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Multigene analysis suggests ecological speciation in the fungal pathogen *Claviceps purpurea*

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Abstract

***Claviceps purpurea* is an important pathogen of grasses and source of novel chemical compounds. Three groups within this species (G1, G2 and G3) have been recognized based on habitat association, sclerotia and conidia morphology, as well as alkaloid production. These groups have further been supported by Random Amplification of Polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, suggesting this species may be more accurately described as a species complex. However, all divergent ecotypes can coexist in sympatric populations with no obvious physical barriers to prevent gene flow. In this study, we used both phylogenetic and population genetic analyses to test for speciation within *C. purpurea* using DNA sequences from *ITS*, a *RAS*-like locus, and a portion of beta-tubulin. The G1 types are significantly divergent from the G2/G3 types based on each of the three loci and the combined dataset, whereas the G2/G3 types are more integrated with one another. Although the G2 and G3 lineages have not diverged as much as the G1 lineage based on DNA sequence data, the use of three DNA loci does reliably separate the G2 and G3 lineages. However, the population genetic analyses strongly suggest little to no gene flow occurring between the different ecotypes, and we argue that this process is driven by adaptations to ecological habitats; G1 isolates are associated with terrestrial grasses, G2 isolates are found in wet and shady environments, and G3 isolates are found in salt marsh habitats.**

Keywords: *Claviceps*, fungi, genealogy, genetic structure, speciation

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Introduction

Species concepts have been intensely debated in the literature, and no one concept is generally accepted among biologists. Traditional species concepts for the classification of fungi are based on morphology and reproductive biology, but phylogenetic approaches have become increasingly popular in recent years to resolve species identification (Harrington & Rizzo 1999; Taylor *et al*. 2000). Moreover, phylogenetic analyses have challenged morphological species concepts and have been especially helpful in delineating fungal species with few morphological characters. Phylogenetic approaches have also been sought for the many fungi in which sexual reproduction is not known to occur, making biological species concepts impossible to implement. Phylogenetic studies have routinely identified cryptic species within morphological species in various fungal genera including *Fusarium* (O'Donnell *et al*. 2000; Skovgaard *et al*. 2002), *Stachybotrys* (Cruse *et al*. 2002), *Tricholoma* (Horton 2002), *Coccidioides* and close relatives (Koufopanou *et al*. 2001), *Cenococcum* (Douhan & Rizzo 2005), *Neurospora* (Dettman *et al*. 2003) and lichenized genera such as *Physcia* (Myllys *et al*. 2001) and *Letharia* (Kroken & Taylor 2001).

What has not been determined in most of these studies is the mechanism or driving force behind the speciation process. In some instances, geographical isolation has been suggested, as in *Coccidioides* (Koufopanou *et al*. 2001) and *Fusarium* (O'Donnell *et al*. 2000), whereas in other cases

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these putative cryptic species may be found in the same geographical location and, in some instances, even isolated from the same soil core (Skovgaard *et al*. 2002; Moyersoen *et al*. 2003; Douhan & Rizzo 2005). Ecological theory predicts that the stable coexistence of identical competitors will not occur (Hardin 1960), suggesting that cryptic species occupying the same apparent niche may play different ecological roles. Therefore, ecological factors likely play a significant role in the speciation process of co-occurring organisms. This 'ecological speciation' process has received little attention so far in the fungi. We will adopt the terminology of Rundle & Nosil (2005) that defines 'ecological speciation' as the 'process by which barriers to gene flow evolve between populations as a result of ecologically based divergent selection'. One group of fungi where ecological aspects may have influenced speciation is within the *Claviceps purpurea* complex. These are plant-pathogenic fungi that infect the flowers of grasses and cause a disease known as 'ergot'. The infection results in a replacement of the grass floral tissue with fungal mycelium that grows to form a darkly pigmented survival structure called a sclerotium (Alexopolous *et al*. 1996). Sclerotia of *C. purpurea* contain potent alkaloids that are toxic to humans and other animals (Hudler 1998). This has been important during human history because sclerotia have been unwittingly mixed with grain and the fungal toxins have contaminated bread flour. The consumption of bread tainted with *C. purpurea* causes the debilitating disease known as 'Holy Fire' or 'St Anothony's Fire', which includes a range of symptoms such as itching, headache, hallucinations, gangrene, seizures and even death (Hudler 1998). Despite the negative impacts of *C. purpurea* alkaloids, interesting and beneficial chemical compounds have also been derived from these fungi, including the infamous lysergic acid diethylamide (LSD) and pharmaceuticals used to treat both postpartum bleeding and migraine headaches (Rehacek & Sajdl 1993; Hart 1999).

Pazoutová *et al*. (2000) synthesized previous research on *C. purpurea* morphology, alkaloid chemistry and genetics and identified three distinct groups within the species, describing them as 'chemoraces.' Rather than species delimitation based on host range, which was historically common in *C. purpurea* taxonomy, intraspecific groups were defined based on habitat specialization. The largest group, G1, is associated with land grasses. G2 isolates are associated with grasses in 'wet and shady' environments, whereas G3 isolates are found only on grasses in salt marsh habitats. G3 isolates are synonymous with *C. purpurea* var. *spartinae* (Duncan *et al*. 2002) and are referred to herein as G3. Sclerotia of both G2 and G3 *C. purpurea* float in water while sclerotia from terrestrial *C. purpurea* (G1) sink, clarifying the significance of earlier reports that sclerotia of some isolates float, and the association of this trait with host habitat (Stager 1922).

Our objective in this study was to investigate speciation within the *C. purpurea* complex using phylogenetic and population genetic approaches based on representative samples. Isolates included in this study were selected as representative of the variation within *C. purpurea sensu lato* and chosen to cover a large geographical area, a wide host range within the Poaceae and all three habitat types. The group identity of each isolate used in this study had been determined previously by Random Amplification of Polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Pazoutová *et al*. 2000; Fisher *et al*. 2005b, c). RAPD and AFLP analyses of these, and of additional isolates, supports the recognition of three discrete groups within *C. purpurea* and revealed high genetic variability between groups, with less than 2% of polymorphic markers shared across all isolates. Similarly, analysis of molecular variation (AMOVA) revealed that genetic variability was mainly due to variations between groups rather than within groups or populations, revealing a high probability of phylogenetic substructure (Fisher *et al*. 2005b, c). However, no phylogenetic investigations of independent loci have been conducted in this putative species complex to confirm the previous studies using RAPD and AFLP. Support of distinct species based on multiple loci would support a more ancient divergence among these fungi. For example, closely related species may not show significant divergence among independent loci whereas multilocus markers such as AFLP may differentiate them. This was shown by Després *et al*. (2003) who demonstrated that AFLP markers could resolve phylogenetic relationships among *Trollius* species when both nuclear and chloroplast phylogenies could not. Determining if the *C. purpurea* complex is made up of one or multiple species is important because it is common practice in wide-ranging studies, from constructing phylogenies to characterizing metabolites, to make no distinction between the three different *C. purpurea* lineages (Tooley *et al*. 2001; Scheffer *et al*. 2005; Stensrud *et al*. 2005; Dabkevicius & Mikaliunaite 2006; Scheffer & Tudzynski 2006).

Materials and methods

Molecular analyses

Fungal isolates and DNA extraction. Claviceps purpurea isolates used in this study and their origins are listed in Table 1. For field-collected isolates, sclerotia were collected during the fall of 2000, 2001 and 2002 and brought to University of California, Davis for culturing. Sclerotia were surface sterilized, cultured, dried and refrigerated as described in Fisher *et al*. (2005c). Numbered isolates were obtained as pure cultures from S. Pazoutová (Institute of Microbiology, Czech Republic) including: G1 isolates 165, 204, 428 and G2 isolates 236 and 434 (Pazoutová *et al*. 2000; Fisher *et al*. 2005c).

Table 1 *Claviceps purpurea* isolates used in this study

Code	Group	Location	Year collected	Host
165	G1	Zubri, Czech Republic	1994	*Poa pratensis*
204	G1	Lauderdale, Alabama	1996	*Festuca arudinacea*
428	G1	Hohenheim, Germany	?	*Secale cereale*
NGE1	G1	Newfoundland, Canada	2001	*Leymus mollis*
WFA	G1	Nahcotta, Washington	2002	*Festuca arundinacea*
WLS	G1	Nahcotta, Washington	2002	*Lolium* spp.
236	G2	Vlei Pole u Bousova, Czech Republic	?	*Molinia coerulea*
434	G2	Phillipsreuth, Germany	1998	*Dactylis* spp.
WAB-1	G2	Long Beach, Washington	2002	*Ammophila breviligulata*
WCN2	G2	Willapa Bay, Washington	2002	*Calamagrostis nutkaensis*
WDG	G2	Leadbetter State Park, Washington	2002	*Dactylis glomerata*
WDS	G2	Willapa River, Washington	2002	*Deschampsia caespitosa*
WHS	G2	Long Beach, Washington	2002	*Holcus lanatus*
ARG1	G3	Argentina Celpa Marsh, Argentina	2002	*Spartina densiflora*
CDE1	G3	Point Reyes, California	2002	*Spartina foliosa*
CMD1	G3	MacDoel, California	2002	*Secale cereale*
CPE10	G3	Palo Alto, California	2001	*Spartina foliosa*
FSA1	G3	St. Augustine, Florida	2000	*Spartina alterniflora*
IRE12	G3	Dublin, Ireland	2001	*Spartina anglica*
RH2	G3	Rhode Island	2001	*Spartina alterniflora*
WDI-1	G3	Willapa River, Washington	2002	*Distichlis spicata*

Tissue for DNA extraction was obtained by growing isolates on cellophane overlaid on Potato Dextrose Agar (PDA) (Difco, Detroit, MI, USA) as described by Fisher *et al*. (2005c) or by scraping fungal mycelium directly from PDA plates. Total DNA was extracted using the methods described by Daehler *et al*. (1999) or slightly modified from Gardes & Bruns (1993). After DNA was recovered in solution, 75 L of supernatant was withdrawn and mixed with 200 L Tris-EDTA buffer (10 mM Tris, 1 mM EDTA (pH 7.8). DNA concentration was quantified by spectrophotometry.

PCR, Sequencing and phylogeny construction. Polymerase chain reaction (PCR) was performed in 40 L reaction mixtures containing 2 L of a 1 : 10 to 1 : 25 dilution of template DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen), 7.5 M of each primer and 0.5 U of Taq polymerase (Invitrogen). Three loci were amplified and sequenced; ITS rDNA using the primers ITS1F and ITS 4 (White *et al*. 1990; Gardes & Bruns 1993), a portion of a beta-tubulin gene using the primers described in Annis & Panaccione (1998), and a putative RAS-like protein using primers described by Carbone & Kohn (1999). Thermocycling conditions consisted of an initial hold at 94 C (4 min), followed by 30 cycles with a denaturing step of 94 C (30 s), annealing temperature of 50–55 C (30 s), and an extension temperature of 72 C (1 min). All amplifications were performed in a PE-9700 thermocycler (Perkin Elmer Corp., Norwalk, CT) or a MyCycler (Bio-Rad Laboratories Inc., Hercules, CA).

Each locus was sequenced in both directions using Big Dye® Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA). The PCR products were cleaned using ExoSapit (USB Amersham, Uppsala, Sweden), following the manufacturer's instructions and sequenced at the Core Instrumentation Facility (CIF) of the University of California's Institute of Integrative Genome Biology at UC Riverside. The sequences were edited using SEQUENCHER (version 4.1.2, Gene Codes Corporation, Ann Arbor, MI), aligned using Clustal X (version 1.81) (Chenna *et al*. 2003) and manually edited in MACCLADE version 4 (Maddison & Maddison 2001). Maximum Parsimony (MP) analysis, using the heuristic search procedure with 1000 random-addition-sequence replicates and tree-bisection-reconnection branch swapping, were conducted using PAUP* version 4.0 beta 10 (Swofford 2002). Confidence in tree topology was examined using bootstrap with 1000 replicates under the heuristic option. All trees were midway rooted.

Population genetic analyses. We investigated the genetic separation of the three ecotypes (G1, G2 and G3) using the program MIGRATE-N (version 2.3.3: Beerli & Felsenstein 1999, 2001; Beerli 2004, 2006) which is based on coalescence theory (cf. Kingman 2000). The three ecotypes were treated as independent genetic units where only migrants and mutation could import new alleles into a unit. We compared two different models: one single population vs. three populations with six different migration rates between all populations. This allowed us to test whether the three loci were powerful enough to reject the hypothesis that these

three ecotypes were generated by a single, randomly mating population.

MIGRATE-N does not take into account potential splitting of the ancestral population. Under such a model, the different ecotypes should show low migration rates if there was no recent divergence or no ongoing gene flow. We tested whether high gene flow between the ecotypes could be rejected using a Bayes Factor approach (Kass & Raftery 1995), by running the Bayesian module of MIGRATE-N for both models and compared the marginal likelihoods.

We used the default settings for MIGRATE-N, except for the following run options: (i) the Bayesian inference module; (ii) one single long run using heating with temperatures of 1.0, 1.5, 3.0 and 10 000, totaling 50 100 000 visited parameter and genealogy changes in the cold (1.0) chain; (iii) sampling 10 replicates of each 50 000 in intervals of 100; and (iv) after discarding the first 100 000 visits. We used uniform prior distributions with ranges from 0 to 0.1 for the mutation scaled population size Θ; that is, the effective population size N_e times the mutation rate per site and generation, and ranges from 0 to 5000 for M; that is, the mutation scaled immigration rate m/ . The mutation model used was the F84 model (Felsenstein 2004) with a transition–transversion ratio set to 2. We report the mode and median, and the 95% credibility set of the posterior distribution for all estimated parameters of the two models.

Estimates of the average number of nucleotide differences, and shared, fixed and unique number of mutations between the G1, G2 and G3 ecotypes were calculated in DNAsp 4.0 (Rozas *et al*. 2003). F_{ST} values were also calculated in DNAsp 4.0, with permutation tests used to test significance using 1000 randomizations.

Results

Phylogenetic inference

For beta-tubulin, 426 characters were analyzed with five parsimony-uninformative and 13 parsimony-informative characters. Figure 1 shows one of the 29 most parsimonious trees that were found with a length of 19. Consistency index, retention index and the rescaled consistency index were 0.947, 0.987 and 0.935, respectively. All G2 isolates except 236 had two copies of the repeat AACTG, starting at position 113 in the alignment, whereas all G1 and G3 isolates had a single copy of the AACTG repeat. All G1 isolates clustered in a clade with a bootstrap support of 100. The G2 and G3 isolates clustered together into one weakly supported clade. Although all G3 isolates clustered together with weak boostrap support, G2 isolates were more variable, with two isolates, 434 and 236, notably different from the rest (Fig. 1).

For *ITS*, 586 characters were analysed with four parsimony-uninformative and eight parsimony-informative characters. Figure 1 shows the single most parsimonious tree with a length of 14. Consistency index, retention index and the rescaled consistency index were 0.929, 0.977 and 0.907, respectively. The G1 clade was supported with a bootstrap score of 83. The G2 and G3 isolates clustered together with a bootstrap value of 88. Although G2 and G3 isolates clustered separately into their own subclades, these subclades had weak bootstrap support.

For *RAS*, 375 characters were analysed with five parsimony-uninformative and 13 parsimony-informative characters. Figure 1 shows one of a total of 354 most parsimonious trees that were found with a length of 15. Consistency index, retention index and the rescaled consistency index were 0.800, 0.955 and 0.764, respectively. The G1 isolates clustered together with a bootstrap support of 99. The G2 and G3 isolates clustered together with poor bootstrap support for any subgroupings.

In the combined dataset including all three genes, 1294 characters were analyzed with eight parsimony-uninformative and 32 parsimony-informative characters. The consistency index, retention index and the rescaled consistency index were 0.840, 0.956 and 0.803, respectively. The G1, G2 and G3 clades were supported by bootstrap values of 83, 68 and 82, respectively. The G2/G3 clade was supported with a bootstrap value of 100. No obvious genetic structure based on geography was found for any of the three loci or the combined dataset.

Population genetic analyses

Three-population model (each ecotype is treated as a population): results on migration rates are all low between all populations with large credibility intervals that, for individual loci, always include zero; the combined estimates exclude zero migration rates. The posterior distribution for the scaled migration rates are all heavily skewed to the right and all peak near zero compared to the whole distribution. We are confident that the prior distribution, a uniform distribution, had little influence on our results. The population sizes Θ reveal that the ecotype G3 has more variability and a larger population than the other types, G1 and G2. Table 2 gives credibility intervals and mode and medians. Typical values for the number of immigrants Nm (ΘM/4) were one immigrant every four generations.

One-population model (all ecotypes are pooled into one population): the population is much larger than the combined population size of the Three-population model suggesting that, because we ignored migration, the length of the genealogies are lengthened which results in an overestimate of the population size (Excoffier 2004). We compared our results from the Three-population model with those from the One-population model using a Log-Bayes factor (BF). Using the reported marginal likelihoods from the two MIGRATE-N analyses: log *L*(Three-population model) = –2073.887689; log *L*(One-population model)

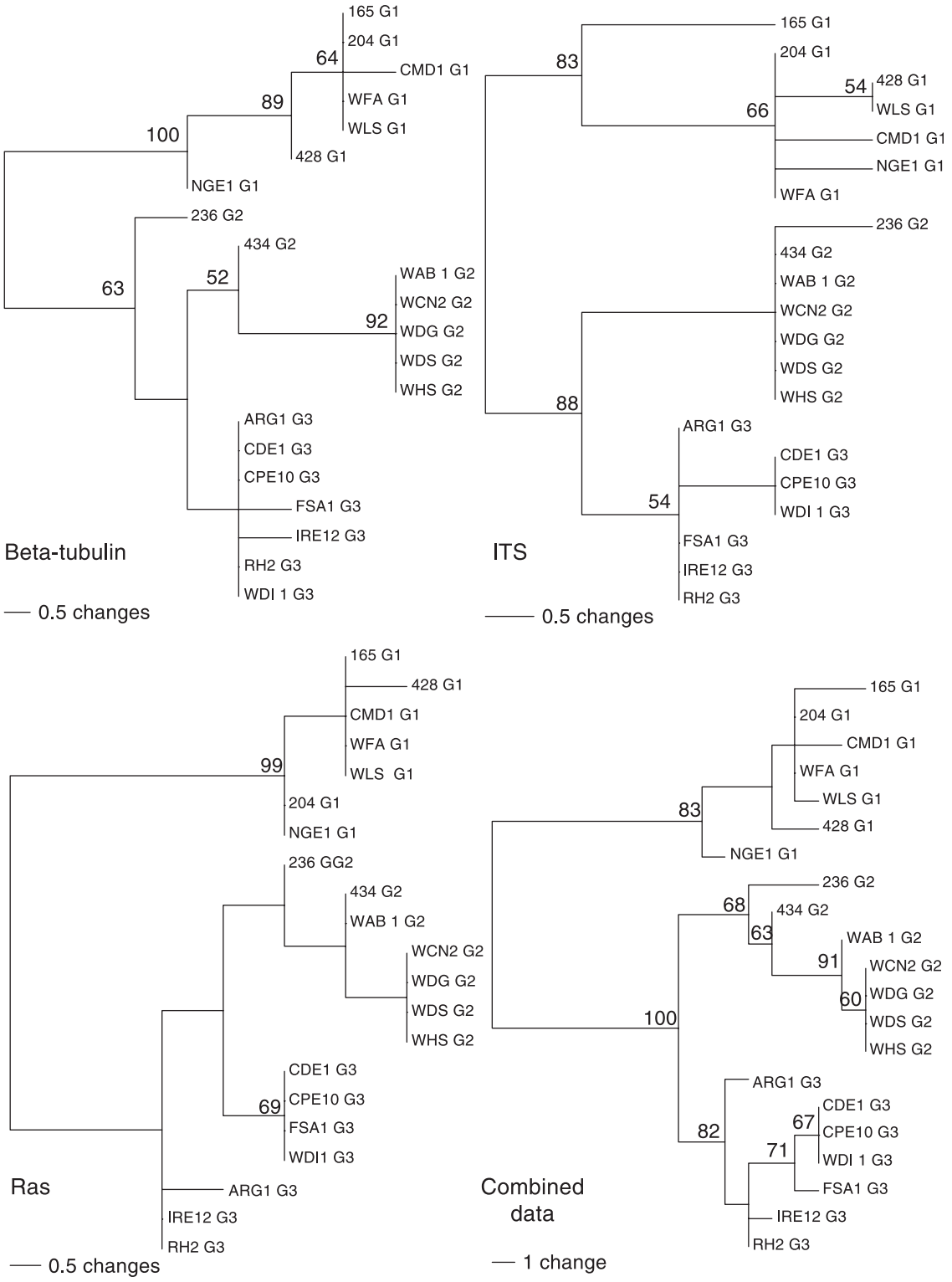

Fig. 1 Maximum parsimony analyses of three individual loci (*ITS*, Betat-tbulin, *RAS*) and the combined data set from G1, G2 and G3 isolates of *Claviceps purpurea*. Bootstrap support of 50% and above are indicated above nodes based on 1000 replicates. Sequences have been deposited in GenBank under Accession nos (EU558978–EU559040).

Table 2 Combined estimates over three loci of mutation scaled population size Θ and mutation scaled migration rates M. The estimates were found using the Bayesian inference module in MIGRATE-N

Model	Parameter	Mode	Median	95% Credibility interval
Three-population	Θ_{G1}	0.00062	0.001	0.00008–0.00356
	Θ_{G2}	0.00049	0.00087	0.00008–0.00302
	Θ_{G3}	0.00353	0.00478	0.00137–0.00923
	$M_{G2\ to\ G1}$	543	868	25–2370
	$M_{G3\ to\ G1}$	508	848	5–2405
	$M_{G1\ to\ G2}$	663	948	30–2530
	$M_{G3\ to\ G2}$	688	982	10–2695
	$M_{G1\ to\ G3}$	333	528	0–1490
	$M_{G2\ to\ G3}$	283	548	5–1560
One-population	$\Theta_{G1+G2+G3}$	0.01125	0.01185	0.00670–0.01760

Table 3 DNA divergence between G1, G2 and G3 *Claviceps purpurea* isolates in the three analyzed loci and the combined data set measured as number of segregating sites, fixed and shared polymorphic sites, average number of nucleotide differences (*k*), average number of nucleotide substitutions per site between habitat types (π) and population differentiation values between habitat types (F_{ST})

	No. of polymorphic sites	Fixed differences	No. of polymorphic compared to monomorphic*	No. of polymorphic compared to monomorphic†	Shared differences	*k*	π	F_{ST}
Beta-tubulin								
G1–G2	15	0	10	3	2	6.385	0.0152	0.8216
G2–G3	9	1	2	6	0	3.121	0.0083	0.6875
G1–G3	13	7	4	2	0	5.747	0.0137	0.9034
ITS								
G1–G2	11	5	1	6	0	4.121	0.0072	0.8369
G2–G3	5	3	1	1	0	2.121	0.0037	0.8800
G1–G3	9	3	1	6	0	3.495	0.0061	0.7719
RAS								
G1–G2	7	4	1	2	0	3.648	0.0134	0.9091
G2–G3	4	0	2	3	0	1.802	0.0065	0.5172
G1–G3	7	5	0	2	1	3.692	0.0135	0.8600
Combined								
G1–G2	33	14	11	8	0	14.08	0.0115	0.8504
G2–G3	17	4	6	8	0	6.516	0.0053	0.7042
G1–G3	29	15	10	5	1	12.93	0.0102	0.8576

*No. of sites that are polymorphic in the first species compared and monomorphic in the second species.
†No. of sites that are polymorphic in the second species compared and monomorphic in the first species.

= –2204.2369. Then the Log(BF) = log *L*(3) – log *L*(1) = 130.34. Values higher than 10 suggest that we should strongly prefer the first model, in our case the Three-population model (Kass & Raftery 1995).

Table 3 shows the summary statistics in the comparison between the G1, G2 and G3 isolates of *C. purpurea*. In the G2–G3 comparisons, the average number of nucleotide differences and nucleotide diversity were consistently lower than in the comparisons of G1–G3 and G1–G2. This was also consistent with lower F_{ST} values for G2–G3 comparisons as compared with G1–G3 and G1–G2 comparisons, except for the *ITS* locus in which the lowest F_{ST} value was for the G1–G3 comparison. All F_{ST} comparisons were significant ($P < 0.001$), suggesting limited gene flow between the three *C. purpurea* lineages from different habitats, which was consistent with the coalescence analyses.

Discussion

In this study, phylogenetic and population genetic analyses showed marked genetic differences among the different ecotypes and suggest little or no gene flow among the

different ecotypes. We can also definitely reject models that assume random mating between the different ecotypes based on the analyses. The G1 types were significantly divergent from the G2/G3 habitat types based on each of the three loci and the combined dataset, whereas the G2/G3 types were more integrated with one another. However, although the G2 and G3 lineages have not diverged as much as the G1 lineage based on DNA sequence data, the use of three DNA loci did reliably separate the G2 and G3 lineages. The fact that the G2 and G3 lineages are more closely related to each other than to the G1 lineage is strongly supported by the fact that sclerotia from G2 and G3 isolates float in water while those of G1 isolates sink (Pazoutová *et al*. 2000; Fisher *et al*. 2005a). Results from this study are in agreement with previous conclusions based on AFLP and RAPD data, in which only 2% of genetic markers were shared among the three lineages of *Claviceps purpurea*. Perhaps because of their high rate of polymorphism or because of their assessment of variation across the entire genome, AFLP and RAPD data were more informative than DNA sequences for separating the different ecotypes. A similar situation was recently shown in two species complexes of mycoparasites, *Hypomyces microspermus* and *H. chrysospermus* (Douhan & Rizzo 2003). In this case, AFLP clearly differentiated cryptic lineages within both *H. microspermus* and *H. chrysospermus*, whereas ITS rDNA sequence data recovered the same cryptic lineages, but in some cases with weak bootstrap support (Douhan & Rizzo 2003).

Should three distinct species within the *C. purpurea* complex be recognized taxonomically? As previously mentioned, traditional species concepts for the classification of fungi are based on morphology and reproductive biology, but phylogenetic approaches may be more powerful to accurately resolve fungal lineages (Harrington & Rizzo 1999; Taylor *et al*. 2000). During the speciation process, reproductive barriers arise between groups of individuals. The two groups at first share allelic polymorphisms until one of the two groups becomes fixed for certain alleles whereas the other group remains polymorphic (Avise 1994; Geiser *et al*. 1998). Therefore, based on previous AFLP and RAPD data, it would appear that the justification for species recognition would be warranted. However, Taylor *et al*. (2000) advocate identifying phylogenetic species of fungi by analyzing evolutionary relationships among multiple genes, which they call the Genealogical Concordance Phylogenetic Species Recognition (GCPSR). They suggest the use of multiple genes to determine the transition from concordance to conflict among taxa, which can be used to determine species boundaries. The conflict is thought to be due to recombination occurring between individuals. Based on the GCPSR criteria we would accept that there are three species within the *C. purpurea* complex, but there was weak bootstrap support for the three terminal lineages (G1, G2 and G3) in contrast with the previous data based on RAPD and AFLP, potentially due to shallow speciation between the taxa (Fisher *et al*. 2005b, c). For more recently derived taxa, a marker system such as AFLP may be more powerful than DNA-sequence loci to determine species boundaries because methods such as AFLP screen many loci across a genome. Despite the strengths of markers such as AFLP, inferring phylogenies based on randomly amplified fragments can be problematic, and there are arguments both for (Kardolus *et al*. 1998; Buntjer *et al*. 2002; Després *et al*. 2003) and against this approach (Seberg & Peterson 1998).

The overall evidence from our phylogenetic and population genetic analyses and previous studies suggest that the three lineages (G1, G2 and G3) should be recognized as unique species, or at least as varieties. Duncan *et al*. (2002) proposed renaming the G3 lineage as *C. purpurea* var. *spartinae* based on a combination of host identity (*Spartina* spp.), sclerotia ecology (floatation) and conidial morphology. However, since the variety status was proposed, G3 isolates were found infecting a non-*Spartina* host in nature (*Distichlis spicata*, Fisher *et al*. 2005b). Therefore, the named variety based on host association does not seem warranted. Regardless of what these fungi are called, it is important that some distinction be made since *C. purpurea sensu lato* is widely studied as a model plant pathogen of economic importance (Tudzynski & Scheffer 2004). Several lines of evidence suggest three divergent lineages within *C. purpurea*, and these lineages likely have important biological differences relevant for studies in the wider scientific community. Currently, it is common practice in wide-ranging studies of everything, from pathogenicity of *C. purpurea* on various hosts to molecular studies of virulence genes to phylogenetic assessments of the genus *Claviceps*, to make no distinction between the three different *C. purpurea* lineages (Scheffer *et al*. 2005; Stensrud *et al*. 2005; Dabkevicius & Mikaliunaite 2006; Scheffer & Tudzynski 2006).

Some authors have argued that species concepts should have an ecological basis as well as a genetic one (Harrington & Rizzo 1999), and we suggest that ecological factors have driven the speciation process in *C. purpurea.* In estuarine habitats, the transition from salt-water tolerant species such as *Spartina* spp. and *Distichlis spicata*, to riparian and terrestrial grasses, is often gradual, with no physical barrier between habitat types (Mitsch & Gosselink 1993). The boundaries of the high tide and the slope of the terrain delimit the change from one habitat to the next, and this will differ by site. For example, in Willapa Bay, Washington, USA, the G1, G2 and G3 lineages coexist within a distance of less than 100 m, with no physical barriers to spore dispersal by rain splash or insect vectors (Fisher *et al*. 2005b). It is not known if G1, G2 and G3 *C. purpurea* diverged sympatrically in a habitat such as a coastal estuary, or allopatrically with geographical barriers to gene flow.

However, since there was no phylogeographical structure found in this diverse sampling of isolates and host grass species, it seems likely that ecological factors were more important in the speciation process of these fungi. The genus *Claviceps* is thought to have a Gondwanan orgin, and most species in the genus are tropical or subtropical. It has been hypothesized that species close to *C. purpurea* migrated from South America to North America after the formation of the Panama land bridge, and that they later spread to Europe and Africa (Pazoutová 2003). It is only these species that developed the ability to deal with cold winters and semiarid conditions, which is potentially more evidence suggesting that ecological habitat helped shaped this species complex.

Although it remains to be determined if the three habitat-associated lineages are reproductively isolated, results from our phylogenetic analysis suggest that reproduction among G2/G3 isolates would be more likely than between either G1 and G2 or G1 and G3 isolates. However, the population genetic analyses strongly suggest little to no gene flow occurring between the different ecotypes. In order for reproduction to occur, theses different ecotypes must be able to infect the same hosts. The results of a host-range study showed that G3 isolates can infect both riparian and terrestrial grasses after artificial inoculation (Pazoutová *et al*. 2002), though its range in nature is so far limited to the C4 grasses *Spartina* spp. and *Distichlis spicata*. Similarly, Pazoutová *et al*. (2000, 2002) reported that G2 isolates can infect both riparian and terrestrial grasses in the greenhouse, but this has not been documented under natural conditions. Currently, we have not identified the barriers to gene flow in sympatric G1, G2 and G3 *C. purpurea* populations like in the Willapa Bay's upper marsh, but possibilities include differences in flowering time or flowering duration among hosts, or differences in plant biochemistry which might preclude infection of C3 grasses by a pathogen adapted to C4-grass hosts. Thus, as in other fungi, there may be multiple habitat-related factors that drive the speciation process. For example, it has been suggested that the behaviour of insect vectors or physiological barriers to mating or infection may prevent gene flow among host races of the anther smut fungus *Microbotryum violaceum* in sympatric populations of three host plant species (Shykoff *et al*. 1999).

Regardless of whether the divergence among *C. purpurea* lineages occurred in allopatric or sympatric populations, selection pressures in maritime habitats are distinct from those in terrestrial habitats, and *C. purpurea* from *Spartina* and *D. spicata* (G3) exhibit characteristics uniquely suited for maritime environments. For example, sclerotia from G2 and G3 *C. purpurea* float on water (Pazoutová *et al*. 2000), presumably due to large intercellular spaces (Duncan *et al*. 2002), and flotation presumably aids both survival and dispersal in areas with flooding or tides. In addition, G3 sclerotia do not require a cold stratification prior to germination, a requirement common in *C. purpurea* from terrestrial habitats (G1) not likely to be met in coastal environments (Duncan *et al*. 2002). Although data is lacking, tolerance for highly salinated leaves, water and soil is likely involved in directional selection leading to the isolation of the G3 lineage.

Host range within *C. purpurea* also suggests that ecology is more important than the host in the evolution of these fungi, which is different from many plant-associated fungi. For example, within the plant-pathogenic *Magnaporthe grisea* species complex there is strong evidence that speciation and genetic divergence are highly coupled with virulence on particular species or even varieties of host grasses. Although host switches have been documented, radiation events in this group of fungal pathogens appear to primarily follow the evolutionary history of hosts (Couch & Kohn 2002; Couch *et al*. 2005; Kohn 2005). A similar pattern of host-associated speciation has also been suggested for some symbiotic fungi, such as in the *Pinus*-associated ectomycorrhizal mushroom genus *Suillus*. In a study of disjunct *Suillus* species complexes from East Asia and Eastern North America, Wu *et al*. (2000) suggested that high host fidelity for species or subgenera of *Pinus* has led to speciation through comigration.

'Ecological speciation' has previously been suggested as the mode of speciation among many diverse animal groups (Via 2001) including fishes (Hatfield & Schluter 1999; Rocha *et al*. 2005), lizards (Ogden & Thorpe 2002; Richmond & Reeder 2002; Rosenblum 2006), toads (Kruuk & Gilchrist 1997), brittle stars (Muths *et al*. 2006), snails (Cruz *et al*. 2004) and various phytophagous and nonphytophagous insects (Via *et al*. 2000; Rundle & Nosil 2005). Similarly, at least six examples of putative ecological speciation have been inferred among angiosperm plants from diverse habitats on several continents (Nagy 1997; Wang *et al*. 1997; Rieseberg 2000; Lamont *et al*. 2003; Fine *et al*. 2005; Hall & Willis 2006). Ecological speciation in fungi has not received much attention and speciation is usually attributed to hosts for pathogenic fungi or to geographical vicariance, both of which may or may not have ecological similarities. However, as far as we know, *C. purpurea* is only the second specific example of a fungus where ecological speciation is the main proposed mode of speciation. The other example of possible ecological speciation in fungi is in the insect-pathogenic fungus *Metarhizium anisopliae*. In a study of *M. anisopliae* in Ontario, Canada, Bidochka *et al*. 2001 showed that this fungus was clearly segregated into two genetic groups; one common in agricultural areas and the other in forested habitats. The authors did not find consistent differences in host preference between the cryptic species, but they detected distinct differences in tolerance to UV light and temperature sensitivity. Although these are the only examples that we know of where ecological speciation has been indicated in fungi specifically, we expect that this is a

product of the lack of study and we expect that many more cases of ecological speciation will be found among fungi in the future.

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