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Small genets of *Lactarius xanthogalactus*, *Russula cremoricolor* and *Amanita francheti* in late-stage ectomycorrhizal successions

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Abstract

We determined the size of genets of late-stage ectomycorrhizal fungi in field sites in coastal Northern California. Basidiocarps were collected, mapped and subjected to genetic fingerprinting using amplified fragment length polymorphisms (AFLPs). The minimum size estimates for the largest genets of *Amanita francheti*, *Lactarius xanthogalactus* and *Russula cremoricolor* were 1.5, 9.3 and 1.1 m², respectively. The molecular markers also showed that *R. cremoricolor* is dimorphic, with red- and white-capped morphotypes of this species forming a continuous population. Our results suggest that spore propagation plays a much more important role in the life history of the Russulaceae in undisturbed forest settings than previously recognized. Fungi appearing late in the succession sequence and systems without obvious disturbance therefore do not necessarily colonize primarily by mycelium.

Keywords: AFLP, *Amanita*, ectomycorrhiza, genet size, *Lactarius*, *Russula*

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Introduction

Ectomycorrhizae are a mutualistic association between plants and fungi which are crucial for the ecology of temperate forests. The symbionts are mostly woody plants on the host side, and basidiomycetes or ascomycetes on the fungal side (Smith & Read 1997). Ectomycorrhizal basidiomycetes propagate by sexual basidiospores or hyphal spreading. The formation of a dikaryotic mycelium from two homokarya is required to produce basidiocarps, which produce homokaryotic basidiospores after meiosis.

For most organisms the determination of their size is very obvious, but not for fungi. Typically, the fungal vegetative body is a network of hyphae spread out and concealed within the substrate. Hyphae usually do not bear sufficient morphological characters for species recognition, and distinguishing 'individuals' is not feasible. Therefore, basic biological attributes of fungi, such as breeding strategies and growth rates, are not as easily observed as in many plants or animals.

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Traditionally, vegetative compatibility techniques that are based on culturing, have been used to determine identity or nonidentity of fungal 'individuals' (Dahlberg & Stenlid 1994). The recent development of a variety of molecular techniques brought a breakthrough in determining the fine-scale structure of fungal populations. It became feasible to distinguish the genotypes of fungal 'individuals' using sensitive DNA-fingerprinting techniques (Smith *et al*. 1992). Individuals of a given genotype are usually referred to as 'clones' or 'genets'. Some genets of parasitic fungi were reported to be surprisingly large (15 ha) and old (1500 years, Smith *et al*. 1992).

From the size and persistence of genets, conclusions about the relative role of vegetative spread vs. spore establishment of ectomycorrhizal fungi can be drawn. This is crucial to understanding the dynamics of fungal successional sequences and to assign fungal species to functional guilds. To ascertain independent observations in ecological or physiological experiments it is also important to determine the minimum spatial distance to efficiently exclude encountering the same fungal genet on a host plant.

In field experiments it has been frequently observed that certain ectomycorrhizal fungal genera appear early in the successional sequence and are found to constitute a major component of disturbed systems, whereas others dominate later stages of succession (Deacon & Fleming 1992).

The early colonizing species typically colonize efficiently by spores under laboratory conditions (Deacon *et al.* 1983; Fox 1983) and in disturbed areas. Many early colonizers appear to have relatively small nonpersistent genets (Gryta *et al*. 1997; Gherbi *et al*. 1999). In contrast, fungi appearing late in succession are expected to colonize initially by spore but then spread primarily from hyphal networks, and their genets should be large and temporally persistent (Dahlberg & Stenlid 1990). Deacon & Fleming (1992) point out that early stage fungi can essentially be considered ruderal or R-selected species, while late-stage exhibit features of combative (C) or stress tolerant (S) strategies as defined by Grime (1979) and Cooke & Rayner (1984).

The genet size of several ectomycorrhizal fungi has been analysed in recent studies. *Hebeloma cylindrosporum* (Gryta *et al*. 1997) and *Laccaria bicolor* (Baar *et al.* 1994), which are members of well-known pioneer genera, have small, nonpersistent genets. Similarly, genets of *Laccaria amethystina* in a 150-year-old hardwood forest turned out to be numerous and small (< = 1.5 m diameter; Gherbi *et al*. 1999). *Suillus* is believed to follow a mixed strategy, colonizing disturbed areas but also persisting in later successional stages (Dahlberg & Stenlid 1990; Dahlberg *et al.* 1997; Bonello *et al*. 1998). *S. bovinus* (Dahlberg & Stenlid 1994), *S. variegatus* (Dahlberg 1997) and *S. pungens* (Bonello *et al*. 1998) were reported to form relatively large genets (20–40 m diameter). Genets of *Pisolithus tinctorius*, which might be considered an early colonizer of disturbed sites, were reported to reach similar sizes (Anderson *et al*. 1998). *Cortinarius rotundisporus*, the first typical ectomycorrhizal fungus appearing in later successional stages to be analysed, was found to have genets of up to 30 m in diameter forming numerous basidiocarps (Sawyer *et al.* 1999).

The Russulaceae and *Amanita* are considered typical protagonists of the late stages of succession (Deacon & Fleming 1992; Keizer & Arnolds 1994). Their spores are difficult to germinate and they are almost never found in seedling assays under laboratory conditions, suggesting that they do not colonize readily from spores. They are also a dominating constituent of some of the field sites we studied (Point Reyes, Rock Springs) and therefore promising candidates for propagation by hyphal spreading. Somatic incompatibility (SI), the technique traditionally used to resolve individual basidiomycete genotypes (Dahlberg & Stenlid 1994), is not feasible for most typical 'late-stage fungi' because of their very limited growth in culture. We therefore developed sets of amplified fragment length polymorphism (AFLP) markers to genotype *A. francheti*, *Lactarius xanthogalactus* and *R. cremoricolor* in field sites in coastal Northern California. AFLP has been used to obtain genetic fingerprints of a wide variety of organisms and is known for its robustness and good reproducibility (Vos *et al*. 1995; Majer *et al*. 1996). Using these molecular markers we addressed the question of whether ectomycorrhizal fungi from late stages of succession predominantly propagate by hyphal spreading. In addition, the markers revealed that two pileus morphotypes of *Russula* co-occurring in the field sites are conspecific colour variants and that genets are much smaller than would be expected from current models.

Materials and methods

Sites

Basidiomes of ectomycorrhizal fungi were collected from field sites in coastal Northern California. *Russula cremoricolor* and *Lactarius xanthogalactus* were studied in three and two independent sites, respectively. *Amanita francheti* was collected from one field site.

Basidiomes of *A. francheti* were collected from a field site at the Point Reyes National Seashore (for details see Gardes & Bruns 1996; Bonello *et al*. 1998). The locations of the basidiomes were mapped to the nearest 0.5 m within an area of 1200 m². The field site was located within a mature stand of bishop pine (*Pinus muricata*). The age of the trees was estimated to be ≈ 40 years (Gardes & Bruns 1996), which represents a mature forest for this short-lived species. Basidiomes were collected in 1994 and 1995 before the forest was destroyed by a wildfire in 1995. As an outgroup, one basidiocarp from Mt. Tamalpais was used, at an aerial distance of ≈ 30 km from the Point Reyes field site.

L. xanthogalactus and *R. cremoricolor* were collected over 2 years (1998–1999) in the Rock Springs study site on Mt. Tamalpais in the Marin Water District (37°54.963′ N, 122°36.833′ W, elevation 630 m). The forest there consisted of *Pseudotsuga menziesii* (Douglas fir) and *Lithocarpus densifolia* (tan oak). This site borders an open chaparral (10 m west of the map area in Fig. 4a,b), mainly composed of *Arctostaphylos glandulosa* ssp. *glandulosa* (Manzanita) and *Adenostoma fasciculatum* (chamize). Dead burls of *Arctostaphylos* in part of the site that roughly corresponds to where the *L. xanthogalactus* basidiocarps were found, provide evidence that the forest invaded the chaparral at this site. The site burned in the 1890s and 1945, therefore the oldest trees were just over 50 years old (V. T. Parker, personal communication; Sparling 1994).

A second field site for *R. cremoricolor* on Mt. Tamalpais, the Trail Junction site, was situated 300 m from the Rock Springs site (37°54.761′ N, 122°36.853′ W, elevation 590 m) at the intersection of Benstein and Simmons trails. The forest there consisted of Douglas fir and tan oak and bordered a meadow. Trees were of different ages, apparently taking over the meadow. A large dead old Douglas fir indicated that parts of the forest were fairly old.

L. xanthogalactus and *R. cremoricolor* were also collected between January and March 2000 in Salt Point State Park (38°35.452′ N, 123°20.384′ W, elevation 40 m), ≈ 90 km

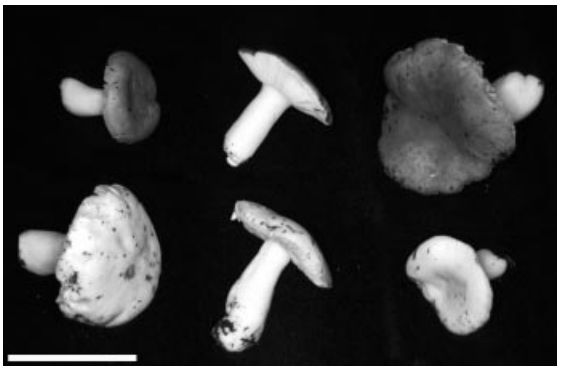

Fig. 1 Basidiocarps of the red (top row) and white (bottom row) morphotypes of *Russula cremoricolor* from Mt. Tamalpais. Size bar, 5 cm. A colour version of the image can be obtained from our website at http://plantbio.berkeley.edu/~bruns/ftp/russula.jpg

aerial distance from Mt. Tamalpais, in a mixed conifer forest consisting of *Abies grandis*, *Pinus muricata* and *Sequoia sempervirens*.

Cartesian coordinates of the basidiomes were determined using an ultrasonic distance measuring system (Forester, Haglöf Sweden AB, Långsele, Sweden) and a custom-designed sighting platform to measure angles. Angle and distance of the basidiomata from single reference points marked by stakes were determined in the Rock Springs site. At the Salt Point and Trail Junction sites, coordinates for each basidiocarp were calculated from distances to two reference points. By repeated measurements we estimate our accuracy to be ±10 cm. Basidiomes were freeze-dried and stored at room temperature. Only fresh specimens without any signs of decay were used.

Morphological identification of the species under study

Species were initially determined using morphological characters of the basidiocarps according to the key by Thiers (1997) and later compared with internal transcribed spacers (ITS)/random fragment length polymorphism (RFLP) analyses. The red-capped basidiocarps of *Russula* from Mt. Tamalpais (Fig. 1) fit the description of *R. silvicola* Shaffer. The white- to cream-coloured basidiocarps (occasionally with yellow–brown spots) were identified as *R. cremoricolor* Earle. Microscopic characters did not distinguish the white-capped from the red-capped morphotype. Apart from obvious cases of washed-out red colour after rainfall, no intermediate pileus colours between dark rosy red and creamy white were found; young, fresh basidiocarps were red or white.

DNA extraction. DNA was extracted from ground fungal tissue using the CTAB/glass milk method as described by Bonello *et al*. (1998). DNA stocks were used directly for the AFLP procedure.

AFLP. For technical reasons, two kits were used in different parts of this project. Protocol 1 was used in earlier stages before the reagent kit and software for protocol 2 became available.

Protocol 1 employed radiolabelled fragments and visual comparisons and used the AFLP Analysis System II kit from Life Technologies (Rockville, MD, USA) according to the manufacturer's instructions, except that half reactions were used for restriction digests and ligations, and 1/5 reactions for the pre-amplification step. [γ^{33}P]-ATP was purchased from Amersham Pharmacia (Piscataway, NJ, USA). Denatured samples were run on 6% acrylamide gels containing 50% urea (w/v) in 1× Tris/Borate/EDTA buffer for 3–4 h at 55 W. Dried gels were exposed on X-ray film for 1–3 days.

Protocol 2 employed fluorescent labels and automated size estimations and used a Perkin–Elmer Applied Biosystems (Foster City, CA, USA) plant mapping kit for small genomes. Reactions were prepared according to the manufacturer's manual with the following exceptions: for restriction ligation, a master mix was prepared that contained all components except DNA. For 10 samples: 10 µL ligase buffer (New England Biolabs, Beverly, MA, USA), 10 µL 0.5 M NaCl, 5 µL bovine serum albumin (1 mg/mL), 10 µL *Mse*I adaptor, 10 µL *Eco*RI adaptor, 2.5 µL *Mse*I (4000 U/mL, New England Biolabs), 0.5 µL *Eco*RI (New England Biolabs, 100 000 U/mL), 0.3 µL Ligase (New England Biolabs, 2×10^6 U/mL), 6.7 µL double-distilled water. For each reaction, 5.5 µL of this mix was combined with 4.5 µL DNA solution. Preselective and selective polymerase chain reactions (PCR) were performed with half the volumes indicated in the manual. The primer combinations were: *Eco*RI-AT/*Mse*I-CT, *Eco*RI-AA/*Mse*I-CT (*R. cremoricolor*), *Eco*RI-AT/*Mse*I-CT, *Eco*RI-AC/*Mse*I-CC (*L. xanthogalactus*). Aliquots of 0.75 µL of the selective PCRs were analysed in each lane of the gel with 0.3 µL of GeneScan [ROX] 500 size standard (PE Biosystems). Data were collected on an ABI 377 sequencer with GENESCAN software.

Gels or electropherograms were scored manually for the presence or absence of bands of the same apparent size. Markers that did not show a clear presence/absence pattern were excluded. The data matrices obtained using this procedure were checked in PAUP for their similarity using y parsimony analyses to identify identical patterns.

Pairwise Jaccard similarity indices (S_J) were obtained using the program package LE PROGICIEL R (version 4, Casgrain & Legendre 1999). Transformed Jaccard matrices (1—similarity coefficient) were analysed in PAUP* 4b2 (Swofford 1999) using the neighbour-joining method. Histograms of the pairwise Jaccard indices were obtained using MICROSOFT EXCEL 98 for Macintosh (Microsoft Corporation Redmond, WA, USA). Similar or identical samples were checked against each other by comparing their electropherograms side by side.

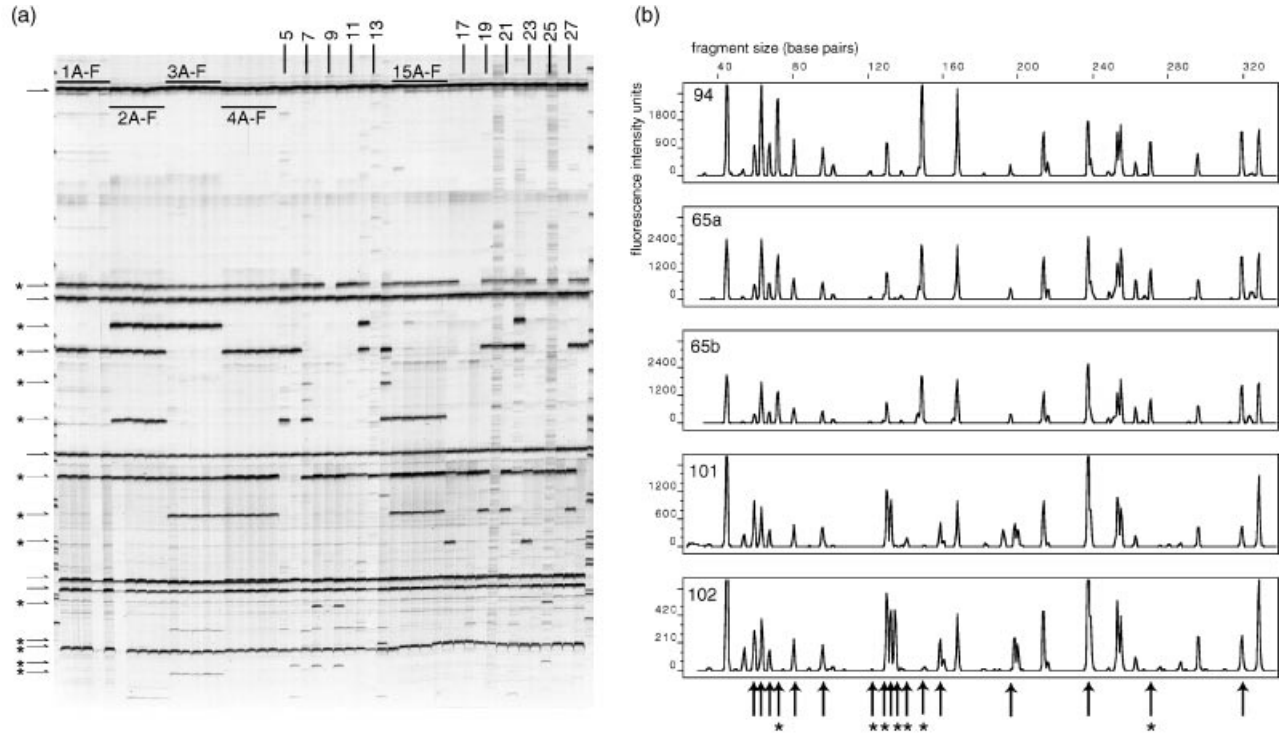

Fig. 2 (a) Amplified fragment length polymorphism gel of *Amanita francheti* obtained using protocol 1 (radioactive labelling) with primer pair *Eco*RI-AC/*Mse*I-CAG. A-F denote repeated extractions from the same specimen. Arrows show scored markers, * indicates polymorphic markers. (b) Electropherograms of AFLPs of *Russila cremoricolor* obtained by protocol 2 (fluorescent labelling) with primer pair *Eco*RI-AT *Mse*I-CT. Samples 65a and 65b are repeated extractions from the same sample. 94 and 65 belong to the same genet, 101 and 102 are relatively similar to each other but different from 65/94. Arrows show marker bands which were present and scored in at least one of the samples shown, markers polymorphic in the samples shown are indicated by *.

The probability of obtaining a given genotype by chance was estimated as following: the frequency of the recessive allele was derived directly from the frequency of the recessive genotype q (square root of recessive genotype frequency). The dominant allele was estimated as $1 - q$. The probability of obtaining a given multilocus genotype was the product of the allele frequencies and the probability of a given phenotype was the sum of the component genotypes.

Reproducibility of AFLP data

A typical gel picture using protocol 1 is shown in Fig. 2a. Electropherograms obtained with the fluorescent labeling procedure (protocol 2) are shown in Fig. 2b. To test reproducibility, samples were extracted independently and analysed several times. Protocol 1 was used with *A. francheti* from Point Reyes and *L. xanthogalactus* from Rock Springs. With protocol 1, 99 markers from 28 samples were obtained for *A. francheti* and 108 markers from 44 samples for *L. xanthogalactus*. To test reproducibility, independent extractions of five samples of *A. francheti* were repeated five times, and *L. xanthogalactus* independent extractions of seven samples were repeated three times. Data for a subset of 22 markers that proved the most reliable were obtained for 32 more samples of *L. xanthogalactus*. Protocol 2 was used for *R. cremoricolor* from three field sites (Rock Springs, Trail Junction, Salt Point, 51 markers from 108 samples) and *L. xanthogalactus* from Salt Point (57 markers for six samples). Reproducibility was checked by two to three independent extractions and AFLP analyses from seven samples. Generally, even the relative peak size was conserved across repetitions.

ITS sequencing. PCR products of *R. cremoricolor* obtained with the primers ITS1F and ITS4B were sequenced directly using a PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA). Electrophoresis and data collection were carried out on a ABI model 377A DNA Sequencer (ABI). DNA sequencing analysis (version 3.4.1) and sequence navigator (version 1.01) were used for processing the raw data. DNA sequences were submitted to the GenBank database under Accession nos AJ277910 (white morphotype) and AJ277911 (red morphotype).

Results

Distinguishing genets

Two potential problems needed to be addressed to infer genetic identity: (i) nonidentical genotypes may be identical with respect to the markers tested if the markers are not sufficiently variable or numerous; (ii) genotypes derived from a single mating may be scored as different due to scoring errors, reproducibility problems or somatic mutations. The first problem was addressed by estimating the probability of obtaining a given multilocus genotype by chance, and the second was addressed by including statistical criteria to decide whether two nonidentical samples belonged to the same genet.

The first potential problem was not a serious issue with the data because AFLPs yield a large number of variable loci for all three species. The highest probabilities for obtaining identical multilocus genotypes by chance were 2.3×10^{-7}, 8.7×10^{-5} and 2.0×10^{-4} for the 99, 22 and 51 markers of *Amanita francheti*, *Lactarius xanthogalactus* and *Russula cremoricolor*, respectively.

From these figures it is obvious that relatively few markers are needed to rule out that the genotypes arose just by chance. However, these calculations assume random mating. If inbreeding was common, the probability of obtaining identity could be substantially higher, and because these are dominant markers the precise probabilities cannot be determined. As discussed below, this possibility is a very minor problem within our current results, as sample identity was rare between samples and overestimating would only make our estimates of the genets smaller than they already are.

The second potential problem, nonidentity due to mutation or error, was addressed in two ways. Parsimony trees were constructed from score matrices and used as a quick visual way to identify all identical and near-identical genotypes. All samples that did not differ by at least one step were counted as belonging to one genet. For data obtained using AFLP protocol 2, identical and near-identical samples were checked by comparing electropherograms side by side. The pairwise Jaccard similarity coefficients were displayed as histograms (Fig. 3). The histograms allowed a visual statistical evaluation of the data and the identification of outliers that can be further examined. The similarity coefficients of genotypes within a randomly mating population should form a normal distribution. The coefficients of identical genotypes forming one genet should be outside of this distribution. Three standard deviations, indicative of a probability of 99% were empirically found to be a useful limit for *A. francheti* and *L. xanthogalactus*. It included all comparisons with the lowest similarities, but excluded identical comparisons and a small number of almost identical comparisons. The latter were clearly separated from the tails of the bell-shaped distributions (Fig. 3a,b). These outlying comparisons were also from adjacent basidiocarps. For these reasons we classify these 'nearly identical' samples as belonging to the same genet. Only a small number of basidiocarps were reclassified in this way, two for *A. francheti* and six for *L. xanthogalactus*,

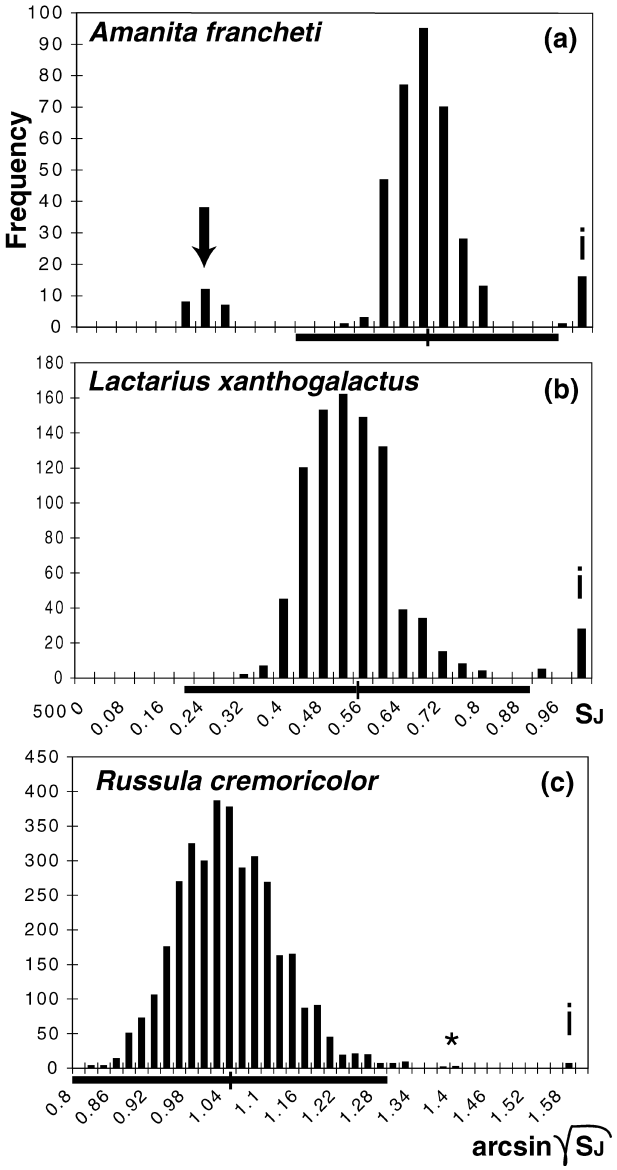

Fig. 3 Histograms of pairwise S_J similarity coefficients of (a) *Amanita francheti* (Point Reyes and outgroup from Mt. Tamalpais), (b) *Lactarius xanthogalactus* (Mt. Tamalpais, Rock Springs site) and (c) *Russula cremoricolor* (Mt. Tamalpais, Rock springs site). The bars under the *x*-axis denotes the mean ± 3 SD. (a) and (b) show untransformed data, data were arcsin-transformed for (c). The axis under (b) is valid for (a) and (b). Identical genotypes (marked by 'i') have an S_J of 1 or an arcsin-transformed S_J of 1.57. Note the second peak of pairwise distances caused exclusively by the *A. francheti* outgroup sample from Mt. Tamalpais (arrow in a). The asterisk in (c) denotes nearly identical samples discussed in the text.

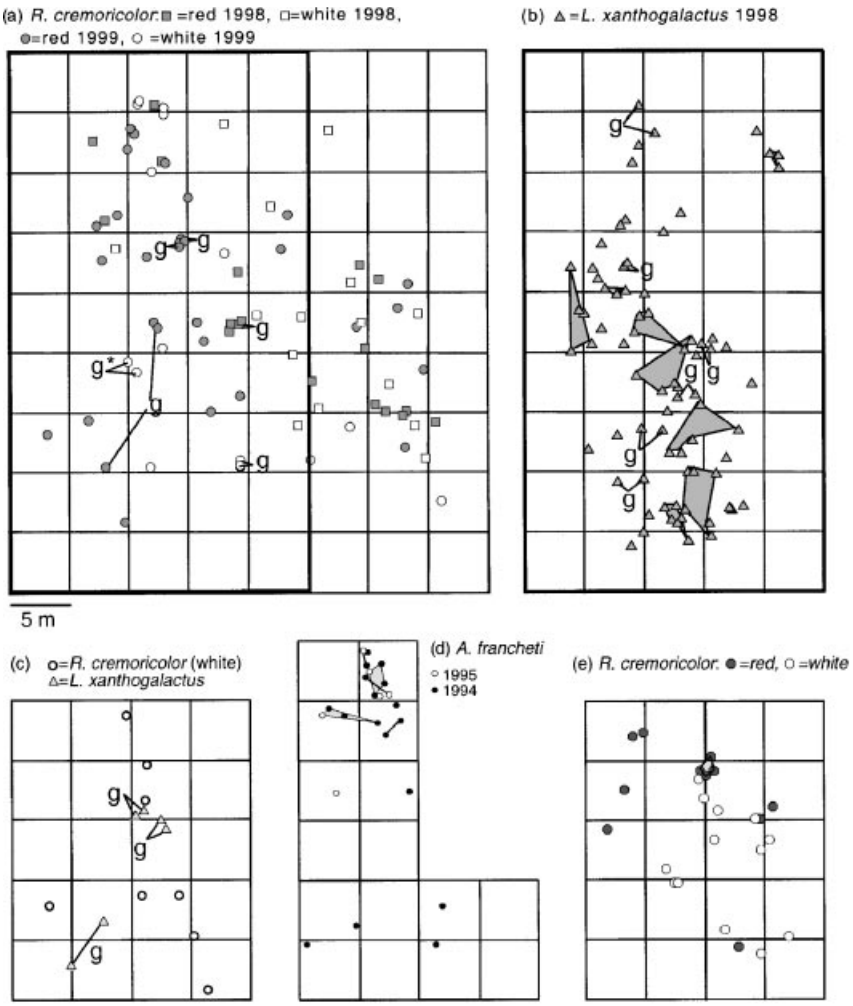

Fig. 4 Basidiocarp maps from the five field sites. Basidiocarps of identical genotypes are connected by lines, marked by 'g' or connected by polygons. All maps are the same scale. (a) *Russula cremoricolor* in the Rock Springs site on Mt. Tamalpais. 'g*' denotes two nearly identical genotypes (for details see text). Two more basidiocarps of a common genotype were located 20 m outside the lower border of the map at a distance of 27 cm from each other. (b) *Lactarius xanthogalactus* at the Rock Springs field site. The mapped area fits inside the area of Fig. 4a marked by a bold outline. (c) *R. cremoricolor* and *L. xanthogalactus* at the Salt Point site. (d) *Amanita francheti* at the Point Reyes site. Three more basidiocarps not grouped in genets were ≈ 15 m off the bottom-right corner of this map and one outgroup sample was collected from Mt. Tamalpais. (e) *R. cremoricolor* at the Trail Junction site on Mt. Tamalpais.

and the effect of this decision was to slightly increase the perceived genet sizes in these species.

The 3 SD limit did not appear to be sufficient for *R. cremoricolor.* In the respective histogram of *R. cremoricolor* distances (Rock Springs site, Fig. 3c), the mean of the similarity index was higher than in the other species (0.73) and the distribution was more continuous beyond the 3 SD limit. Because of the high mean, the data were arcsin-transformed, a method recommended for datasets containing proportions falling mostly outside the range 0.3–0.7 (Snedecor & Cochran 1967).

This transformation had the effect of spreading out the distribution more, but the tail was still continuous beyond the 3 SD limit. Comparisons just outside the limit were not scored as identical because they obviously belonged to the distribution. Comparisons that were further removed from the mean (marked by * in Fig. 3c and g* in Fig. 4a) represented two basidiocarps that were within 1.3 m of each other, and could, therefore, have been members of a single genet, but two other comparisons in this range grouped red and white cap variants together. For these reasons we did not reclassify any of these nearly identical samples as a common genet. The respective histogram for the Trail Junction site shows statistical parameters similar to the Rock Springs site, but only completely identical comparisons were situated outside the 3 SD limit (not shown).

To further test whether the surprisingly small number of identical samples of *R. cremoricolor* was due to the AFLP protocol, some samples of *L. xanthogalactus* were also analysed with AFLP protocol 2. Identical conclusions were derived for both protocols.

Genet size of L. xanthogalactus

Minimum genet sizes of the six detected genets comprising more than one sporocarp in the Rock Springs site were between 0.4 and 9.3 m² (Fig. 4b). The largest detectable distance across any genet was 7.3 m. Genets were generally compact in shape with little evidence for fragmentation. Even in areas of high sampling density, most genotypes were found only once. The temporal persistence of the genets could not be analysed, because basidiocarps were

only found in 1998 and not in the two following years. In Salt Point, three genets each comprising two fruit bodies were found (Fig. 4c). The largest distance within those pairs was 4.3 m.

Genet size of A. francheti

Minimum genet sizes of the two largest detected genets comprising more than two basidiocarps were 1.5 m^2 (Fig. 4d). The largest detectable distance across any genet was 4.7 m. Interestingly, most larger genets included basidiocarps from both sampling years, introducing a time component to be considered. This allows for the possibility that genets were even smaller at a given time and extended or translocated over time. It is not clear whether the two apparently overlapping genets in fact occupied the same space, or if one was fragmented, or if their actual shape was different from that shown in Fig. 4d.

Both histograms and phylogenetic trees give evidence for geographical population differentiation within *A. francheti*. The outgroup sample from Mt. Tamalpais clearly stood out in the histogram (Fig. 3) and also in the phylogenetic tree derived from Jaccard coefficients (not shown).

Conspecificity of red-capped and white-capped morphotypes of R. cremoricolor

Although the red- and white-capped basidiocarps (Fig. 1) from the Rock Springs site were morphologically assignable to two different species, *R. silvicola* (red) and *R. cremoricolor* (white), they have identical sequences of the ribosomal (r)DNA ITS. ITS sequences of *R. cremoricolor* from the Salt Point site are also identical. The ITS2 sequences we determined also matched sequences of both morphotypes that were found close to the Rock Springs site 6 years ago (Tom Horton, personal communication). Apart from that, the sequences indicate that the closest known relative of *R. cremoricolor* is *R. fragilis* (Lee Taylor, personal communication). However, ITS sequence identity alone is not compelling evidence of conspecificity; it only provides evidence that they are very closely related.

The histogram of the pairwise Jaccard similarity coefficients shows that the two morphotypes appear as one randomly mating population (Fig. 3). If two separate populations were present, two peaks would be expected. Separate histograms of pairwise distances among the red morphotypes, among the white morphotypes and between both (not shown) also produced no evidence of reproductive isolation. Phylogenetic trees derived from AFLP data by parsimony or distance analysis (Fig. 5) did not differentiate red- and white capped morphotypes from each other. By contrast, specimens of other species from the Russulaceae tested (*R. brevipes*, *R. amoenolens*, *L. xanthogalactus*, L. fragilis) did not share a single AFLP band with *R. cremoricolor*.

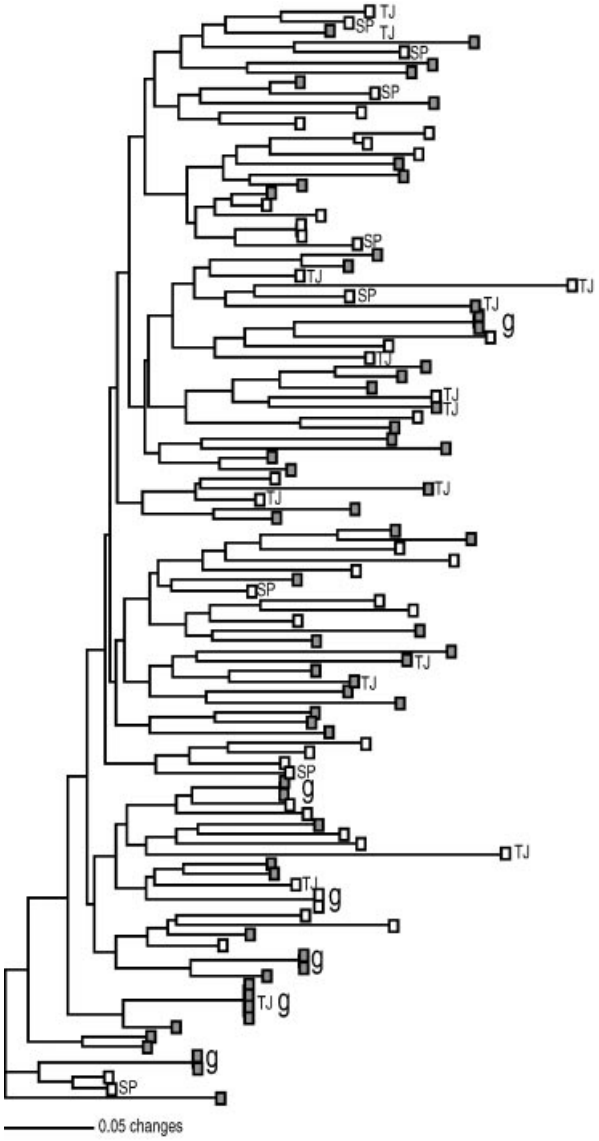

Fig. 5 Neighbour-joining tree obtained from S_J similarity coefficients of *Russula cremoricolor* from three field sites. Filled or hollow squares on branches show the pileus colour of the sample (red or white, respectively). The red and white morphotypes do not show separation. TJ, Trail Junction site; SP, Salt Point site; no letters, Rock Springs site.

Thus, there is no evidence for reproductive separation between the two morphotypes that differ mainly by the pileus colour. We conclude that they form a continuous population and are conspecific. As *R. cremoricolor* is an older name than *R. silvicola*, we use the first name when referring to the red- and white-capped morphotypes. At the Salt Point site, only white-capped types were found. Unlike *A. francheti*, no evidence for geographical differentiation between the sites was found in the trees or the histograms.

Genet size of R. cremoricolor

Relatively few basidiocarps of *R. cremoricolor* from the Rock Springs site show identical marker combinations (Fig. 5) and no genets comprising more than two basidiocarps were found. Most genets with more than one basidiocarp were very small, with the distance between basidiocarps between 38 cm and 1.27 m. The only exception were two basidiocarps situated 12 m from each other. However, no connecting basidiocarps were found over this distance, leaving the possibility open of an error during handling of the specimens. In spite of this possible exception, the overall picture is that the genets of *R. cremoricolor* are surprisingly small and this conclusion would not change if the small number of nearly identical samples were assumed to be genets. A Mantel test did not show a significant correlation between genetic and spatial distances, which might be expected if inbreeding was occurring on a fine spatial scale.

At Salt Point, genotypes from eight samples were all different (Fig. 4c). In the Trail Junction site, one genet comprising four fruit bodies of a total of 21 was found (Fig. 4e). The area between those basidiocarps was 1.05 m^2.

Discussion

The S_J indices of specimens showing identical AFLP patterns fell outside the normal distribution of the randomly mating population with 99% probability. A very small number of nearly identical genotypes fell outside the 3 SD criteria. These may result from minor scoring errors, mutation or perhaps inbreeding.

Our molecular markers provided conclusive evidence that the red- and white-capped basidiocarps initially identified as *R. cremoricolor* and *R. silvicola*, respectively, belong to the same species. These findings demonstrate that pileus colour is simply a variable trait at the population level. The absence of any intermediates between the red and white types and the absence of a correlation between genetic distance and colour suggests a simple genetic switch, perhaps even a single gene codes for pileus colour. The possible pitfalls of relying on pileus colour alone for species determination seem obvious, but in fact, keys of *Russula* depend heavily on this character (Shaffer 1975; Thiers 1997). Pileus colour has been used because it is obvious, and because microscopic characters separate some species but intergrade with others. For example, Shaffer (1975) noted that *Russula raoultii*, a white-capped species very similar to *R. cremoricolor*, is close to *R. silvicola*, with some specimens possibly showing intermediate characters. The taxonomy of *Russula* has traditionally been the source of confusion. The question of whether *R. silvicola* is a valid taxonomic entity goes beyond the scope of this study. The fact that it was described as an eastern US species (Shaffer 1975) means that the name could be misapplied in the west.

The largest fungal genets to date (15 ha) have been found in *Armillaria*, a parasitic fungus (Smith *et al*. 1992). From these observations it seemed plausible that a fungal individual could potentially be of indeterminate size (Dahlberg & Stenlid 1995). Ectomycorrhizal fungi have shown consistently smaller genet sizes, one of the largest to date being *Suillus pungens* with 300 m^2 (Bonello *et al*. 1998).

Ruderal ectomycorrhizal species colonizing disturbed sites do so mainly by spores, following a strategy of transient establishment and rapid reproduction (Deacon *et al*. 1983; Fox 1983). As a consequence, they tend to be overrepresented in bioassays of soils favouring spore colonization. Typically they are replaced in later stages of forest development by other fungi that are stronger competitors.

This strategy should result in numerous small genetic individuals, whereas large genets result from hyphal growth over long periods (Dahlberg & Stenlid 1990). However, it has been hypothesized that intact mycelium is the primary, perhaps exclusive EM inoculum in mature, undisturbed forests (Read 1992)

Hebeloma cylindrosporum, a typical pioneer species, showed genet sizes of not more than 3.5 m diameter with the majority being much smaller (Gryta *et al*. 1997, 2000). The genus *Laccaria is* also regarded as predominantly ruderal (Deacon & Fleming 1992). *Laccaria amethystina* had numerous small genets of < 1 m^2 (Gherbi *et al*. 1999), and the maximum size for *Laccaria bicolor* was 12.5 m^2 (Baar *et al*. 1994). *Suillus* genets were reported to reach substantially larger sizes of up to 300 m^2 (Dahlberg & Stenlid 1990; Bonello *et al*. 1998). These authors concluded that this indicates a mixed strategy, combining characteristics of ruderal (R), combative (C) and stress (S) strategies. Within this conceptual framework, large, persistent genets would be expected for typical protagonists of the late stages of ectomycorrhizal succession. *Cortinarius rotundisporus*, the only late-stage ectomycorrhizal fungal species analysed to date, seemed to confirm that notion with relatively large genets of up to 30 m in diameter (Sawyer *et al*. 1999).

Contrary to these expectations, none of the three species studied here showed large genet sizes, although all three typically appear in the late succession. In this study *Amanita francheti* was studied in the same field site in which the largest *Suillus* genet was found (Bonello *et al*. 1998), but genets of the first species were smaller by an order of magnitude. *Lactarius xanthogalactus* and *R. cremoricolor* were studied in two to three other, independent field sites. *L. xanthogalactus* genets were up to 7.3 m in diameter, whereas *R. cremoricolor* showed a surprising variety of small genets, mostly not exceeding 40 cm in diameter. This is not compatible with the notion of predominant hyphal spread in these species but suggests instead that spores are the main method by which local colonization occurs. The statistical precautions that we took to avoid mistaking somatic mutations for genetic variation between genets, an

error reported by other authors (Gryta *et al*. 2000), ensure that we rather overestimate rather than underestimate genet sizes.

The mapping method used on the Mt. Tamalpais and Salt Point study sites allowed us to adjust the borders of the field site according to the occurrence of basidiocarps, therefore minimizing the risk of missing parts of a large genet fruiting outside a previously defined grid. A fruiting bias cannot be excluded completely, but it is very unlikely, at least in the densely sampled Rock Springs study site, that this had any influence on the results. Considering that fungi are known to be territorial (Rayner 1991), it is unlikely that larger genets could be concealed among the small ones we detected.

Dahlberg & Stenlid (1995) found a correlation between the age of the forest and the size of the genets. None of the studied sites was an old growth forest. However, the mature bishop pine stand was old enough to potentially allow the formation of large genets of *A. francheti*, as Horton *et al*. (1998) showed at a nearby site that *Amanita* and *Russula* species were already forming ectomycorrhizae within the first 6 months after a wildfire. In fact, surveys of root tips from the site we studied indicated that *A. francheti* was widely established at sampling time (Gardes & Bruns 1996). Although the Douglas fir/tan oak forest at the Rock Springs site is situated at the border between forest and chaparral this is not necessarily a transitory environment from the fungal standpoint. Both fungal species studied here (*L. xanthogalactus* and *R. cremoricolor*) are also constituents of the chaparral mycoflora. *L. xanthogalactus* was shown to form mycorrhizas with *Arctostaphylos*, and *R. cremoricolor* fruit bodies were found in pure *Arctostaphylos* stands adjacent to our field site (Horton *et al*. 1999). Potentially, genets of both species could, therefore, have predated the Douglas fir forest at this site, allowing enough time for the formation of much larger genets than we found.

One consequence of the small genet sizes is that most of the fruiting observed for these three species can now be viewed as an indication of separate individuals. That means that diversity indices such as Shannon-Weiner's and Simpson's which depend on the number of individuals (Barbour *et al*. 1987), can be applied to these species with some confidence within a defined spatial scale. Figure 6 shows that for these three species, observed fruiting > 7 m would rarely be expected to be a result of a single genetic event.

Our results indicate that proliferation by sexual spores plays a much more important role than previously expected in the life history of these fungi and in mature forests in general. The mere appearance of a species in late successional stages seems to be a poor predictor for the actual colonization strategy. Other factors such as the capability to form rhizomorphs for long-distance transport probably provide greater opportunity for large genet

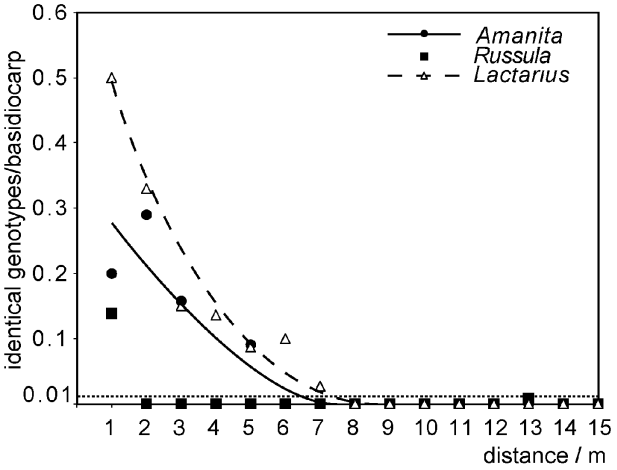

Fig. 6 Proportion of identical genotypes of total basidiocarps in relation to spatial distance. *Russula cremoricolor* and *Lactarius xanthogalactus* data are from the Rock Springs site.

production. This trait is found in *Armillaria*, *Suillus*, *Pisolithus* and *Cortinarius*, but not in *Amanita* and the Russulaceae.

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