tulloss, Else Vellinga, Peter Werner, Nathan Wilson and Benjamin Wolfe (USA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Development of the IGS locus

Appendix S2 DNA extraction, PCR amplification and sequencing: the IGS locus

Appendix S3 Protocols used to amplify DNA from herbarium accessions

Table S1 IGS primers used with *Amanita phalloides*

Table S2 Species included in a 5S alignment used to verify the identity of 5S alleles

Table S3 Primers were designed to specifically target IGS 2 types. Here we list the most effective primers for amplifying each IGS 2 type

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