Progress and Prospects for the Ecological Genetics of Mycoheterotrophs

6

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6.1 Introduction

Mycoheterotrophic plants provide fascinating evolutionary narratives. Examples include convergent evolution of traits such as miniscule seeds, shortened and thickened roots, narrow mycorrhizal specificity, and self-fertilization (Leake 1994). Another example is provided by the patterns of decay of the photosynthetic machinery (DePamphilis and Palmer 1990; Barrett and Freudenstein 2008; Delannoy et al. 2011; Logacheva et al. 2011). Finally, the dynamics of specialization and host-jumps among various clades of fungal hosts (Taylor et al. 2004; Kennedy et al. 2011) offer compelling cases for comparison with more "main-stream" parasites, such as lice (Hafner and Page 1995), phytophagous insects (Hawthorne and Via 2001), or rust fungi (Jarosz and Burdon 1991). Another interesting feature of mycoheterotrophs, which is perhaps less often considered, is that multiple traits, including most of those mentioned above, appear to be evolving rapidly across numerous independent lineages. For example, from the limited sampling available at present, it appears that sister species of mycoheterotrophic plants always target different fungal clades (Taylor and Bruns 1999; Bidartondo and Bruns 2001, 2002; Kennedy et al. 2011). This phenomenon is too widespread to be merely coincidence. But we are currently without any clear understandings of the evolutionary dynamics and selective pressures that underlie this phenomenon. Investigation of selective pressures and rapidly evolving traits falls principally under the purview of population genetics, quantitative genetics, genomics, and molecular evolution. When applied to ecological questions, these fields form the basis for ecological genetics, the subject of this review. Ecological genetics of mycoheterotrophic plants is only in its infancy, hence in this review we suggest some ideas about ways in which ecological genetics might enlighten the field of mycoheterotrophic plant research going forward. We also present case studies of several fully mycoheterotrophic

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species, to illustrate some emerging trends, and further questions raised by these early findings.

Mycoheterotrophic plants present several challenges as potential model systems in ecological genetics. The most significant is the fact that they cannot routinely be grown in cultivation. Hence, standard methods such as controlled crosses (and resulting tools, such as recombinant inbred lines), common gardens, and most experimental manipulations are difficult or impossible to apply to mycoheterotrophic plants. On the other hand, methods for the study of the ecological genetics of wild populations have advanced significantly in recent years (Travers et al. 2007; Baird et al. 2008; Nadeau and Jiggins 2010; Helyar et al. 2011; Baxter et al. 2011). Today, ecological genetics and genomics have much to offer the study of the biology of mycoheterotrophic plants. Mycoheterotrophic plants are usually rare plants, with patchy local distributions, yet sometimes rather wide geographic distributions (see Chap. 3) as well as occasional cleistogamy, and other forms of inbreeding (Chap. 7). Hence, studies of mating systems are fundamental to an understanding of the microevolution of mycoheterotrophic plants. However, traditional methods of studying breeding systems, such as observations of pollinator behavior and experimental manipulation of pollination, are not adequate to characterize mating systems in many mycoheterotrophic plants. To understand mating and gene-flow, molecular methods based on multiple, independent, highly variable markers offer the best way forward (e.g., Klooster and Culley 2010). Population genetics summary statistics (Table 6.1) such as Hardy–Weinberg, heterozygosity, F_{st} can then be calculated to infer patterns of gene-flow and answer key questions about the degree of genetic differentiation within and among populations at various spatial scales. Marker data can also be subject to methods such as parentage analysis (Blouin 2003) and population assignment (Pritchard et al. 2000) to provide additional insights into within-population mating patterns, as well as population boundaries where they are not obvious from morphology or geography. As described in several of the case studies below, even species boundaries are often ambiguous in mycoheterotrophic plants, due to highly variable floral morphologies and the occurrence of nearly continuously variable intermediate forms. An absence of gene-flow between populations based on molecular markers can provide strong evidence for the existence of distinct biological species. This contemporary perspective is complementary to a historical perspective based upon multilocus, sequence-based phylogenetic analyses (e.g., Kennedy and Watson 2010) for distinguishing lineages that are on independent evolutionary trajectories.

Understanding species and population boundaries and elucidating patterns of gene-flow and genetic variation set the stage for investigation of adaptation and natural selection. Numerous approaches have been developed, only a few of which will be mentioned here. One fundamental definition of evolution is change in allele frequencies in a population over time. Hence, investigation of the genetic constituency of mycoheterotrophic plant populations over life stages, generations, space and time can alert us to situations in which evolution has taken place. If, for example, certain alleles or genotypes are consistently lost in the transition from zygotes to adult mycoheterotrophic plants, selection against those genotypes is likely. A major emphasis in ecological genetics is the identification of genes that influence traits of interest, especially those that are related to fitness differences underlying ecological adaptation (Hohenlohe et al. 2010). Furthermore, reconstruction of the geographic and demographic histories of populations and species is a rapidly advancing, energetic field (Huang et al. 2011). All of these subdisciplines are becoming exponentially more informative as the genome-wide distribution of variable markers increases in a greater diversity of taxa. In tandem with improvements in molecular tools, advances in related "–omics" technologies are providing radical new opportunities to investigate functional genomics, even in non-model organisms. For example, next-generation sequencing of cDNAs can provide a detailed snapshot of gene expression in any organism (Mortazavi et al. 2008).

Table 6.1 Summary genetic statistics for selected mycoheterotroph species and varieties

Family (Subfamily)	Species	Sampling distirbution	Natural forms/variants	Breeding system	*N*	*S*	π	*#h*	*hd*	He	Ho	F_{is} (f)	F_{st} (theta)	Data type	Citation
Ericaceae (Monotropoideae)	*Monotropa hypopitys*	Eastern North America	Red color form	Facultatively xenogamous; partial autogamy	41.9					0.25	0.33	−0.364–0.286	0,12	microsatellites	Klooster and Culley (2010)
			Yellow color form	Mixed breeding; primarily autogamous	31.6					0.2	0.14	−0.358–0.503	0.13–0.61		
		Northern Ireland	Yellow color form		141					0.492–0.507		0.339–0.497	n/a	microsatellites	Beatty and Provan (2011a)
Orchidaceae (Epidendroideae: Calypsoeae)	*Corallorhiza striata* complex (nuclear DNA)	Southwestern USA, Mexico	var. *vreelandii*	Facultatively xenogamous; partial autogamy	46	6	0.0016	3[a]	0.4	0.39	0.11	n/a	n/a	Nuclear intron sequences	(Barrett et al. 2010); Barrett and Freudenstein (2011)
		Northern USA, southern Canada	var. *striata*	Primarily xenogamous; some autogamy	75	10	0.004	4.33	0.51	0.54	0.5	n/a	n/a		
		Sierra Nevada, California, USA	Californian populations	Primarily xenogamous; some autogamy	34	18	0.0066	5.67	0.69	0.69	0.61	n/a	n/a		
		Mexico	*Corallorhiza involuta*	Primarily autogamous, xenogamy unobserved	7	0	0	1	0	no var	no var	no var	no var		
		Virginia, West Virginia, USA	*Corallorhiza bentleyi*	Primarily autogamous, xenogamy unobserved	6	0	0	1	0	no var	no var	no var	no var		
												0.04 (phi-is, overall)	0.45[b] (phi-ct, overall)		
	C. striata complex (plastid DNA)	Southwestern USA, Mexico	var. *vreelandii*	Facultatively xenogamous; partial autogamy	54	11	0.0011	14	0.86	n/a	n/a	n/a	n/a	Sequences from 2 plastid regions	Barrett et al. (2010); Barrett and Freudenstein (2011)
		Northern USA, southern Canada	var. *striata*	Primarily xenogamous; some autogamy	94	18	0.0019	15	0.8	n/a	n/a	n/a	n/a		
		Sierra Nevada, California, USA	Californian populations	Primarily xenogamous; some autogamy	39	4	0.0005	5	0.71	n/a	n/a	n/a	n/a		
		Mexico	*C. involuta*	Primarily autogamous, xenogamy unobserved	8	1	n/a	2	0.24	n/a	n/a	n/a	n/a		
		Virginia, West Virginia, USA	*C. bentleyi*	Primarily autogamous, xenogamy unobserved	7	0	0	1	0	n/a	n/a	n/a	n/a		
												n/a	0.45[b] (phi-ct, overall)		

(continued)

Table 6.1 (continued)

Family (Subfamily)	Species	Sampling distirbution	Natural forms/variants	Breeding system	*N*	*S*	π	*#h*	*hd*	He	Ho	F_{is} (f)	F_{st} (theta)	Data type	Citation
Orchidaceae (Epidendroideae: Bletiinae)	*H. arizonica*	Texas, New Mexico, Arizona		Mostly autogamous, some falcultatively xenogamous in Arizona	15	1	0.0002	2	0.16	n/a	n/a	n/a	0.9923 (4)	Sequences from 6 plastid regions	Unpublished, sequence data from Kennedy and Watson (2010)
	H. colemanii	Arizona		facultatively xenogamous	4	1	0.0002	2	0.5	n/a	n/a	n/a	0.9926		
	H. nitida	Sierra Madre Oriental, Mexico; Texas, New Mexico		mostly autogamous, some falcultatively xenogamous	5	2	0.000399	3	0,7	n/a	n/a	n/a	0.9923		
	H. parviflora	Western Mexico and northern Guatemala		Xenogamy observed, autogamy not observed	3	4	0.001197	3	1	n/a	n/a	n/a	0.9914		
	H. revoluta	Sierra Madre Oriental, Mexico; New Mexico, Texas; USA		Facultatively xenogamous	5	0	0	1	0	n/a	n/a	n/a	0.9935		
	H. spicata	Northern Mexico; Arizona east through Texas and the eastern US north to Virginia		Only xenogamy observed	11	3	0.0004	4	0.69	n/a	n/a	n/a	0.9917		
													Overall *F*st=0.9923		
Orchidaceae (Epidendroideae: Calypsoeae)	*Corallorhiza maculata*	USA except the Great Plains and Southeast, Northern Canada		Primarily autogamous, facultatively xenogamous	47					0.575	0.140[b]	0.7622	0.2821	Microsatellites	Hopkins and Taylor (2011)
	Corallorhiza mertensiana	Western USA, Western Canada and Alaska		Partially autogamous; little data	10					0.527	0.575				

N sample size, *S* # segregating sites, π nucleotide diversity, *#h* number of alleles/haplotypes (nuclear DNA/organellar DNA), *hd* haplotype diversity, *He* expected heterozygosity, *Ho* observed heterozygosity

[a]=for *C. striata* nrDNA, mean of F3H intron II, RPB2 5′ region, RPB2 3′ region

[b]Indicates significant deviation from H–W frequency, F_{is} (*f*)=inbreeding coefficient, F_{st}=proportion of variation between demes (phi-ct=proportion of variation between groupings of CLADES (4 hierarchical levels))

6.2 Population Genetic Analysis of *Hypopitys monotropa* (Ericaceae)

The Monotropoideae are eudicots belonging to the Ericales, and include the fully mycoheterotrophic genera *Allotropa, Pleuricospora, Pterospora, Sarcodes, Monotropa, Hypopitys, Monotropastrum, Monotropsis, Pityopus* and *Hemitomes* (Chap. 2). Though the green genus *Pyrola* has traditionally been placed within the Monotropoideae, molecular systematic studies have not yet resolved the closest photosynthetic relatives to the fully mycoheterotrophic Monotropoideae (Cullings 2000; Kron et al. 2002).

Studies of the Monotropoideae have historically played a fundamental role in enhancing our understanding of the biology of mycoheterotrophic plants and their fungal associates. Specifically, *Hypopitys monotropa* Crantz (syn. *Monotropa hypopitys* L.), has functioned as a model system for investigating many aspects of the biology of mycoheterotrophic plants, with pioneering investigations including the nature of mycorrhizal infections (Björkman 1960), mycorrhizal symbioses (e.g., Bidartondo and Bruns 2002, 2005; Leake et al. 2004), developmental biology (Olson 1990, 1993), reproductive ecology (Klooster and Culley 2009), and the life history chronology of mycoheterotrophic plants from seed to reproductively mature adult (Leake et al. 2004; Bidartondo and Bruns 2005). Despite the importance of this species to our understanding of mycoheterotrophic plants, *H. monotropa* has also created much confusion among scientists since it was first described by Carl Linnaeus over 250 years ago as *M. hypopitys*. Ironically, the taxonomic fate of this species was predestined for quandary from its very inception, as even Linnaeus misspelled the species name "*hypopithys.*"

Taxonomists have long struggled to resolve the relationships among various color forms and morphs of *H. monotropa* throughout its circumboreal distribution. In fact, the species has undergone over 85 taxonomic rearrangements dating back to Linneaus' initial, errant circumscription, with classifications ranging from multiple species within the genus *Hypopitys*, to a single species within the genus *Monotropa*, with ascribed subspecies, variety, and form epithets. Much of this confusion arises from the lack of thorough ecological investigations in this system. Additionally, many taxonomic arguments have been constructed from assessments of pressed herbaria specimens, with the dramatic changes in color and loss of diagnostic morphological features of dried tissues clouding our resolution to effectively assign taxonomic identities. The taxonomic dilemma of *H. monotropa* was further investigated over the past 10 years through the use of molecular systematics, with studies by Cullings (2000), Bidartondo and Bruns (2002), and Neyland (2004). Despite these thoughtful attempts to elucidate the true taxonomic identity of *H. monotropa*, our understanding of the evolutionary ecology of the various color forms and morphs has remained unresolved.

A recent study of the reproductive ecology of two genera within the Monotropoideae (Klooster and Culley 2009) identified distinct ecological differences between color forms within *H. monotropa*. Specifically, this study reconfirmed the presence of discrete blooming periods presented by Neyland (2004), with the yellow color form exhibiting a summer blooming phenology (June–August) and the red color blooming in the fall (September–October). Also, breeding system differences were identified between populations of each color form, with the yellow form exhibiting a mixed breeding system with high rates of autogamous, self-pollination, and the red form approaching herkogamy (spatial separation between anthers and stigma) and facultative xenogamy (movement of pollen among genetically distinct plants; see Chap. 7). The functional data presented in this study supported the hypothesis that forms within *H. monotropa* possess some ecological differences that extend beyond natural plasticity in colouration and that may be attributable to genetic variation.

Because phylogenetic analyses had previously been somewhat unsuccessful at assigning a conclusive genetic identity to the color forms of *H. monotropa*, Klooster and Culley (Klooster and

Culley 2010) utilized population genetic techniques to investigate *H. monotropa*. Using seven populations consisting of red and yellow color forms of *H. monotropa* growing in sympatry and allopatry in the Ohio River Valley, USA, Klooster and Culley assessed levels of genetic variation within populations, genetic differentiation among populations, and genetic structuring by color form. Results from this investigation demonstrated low to moderate levels of within-population genetic variation, with higher variation present in the facultatively xenogamous, red color form, and relatively low levels occurring within the primarily autogamous yellow form (see Table 6.1). Furthermore, pair-wise comparisons of some populations of the yellow color form exhibited high levels of genetic differentiation, although this did not significantly correlate with geographic distance, demonstrating the possibility that various extrinsic and/or intrinsic factors may be contributing to genetic substructuring within the yellow color form. All pair-wise comparisons between yellow and red color forms growing in both allopatry and sympatry indicated high levels of genetic differentiation (Table 6.1). Finally, population genetic spatial analyses revealed high levels of genetic structuring by color form, indicating minimal gene-flow and strong genetic divergence between color forms, suggesting reproductive isolation and corresponding speciation between "color forms."

The synergistic culmination of historical, ecological, reproductive, and genetic analyses of *H. monotropa* conclusively demonstrates that forms of *H. monotropa* possess discrete traits which merit taxonomic recognition as separate species. Also, these multifaceted analyses illuminate "hidden" divergence between populations that might have otherwise gone ignored given the minimal and sometimes ambiguous morphological differences present in this system. In both of the aforementioned studies, minimal to no morphologically discrete features revealed the existence of intriguing genetic differences and informative patterns. Clearly, merging fine-scale population genetic analysis with broad scale phylogeographic assessments will provide a vastly enhanced understanding of the evolutionary history of *H. monotropa*, as we have only begun to scratch the surface of ecological genetic discoveries.

In 2011a, Beatty and Provan published a complimentary study examining peripheral populations of the yellow form of *H. monotropa* in Northern Ireland (Beatty and Provan 2011a). These remnant populations are located at the western edge of the species European range and are small and highly fragmented. Microsatellite analysis of the 21 extant populations, which occur in two separate areas, revealed high levels of genetic diversity, genetic structuring and inbreeding. *H. monotropa* is a highly self-compatible species (Klooster and Culley 2009) and with numbers of individuals in patches generally low, the high incidence of inbreeding is probably due to increased self-pollination. Reproduction in these populations was found to be predominantly sexual, but several small clones were detected. This is in contrast to *Orthilia secunda*, another member of the Monotropoideae that exhibits a similar distribution to *H. monotropa* in Northern Ireland, where all populations studied each comprised a single clone (Beatty et al. 2008). Although typical range-edge population dynamics (small and fragmented populations) were evident for both species, it is thought that the observed switch to clonal growth in *O. secunda* might reflect the response of this boreal species to global climate change.

A larger scale phylogeographic study by Beatty and Provan (Beatty and Provan 2011b) investigated the glacial history of *H. monotropa* in North America. The species exhibits an East–West disjunct distribution, with additional pockets of the species found in central North America. Phylogeographic analysis using the chloroplast *rps*2 gene, the nuclear internal transcribed spacer (ITS) region and eight microsatellite loci revealed that the present-day distribution is due to persistence in separate eastern and western refugia during the last glaciation. Patterns of genetic variation, namely levels of diversity and the occurrence of unique haplotypes, indicated two western refugia in Oregon and further north in the Alexander Archipelago. In the eastern part of the species range, refugia were identified in

the south, around the area of the Carolinas, as well as further north in the "driftless region," just south of the ice sheets. Due to the parasitic nature of *H. monotropa*, presence of a host tree and associated fungal species would have been fundamental to existence in any refugial location, and palynological records confirmed the existence of host tree species in all areas during the glaciations. Unlike *H. monotropa, O. secunda*, which has a similar contemporary East–West disjunct distribution, was confined to exclusively western refugia during the LGM based on chloroplast sequencing and nuclear microsatellites (Beatty and Provan 2010). Its present-day distribution has resulted from eastward postglacial recolonization following the retreat of the ice sheets, and loss of central populations as forests were replaced by the grasslands of the Great Plains.

Phylogeographic studies in Europe again revealed some differences between the two species (Beatty and Provan 2011c). In this case, patterns of genetic variation generally corresponded more to the classic scenario of "southern richness vs. northern purity" observed in numerous European phylogeographic studies (Taberlet et al. 1998; Hewitt 1999; Provan and Bennett 2008). Southern refugia were identified in the Balkans/southeast Europe for *H. monotropa*, and in the French and Austrian Alps and Slovakia for *O. secunda*. Populations of *H. monotropa* in recolonized areas, however, were much more genetically depauperate than those of *O. secunda*, possibly indicating more northerly persistence of the latter species, which is cold-tolerant, during the glaciations. Models of future species distributions under climate change scenarios suggested that loss of rear-edge populations will have a disproportionally greater effect on *H. monotropa* than in *O. secunda*, since these populations harbor the majority of the genetic variation. A similar scenario has recently been reported in other species (Alsos et al. 2012; Provan and Maggs 2012), and these studies highlight the importance of taking into account the distribution of genetic variation across species ranges when considering the potential effects of climate change and population extinction (Hampe and Petit 2005).

The extrinsic and intrinsic mechanisms underlying the observed population genetic patterns and possible cryptic species in *H. monotropa* still remain primarily inferential. Continuing such analyses, coupled with a corresponding investigation into the identity and diversity of *Tricholoma* fungal associates will more completely elucidate both historic and contemporary factors influencing the evolutionary ecology of this fascinating symbiosis. Given the intrinsic reliance of mycoheterotrophic taxa upon their fungal associates, it is possible that a substantial portion of the population genetic patterns discovered in the aforementioned studies will correspond with equally intriguing evolutionary dynamics of interactions with their fungal partners. Also, human-mediated habitat destruction and fragmentation has likely exacerbated the degree of genetic structuring among populations, contributing to further reproductive isolation and divergence of populations. Consequently, ecological investigations coupled with genetic analyses are required to better understand the evolutionary processes behind speciation within and among lineages of the Monotropoideae.

6.3 Hybrid Origin of Monotropoid Taxa

The process of hybridization as a mechanism of speciation within the Monotropoideae has been proposed numerous times throughout the literature. Specifically, Wallace (1975) observed intermediate pinkish color forms of *H. monotropa* growing among yellow and red forms in the western United States, suggesting the possibility that hybridization may be responsible for producing intermediate, hybrid color forms. Also, Cullings (2000) found polyphyletic placement of *H. monotropa* within his phylogenetic reassessment and suggested the possibility of having sampled individuals arising from hybridization between *H. monotropa* and *Pterospora* growing in sympatry in Yellowstone National Park, Wyoming, USA or, alternatively, he argued that the samples represented a cryptic new species that is morphologically similar to *H. monotropa*.

It has also been suggested that *Pityopus californicus* represents a possible hybrid between the parent lineages of *Hemitomes congestum*, *Pleuricospora fibriolata*, and *H. monotropa* (G. Wallace pers. comm., in Cullings 2000; Neyland 2005). Given the high degree of morphological similarity between *P. californicus* and *H. monotropa*, both species were once classified within the genus *Monotropa* and *P. californicus* was later given the name *Hypopitys californica* (Eastwood 1897, 1902). Cullings (2000) and Neyland (2005) further entertained the suggestion that *Pityopus* arose from hybrid origin, although subsequent molecular analyses failed to conclusively support this theory.

Although hybridization remains a viable mechanism for speciation in the Monotropoideae and may account for some of the morphological and molecular variation observed within particular lineages, it has not yet been empirically shown to occur. Additionally, it is unclear how hybridization might impact the ability of a mycoheterotroph to successfully recruit and associate with fungal hosts. The many factors that have limited empirical assessment of hybridization in this and similar systems include high level of molecular divergence among lineages, discrete reproductive phenologies that preclude cross-pollination manipulations, and our inability to cultivate these mycoheterotrophic species in controlled settings.

6.4 *Hexalectris spicata* Species Complex

Hexalectris is a New World genus of nine fully mycoheterotrophic terrestrial orchid species belonging to the derived "higher" Epidendroideae, and appears to be most closely related to the photosynthetic genera *Basiphyllaea* and *Bletia*, collectively the Bletiinae (Goldman et al. 2002; Sosa 2007). Within *Hexalectris* are six species that comprise the *H. spicata* species complex and share a floral architecture that is distinctive from those of the remaining species, *H. brevicaulis*, *H. grandiflora*, and *H. warnockii* (Goldman et al. 2001). Morphological variation among members of the *H. spicata* complex is primarily in terms of floral characteristics, although height, thickness, and color are also informational. Floral variation within these species is relatively low; however higher for the more wide-ranging members, such as *H. arizonica* and *H. nitida* which each exhibit chasmogamous (open-flowered) and cleistogamous (closed-flowered) floral forms with an accompanying loss of rostellar flaps and reduced flower size.

Members of the *H. spicata* species complex range throughout the distribution of *Hexalectris*, which generally follows the mountainous regions of Mexico (Sierra Madre Occidental, Sierra Madre Oriental, Sierra Madre del Sur, and the Trans-Mexican Volcanic Belt) and their extensions into the United States. Observed species richness is at its height in these mountainous regions near the borders between Texas, USA and Coahuila, MX; and Arizona, USA and Sonora, MX. Overlaying species-level distributions while considering phylogenetic relationships within this complex (Kennedy and Watson 2010) reveals a high degree of sympatry between well-supported sister species and suggests that the Sierra Madre Oriental and the Sierra Madre Occidental have facilitated independent northward migration routes for this radiation and potential contact zones for hypothesized hybrid progenitors (Kennedy and Watson 2010).

Members of the *H. spicata* species complex persist mostly undetected due to rarity and inconspicuous and inconsistent flowering patterns across this distribution. Collectively this group exploits a wide array of habitats from tropical dry forests of western Mexico, oak-lined desert canyons of the Santa Rita Mountains, juniper woodlands of the Edwards Plateau, mixed conifer-hardwood forests on the peaks of the Chisos Mountains in the Big Bend, to the mixed mesophytic hardwood forests of Appalachia (Liggio and Liggio 1999; Coleman 2002; Kennedy and Watson 2010).

Independent and combined phylogenetic analyses of the nuclear ITS region and six plastid

DNA regions (*trnL*$^{(uaa)}$ intron (*trnL*), *trnL*$^{(uaa)}$—*trnF*$^{(gaa)}$ intergenic spacer (*trnL-F* IGS), *matK*, *psbA*, *atpA*, and *accD*) revealed strong support for the monophyly of this complex and six phylogenetic species, *H. spicata*, *H. arizonica*, *H. nitida*, *H. revoluta*, *H. colemanii*, and *H. parviflora* (Kennedy and Watson 2010). The derived phylogenetic position of the *H. spicata* complex within *Hexalectris*, a small number of morphological synapomorphies for each species, a low level of differentiation in ITS sequences, and poor support for the position of some clades in all trees suggests a recent, rapid, and continuing radiation in this lineage (Kennedy and Watson 2010). Hybridization has been proposed as an explanation for morphological intermediacy in floral traits in some species (Catling and Engel 1993), and for incongruent phylogenetic positions of some clades between plastid and nuclear gene trees (Kennedy and Watson 2010). However, strong evidence for hybridization remains elusive. For example, Kennedy and Watson (2010) detected no evidence for hybridization after statistically testing for recombination among ITS clones from putative hybrid species. Also, population genetic analyses of several plastid DNA regions reveals that genetic diversity is almost entirely distributed among species (Table 6.1), providing evidence that although hybridization may have occurred historically, each modern lineage is likely reproductively isolated and, therefore, an independent biological species (Kennedy et al. unpublished data).

A particularly intriguing example of potential historical hybridization may be found within the morphological species *H. spicata* s.l., which contains the cryptic phylogenetic species *H. spicata* and *H. arizonica* (Fig. 6.1; Kennedy and Watson 2010). *H. spicata* s.l. ranges from Virginia, south to central Florida, and west to Missouri and Arizona. Its varieties, *spicata* and *arizonica*, may be distinguished based on the presence or absence of a rostellar flap, respectively (Catling and Engel 1993). In the eastern portion of this species' range (i.e., east of Texas) only chasmogamous plants with a well-developed rostellum may be found, whereas cleistogamous and chasmogamous plants with a variety of rostellum development may be found throughout Texas and Arizona (Kennedy and Watson 2010). In Texas, differentiating between these taxa is clear; closed-flowered plants lack rostellar flaps and open-flowered plants have well-developed flaps. However in Arizona, plants may be found with almost any combination of these characteristics, including open flowers and no rostellar flap, making identification difficult. Phylogenetic analyses provided strong support for two clades, one containing only plants with open flowers and a well-developed rostellum from the eastern portion of the distribution and Texas (i.e., *H. spicata* s.s.), and another comprised exclusively of plants with cleistogamous flowers lacking a rostellum from throughout Texas and all plants from Arizona (i.e., *H. arizonica*). Despite this clarity, incongruent positions of the *H. arizonica* clade between nuclear and plastid gene trees suggested that this lineage may be the product of hybridization between *H. spicata* s.s. or *H. nitida* and some other member of the *H. spicata* species complex (Kennedy and Watson 2010).

Hexalectris species maintain long-lived rhizomes with highly reduced roots that are the sites for mycorrhizal colonization (Taylor et al. 2003). Members of the *H. spicata* complex associate nearly exclusively with fungi from Sebacinaceae subgroup A (Taylor et al. 2003; Kennedy et al. 2011), a group of ectomycorrhizal fungi known to simultaneously form endomycorrhizas with orchids (Selosse et al. 2002a, b, 2009). Specificity, as measured by the average genetic pair-wise distances between fungal associates of a particular *Hexalectris* species (i.e., π; Nei and Tajima 1981), revealed narrow associations within each species, ranging between 0.012 in *H. arizonica* and 0.047 in *H. spicata* (Kennedy et al. 2011). Interestingly, the two primarily self-pollinating species in this group, *H. arizonica* and *H. nitida*, are the most highly specialized toward their mycorrhizal fungi (0.012 and 0.021, respectively), suggesting that specialization toward mycorrhizal partnerships may be narrowed with the loss of genetic diversity that accompanies self-pollination (Kennedy et al. 2011). From a phylogenetic perspective, each species within this complex was identified to associate with a unique clade or group of clades within

Fig. 6.1 Photos of the phylogenetic species that comprise the *Hexalectris spicata* species complex (*sensu* Kennedy and Watson 2010). *H. spicata* (a-b, Harrison Co., Indiana, USA) and the open-flowered form of *H. arizonica* (c, Pima Co., Arizona, USA) are difficult to distinguish morphologically, but fortunately for identification purposes only the closed-flowered form of *H. arizonica* (d, Santa Cruz Co., Arizona, USA; e, Dallas Co., Texas, USA) is sympatric with *H. spicata*. *H. nitida* also has open- and closed-flowered forms (f, Brewster Co., Texas, USA; g, Dallas Co., Texas, USA; respectively). *H. parviflora* (h, Cuquío, Jalisco, Mexico), *H. colemanii* (i, Pima Co., Arizona, USA), and *H. revoluta* (j, Brewster Co., Texas, USA) complete this species complex. Photographs by Aaron H. Kennedy

Sebacinaceae subgroup A, suggesting that specialization is not only narrow within this complex, and narrower within each of its species, but that specificity is a rapidly evolving characteristic.

Finally, these data also revealed that many of the mycorrhizal fungi that *H. spicata* species complex members specialize on are widely distributed across North America and have largely sympatric distributions. This finding supported the conclusion that the geographic distributions of *Hexalectris* species may not be influenced by their fungal associates' distributions, a finding also made in *Cypripedium* (Shefferson et al. 2007). For example, the sebacinaceous fungi identified from *H. spicata* in North Carolina alone span nearly the entire breadth of associations formed by this species across its total geographic distribution, revealing wide distribution for these sebacinaceous fungi. Also, even though the extremely rare *H. colemanii* is restricted to only a few populations in southern Arizona, the fungi identified from two populations in highly similar habitats and only a few dozen kilometers apart were widely distant members of Sebacinaceae subgroup A, and identified from other members of the *H. spicata* species complex ranging from western Mexico to the eastern United States (Kennedy et al. 2011). These findings therefore also suggest that mycorrhizal host-jumps and preferences have evolved largely where *Hexalectris* species and their potential mycorrhizal fungi exist in sympatry.

6.5 The Genus *Corallorhiza*

Corallorhiza is a new world genus of about ten mycoheterotrophic species that falls within the Epidendroideae, with the closest leafy relatives belonging to the genera *Cremastra*, *Oreorchis* (both Asian), and *Aplectrum* (North American) (Freudenstein and Senyo 2008). *Corallorhiza* species occupy temperate forests across North America; only *Corallorhiza trifida* has spread widely across boreal regions, achieving a pan-Arctic distribution.

6.5.1 *Corallorhiza striata* Complex

From a phylogeographic perspective, *Corallorhiza striata* (Orchidaceae) is one of the most comprehensively studied fully mycoheterotrophic species complexes. This wide-ranging, North American, temperate-montane group displays extensive geographic variation in floral morphology (Fig. 6.2; Freudenstein 1997; Barrett and Freudenstein 2009, 2011). Using plastid DNA

Fig. 6.2 The *Corallorhiza striata* species complex. From the left, *Corallorhiza involuta* (Morelos, Mexico), *Corallorhiza bentleyi* (cleistogamous population, Virginia, USA), *C. striata vreelandii* (New Mexico, USA), *C. striata* from California, USA, and *C. striata striata* (the only known population in New York State, USA). Photographs by Craig Barrett and John Freudenstein

(*rbcL*, *rpl32-trnL*), Barrett and Freudenstein (2009) demonstrated that the *C. striata* complex also displays substantial genetic diversity ($n = 84$ individuals). Four largely allopatric plastid DNA clades were identified across North America, and no populations were found to harbor members of more than one clade, even in potential contact zones. These clades are distributed as follows: clade (a) *Corallorhiza involuta* (Mexico) + *Corallorhiza bentleyi* (Virginia and West Virginia, USA), clade (b) Sierra Nevadan clade (California, USA), clade (c) *C. striata* var. *vreelandii* (southwestern USA, Mexico, and Newfoundland, Canada), and clade (d) *C. striata* var. *striata* (northern USA, Canada). Overall flower size in this complex appears to be clinal, roughly increasing with latitude (Freudenstein 1997). The incorporation of plastid DNA information gives a different perspective on morphological variation, with each clade corresponding to a distinct morphometric grouping.

The four plastid clades associate with overlapping—yet significantly divergent—members of a single, highly variable, ectomycorrhizal fungal taxon, *Tomentella fuscocinerea* (Fig. 6.3; Barrett et al. 2010). This fungal species may, in fact, be composed of 9–12 cryptic species, based on 3 % and 2.5 % nuclear ITS divergence criteria, respectively. Thus, the *C. striata* complex has highly specific nutritional preferences across the entire geographic range. Overall, gene trees based on plastid DNA (*C. striata* complex) and ITS (*T. fuscocinerea*) were incongruent but also significantly nonindependent, suggesting some level of cophylogeographic structure (Fig. 6.3). In particular, the plastid clade endemic to California associates almost exclusively with a single ITS clade of *T. fuscocinerea*; this finding was consistent across multiple populations along a ca. 600 km transect through the Sierra Nevada. Overall, patterns of subspecificity within the *C. striata* complex reveal a "geographic mosaic" (Thompson 1994, 2005), with orchid plastid types associating with divergent sets of *T. fuscocinerea* fungi in different biogeographic regions of North America. This study represents one of the most extensive phylogeographic investigations of host specificity for any mycoheterotroph to include both plant and fungal DNA, and has conservation implications for *C. striata* and the habitats in which these and several other mycoheterotrophic species occur. Analysis of the *C. striata* complex based on nuclear intron sequences (flavanone-3 hydroxylase, RNA polymerase II beta subunit) showed less geographic structuring than did plastid DNA, with alleles shared between plastid groupings/geographic regions. *C. bentleyi* (eastern USA) and *C. involuta* were genetically identical for all loci, forming a clade that was highly divergent from the remaining *C. striata* (= *sensu stricto*). A closer investigation of population structure within *C. striata* s.s. identified three geographically parapatric clusters, corresponding to var. *vreelandii*, var. *striata*, and Californian populations Interestingly, a few individuals of both var. *striata* and Californian populations displayed admixed multilocus genotypes, suggesting either limited gene-flow between groupings or residual ancestral polymorphism. Multilocus distance estimates of relationships within *C. striata sensu stricto* based on nuclear alleles indicated strong evidence for divergence between var. *vreelandii* and var. *striata*, with Californian individuals occupying intermediate positions relative to both. *C. striata*, *C. striata vreelandii*, and the Californian accessions (i.e., var. *californica* ined) are best described at the level of variety, thus comprising a widespread, highly variable (and geographically structured) species, *C. striata sensu stricto*. Furthermore, the varieties therein represent evolutionarily significant units (ESUs) for conservation purposes (*sensu* Dizon et al. 1992; Moritz 1994).

Based on cumulative integration of genetic, morphological, geographic, phenological, and reproductive-mode variation, there is evidence to suggest that the *C. striata* complex is composed of three species: *C. bentleyi*, *C. involuta*, and *C. striata* s.s. There is ample evidence for largely autogamous modes of reproduction in both *C. bentleyi* and *C. involuta* based on: (1) small, drab-colored, partially closed flowers (some populations of *C. bentleyi* are fully cleistogamous), (2) the tendency for all flowers in a raceme to set seed (even before anthesis), and (3) low plastid/

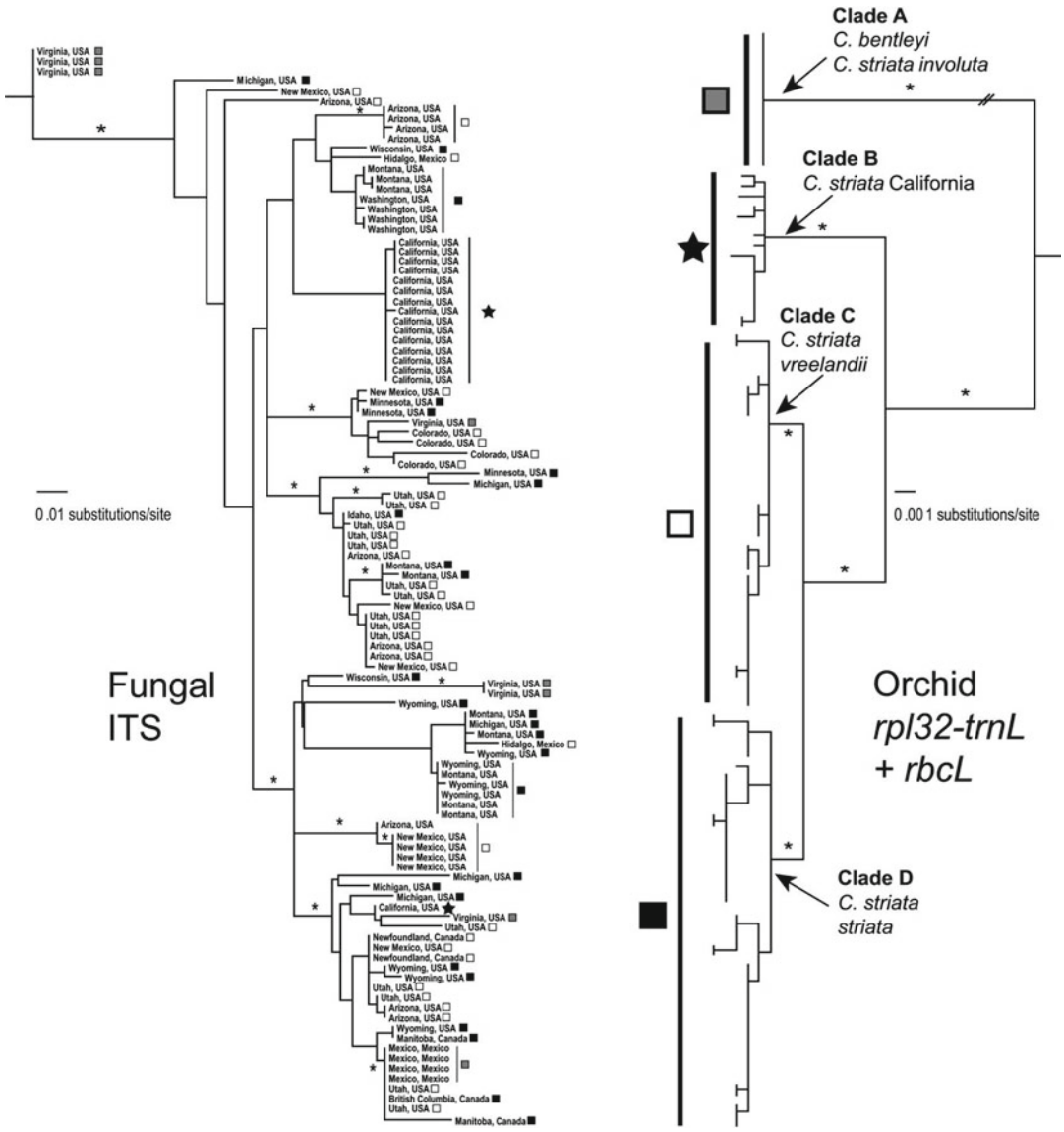

Fig. 6.3 Comparison of fungal internal transcribed spacer (ITS) and orchid plastid *rbcL*+*rpl32-trnL* (right) gene trees for a rangewide sampling of *Corallorhiza striata* complex populations across North America. Trees are represented by highest likelihood topologies under the GTR-Γ model in RAxML (Stamatakis 2006). Asterisks adjacent to branches indicate likelihood bootstrap values > 90 % based on 2,000 pseudoreplicates. *Left scale bar* (fungi) = 0.01 substitutions/site and right scale bar (orchids) = 0.001 substitutions/site. Accessions in fungal ITS tree are coded by US/Mexican State, or by Canadian Province, and with symbols corresponding to orchid DNA clade (also found on orchid DNA tree). Clade A (*Corallorhiza bentleyi, Corallorhiza involuta*) = *gray squares*; Clade B (Californian *C. striata*) = *black stars*; Clade C (*C. striata vreelandii*) = *empty squares*; Clade D (*C. striata striata*) = black squares

nuclear genetic diversity. The latter observation may be a cause for conservation concern, in that adaptive potential (similar to neutral variation) could be reduced in autogamous, genetically depauperate species, rendering them unable to cope with future environmental fluctuations (e.g., climate change). Alternatively, they may represent highly adapted, fixed genotypes in populations

that have been historically purged of deleterious alleles; this certainly deserves further study.

C. striata s.s. displays morphological and genetic attributes concordant with a xenogamous reproductive mode. Several observations have been made of visitation and pollinium transfer to/from flowers by ichneumonid wasps known to pollinate *C. striata* (Freudenstein 1997; C. Barrett, pers. observ.). *C. striata* s.s. is likely to be reproductively isolated from *C. involuta* and *C. bentleyi*, and the same is also true between the latter two entities based on their autogamous reproductive modes and the immense geographic distances separating them.

Members of the *C. striata* complex are of great conservation interest, because they are good candidates as both "indicator" and "umbrella" species. The former concept refers to the idea that *C. striata* represents a window into otherwise elusive soil processes; its fungal host (*T. fuscocinerea*) is rare and extremely ephemeral (in terms of sporocarp formation), having been collected very few times (U. Kõljalg, pers. comm.). Thus, *C. striata* is highly sensitive to habitat perturbations affecting soil microbial processes, and is typically only found in old-growth habitats. This *C. striata* complex is also composed of umbrella species in that any conservation decisions focused toward them will most certainly benefit co-occurring species, many of which are sensitive/rare as well (e.g., other orchids and mycoheterotrophs).

6.5.2 *Corallorhiza wisteriana–odontorhiza* Complex

Corallorhiza odontorhiza ranges from Nicaragua northward through Mexico into the eastern USA/Canada. *Corallorhiza wisteriana* is distributed from Chiapas, Mexico through the Sierras Madre Oriental and Occidental, into the USA (Freudenstein 1997). In the USA, the latter becomes disjunct between the southeastern USA and a portion of the Rocky Mountains to the west, separated by the Great Plains and arid regions of north-central Mexico (Freudenstein 1997). Both species have similar flowering times in Mexico (winter), but diverge phenologically in more northern populations. In the USA, *C. wisteriana* flowers in the spring, while *C. odontorhiza* flowers in the autumn. Both species show evidence for a largely autogamous reproductive mode (e.g., *C. odontorhiza* is mostly cleistogamous), but this remains to be further investigated in the group as a whole. In the eastern US portion of its range, *C. wisteriana* primarily occurs in deciduous forests, whereas in the West and in Mexico, it occurs in higher elevation conifer forests. Plastid DNA analyses based on *rpl32-trnL* and *trnL-F* indicate that *C. odontorhiza* forms a clade, with three Mexican accessions sister to those from eastern US populations (Freudenstein and Barrett, unpublished data). There seems to be little additional phylogeographic structure within this species, with multiple plastid types often occurring in the same population. Sister to all *C. odontorhiza* populations are Mexican accessions of *C. wisteriana*, with the remainder of *C. wisteriana* sister to this collective assemblage. Analyses based on plastid DNA within *C. wisteriana* from the USA ($n = 40$ individuals; *rpl32-trnL* + *trnL-F*) indicate two divergent haplotypes, corresponding to eastern and western populations; however, these loci are invariant within populations. Patterns based on plastid DNA are consistent with an origin of the group in Mexico, followed by migration northward and divergence in flowering times. More variable markers will be needed to better address population genetics and patterns of gene-flow within and between both species.

C. wisteriana presents an intriguing example of allopatric genetic differentiation that is correlated with differences in habitat preferences and a major shift in fungal associations. First, both *C. odontorhiza* and western-US populations of *C. wisteriana* associate with members of the genus *Tomentella* (Thelephoraceae), while in the eastern USA, *C. wisteriana* associates with Russulaceae based on fungal ITS sequencing (Taylor 1997; J. Freudenstein and Barrett, unpublished data). The earliest-diverging lineages of *Corallorhiza* (*C. striata, C. trifida*) typically associate with Thelephoraceae (Taylor 1997; McKendrick et al. 2000b; Barrett et al. 2010), so association with Russulaceae in the eastern USA may represent a geographic/habitat-correlated host shift, but a broader genus-level investigation is warranted to determine the polarity of host

shifts. It remains to be investigated whether these plastid DNA, habitat, and fungal host correlations persist toward the southern extent of the geographic range in Mexico.

6.5.3 *Corallorhiza maculata–mertensiana* Complex

Corallorhiza maculata, or the spotted coral root orchid, is one of the most abundant terrestrial orchids in North America (Coleman 1995) and, in particular, the most likely encountered species of *Corallorhiza* (Freudenstein and Doyle 1994). The species ranges from Mexico to Canada in western North America and can be found throughout the rest of the United States with the exception of the Great Plains region and the deep Southeast (Freudenstein 1997). In general, the plants are found in shaded areas of both deciduous and coniferous old-growth forests. This species can occur singly or in large clumps (Luer 1975). It has long been acknowledged that there is a large amount of floral variation within the species, with many populations encompassing morphologically dissimilar individuals (Freudenstein 1997). While this morphological variation can be quite useful as a proxy for genetic relatedness, it is not reliable enough to supplant the need for DNA sequencing and other molecular markers (Freudenstein 1997).

C. maculata is often referred to as the *C. maculata* complex because it is composed of several recognized varieties: *C. maculata* var. *maculata*, *C. maculata* var. *occidentalis* (both occurring throughout the entire range), and *C. maculata* var. *mexicana* (restricted to Mexico, and possibly bordering states) (Freudenstein 1992, 1997). This complex also includes other closely related species: *C. bulbosa* (Mexico), *C. macrantha* (Mexico), and *Corallorhiza mertensiana* (northwestern North America), the latter of which is likely to be the closest relative to the two aforementioned varieties of *C. maculata*. Analysis of the ITS and the *rbcL* gene, (Barrett and Freudenstein 2008) and other plastid DNA (Freudenstein and Doyle 1994) places *C. mertensiana* within the *C. maculata* clade. Chloroplast RFLP studies have indicated that *C. mertensiana* may be derived from *C. maculata* (Freudenstein and Doyle 1994). The range of *C. mertensiana* is almost entirely contained within the range of *C. maculata* and the two species are often found co-occurring (Taylor and Bruns 1999). In addition to different varieties of *C. maculata*, it has also been observed in the Midwestern and Eastern United States that "early" and "late" flowering forms exist, and correspond to vars. *maculata* and var. *occidentalis* (Freudenstein 1987). The phenology of these individuals is sufficiently distinct that cross-pollination between them is unlikely.

It has long been suspected that *C. maculata* is almost completely autogamous. While capable of outcrossing via pollinators, any flower that has not been pollinated via foreign pollen will self-fertilize, with plants consistently displaying nearly 100 % seed set. Personal observation (S. Hopkins) has provided evidence that plants whose emergence from the soil has been impeded due to a physical obstruction will still flower and experience full seed set underground. Despite its ability to self-pollinate, microsatellite analysis of parents and seeds is revealing more outcrossing than was previously thought to occur (Hopkins and Taylor 2011). Hypothesized pollinators include dance flies (Empididae), Bombyliid flies, and Acroceratid flies (Kipping 1971) and Luer (1975) published a photograph of *C. maculata* being visited by a bee in the genus *Andrena* with pollinia attached to its back.

The question of potential cross-pollination is an interesting one given that *C. maculata* is the first species complex in which diversification of fungal specificity had been shown to exist below the species level. *C. maculata* and *C. mertensiana* both associate with fungi in the Russulaceae, but have never been found to share a *Russula* species, even where the two orchids co-occur (Taylor and Bruns 1999). Furthermore, molecular methods were used to identify six distinct genotypes of *C. maculata* using three single nucleotide polymorphisms (SNPs) in the nuclear ITS region and the nuclear single-copy flavanone beta hydroxylase gene (Taylor et al. 2004). These genotypes, or races, have been shown to associate with separate clades of fungi within the family Russulaceae, with fairly little overlap in fungal associations across races (Taylor et al. 2004). Multiple races have been found growing in the same location but

never utilizing the same fungal partners, demonstrating a genetic control of fungal associations that is not dependent on habitat, and occurs below the species level (Taylor et al. 2004). Current work utilizing 6 polymorphic microsatellite markers on adults and seeds from 15 putative populations with multiple morphotypes suggests that there are a minimum of 6 races present in this species and that considerable population structure exists. Furthermore, these microsatellites show that *C. mertensiana* is genetically distinct from *C. maculata*, despite their paraphyletic relationship in prior chloroplast studies. Preliminary data has indicated that only 1 of the ca. 100 *C. maculata* adults examined may be a hybrid between two different races and that less than 5 % of the seed capsules surveyed display any heterozygosity at the genotyped loci. Along with this distinct lack of gene-flow between populations, individuals of *C. maculata* display extreme fungal specificity. Recent work with microsatellite markers has yielded results in agreement with those above: A single clade of individuals of *C. maculata* will nearly always associate with only a single clade of fungi, or at most two clades of fungi, within the genus *Russula.* In some cases individuals of different genotypes will grow within centimeters of one another, however these different genotypes have never been found to associate with the same fungus or even the same clade of fungi. These results demonstrating fine-scale fungal preference suggest that mycorrhizal specificity is evolving rapidly, with changes occurring among very recently separated biological species or possibly even at the population level.

6.6 Emerging Patterns

The case studies presented above suggest several interesting trends in the ecological genetics of mycoheterotrophic plants. On the other hand, these examples are limited to only a few species, mostly from North American temperate forests (two *Hexalectris* species were sampled in subtropical forests). Therefore, the generality of the patterns discussed below remains to be determined. Nevertheless, the trends are tantalizing. First, we note that species and population boundaries are difficult to discern, even when careful morphological studies and informative molecular markers are combined (Barrett and Freudenstein 2009; Kennedy and Watson 2010). This observation is likely explained, in part, by the mixed mating systems seen in these mycoheterotrophic plants. Nearly all are self-fertile, many have explicit mechanisms for selfing such as cleistogamy (though all appear capable of outcrossing), all have small, widely scattered populations, and all display moderate to high levels of inbreeding. The specter of hybridization as a pathway to novel forms or species has been raised for several taxa, though proven in none. Thus, these plants have complex evolutionary histories, making species and population boundaries extremely dependent on what criteria are used and how they are weighed (Barrett and Freudenstein 2009; Kennedy and Watson 2010). When integrative approaches are applied, the best-studied taxa turn out to be species complexes encompassing one or several cryptic species (Kennedy and Watson 2010; Barrett and Freudenstein 2011; Hopkins and Taylor, unpublished data). From an evolutionary point of view, these plants offer exciting research opportunities, since their particular combination of population dynamics, symbioses and speciation patterns are unusual in the plant world.

Focusing in on the ESUs, host-races, and populations that have been uncovered, we find that genetically discrete demes are often maintained in sympatry or parapatry. Examples include the two color forms of *H. monotropa* (Klooster and Culley 2010), the three western ESUs within *C. striata sensu stricto* (Barrett and Freudenstein 2011), the host-races within *C. maculata* (Taylor et al. 2004), and the western sibling species within the *H. spicata* complex (Kennedy and Watson 2010). However, we do not interpret these patterns to suggest that geographic barriers are unimportant to the diversification of these lineages. There are several very clear examples of geographic patterning of genetic breaks, including East–West disjunctions and associated genetic divergence within *H. monotropa* (Beatty and Provan 2010) and *C. wisteriana*. Thus, we infer that genetic divergence in allopatry has played an

important role in several of these lineages, although the spatial and temporal scales of allopatric diversification remain open questions.

Perhaps the most exciting trend that coincides with the discovery of these cryptic species and ESUs is that these entities have diverged in traits of considerable ecological interest. In some cases, different floral forms or mating systems (color, phenology, cleistogamy) distinguish the lineages. And, in some cases, mycorrhizal specificity has diverged between these closely related lineages. The best examples of the latter trend are the distinct *Sebacina* clades targeted by different members of the *H. spicata* complex (Kennedy et al. 2011) and the distinct *Russula* clades targeted by different *C. maculata* host-races (Taylor et al. 2004; Hopkins, unpublished). These rapid and ongoing evolutionary dynamics open the way for incisive studies of the genes and selective forces underlying ecological diversification. For example, do host-jumps to novel mycorrhizal taxa tend to occur in geographically isolated populations following rare long-distance dispersal? Or do increases in selfing, such as in transitions to cleistogamy, and associated genetic drift and low genetic diversity, precede the evolution of extremely narrow specialization, as suggested for *H. arizonica* and *H. nitida* (Kennedy et al. 2011)?

6.7 Future Questions and Approaches

In the past, developing molecular markers and other tools with which to carry out research on the ecological genetics of a non-model species has been a laborious, costly, and often untenable undertaking. Technological breakthroughs, particularly next-generation sequencing and computational tools, are allowing ecological genetics and genomics to be applied to any organism. We anticipate that this revolution will greatly expand our view of the evolution, ecology and function of mycoheterotrophic plants.

Currently, the major advantage provided by next-generation technologies to studies of non-model organisms is the much greater ease with which variable markers can be discovered and assayed. For example, highly polymorphic microsatellites have been the loci of choice for studies of gene-flow and population structure for the last 20 years. But it has been slow and costly to develop microsatellites for a single new species. Today, a fraction of a single pyrosequencing run, using either random genomic DNA (shotgun sequencing) or microsatellite-enriched genomic DNA, can rapidly yield thousands of loci containing microsatellite motifs (Guichoux et al. 2011). Assays of SNPs are beginning to rival microsatellites in popularity, as methods for genome-wide analysis become more routine. By tagging and pooling individuals prior to a shotgun sequencing run, numerous SNPs can be discovered. In a further refinement of this approach, genomic DNA is cut with a restriction enzyme before tagging, pooling and sequencing (RADseq; Davey and Blaxter 2010). This approach has been used to simultaneously discover and genotype tens of thousands of SNPs spread across the genome in non-model organisms. For studies at or above the species level, where a select set of loci are known to be informative, the target regions can be PCR amplified, ligated to adaptors, pooled and sequenced (De Leeneer et al. 2011), or enriched then sequenced. These target capture methods include molecular inversion probes (MIP; Porreca et al. 2007), solution hybrid selection (SMS; Elshire et al. 2011), and microarray-based genomic selection (MGS; Okou et al. 2007). In this way, hundreds or thousands of defined loci can be sequenced across large numbers of individuals simultaneously.

Improved markers will enable inquiries into numerous aspects of the evolutionary ecology of mycoheterotrophs. In particular, we envision more in-depth investigations of mating systems and gene-flow within and among mycoheterotrophic plant demes, which should provide improved estimates of population and species boundaries. For example, improved likelihood and Bayesian methods for parentage analysis have appeared in recent years (Jones et al. 2010), and could be applied to mycoheterotrophic plants to reveal patterns of pollen movement and gene exchange within and among populations, ESUs and species. Detailed, fine-scale genetic data can

also be used to infer population parameters, such as effective population sizes. This should be particularly informative in the case of mycoheterotrophic plants, in which true population sizes are poorly represented through censuses of above-ground flowering individuals in any one year (Shefferson et al. 2001). Bayesian methods have also been developed for assigning individuals to populations, some of which can be used without any a *priori* population definitions (Pritchard et al. 2000). These methods, in part, have been used to define genetically distinct ESU's within *C. striata sensu stricto*, and to investigate specific cases of admixed multilocus genotypes based on multiple nuclear markers (Barrett and Freudenstein 2011). This will also be particularly useful for the elucidation of population boundaries in *C. maculata*, where widely divergent genotypes occur in sympatry, and population boundaries are thus difficult to define using geographic criteria.

Additional markers will also improve our ability to infer the phylogeographic and demographic histories of mycoheterotrophic plant species. We may be able to infer the migration routes that have led to current geographic ranges, along with the timing and coincident climatic conditions under which changes in distributions occurred. These inferences can be approached by combining geographic reconstruction methods such as nested clade analysis (Templeton 1998), coalescent methods that provide insights into demographic histories in the process of reconstructing the likely time course of mutation events (Donnelly and Tavare 1995; Carbone and Kohn 2001), and niche/climate modeling (Peterson 2001; see Beatty and Provan 2011b for an example). A focal issue may be the geographic, demographic, and temporal scenarios under which mycorrhizal specialization has changed.

New markers and analytical tools also open the way to asking more direct questions about adaptation in non-model organisms. For example, one approach seeks to elucidate the genetic architecture underlying adaptation via estimation of heritability and the use of association tests, which search for statistical correlations between variation in particular alleles and variation in traits of interest. Controlled crosses combined with phenotypic measurements in common environments form the basis for the field of quantitative genetics (Falconer and Mackay 1996). For organisms, such as mycoheterotrophic plants, which cannot be easily crossed or cultivated, related methods have been developed for wild organisms (Ritland 2000). The most powerful of these methods start with at least some pedigree information (e.g., mother-offspring relationships), and attempt to reconstruct missing pedigree information (e.g., via paternity assignment methods), then model trait values as a function of allelic segregation (Garant and Kruuk 2005). Another approach relies on the principles of population genetics and molecular evolution, without reference to observable phenotypic variation. DNA sequences themselves can carry signatures of drift, hitchhiking, population bottlenecks, directional selection, balancing selection, and other evolutionary phenomena (Nordborg 1997; Nielsen 2000; Nielsen and Wakeley 2001; Charlesworth 2006). These methods are best viewed as tools for data exploration, i.e., ways to search for candidate loci, leading to the formulation of testable hypotheses concerning the adaptive significance of particular loci (Storz and Wheat 2010).

In the case of mycoheterotrophic plants, we recommend pursuit of both improved molecular markers and field and/or laboratory manipulations to begin to elucidate the processes and selective pressures underlying ecological variation. For example, hand-pollination experiments combined with seed packet methods (Rasmussen and Whigham 1993) and subsequent genomic scans could be used to begin to quantify the contribution of various mechanisms to reproductive isolation among populations with overlapping geographic ranges, i.e., to ascertain whether isolation is prezygotic or postzygotic, and, therefore, whether there may be selection against hybrids.

Lastly, mycoheterotrophic plant research would benefit from the application of advancing methods in functional genomics. Transcriptional profiling can be used to reveal patterns of gene expression under varying conditions, even in non-model organisms. A flood of gene expression data has come in recent years from the use of

microarrays. However, microarrays are very expensive to design and construct, and, hence, have only been developed for model organisms. There have been several successful uses of microarrays to study gene expression in non-model organisms that are closely related to model species for which microarrays are available (Travers et al. 2007). Unfortunately, to our knowledge, there are no model organisms that are closely related to a mycoheterotrophic plant species. On the other hand, direct next-generation expressed sequence tag (EST) sequencing methods, such as Illumina Hi-seq, can be applied to any organism at costs much lower than microarray design. These approaches could be used to survey the plant and fungal genes that are up or down-regulated at different stages of the interaction (Seddas et al. 2009). Alternatively, gene expression could be compared across species or ESUs with differing fungal specificity, flowering times, or other traits of interest, to search for patterns of gene expression that may underlie these differences.

6.8 Conclusions

While mycoheterotrophic plants may never become common "lab-rat" model organisms, in our view, they have great value as models of specificity and symbiosis-shaped evolution in the unique circumstance in which it is the plant that attacks the fungus, as opposed to the traditional role of plants as victims of attack by numerous pathogens and herbivores. The value as a study system in evolutionary ecology is magnified by the fact that the specialized plant-fungus interaction is evolving rapidly in some mycoheterotrophic plant lineages. The rapid advance of methods in ecological genetics and genomics that can be applied to wild organisms holds tremendous promise for mycoheterotrophic plant research. To most effectively pursue these opportunities, we suggest that one or a few mycoheterotrophic species should receive increased attention from the research community as mycoheterotrophic plant models. *C. trifida* comes to mind, because it has been grown in tripartite symbiotic microcosms (McKendrick et al. 2000a, 2000b) and because a significant amount of physiological work has been carried out (Zimmer et al. 2008; Cameron et al. 2009). However, we still know relatively little about patterns of genetic variation and fungal associations within this taxon (McKendrick et al. 2000b). While the new methods democratize genomics, we cannot yet apply them to every mycoheterotrophic species due to constraints of funding and feasibility. There are still synergistic gains to be made by focusing on species that are most tractable in terms of crossing and growth (in the field, if necessary), and working as a community to develop tools for these species.

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