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SHORT NOTE

Taiana Riviere · K. Natarajan · Bernard Dreyfus

Spatial distribution of ectomycorrhizal Basidiomycete *Russula* subsect. Foetentinae populations in a primary dipterocarp rainforest

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Abstract The spatial distribution of basidiocarps of the ectomycorrhizal Basidiomycete *Russula* subsect. Foetentinae was assessed in a primary forest in the Western Ghâts (India) dominated by the ectomycorrhizal tree species *Vateria indica* and *Dipterocarpus indicus*. Over a 7,700-m^2 sampling area, both trees and basidiocarps of *Russula* subsect. Foetentinae were mapped during the first month of the 2002 rainy season. First-order spatial analysis revealed that the distribution of the 45 collected carpophores was highly aggregated, with 60% of all basidiocarps located at a distance lower than 1 m from the nearest one. The genetic structure of the *Russula* subsect. Foetentinae population was studied by inter-simple sequence repeat polymorphism analysis using three primers. Eighteen of the 45 genotypes were represented by single basidiocarps. Twenty-seven basidiocarps were identified as belonging to 11 genets or separated ramets. Five genets were small, with diameters ranging from 0.5 to 5 m. The six others were large, with a diameter ranging from 31 m to a maximum measured distance of 70 m. In spite of the lack of data concerning the reproductive biology of this species, the presence of large genets suggests that mature stands may shelter well-spread underground mycelium, crucial for durable interaction with plant partner.

Keywords Ectomycorrhizal · Basidiomycete · Genet · ISSR · Primary tropical rain forest · Spatial pattern

T. Riviere · B. Dreyfus
UMR 113, Symbioses Tropicales et Méditerranéennes, LSTM,
34398 Montpellier Cedex 5, France

T. Riviere
Institut Français de Pondichéry,
11 St Louis Street,
Pondicherry, 605001, India

K. Natarajan
Center of Advanced Study in Botany, University of Madras,
Chennai, India

Present address:
T. Riviere (✉)
CIRAD UR 22,
Campus International de Baillarguet TA 30/E,
34398 Montpellier Cedex 5, France
e-mail: taianariviere@yahoo.co.uk
Tel.: +33-6-79412944
Fax: +33-4-67593799

Introduction

Ectomycorrhizal (ECM) fungi are symbiotic partners of most woody plants in forest ecosystems and have a crucial role in terms of nutritional transfer between soil and host plant roots (Smith and Read 1997). The fungal vegetative network forms extensive mycelia that radiate from ECM root tips to explore the soil for resources and permit the transfer of nutrients to associated plants (Finlay et al. 1998; Perez-Moreno and Read 2000). Together with sexual basidiocarps, ECM Basidiomycete populations use mycelial spread to propagate and colonize new habitats. The spatial distribution of individual mycelial systems is therefore of considerable importance to understand ECM population dynamics in forest ecosystems (Dahlberg and Stenlid 1990).

The absence of relevant tools for ECM identification, both at the level of species and individuals, has represented a bottleneck for understanding ECM fungal population dynamics (Redecker et al. 2001; Kretzer et al. 2003). The development of molecular techniques has made it possible to determine the structure of fungal populations on a fine scale, and it is now feasible to distinguish the genotypes of fungal individuals using DNA fingerprinting techniques (Burnett 2003). The concept of genet, a group of individuals of a given genotype, is a useful tool to understand not only the spatial distribution of the populations but also the dynamics of fungal successional sequences (Smith et al. 1992). In general, genets can only be identified after isolating and further investigating the properties of each species. Somatic incompatibility is the technique traditionally used to resolve individual Basidiomycete genotypes (Dahlberg and Stenlid 1994), but it is not feasible for all ECM species (e.g.

Russulaceae) because of their very limited growth in culture. Inter-simple sequence repeats (ISSRs) have therefore been used to characterize the genetic variation within fungal populations as it is a highly reproducible technique which allows the detection of ECM fungal genets from basidiocarps (Hantula et al. 1996; Anderson et al. 1998; Sawyer et al. 1999; Zhou et al. 1999).

The genet size of ECM fungal populations is assumed to vary with species identity, host forest age and environmental conditions (Dahlberg and Stenlid 1994). For example, pioneer genera such as *Hebeloma* and *Laccaria* generally show numerous small (<3.5 m^2), and non-persistent genets (Baar et al. 1994; Gryta et al. 1997). In contrast, fungi appearing late in succession such as *Cortinarius rotundisporus*, spread primarily from hyphal networks, and their genets are large (up to 30 m) and temporally persistent (Sawyer et al. 1999). However, the overall picture is not that clear since certain genera such as *Suillus* may be found in disturbed areas but also occur in mature forests, thus following a mixed strategy (Dahlberg et al. 1997; Bonello et al. 1998; Bruns et al. 2002). As a general principle, a high number of genotypes would be expected as a result of reproduction primarily by spores, whereas the formation of larger clones would be predicted if reproduction occurred primarily by mycelial expansion (Dahlberg and Stenlid 1990).

The Russulaceae are of particular interest for examination of the genetic structure and dynamics of late-stage ECM populations. Despite the fact that they are diverse and abundant in many types of forest ecosystems (Mason et al. 1987), the way they survive and spread in nature is still controversial. On the one hand, they are believed to be typical protagonists of the late-stage field succession (Deacon and Fleming 1992; Keizer and Arnolds 1994) as they represent the majority of basidiocarps found in mature stands of temperate forest (Richardson 1970; Kernaghan et al. 1997). Although little is known about the ability of Russulaceae to colonize tree seedlings in the field, laboratory studies indicate difficulties in germination from spores (Redecker et al. 2001). On the other hand, recent studies have found small genets of *Russula* in late-stage forests, suggesting that the role of sporulation in the life history of the Russulaceae growing on undisturbed forest may play a much more important role than previously recognized (Redecker et al. 2001; Bergemann and Miller 2002). Another interesting aspect of Russulaceae is that they are often dominant in tropical rainforests of Africa, Asia and Madagascar (Buyck et al. 1996; Lee et al. 1997; Watling and Lee 1998). Our knowledge on genet distribution of ECM fungi comes essentially from temperate ecosystems, and virtually nothing is known for tropical rainforests. In these systems, however, ECM tree species might be dominant (Dipterocarpaceae in India and Fabaceae, Caesalpinaceae and Euphorbiaceae in Africa), and the low nutrient availability of soils exacerbates the role of the ECM symbiosis for tree growth.

The aim of this study was to investigate the relative sizes of the individual genotype of *Russula* subsect. Foetentinae in a primary rainforest dominated by dipterocarps species using ISSRs. To minimize the possibility of errors in assigning genotypes to genets (see Redecker et al. 2001), we quantified the probability of obtaining a given genotype by chance and calculated a pairwise Jaccard's similarity among samples.

Materials and methods

Study site

The study site was located in a dense evergreen forest in the Kadamakal Reserve near the village of Uppangala (12° 30′ N; 75° 39′E) in India. The annual rainfall reaches 5,200 mm, with a marked dry season from December to March. Located at an average altitude of 500 m, the Uppangala forest represents one of the rare primary forest of Southern Western Ghâts. The vegetation is dominated by *V. indica* L. (Diperocarpaceae), *Humboltia brunonis* Wall. (Fabaceae), *Myristica dactyloïdes* Gaertn. (Myristicaceae) and *D. indicus* Bedd. (Dipterocarpaceae). They represent more than 48% of the trees and 55% of the basal area (Pascal and Pélissier 1996), with pioneer species accounting for only 1.1% of the trees. Dominant ECM species include *V. indica* and *D. indicus*, which represent 21% of the tree density (Pascal and Pélissier 1996).

Basidiocarps of *Russula* subsect. Foetentinae were collected and mapped to the nearest 0.1 m within a study plot of 7,700 m^2 (110×70 m). During the first month of the rainy season (May–June 2001), the collection was performed every 2 days. A small portion (≈0.5 cm^3) of the flesh of each basidiocarp was removed under sterile conditions and placed on a cotton layer into 10-ml tubes half-filled with Silicagel (Prolabo) for rapid drying.

DNA extraction and ISSR amplification

DNA was extracted from each sample of basidiocarp using a Dneasy Plant Mini kit following the manufacturer's recommendations (Qiagen, France). The samples were purified using the Wisard DNA Clean-Up System (Promega). ISSR-PCR reactions were performed with three primers named ISSR 1 [5′BDB $(ACA)_5$], ISSR 2 [5′DDB $(CCA)_5$] and ISSR 3 [5′DHB $(CGA)_5$], where B is C, G or T; D is A, G or T; and H is an A, C or T (Hantula et al. 1996). The annealing temperature was 50°C for ISSR 1, 60°C for ISSR 2 and 59°C for ISSR 3. The 50 μl ISSR-PCR reaction final volume contained 200 pmol of the primer, 2.5 mM $MgCl_2$, 200 μM of each dNTP 2.5 U of *Taq* DNA Polymerase (Amersham Pharmacia Biotech), 10 mM Tris–HCl, 50 mM KCl and 10–20 ng of genomic DNA. Amplification was realized with a DNA thermal cycler (Gen Amp PCR System 2400, PerkinElmer) programmed as follows: 1 cycle for 10 min at 95°C followed by 37 cycles at 95°C for 30 s, annealing at the temperature dependent on the primer for 45 s, 72°C for 2 min 30 s, and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 3% (wt./vol) metaphor gels (Sigma) in 1× Tris–borate–EDTA (TBE) with ethidium bromide at 10 mg/ml in the running

buffer. DNA bands were visualized by fluorescence under UV light. The size of amplification products was estimated by comparison to a VII molecular size marker (Promega). To test for the reproducibility of the ISSR technique, independent extractions and amplifications were carried out in duplicates.

Polymerase chain reaction and sequencing

The molecular identification of *Russula* subsect. Foetentinae was performed using both nuclear and mitochondrial DNA fragments. First, the ITS1, 5.8 S and the ITS4 rDNA sequences were obtained using ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TAGC-3′) (White et al. 1990) as primer pairs. Second, a fragment of the mitochondrial large subunit rDNA was amplified using the specific primers ML5 (5′-CTCGGCA AATTATCCTCATAAG-3′) and ML6 (5′-CAGTAGAAG CTGCATAGGGTC-3′) (White et al. 1990). The PCR reaction was performed following the procedure described in White et al. (1990). PCR products were separated by electrophoresis in 1% (wt/vol) agarose gels in 1× Tris–acetate–EDTA (TAE) with ethidium bromide at 10 mg/ml in the running buffer. Each PCR product was extracted and purified using a QIAquick gel extraction kit (Qiagen). Sequencing in forward and reverse was performed with both primers using the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, CA) and analysed on an Applied Biosystems model 310 DNA sequencer (PerkinElmer). The GenBank accession numbers for the sequences described here are DQ093423 for the rDNA nuclear sequence and DQ093424 for the rDNA mitochondrial sequence.

Statistical analyses

Spatial randomness

To determine whether the pattern of basidiocarp distribution was random, aggregated or regular, we used the first-order pair correlation function $G(r)$. This function gives the expected number of points at a distance r from an arbitrary point, divided by the intensity λ of the pattern (Diggle 1983). Significance of distribution pattern (random, aggregated or regular) was evaluated by comparing the observed data with Monte Carlo envelopes from the analysis of 1,000 simulations of the null model of complete spatial randomness (CSR) described as follows:

$$P(N(S) = n) = e^{-\lambda S} \frac{(\lambda S)^n}{n!} \quad (1)$$

This model assumes that the intensity of the point pattern is constant over the study region, and that the probability of finding n points in an area S follows a Poisson distribution with a mean λS.

Genotype identification

Inter-simple sequence repeat banding patterns were scored manually for the presence or absence of bands of the same size. Similar or identical samples were checked against each other by comparing the multilocus patterns. Markers that did not show a clear presence/absence pattern were excluded. To test for non-identity due to somatic, miss-scored gels or reproducibility problems, we calculated pairwise Jaccard's similarity (S_j) between genotypes on arcsin-transformed data using the following formula:

$$S_j = \frac{a}{a + b + c} \quad (2)$$

where a is number of bands common to both genotypes, and b and c are the number of bands present in only one of the two genotypes.

For each primer, the data matrices were checked for similarity using cluster analyses (Euclidian distance and simple link) to identify identical patterns. Isolates having an identical fingerprint with three primers were considered to be of the same genotype and considered to belong to the same genet. Because no bands were produced with primer 1 or 3 with certain samples, genets C, H, K and E were identified with only two primers.

All statistical analyses were performed using R 2.0.1 (free software available online (http://www.r-project.org, The R Development Core Team). The histogram of the pairwise S_j was obtained using Grapher 4.00 (Golden Software, Colorado, USA).

Results

In total, 45 basidiocarps of *Russula* subsect. Foetentinae were mapped and collected over 1 month. Spatial analyses of the point pattern revealed a significant aggregation of the basidiocarps in a central zone of the study area, representing less than 30% of the total surface prospected. The first paired correlation function, $G(r)$, showed that 60% of all basidiocarps were located at a distance lower than 1 m from the nearest basidiocarp.

Inter-simple sequence repeat PCR fingerprints generally comprised 4–11 amplification products with ISSR 1, 3–9 amplification products with ISSR 2 and 4–13 amplification products with ISSR 3. Amplification products ranged from 150 to 1,500 bp, depending on the isolate and the primer used. A total of 93 bands were obtained using the three primers, of which 36 (39%) were polymorphic. No bands were obtained with certain isolates using ISSR 1 and ISSR 3. Primer ISSR 2 yielded more numbers of polymorphic bands than ISSRs 1 and 3. Within the three ISSR primers used, ISSR 2 was the most polymorphic. Sixty-two fragments were obtained, of which 23 (37%) were polymorphic.

To test the possibility of reproducibility errors in inferring genets from genotypes, we plotted all pairwise Jaccard's similarity coefficients of genotypes for statistical outliers. Our results showed a bell-shaped distribution typ-

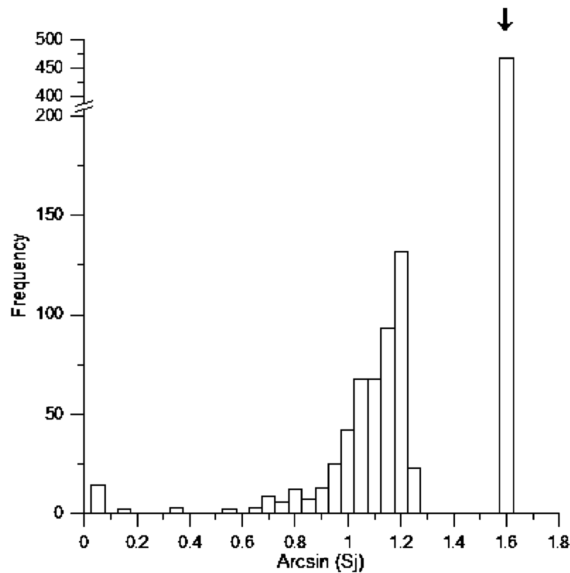

Fig. 1 Histogram of pairwise Jaccard's similarity (S_j) coefficients of *Russula* subsect. Foetentinae basidiocarps. The data are arcsin-transformed. Identical genotypes (↓) have an arcsin-transformed S_j of 1.57

ical of randomly mating population (Fig. 1). Identical genotypes were clearly separated from the right tail of the distribution.

Cluster analyses on the 45 ISSR patterns revealed a total of 29 different genotypes, with 18 genotypes represented by only one basidiocarp and 11 genotypes represented by two to three basidiocarps. Basidiocarps belonging to the same genet were not necessarily collected on the same day.

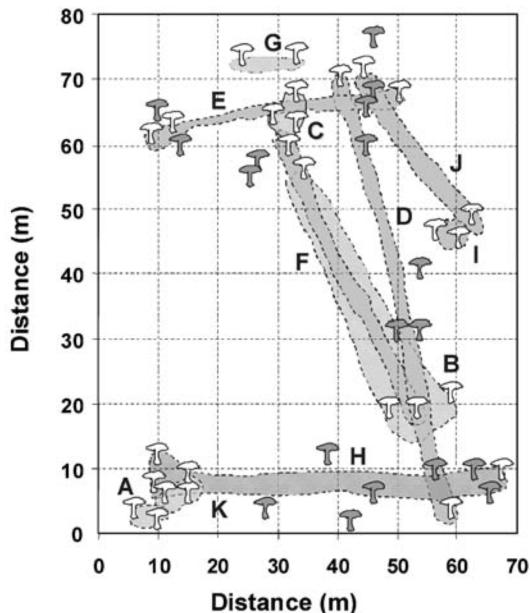

Fig. 2 Schematic relative positions and arbitrary sizes of *Russula* subsect. Foetentinae genotypes plotted on a portion of the Uppangala study site (5,600 m^2). Each individual basidiocarp is identified with an *icon* (*open*, basidiocarps belonging to a genet; *full*, individual basidiocarps). *Dotted lines* represent arbitrary surfaces of the separate genets

The 11 detected genets can be separated into two size classes (Fig. 2). Six genets were large, with a diameter ranging from 31 m (genet J) to 70 m (genet D). The five other genets (A, C, G, I and K) were of a smaller size, with diameters ranging from 0.5 m (genet I) to 5 m (genet G).

Discussion

Inter-simple sequence repeat polymorphism is a useful tool to distinguish otherwise morphologically indistinguishable individuals of fungi (Hantula et al. 1996). This technique was particularly appropriate in our study because the *Russula* genus contains a large number of species notorious for exhibiting high phenotypic plasticity (Miller and Buyck 2002). However, even when the individuals making up a population can be defined phenotypically or genotypically, their breeding behaviour is rarely immediately obvious. In particular, the sampling of basidiocarps above-ground may not be an adequate estimate of the size, frequency or spatial extension of genets below-ground (Gardes and Bruns 1996; Jonsson et al. 1999). In investigations of community structure above- and below-ground for ECM fungi, the abundance of basidiocarps was not always indicative of the mycorrhizal morphotypes (Gardes and Bruns 1996; Dahlberg et al. 1997; Jonsson et al. 1999). Because root tip or soil DNA extract analyses were not feasible under our field conditions, the information available on the distribution of basidiocarps was the only possible indicator of the presence and activity of individual mycelia.

When inferring clones from molecular data, two types of errors need to be tested. The first type may erroneously assign identity because the markers are not variable enough. To test for this error, it is possible to estimate the probability of obtaining a given genotype by chance following Redecker et al. (2001). The frequency of the recessive allele q is estimated as the square root of the proportions of individuals that exhibit this allele. The dominant allele is estimated as 1 − q. The probability of obtaining a given multilocus genotype was the product of the allele frequencies. Assuming random mating, the highest probabilities for obtaining identical multilocus genotypes by chance were 2.3×10^{-5}, 5.7×10^{-4} and 3.6×10^{-4} for ISSRs 1, 2 and 3, respectively. The use of three markers makes it unlikely that the genotype arose just by chance. If inbreeding was common, this probability can be higher. However, this is not a serious problem in our study, as multilocus identity was rare among *Russula* samples. The second type of error is to name identical genets as different. A pairwise distance histrogram showed that identical genotypes of *Russula* subsect. Foetentinae were well separated from the randomly mating population, clearly showing the absence of nearly identical genotypes.

Inter-simple sequence repeats revealed the presence of large *Russula* subsect. Foetentinae mycelial individuals in the mature dipterocarps stand we studied. The largest individual comprises three basidiocarps, two of which were situated 70 m from each other. Caution is needed in the interpretation of this result. First, the size estimates are based only on the presence of above-ground fruiting bodies.

Second, no connecting basidiocarps were found over this distance, leaving the possibility of an error during handling of the specimens. Third, the action of fungivores or physical constraints of soil may have separated ECM mycelia into several genetically identical genotypes or ramets (Dahlberg and Stenlid 1995; Griffiths et al. 1996). It is, however, not possible from our data to assess the extent to which fragmentation has occurred within the mycelial individuals at the site. Despite all these potential limitations, we found five other large genets (>30 m) in the study site, which strongly suggests the capacity of *Russula* subsect. Foetentinae individuals to spread over a long distance.

Russulaceae species are usually believed to spread via vegetative reproduction and to form relatively small genets; e.g. *Russula cremolicolor* (1.1 m^2, Redecker et al. 2001), *Russula brevipes* (3–18 m, Bergemann and Miller 2002) and *Russula vinosa* (<1 m, Liang et al. 2004). Our data indicate genets that are much larger than previously described for other Russulaceae species, but the presence of large genets is not uncommon among ECM fungal species. Large genets have been found for *Suillus bovinus* and *Suillus variegatus* (40 m, Dahlberg and Stenlid 1994; Dahlberg 1997), *Suillus pungens* (40 m, Bonello et al. 1998), *Pisolithus tinctorius* (30 m, Anderson et al. 1998) and *Xerocomus chrysenteron* (110 m, Fiore-Donno and Martin 2001). Comparison with data from the literature is however limited, as the size and extent of the mycelial phase can differ between genera and species or between different genets of the same species (Bonello et al. 1998; Redecker et al. 2001; Bergemann and Miller 2002). The presence of large genets suggests that *Russula* subsect. Foetentinae can colonize by mycelial expansion and may be indicative of more mature mycelial systems that have grown from point sources of individual mating events over a long period (Dahlberg and Stenlid 1990). Because the spatial extent of genets has been correlated with the age of host stands (Dahlberg and Stenlid 1994), our main hypothesis to explain the presence of large genets of *Russula* subsect. Foetentinae would be the absence of disturbance over a long period of time in the studied primary forest (>100 years, Loffeier 1989). However, the presence of smaller genets for 18 basidiocarps suggests that *Russula* subsect. Foetentinae can also spread via sexual reproduction of basidiocarps.

Field knowledge is lacking on the ecology of tropical ECM symbiosis, particularly in primary rainforest ecosystems. To date, most studies on ECM fungi in such ecosystems have consisted in species inventories (Buyck et al. 1996; Béreau et al. 1997; Lee et al. 1997). There is little information about life history strategies of tropical ECM fungi, and only recently, Onguene and Kuyper (2002) revealed in a tropical forest of Cameroon that ECM fungal mycelium might form important networks acting as “nursery zones” for young trees. Our results give preliminary information on reproductive strategies of *Russula* subsect. Foetentinae, which may have important implications in terms of the conservation of Asiatic primary rainforests. Dipterocarps are one of the most important timber species of tropical rain forest in Southeast Asia and are mostly ECM. Furthermore, they are mainly associated with Russulaceae and Amanitaceae species (Alexander and Högberg 1986; Watling and Lee 1998). The failure of dipterocarp regeneration in logged forests has been related to inadequate or inefficient ECM formations (Smits 1983). The latter have further been proven to enhance dipterocarp seedling growth both in nursery and in natural habitats (Lee and Alexander 1994; Lee et al. 1997).

This study represents a first step in our understanding of ECM fungal population dynamics in tropical primary forest ecosystems, but this only represents a snap shot of the genet distribution of *Russula* subsect. Foetentinae. Our results suggest that mature stands may shelter well-spread underground mycelium, crucial for durable interaction with the plant partner. Although the consistency of these results has to be confirmed on additional sites and over several years, they can be of particular importance in light of the current destruction of tropical forests or its degradation into secondary stands. Further studies on the temporal persistence of these large genets and on the consequences of human-induced changes on the dynamics of fungal populations are urgently needed.

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