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Evidence for mycorrhizal races in a cheating orchid

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Disruptive selection on habitat or host-specificity has contributed to the diversification of several animal groups, especially plant-feeding insects. Photosynthetic plants typically associate with a broad range of mycorrhizal fungi, while non-photosynthetic plants that capture energy from mycorrhizal fungi ('myco-heterotrophs') are often specialized towards particular taxa. Sister myco-heterotroph species are often specialized towards different fungal taxa, suggesting rapid evolutionary shifts in specificity. Within-species variation in specificity has not been explored. Here, we tested whether genetic variation for mycorrhizal specificity occurs within the myco-heterotrophic orchid *Corallorhiza maculata*. Variation across three single-nucleotide polymorphisms revealed six multilocus genotypes across 122 orchids from 30 sites. These orchids were associated with 22 different fungal species distributed across the Russulaceae (ectomycorrhizal basidiomycetes) according to internal-transcribed-spacer sequence analysis. The fungi associated with four out of the six orchid genotypes fell predominantly within distinct subclades of the Russulaceae. This result was supported by Monte Carlo simulation and analyses of molecular variance of fungal sequence diversity. Different orchid genotypes were often found growing in close proximity, but maintained their distinct fungal associations. Similar patterns are characteristic of insect populations diversifying onto multiple hosts. We suggest that diversification and specialization of mycorrhizal associations have contributed to the rapid radiation of the Orchidaceae.

Keywords: speciation; specialization; mycorrhizae; evolution; Orchidaceae; host race

1. INTRODUCTION

The concept of ecological specialization rests at the intersection of ecology and evolutionary biology: it encompasses both adaptation and the relationship of an organism to its environment (Levene 1953; Futuyma & Moreno 1988). The degree of ecological specialization of an organism refers to the breadth of its realized niche along an axis of interest, such as temperature, moisture, light availability or prey species (Hutchinson 1958). Adaptation to distinct abiotic or biotic environments often involves specialization. However, theoretical efforts to define the conditions that should lead to specialization or generalization have not yet produced a consensus (Levene 1953; Rice 1984; Brown & Pavlovic 1992; Fry 1996; Johnson *et al.* 1996; Whitlock 1996). In part, a lack of empirical data is holding back a resolution, because we know little about the traits controlling specialization or their genetic underpinnings (Johnson *et al.* 1996; Peichel *et al.* 2001; Via & Hawthorne 2002). In animals, evidence is accumulating that divergent selection on resource acquisition can lead to habitat or trophic specialization and thereby promote speciation and adaptive radiation (Richman & Price 1992; Schluter 1995; Lu & Bernatchez 1999; Via 1999; Rundle *et al.* 2000). A special case occurs in plant-feeding insects when trade-offs (or even simply inequalities) in performance on different hosts create divergent selection (MacKenzie 1996; Via 1999; Filchak *et al.* 2000). These trade-offs may explain the frequency of host-specialized populations, the most divergent of which can be classified as 'host races' (Dres & Mallet 2002).

Unlike most animals, the vast majority of plants acquire nutritional resources via photosynthesis and absorption from the soil. Mineral absorption from the soil is usually mediated by soil microbes, upon which wild plants depend heavily. Little is known about the genetics and microevolution of traits governing resource acquisition in wild plants, especially traits related to interactions with soil microbes (Taylor 2000). Mycorrhizal symbioses, which occur in *ca*. 90% of plant families, mediate uptake of the generally most limiting nutrients phosphorus and nitrogen (Smith & Read 1997). It has been hypothesized that this symbiosis was a key to the invasion of land by plants (Malloch *et al.* 1980). The mutualism is based on an exchange: plants provide sugars to the fungi in return for nutrients scavenged from the soil (Smith & Read 1997). Specificity of plants towards mycorrhizal fungi is generally low (Molina *et al.* 1992), as might be expected of an ancient mutualism (Law & Koptur 1986). However, it has recently become clear that the distinct guild of plants known as myco-heterotrophs (Leake 1994) are specialized towards narrow clades of fungi (reviewed in Taylor *et al.* 2002; and see Bidartondo & Bruns 2002; Bidartondo *et al.* 2002, 2003).

Myco-heterotrophs are non-photosynthetic plants that do not directly parasitize other plants as do, for example, mistletoes (Santalales) and broom rapes (Orobanchaceae), but instead depend upon vigorous mycorrhizal associations (Leake 1994). The more than 400 species of myco-heterotrophic plants have reversed the usual plant-to-fungus direction of carbon flow and depend on fungal symbionts not only for mineral nutrition, but also for their energetic requirements (Leake 1994). The facts that

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myco-heterotrophs consume rather than supply sugars and digest the hyphae of the fungi that colonize them (Bjorkman 1960; Dudderidge & Read 1982; Taylor & Bruns 1997) suggest that these plants are parasites upon their mycorrhizal fungi. However, this suggestion will remain speculative until the effects of myco-heterotrophs on fungal fitness are measured. Related species of myco-heterotrophs often associate with related groups of fungi, a pattern reminiscent of more conventional parasites, especially plant-feeding insects (Futuyma & Moreno 1988; Thompson 1994; Janz & Nylin 1998). The nutritional mode of myco-heterotrophic plants is reflected in many aspects of their life histories (Leake 1994). The seeds carry scant energy reserves and depend on encountering suitable fungi immediately upon germination (McKendrick *et al.* 2000). The small investment allows the production of vast quantities of miniscule dust-seeds. While it is the plant that depends upon the fungi, the fungi are somehow induced to invade and proliferate within the mycorrhizal organs of the orchid (see fig. 2 of Taylor & Bruns 1997). This 'inside-out' parasitism can be seen as analogous to the situation in predatory snapping turtles, which lure prey to enter their camouflaged mouths. At present, the microevolution of mycorrhizal specialization in myco-heterotrophs has been little studied.

Corallorhiza maculata is a perennial terrestrial myco-heterotrophic orchid. Though population densities are usually low, it is one of the most widespread orchids in North America, growing in the shade of closed-canopy forests from Guatemala, through Mexico, to British Columbia on the west coast and Newfoundland on the east coast (Freudenstein 1997). In the USA, it is absent from only the Deep South and the Great Plains (Freudenstein 1997). Previous studies employing culture-independent molecular methods for fungal identification have shown that *C. maculata* associate only with ectomycorrhizal fungi within a single basidiomycete family, the Russulaceae (Taylor & Bruns 1997). This represents high specificity relative to typical photosynthetic plants, which often associate with fungi from tens of families in both the Ascomycota and Basidiomycota (Molina *et al.* 1992). However, across 104 *C. maculata* individuals, 20 different *Russula* species were found; hence, the association is not one-to-one at the fungal species level (Taylor & Bruns 1999). The latter study also suggested that specificity is structured within *C. maculata*. Distinct sets of fungi were found consistently in particular habitats, such as geographically intermixed oak and conifer forests. Moreover, orchid individuals with distinct floral features (e.g. colour intensity, spotting, labellum shape) growing together at the same site nearly always had different fungal associates. We hypothesize that, if these patterns are caused by genetic variation in plant specificity, then this orchid species may consist of distinct groups each with its own compatibility with a particular fungal taxon. These 'mycorrhizal races' would be much like 'host races' in more conventional parasites.

Owing to the potential importance of race formation in microevolution and specialization, we sought to test this hypothesis rigorously. By extrapolation from studies of insects, we suggest that mycorrhizal races should:

(i) be more narrowly specialized in fungal associations than is *C. maculata* as a whole;
(ii) differ from one another in their target fungal clades;
(iii) maintain these patterns in both sympatric and allopatric settings; and
(iv) exhibit limited but non-zero levels of gene flow.

Here, we test the first three predictions through analyses of molecular variation in orchids and their associated fungi.

2. MATERIAL AND METHODS

(a) *Orchid collections*

From 1992–1998, we sampled small portions of mycorrhizal rhizome tissue from 122 *C. maculata* individuals across 30 sites, mostly from the Rocky, Cascade and Sierra Nevada mountain ranges of the western USA (table 1). DNA of plants and endogenous mycorrhizal fungi were simultaneously extracted from rhizome tissue and stored at −20 °C over GeneClean glassmilk as described in Taylor & Bruns (1999).

(b) *Plant genotyping*

To identify polymorphic plant-specific molecular markers for *C. maculata*, we sequenced several potential marker loci from three to six morphologically variable orchid individuals. The internal transcribed spacer (ITS) region of the nuclear ribosomal repeat was amplified using the plant-specific primer ITS 1P (CTTTATCATTTAGAGGAAGGAG) together with ITS 4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990; Taylor & Bruns 1997); the intron between the 10th and 11th coding regions of the RNA polymerase II gene was amplified using the primers P10F (CATGATGATATGCCATGGAC) and P-11A-R (GTGAATCTTGTCATCMACCATATGC) (Denton *et al.* 1998); and a region including the second intron of the flavanone-3-beta-hydroxylase gene (Britsch *et al.* 1994) was amplified using new primers FlavF2 (TTCATYGTYTCC AGCCATCT) or FlavF (GGYGRRACCTGGATCACCGT) and FlavR (ATGGTCTGCGTTCTTGAAGC). When the resulting sequences were queried against GenBank using BLASTN (Altshul *et al.* 1997), the most similar accessions were sequences from orchids in every case. We found single-nucleotide polymorphisms (SNPs) at positions 148, 174, 464 and 617 in the ITS and at position 768 of the *Bromheadia finlaysoniana* flavanone-3-beta-hydroxylase coding sequence (GenBank X89199); no changes were found in the RNA polymerase II intron. The first two ITS SNPs overlapped sites for the restriction enzymes BsmA1 (ITS SNP 1) and Mbo 1 (ITS SNP 2), respectively, while the flavanone SNP overlapped a BsaMI site, allowing us to score these three SNPs efficiently as cleavable amplified polymorphic sequence markers.

For a single locus in a diploid organism, one would expect complete presence or absence of the band corresponding to a restriction site in a homozygote, and roughly equal intensities of the two bands in a heterozygote. Only simple homozygous presence/absence patterns were obtained for the flavanone SNP. However, in the case of the ITS amplicon, the intensities of the two diagnostic bands were never equal, and the intensity ratios of the two bands varied widely across individuals for both restriction sites. The nuclear ribosomal gene region, in which the ITS resides, occurs in higher plants in thousands of copies that are tandemly repeated on one or more chromosomes (Saghai-Maroof *et al.* 1984). Hence, we suspect that the variable restriction-digest band intensities were the result of intra-individual sequence variation among ITS copies. We tested this

Table 1. Restriction digest patterns and geographical distributions of the six plant genotypes.
(Note that the first four geographical regions each encompass diverse habitats and multiple populations. Parentheses indicate minor bands.)

orchid genotype locus:	1	2	3	4	5	6
ITS SNP 1	−−	(+)−	+(−)	(+)−	+(−)	+(−)
ITS SNP 2	−−	−−	(+)−	−−	(+)−	−−
flavanone SNP	++	++	++	−−	−−	−−
distribution:						
Sierra Nevada Mountains	4	11	2	—	1	2
Western Coast Range	20	3	—	2	2	41
Cascade Mountains	2	1	—	1	—	4
Rocky Mountains	10	10	1	—	1	—
Arizona	2	—	—	—	—	—
Ohio	—	—	1	—	—	—
Wisconsin	—	—	1	—	—	—
number of individuals:	38	25	5	3	4	47

by cloning PCR products from five plants representing the range of band-intensity ratios using high-fidelity *Pfu*-Turbo polymerase and the PCR-Script Amp Cloning kit (Stratagene, La Jolla, CA, USA). Cloned fragments were reamplified from 254 colonies and digested with the same enzymes. Each clone amplicon displayed a single band diagnostic of either presence or absence of the restriction site, and the ratio of cutting to non-cutting clones obtained from a given plant was strongly correlated with the ratio seen in restriction digests of PCR products amplified from genomic DNA ($r = 0.99$). Hence, the unequal band intensities seen in PCR–restriction fragment length polymorphisms (RFLPs) from genomic DNA are not caused by incomplete digestion reactions. To score genetic variation in the ITS region, we placed individuals in one of two categories for each restriction site. For both SNP 1 and SNP 2, individuals with only an uncut band were labelled '−−', individuals with a predominance of the cut band were labelled '+(−)' and individuals with a predominance of the uncut band were labelled '(+)−'. No '++' homozygotes were observed.

(c) *Fungal identities and relationships*

We identified the fungi associated with 22 plant samples by the PCR–RFLP method, and added these samples to the ITS–RFLP dataset of Taylor & Bruns (1999). Previous work has shown that each *C. maculata* individual is usually colonized by a single fungus (Taylor & Bruns 1999). We sequenced the fungal ITS obtained from a representative of each of the 22 fungal ITS–RFLP types found associated with *C. maculata*, as well as from 21 identified sporocarps in the Russulaceae. Some of these sporocarps were collected and provisionally identified by Harry D. Thiers (San Francisco State University (SFSU) accessions). Other specimens were collected and identified by the authors (LT, TDB, TRH and SNF herbarium accession codes). Sequences from this study have been submitted to GenBank. The sequence alignment is available at http://mercury.bio.uaf.edu?~lee_taylor/. For subsequent statistical analyses, we assumed that fungi with the same ITS–RFLP patterns obtained from different orchid individuals had identical ITS sequences. We also submitted each of the *C. maculata* fungal sequences to an BLASTN search of GenBank (Altshul *et al.* 1997), and downloaded closely related sequences. An alignment of 825 characters was created using CLUSTALX (Thompson *et al.* 1997) and improved by eye. Heuristic maximum-likelihood searches were completed in METAPIGA (using four populations, strict consensus pruning, the HKY85 substitution model and other settings at default values; Lemmon *et al.* 2002). Bootstrap support values for branches were estimated by neighbour-joining under the minimum-evolution criterion with distances estimated by the general time-reversible model with rate heterogeneity (gamma = 0.5) and the proportion of invariant sites estimated from the data in PAUP* v. 4.0b10 (Swofford 2000).

(d) *Statistical analyses*

To test prediction (i) from § 1, narrow specificity, statistically, we compared the extent of phylogenetic clustering of samples of fungi using Monte Carlo resampling (Manly 1997). The statistic used to describe the overall phylogenetic diversity in a sample was the mean of all pairwise fungal ITS sequence distances, π (Tajima 1983). Two types of sample were compared: (i) the set of fungi that was associated with a particular orchid genotype; and (ii) random samples of the same size ($n = 3$, 4, 5, 25, 38 or 47) drawn from the total population of 122 fungi (one fungus for each *C. maculata* individual). The random samples were drawn 1000 to 10 000 times to provide a frequency distribution under the null hypothesis of no sub-specialization of orchid genotypes. Because we predicted smaller values of π within genotypes than in random draws, the *p*-values are one-sided. We performed a sensitivity analysis by conducting the resamplings using three different distance measures in the pairwise comparisons: (i) raw numbers of substitutions, with all sites included; (ii) raw numbers of substitutions, with parsimony-uninformative sites excluded; and (iii) distances from all sites corrected for unequal transition–transversion ratios using the Kimura 2-parameter (K2P) model.

We next tested prediction (ii) from § 1, different target clades, by treating the sets of fungi from each genotype as 'populations' and estimating the degree of differentiation within and between orchid genotypes in an analysis of variance framework (Cockerham 1969), taking into account molecular divergence among fungal haplotypes as implemented in the analysis-of-molecular-variance method of ARLEQUIN v. 2.01 (Excoffier *et al.* 1992), using fungal sequence distances corrected under the K2P model and allowing rate variation across sites ($\gamma = 0.5$). Pairwise tests of differentiation between orchid genotypes based on the

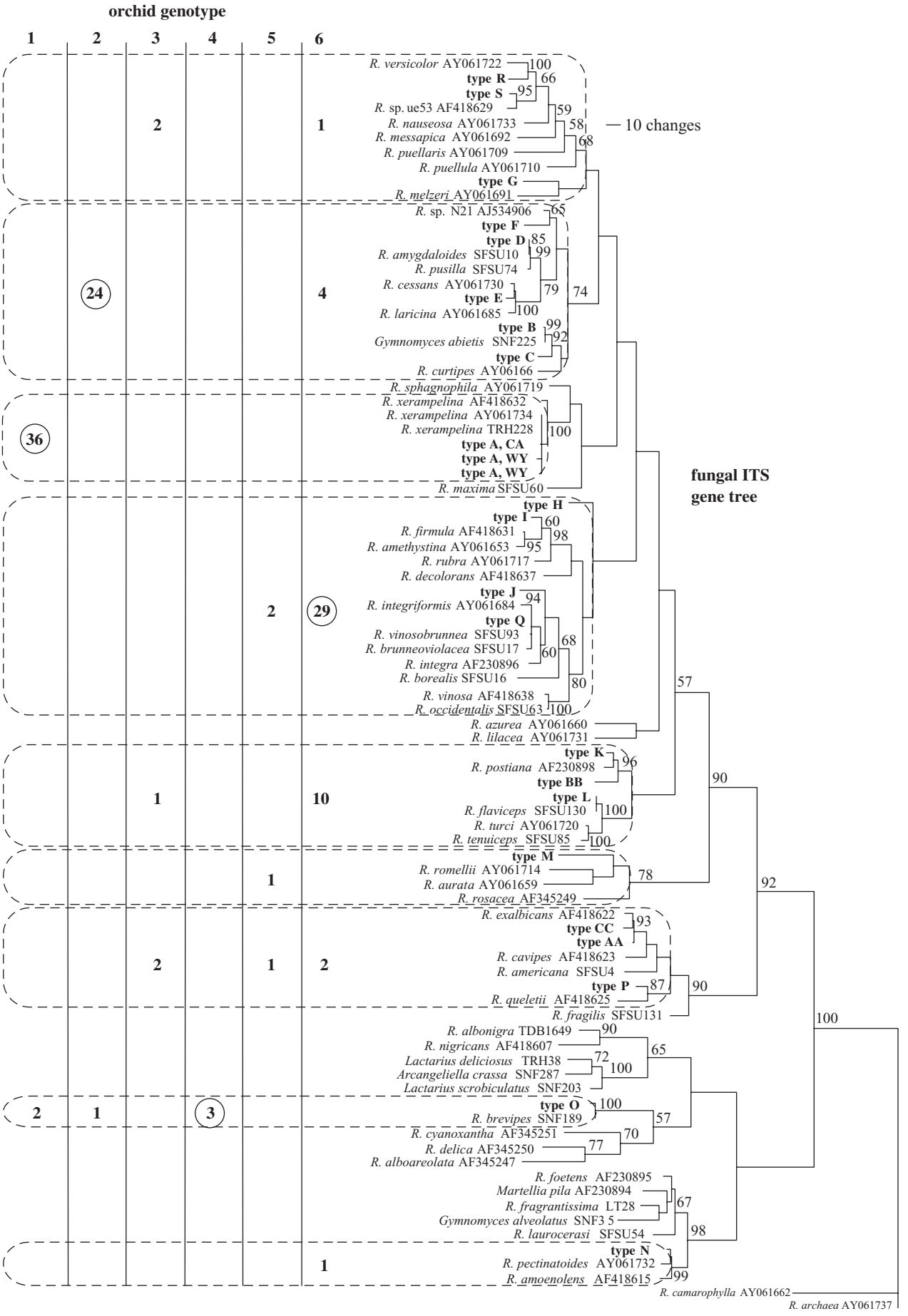

Figure 1. (*Caption opposite*.)

Figure 1. Evidence for sub-specialized genotypes within *Corallorhiza maculata*. The right side shows an ITS gene tree of the Russulaceae derived by maximum likelihood. Neighbour-joining bootstrap support values above 50% are given above or below branches. The abbreviation '*R.*' is given for members of the genus *Russula*. The fungi associated with *C. maculata* are indicated as Russulaceae types A through to CC on the fungal gene tree. Each letter code designates a distinct fungal ITS–RFLP type, for which one or more representatives have been sequenced. For fruitbodies, GenBank accession numbers or herbaria abbreviations are given. SNF, TDB, LT and TRH collections reside in the Bruns laboratory at the University of California, Berkeley. The numbers of individuals of each orchid genotype associated with fungi from a particular clade are provided in the centre of the figure. Narrow clades targeted by more specialized genotypes are circled. Notice that different orchid genotypes associate primarily with different fungi, which often fall into a discrete subclade within the Russulaceae.

variances of fungal sequence distances were also conducted, and significance levels were determined by exact permutation tests.

Last, to test prediction (iii) from § 1, maintenance of specificity in sympatry, exact likelihood ratio tests of homogeneity were applied to fungal species counts from sympatric orchid genotypes using StatXact v. 3.0.

3. RESULTS AND DISCUSSION

(a) *Phylogenetics of* Corallorhiza *fungal associates*

As previous work has suggested, the 22 new orchid samples were associated exclusively with russuloid fungi. Across the 122 previous and new samples, there were 22 different russuloid species diagnosed by unique ITS–RFLP patterns. Phylogenetic analyses of ITS sequences obtained from an array of sporocarps and from other studies of the Russulaceae (Calonge & Martin 2000; Eberhardt 2002; Miller & Buyck 2002) were used to infer the positions of the 22 russuloid ITS–RFLP types associated with *C. maculata* within the Russulaceae. We sequenced multiple representatives of two ITS–RFLP types (see type A, which matches *Russula xerampelina*, in figure 1) and found 0–2 substitutions within types, while different RFLP types differed by 9–121 substitutions and indels, showing that variation within types is a minor component of overall sequence variation across the 22 ITS–RFLP types. The high levels of sequence divergence across the Russulaceae made alignment of several regions difficult, suggested saturation of changes at a number of sites and may explain the low bootstrap support for some basal branches. However, distal clades, which identify close russuloid relatives of nearly every *C. maculata* associate, are strongly supported. Furthermore, congruent major clades were identified by parsimony, neighbour-joining and maximum likelihood, and similar clades were identified in two recent molecular–systematic studies (Eberhardt 2002; Miller & Buyck 2002). Inspection of the inferred fungal phylogeny (see the right side of figure 1) reveals that the fungi with which *C. maculata* associates span much of the phylogenetic breadth of the genus *Russula*, but are concentrated in the crown group defined as 'clade 6' by Miller & Buyck (2002) and as 'clade III, remaining Genuinae' by Eberhardt (2002).

(b) *Fungal diversity across orchid genotypes*

Variation across the three plant SNPs allowed us to discriminate six multilocus genotypic combinations among our 122 orchid samples. Three of the observed genotypes were common (25–47 individuals) and widespread, while three were rare (three to five individuals; table 1 and figure 1). The two ITS SNPs are in linkage disequilibrium, as one would expect for positions 26 bases apart, but each SNP contributes unique information. Neither ITS SNP was in disequilibrium with the flavanone SNP. The occurrence of russuloid fungi in each orchid genotype is plotted against the inferred fungal tree in figure 1. This graphic suggests a non-random relationship between orchid genotype and fungal type. Notice that, in the case of orchid genotypes 1, 2, 4 and 6, the majority of the russuloid associates fall into subclades within the Russulaceae. Permutation tests showed that the fungi associated with each of these four orchid genotypes had smaller mean pairwise genetic distances (π) than could be explained by chance ($p = 0.03$ to $p < 0.001$; table 2). Similar distributions and significance levels were obtained for raw distances, parsimony-informative sites and distances corrected under the K2P model (table 2). These results agree with prediction (i) that mycorrhizal races have narrower specificity than does *C. maculata* as a whole.

Comparisons of fungal pairwise sequence distances within and between orchid genotypes by analysis of molecular variance showed that, overall, the six orchid genotypes differ significantly in their fungal associations ($p < 0.001$). About 52% of the fungal molecular diversity was contained within orchid genotypes while 48% was partitioned among genotypes (calculated as F_{ST} through our 'genotype equals population' analogy). Pairwise comparisons showed that genotypes 3 and 5, and 5 and 6 were not statistically different in their fungal associations (i.e. F_{ST} did not differ from zero), but that all other genotype pairs were differentiated (table 3). These analyses provide statistical support for the indication in figure 1 that four out of the six genotypes target distinct fungal clades, and, therefore, show that these genetic entities satisfy prediction (ii). We obtained no evidence for specialization towards distinct clades in genotypes 3 and 5. Strong conclusions about specificity in genotypes 3, 4 and 5 cannot be drawn because they appeared so rarely in our samples of *C. maculata*. Only one or two samples were collected at most of the sites where these three rare genotypes were found, whereas up to 20 samples were collected at sites occupied by the other genotypes. Hence, the relative proportions of the six genotypes in our sample may not accurately reflect their true frequencies.

Our finding that four out of six orchid genotypes, accounting for 113 out of 122 orchid collections, associate consistently with discrete russuloid clades could be caused simply by correlated spatial distributions of plant genotypes and fungal clades, i.e. large-scale coincidence of geographical ranges, or smaller-scale correspondence of habitat preferences. Different orchid genotypes were often found growing together at the same site, which allowed us to test this possibility rigorously. Exact likelihood ratio tests strongly rejected the null hypothesis of homogeneity of fungal associations for the two pairs of genotypes that were found together at multiple sites, in support of prediction (iii) (table 4). Moreover, by comparison of table 4 and

Table 2. Mean pairwise fungal sequence differences (π) between real and simulated samples.
(When real samples have smaller π-values than simulated samples, it indicates phylogenetic clustering of the fungi associated with that orchid genotype and, therefore, sub-specialization of that genotype.)

orchid genotype	1	2	3	4	5	6
sample size	$n = 38$	$n = 25$	$n = 5$	$n = 3$	$n = 4$	$n = 47$
number of randomizations	1000	1000	10 000	10 000	10 000	1000
all substitutions						
mean randomized π	51.71	51.74	51.67	51.62	51.56	51.76
observed π	10.24	29.97	51.10	0.00	64.83	45.68
p-value	< 0.001	< 0.001	0.546	0.0295	0.815	< 0.001
parsimony-informative sites						
mean randomized π	42.98	43.16	43.04	43.14	43.18	43.23
observed π	7.27	24.46	47.30	0.00	52.50	39.75
p-value	< 0.001	< 0.001	0.693	0.0282	0.833	0.002
Kimura 2-parameter corrected substitutions						
mean randomized π	94.22	93.60	94.33	93.80	94.33	93.88
observed π	22.25	52.53	91.32	0.00	122.53	80.17
p-value	< 0.001	< 0.001	0.584	0.0248	0.812	0.030

Table 3. Pairwise divergences (F_{ST}) between orchid genotypes calculated from fungal pairwise molecular distances (π).
(Significant F_{ST} values indicate that the two orchid genotypes associate with phylogenetically distinct sets of fungi.)

	1	2	3	4	5	6
1	0.000 00					
2	0.679 19***	0.000 00				
3	0.631 84***	0.330 74***	0.000 00			
4	0.900 57***	0.737 27***	0.664 34*	0.000 00		
5	0.666 14***	0.435 88***	−0.007 60 n.s.	0.670 53*	0.000 00	
6	0.487 82***	0.284 12***	0.115 14*	0.656 01***	0.089 19 n.s.	0.000 00

*$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$; n.s., not significant.

Table 4. Likelihood ratio test for homogeneity of fungal associates between sympatric orchid genotypes.
(The significant p-values show that these pairs of sympatric orchid genotypes associate with categorically distinct sets of fungi. The number of sympatric sites where a pair of orchid genotypes were found is given.)

	counts of fungal types								
sympatric orchids	A	C	D	E	L	R	number of sites	G likelihood	p
genotype 1 and	10	0	—	0	0	0	3	26.29	< 0.0001
genotype 6	0	2	—	1	5	1			
genotype 1 and	8	0	0	—	—	—	2	15.28	0.002
genotype 2	0	3	1	—	—	—			

figure 1, it can be seen that these sympatric orchids remained specific to the russuloid clades found in that genotype elsewhere, arguing against broad geographical variation as the cause of the correlations. The form of the sympatry also casts doubt on shared microhabitat preferences as the cause of the correlations, because different orchid genotypes grew within metres of one another and were spatially intermixed throughout the sympatric populations. Furthermore, sporocarp and ectomycorrhiza collections show that many of these sites support diverse species of Russulaceae (Taylor & Bruns 1999). Several of the genotypes were found at distant sites but maintained consistent specificity, such as genotype 1, which was common in California, Oregon, Washington and Montana and had only a single fungal associate in all these locations. Thus the four genotypes that differed from one another in specificity were consistent in their fungal associations regardless of the geographical distance between orchid samples or the presence at a site of other orchid genotypes and a variety of other *Russula* species. Based on the consistency of the associations, we conclude that orchid genotypes 1, 2, 4 and 6 differ genetically in their mycorrhizal

specificity. Note that the results reported here are based upon only three SNPs. Studies using additional plant genetic markers might reveal additional complexity in the relationship between plant genotype and mycorrhizal specificity.

(c) *Specificity and diversification*

The formation of host races is very common in plant-feeding insects and is thought to have played an important role in the diversification of this species-rich group (Mitter *et al.* 1991; Farrell 1998). The patterns of specificity across these orchid genotypes closely resemble patterns found in many insects, although prediction (iv) was not tested because gene flow between the orchid genotypes has not been measured. Under a scenario of high gene flow, these orchid genotypes would appear to be equivalent to pathogen 'biotypes', while, if gene flow is quite limited, they may equate to 'host races', and, if it is absent, they represent cryptic species (Dres & Mallet 2002). Because variation in morphological traits is continuous (Freudenstein & Doyle 1994) and there is evidence for subdivision of these six genotypes into further subspecialized entities (D. Lee Taylor, unpublished data), we view a complete absence of gene flow indicating numerous cryptic species as unlikely.

Many insects mate on their host plant, meaning that a change in host preference can immediately produce assortative mating and reduced gene flow. Reproductive isolation can also be caused by shifts in developmental timing, which occur as a by-product of colonization of a different host plant. Hence, in many insect groups, the evolutionary path from biotypes to new species is easy to conceptualize, though often difficult to document. It is unclear how the putative mycorrhizal races in *C. maculata* can persist in sympatry because any link between mycorrhizal specificity and reproductive isolation is obscure. However, strong correlations between flower morphology, chloroplast haplotype and flowering time have been documented in *C. maculata* (Freudenstein & Doyle 1994), especially in eastern populations. Flowering-time differences provide a clear mechanism for assortative mating. Mathematical models incorporating genes for habitat preference (analogous to mycorrhizal specificity, in the present case), habitat-based fitness and assortative mating produce sympatric speciation under a broad range of conditions (Johnson *et al.* 1996). Whether assortative mating resulting from phenological differences contributes to the persistence in sympatry of *C. maculata* genotypes with distinct mycorrhizal specificity, and whether fitnesses vary across different genotype–fungus pairings, remain to be determined.

If the orchids and fungi had radiated in synchrony (i.e. co-speciated), we would expect equal numbers of substitutions in both lineages for genes that they share in common, under a molecular-clock hypothesis. Because we sequenced the ITS in both orchids and fungi, we were able to test for co-speciation. We found only a few base changes across the orchid individuals, while the associated fungi differed by up to 121 base changes, i.e. a difference of two orders of magnitude. While substitution rates can vary significantly across lineages (Gillespie 1991), we are unaware of rate differences large enough to explain these results under a co-speciation scenario. Instead, it seems more probable that this orchid has radiated onto a significantly older fungal lineage. Thus, coevolution may be occurring (e.g. the evolution of defences and counter-defences), but it has not resulted in co-speciation (see also Bidartondo *et al.* 2002). This trend in myco-heterotrophs also parallels emerging trends in phytophagous insects (Janz & Nylin 1998).

A major distinction between myco-heterotrophs and insects is the importance of a third player in the system: the autotrophic plant that supports the growth of the mycorrhizal fungi and the linked myco-heterotrophic plant. While *Corallorhiza* is highly specific towards particular *Russula* fungi, these fungi have wide ranges of autotrophic hosts, including species of *Quercus*, *Betula*, *Salix*, *Poplar*, *Abies*, *Pinus*, *Tsuga* and others. These autotrophs form ectomycorrhizal relationships with thousands of fungal species outside the Russulaceae. Furthermore, the *Russula* species occur in many locations that lack *Corallorhiza*. The diffuse interactions between fungi and autotrophs combined with intermittent interactions between fungi and myco-heterotrophs suggest that any selection imposed by cheating myco-heterotrophs on the linked autotrophs is likely to be weak and diffuse.

Statistical analyses confirm unusually rapid diversification in the Orchidaceae (Dodd *et al.* 1999), a relatively young family with an estimated 30 000 species. Many orchids have highly specialized pollination syndromes and these can differ even between sister species. Biologists from the time of Darwin to the present have proposed that diversification of highly specialized pollination syndromes may help explain the species-richness of the Orchidaceae and other groups (Darwin 1884; Van der Pijl & Dodson 1966; Hodges & Arnold 1995), but many orchids do not have specialized pollination mechanisms. The capacity for mycorrhizal specialization may be widespread in orchids (Otero *et al.* 2002; Taylor *et al.* 2002) and the results presented here show that specialization can evolve quite rapidly. We suggest that diversification of mycorrhizal associations, in addition to pollination syndromes, has contributed to the radiation of the Orchidaceae. This broader view of the Orchidaceae highlights a remarkable tendency towards specialized and deceptive interactions both above and below ground. The impacts of mycorrhizal interactions on plant microevolution in general are deserving of greater study.

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