Journal of Heredity 2006:97(3):270–278
doi:10.1093/jhered/esj031
Advance Access publication April 13, 2006

High Levels of Genetic Variation Exist in *Aspergillus niger* Populations Infecting *Welwitschia mirabilis* Hook

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Abstract

Aspergillus niger is an asexual, haploid fungus which infects the seeds of Namibia's national plant, *Welwitschia mirabilis*, severely affecting plant viability. We used 31 randomly amplified polymorphic DNA markers to assess genetic variation among 89 *A. niger* isolates collected from three *W. mirabilis* populations in the Namib Desert. While all isolates belonged to the same vegetative compatibility group, 84% were unique genotypes, and estimates of genotypic evenness and Simpson's index of diversity approached 1.0 in the three populations. Analysis of molecular variance revealed that 78% of the total variation sampled was among isolates from individual *W. mirabilis* plants. Lower, but significant, amounts of variation detected among isolates from different plants (12%) and different sites (10%) also indicated some site- and plant-level genetic differentiation. Total gene diversity ($H_T = 0.264$) was mostly attributable to diversity within populations ($H_S = 0.217$); the relatively low level of genetic differentiation among the sites ($G_{ST} = 0.141$) suggests that gene flow is occurring among the three distant sites. Although sexual reproduction has never been observed in this fungus, parasexuality is a well-known phenomenon in laboratory strains. We thus attribute the high levels of genetic variation to parasexuality and/or wind-facilitated gene flow from an as of yet undocumented broader host range of the fungus on other desert vegetation. Given the apparent ease of transmission, high levels of genetic diversity, and potentially broad host range, *A. niger* infections of *W. mirabilis* may be extremely difficult to control or prevent.

Welwitschia mirabilis Hook. is a large, leafy gymnosperm endemic to the arid and semiarid environments of Namibia and Angola. As the protected national plant of Namibia, *W. mirabilis* is of great conservation value, both as an important tourist attraction and as a biological curiosity (Barnard 1998). The range of *W. mirabilis* extends over a 150,000-km^2 area along the southwestern Namib coast from the Nicolau River in southern Angola to the Kuiseb River and the central Namib Sand Sea in central Namibia (Kers 1967) (Figure 1). Geographically discrete populations of 2 to over 2,000 individuals (Henschel and Seely 2000) are typically found in and along ephemeral watercourses and shallow plains (Jacobson et al. 1993a). A recent study by Wetschnig and Depish (1999) eliminated wind as a possible pollination vector for *W. mirabilis* and established flies as the primary pollination agent. Reflecting likely dispersal patterns by these pollinators, as well as the large endosperm-rich seeds, Jacobson and Lester (2003) found that genetic differences between populations of *W. mirabilis* correlate with geographic distances between them, with gene flow occurring between populations 6 km apart but not between populations located 18 or more kilometers apart.

Individuals of *W. mirabilis* are estimated to be up to 3000 years old (Bornman et al. 1972; von Willert and Wagner-Douglass 1994), and recent fossil evidence suggests that *Welwitschia*-related plants were present during the lower Cretaceous, approximately 112 million years ago (Dilcher 2005). There is concern, however, about the long-term viability of the species (Barnard 1998) because the seeds of many *W. mirabilis* populations are infected by a fungal seed pathogen in the *Aspergillus niger* van Tiegh aggregate. Initially noted by Hooker (1863) as a dark fungal infection of female cones in his original description of *W. mirabilis*, this pathogen severely reduces the reproductive viability of infected plants, with average infection rates ranging from 64% to 100% at 13 sites (1,013 plants sampled) (Cooper-Driver et al. 2000). While uninfected plants are highly fertile, juveniles are rare (Bornman 1978; Bustard 1990), and the fungal pathogen has recently been implicated as a primary cause of low recruitment.

A. niger conidia are small with thick, darkly pigmented walls that prevent desiccation and provide protection from UV radiation (Al-Musallam 1980). While it is likely that the conidia are wind dispersed over long distances, Bornman (1978) suggested that they are dispersed locally by a flightless,

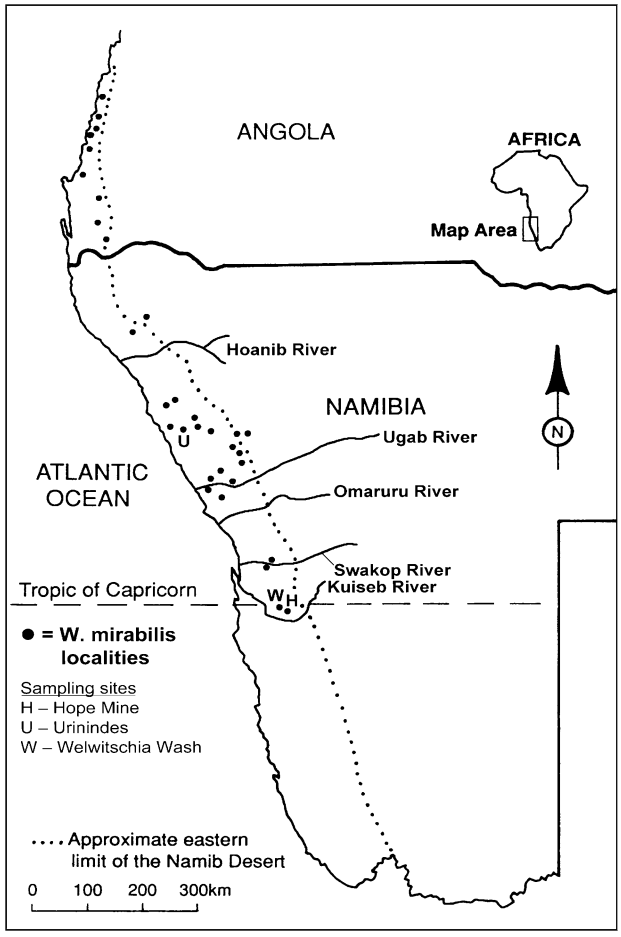

Figure 1. The distribution of *Welwitschia mirabilis* in Namibia and Angola according to Kers (1967). Samples of *Aspergilllus niger* were isolated from plants at labeled sites.

herbivorous insect, *Probergrothius sexpunctatis* (Pyrrhocoridae, Heteroptera). The ability of this insect to carry and introduce fungal conidia to female cones with its long proboscis has been demonstrated in the laboratory (Cooper-Driver et al. 2000). In addition, a recent spatial autocorrelation study of *A. niger* infection levels in *W. mirabilis* populations (Cooper-Driver et al. 2000) found spatial clustering of the pathogen in patterns that support this hypothesis of transmission by flightless insects with limited individual ranges.

While *A. niger* is classified as an asexual deuteromycete (Fennell 1977; Raper and Fennell 1965), its parasexual behavior has been extensively studied in the laboratory (Debets 1998; Lhoas 1967; Pontecorvo 1956). The relevance of parasexuality in maintaining genetic variation in natural populations of *A. niger* is not known, but in laboratory studies the rates of mitotic recombination (crossing over and haploidization) in *A. niger* were calculated to be 100× higher than meiotic crossing over in the sexual species *Aspergillus nidulans* (Lhoas 1967). While the exact significance of this calculation is unclear because no one has observed the frequency of heterokaryon formation (the initial step in the parasexual process) in wild populations (Debets 1998), recent studies of populations of a variety of fungal pathogens have shown that even strictly asexual species often have recombinant population structures. Examples of putatively asexual fungi with genetic evidence for recombination include *Alternaria brassicae*, *Alternaria brassicicola*, and *Alternaria tenuissima* (Berbee et al. 2003); *Aspergillus flavus* (Geiser et al. 1998); *Beauveria bassiana* (Paccola-Meirelles et al. 1991); *Candida albicans* (Gräser et al. 1996); *Coccidioides immitis* (Burt et al. 1996); *Cochliobolus carbonum* (Lodge and Leonard 1984); *Cochliobolus sativus* (Zhong and Steffenson 2001); *Colletotrichum sublineolum* (Souza-Paccola et al. 2003); *Fusarium culmorum* (Miedaner et al. 2001); *Magnaporthe grisea* (Zeigler et al. 1997); and *Ustilago scabiosae* (Garber and Ruddat 1992).

In order for parasexual recombination to occur between isolates, they must belong to the same vegetative compatibility group (VCG). Isolates that are vegetatively compatible have identical alleles at loci that facilitate hyphal fusion (plasmogamy), heterokaryon formation, and nuclear exchange analogous to histocompatibility loci in vertebrates (Brasier 1992). Fungal species and populations vary tremendously in the number of VCGs present. For example, only one to two VCGs were found in *Cryphonectria parasitica* populations in Europe, whereas a single population of this fungus in the eastern United States comprised 35 VCGs (Anagnostakis 1992).

Herein we report the results of our investigation of genetic variation among 89 isolates of *A. niger* from three geographically discrete populations of *W. mirabilis* (Figure 1). The Welwitschia Wash and Hope Mine sites are 6 km apart and located 500 km south of the Urinindes site. In a preliminary study of vegetative compatibility among the isolates, we found that all isolates (29–30) from within a site were vegetatively compatible. Subsequent interpopulation crosses using five isolates randomly chosen from each population revealed that all isolates from all three populations were vegetatively compatible and that there were no genetic barriers precluding parasexuality (Donovan and Jacobson 2001). All crosses were performed according to a protocol detailed in Jacobson et al. (1993b), in which each isolate is paired with the test isolate and itself (control) on CYM medium (ATCC medium 987). All crosses were conducted two times and interactions were unambiguous, with all testcrosses showing no barriers between the mycelia, as was observed for the control crosses.

The objectives of the study reported here were to describe the genetic variation and diversity within each site and to determine whether patterns exist in the distribution of variation within and between sites. To assess levels of genetic variation, we used randomly amplified polymorphic DNA (RAPD) markers derived by polymerase chain reaction using 10-base primers, which amplify random fragments throughout the genome (Williams et al. 1990). RAPD markers have provided an efficient initial assessment of genomic variation within and between fungal populations and closely related individuals of fungi (e.g., Dalgleish and Jacobson 2005; Jacobson et al. 1993b; McDonald and McDermott 1993), including molecular typing of *A. niger* and related species (Birch et al. 1995).

If populations of *A. niger* at our study sites were predominately clonal, we expected to see low levels of genetic variation among isolates from a single plant and also among all isolates from a single site. If, on the other hand, parasexual recombination were occurring among isolates within plants and sites, we expected to see higher levels of genetic variability within plants, although just how high could not be predicted as no work had been done that would indicate the level of genetic variability that parasexuality can produce in a natural population of *A. niger* (Debets 1998; Taylor et al. 1999). If conidia dispersal was limited to flightless insects, thus limiting gene flow between populations, we expected to see high levels of genetic differentiation between the isolates from geographically distinct sites because *P. sexpunctatis* is believed to be incapable of crossing the distances between populations (up to 500 km with no intervening *W. mirabilis* populations for at least 250 km between the northern and southern sites) (Cooper-Driver et al. 2000). In contrast, if this is a wind dispersed, panmictic organism, we expected to see little differentiation between sampling sites.

Materials and Methods

Taxon Description

Two collections of black mold fungi from *W. mirabilis* seed cones were previously identified as one of the following four taxa: *A. niger* V. Tiegh., *A. phoenicus* Corda, a species within the *A. niger* aggregate, or *A. niger* var. *phoenicus* Corda (Al-Musallam 1980; Schutte 1994). In her revision of black *Aspergillus* species, Al-Musallam (1980) used morphological and cultural characteristics to describe the *A. niger* aggregate as being composed of six varieties differentiated by conidia roughness and ornamentation. Specifically, the distinguishing features of *A. niger* var. *phoenicus* were conidia, 3–4.5 (5) μm in diameter, "usually rough, showing regular surface ornamentation with ridges in longitudinal striation." In 1996, L. Shutte and H. Kolberg identified infections from the Messum River as *A. niger* var. *phoenicus*, and as a result Cooper-Driver et al. (2000) referred to the infections they mapped as this taxon. We examined conidia from nine of our isolates ($N = 20$) from each of the three populations and found they were uniformly globose, 4–5 μm in diameter, irregularly roughened to punctuate, but entirely lacking longitudinal ridges. Thus, our isolates, notably all belonging to the same VCG (Donovan and Jacobson 2001), do not fit Al-Musallam's strict definition of *A. niger* var. *phoenicus* and are more appropriately described by the broader description of Raper and Fennell (1965) of the *A. niger* aggregate. Recent molecular studies (see review by Abarca et al. 2004) of black *Aspergillus* species have confirmed that the *A. niger* aggregate is a distinct phylogenetic entity but have not yet resolved taxonomic definitions within the group.

Site Description

The Welwitschia Wash (23°36′54.5″S, 15°10′45″E) and Hope Mine (23°34′273″S, 15°15′533″E) sites are located at the southern edge of the range of *W. mirabilis*, just north of the ephemeral Kuiseb River in the central Namib Desert, and are approximately 6 km apart (Jacobson and Lester 2003). The Urinindes (19°51′667″S, 13°39′075″E) site is located approximately 500 km north of these sites in the roadless area between the Ugab and Hoanib Rivers. While there are numerous well-known *W. mirabilis* populations between these northern and southern sites, all intervening northern and southern populations are separated by at least 250 km (Jacobson and Lester 2003).

Collection, Isolation, and Harvesting

Female *W. mirabilis* cones infected with *A. niger* were collected in July 1999 from three individual plants at each of the three sites and stored dry in sterile bags. Conidia were isolated by agitating an infected cone scale from each plant in sterile water. The suspended conidia were diluted 100- to 1000-fold and then plated on 100 μl CYM agar and incubated overnight at 20°C. Germinating conidia were isolated using a dissecting microscope, transferred on to separate CYM agar plates, and incubated approximately 1 week at 20°C. Each isolate was subcultured to two CYM agar plates and incubated for 10 days at 20°C. The mycelium and conidia were scraped from the surface of both plates with a sterile razor blade and stored frozen in CTAB buffer at −20°C until DNA was extracted. Nine to ten conidial isolates were isolated from three individual *W. mirabilis* plants at each of the three sites for a total of 89 isolates.

Molecular Methods

DNA was extracted from the fungal tissue using a CTAB DNA extraction protocol (Gardes and Bruns 1993) followed by use of the Omega Bio-tek (Doraville, GA) EZNA® Fungal DNA Kit to remove the remaining pigment when necessary. The DNA samples were then quantified with a fluorimeter and DNA-specific dye (Hoechst). RAPD reactions (25 μl) consisted of 12 ng genomic DNA, 25 mM $MgCl_2$, 250 μM each of dATP, dTTP, dGTP, and dCTP, 5 units *Taq* DNA polymerase (Promega, Madison, WI), and 40–50 ng of each 10-mer (Operon Technologies, Alameda, CA). Primers OPA 1–20 were screened to yield a total of 30–40 loci. RAPD amplification was performed in an MJ Research (Watertown, MA) PTC-150 Minicycler™ under conditions specified by Williams et al. (1990). The RAPD products were separated by agarose gel electrophoresis, and gels were stained with ethidium bromide and photographed under UV illumination using the Kodak Electrophoresis Documentation and Analysis System 290. Gel products were sized relative to a 100-bp DNA ladder standard (100–1500 bp) (Promega, Madison, WI) using Kodak ID Image Analysis software to allow objective and consistent sizing and quantification of bands. In addition to running water controls with each setup, four reactions, using 66 samples, were performed twice to confirm that RAPD markers scored (sizes ranges 200–1700 bp) were reproducible (Hadrys et al. 1992; Perez et al. 1998).

Data Analysis

All RAPD bands were scored as loci with two alleles, with one allele indicated by the presence of a fragment and the

other by the absence of a fragment (scored, respectively, as 1 or 0 in the Excel data file). Population structure at the three sites was studied in three ways. Firstly, we used data from all 89 isolates to quantify total gene diversity (H_T), the genetic diversity of individuals relative to their subpopulation (H_S), and genetic differentiation relative to the total population (G_{ST}) (Nei 1973). Each genetic parameter value was corrected for small, unequal sample sizes according to Nei and Chesser (1983). These values were also calculated for 29–30 isolates from each site to further quantify population structure at the site level.

The data set for all 89 isolates was used to create a similarity matrix in NTSYS using the Jaccard algorithm (Rohlf 1988). A phenogram of the resulting matrix was generated using the unweighted pair group method with arithmetic mean. The cophenetic correlation statistic was computed using the COPH function, and the goodness-of-fit of the phenogram for the similarity matrix was calculated using MXCOMP.

Finally, an analysis of molecular variance (AMOVA), which uses haploid molecular variance data rather than marker frequency (Excoffier et al. 1992), was also performed. This analysis was performed on a matrix created using the Euclidean distance algorithm (Liao and Hsiao 1998). AMOVA, which has been used effectively to obtain a first assessment of genetic variation in a number of other fungal species (Gosselin et al. 1999; Huff et al. 1994), was used to partition the total genetic variation into three specific hierarchical levels: among isolates on a *W. mirabilis* plant, among isolates on different plants at each site, and between sites. Significance levels for the resultant variance components were computed by nonparametric permutation procedures (Excoffier et al. 1992).

Results

Four primers (OPA2 = 5′-TGCCGAGCTG-3′, OPA4 = 5′-AATCGGGCTG-3′, OPA10 = 5′-GTGATCGCAG-3′, and OPA20 = 5′-GTTGCGATCC-3′) yielded 35 unambiguous RAPD loci from the 89 isolates. Four of these loci were invariant for the marker allele in all three populations, and of the remaining 31 loci, 60%–77% were variable within each site (Tables 1 and 2). The frequencies of only five of the 31 loci varied significantly ($P < .05$) among the three populations (Table 2), and Simpson's index of diversity and the estimate of genotypic evenness (Table 1) both approached values of 1.0. All three sites were composed primarily of isolates with unique genotypes (72%–93%) (Table 1), and despite the intense sampling of isolates within a single plant, only seven pairs of isolates had identical genotypes (Figure 2).

The analysis of population structure at the site level revealed that genetic diversity within sites accounted for most of the total genetic diversity. Adjusted values of genetic diversity within sites (H_S) ranged from 0.02 to 0.49 and averaged 0.22, accounting for 82% of the total genetic diversity (H_T). The proportion of the total diversity attributable to differentiation at the site level (Table 2: G_{ST}) ranged from 0.01 to 0.47 and averaged 0.14, indicating little population subdivision at the site level.

Table 1. Description of genetic variation and genetic diversity in *Aspergilllus niger* isolates from three sites, Urinindes (U), Hope Mine (HM), and Welwitschia Wash (WW), using RAPD markers

	Sites		
	U	HM	WW
N (number of isolates)	29	30	30
No. of loci/population	35	35	35
Percent variable loci/population	60%	66%	77%
G [percent unique genotypes/ population (PD)]	72	87	93
D (Simpson's index of diversity)	0.99	1.00	1.00
E (genotypic evenness)	0.99	0.99	0.99

The analysis of population structure at the plant level (Table 3) further revealed that genetic diversity of isolates within *W. mirabilis* plants accounted for most of the total genetic diversity within each site. Adjusted values of genetic diversity (H_S) within plants averaged 0.26 at Hope Mine, 0.24 at Welwitschia Wash, and 0.22 at Urinindes accounting for, respectively, 83%, 76%, and 67% of the total genetic diversity within each site (H_T). In addition, the average G_{ST} values for *A. niger* isolates among the three plants at each site were HM = 0.17, WW = 0.21, and U = 0.25, respectively, indicating low and intermediate levels of population subdivision at even the plant level (Table 3).

The AMOVA confirmed that most of the total genetic variation was accounted for among the isolates from individual *W. mirabilis* plants (78% of the total variance; $P < .001$) but also revealed that the much smaller proportional variances attributed to within-site (12%) and between-site (10%) differences were highly significant ($P < .001$) (Table 4).

Further illustrating the relationships among the 89 isolates, the phenogram (Figure 2) showed that few isolates formed site-specific genetic clusters. While 18/29 isolates from the Urinindes site formed a cluster sharing 82% similarity, the other 11 Urinindes isolates clustered with isolates from Hope Mine and Welwitschia Wash sharing up to 90% similarity. Similarly, 16/30 isolates from Welwitschia Wash formed small clusters (two to eight isolates per cluster) sharing 67%–76% similarity, but like the Urinindes isolates, the remaining 14 Welwitschia Wash isolates clustered with Urinindes and Hope Mine isolates with up to 96% similarity (Figure 2).

Discussion

Levels of genetic diversity both among and between isolates from the sampled sites were much higher than expected for an asexual organism occurring on a single host species in isolated locations. These populations exhibited no clonal population structure. In fact, values obtained for total gene diversity (H_T), genetic diversity of individuals relative to their subpopulation (H_S), and genetic differentiation relative to the total population (G_{ST}) are similar to those observed in fungi with known sexual life cycles (e.g., Goodwin et al. 1992, 1993; Gosselin et al. 1999; Johannesson et al. 2001; McDermott and McDonald 1993; Peever and Milgroom 1994).

Table 2. Frequencies of 31 RAPD loci and genetic diversity estimates of 89 *Aspergilllus niger* isolates from three sites: Hope Mine (HM), Welwitschia Wash (WW), and Urinindes (U). Genetic parameters were corrected for small, unequal samples sizes according to Nei and Chesser (1983)

	Frequency of the marker allele			Genetic diversity			
	HM	WW	U	All populations (HM, WW, U)			
Locus number	$N = 30$	$N = 30$	$N = 29$	Probability	H_S	H_T	G_{ST}
OPA2:475	0.53	0.40	0.83	*	0.47	0.48	0.03
OPA2:500	1.00	0.93	0.97	ns	0.06	0.06	0.03
OPA2:700	0.47	0.17	0.34	ns	0.40	0.44	0.10
OPA2:850	0.90	0.97	1.00	ns	0.08	0.09	0.07
OPA2:900	1.00	0.47	0.55	*	0.28	0.44	0.37
OPA2:1000	0.90	0.87	1.00	ns	0.13	0.14	0.07
OPA4:275	0.83	0.87	1.00	ns	0.16	0.18	0.08
OPA4:300	0.90	0.97	1.00	ns	0.08	0.08	0.07
OPA4:350	0.47	0.23	0.11	ns	0.32	0.39	0.17
OPA4:450	0.97	0.90	0.34	*	0.15	0.39	0.62
OPA4:480	0.63	0.80	0.24	*	0.33	0.49	0.33
OPA4:580	0.93	1.00	1.00	ns	0.04	0.05	0.09
OPA4:750	0.50	0.37	1.00	*	0.25	0.47	0.47
OPA4:775	0.47	0.47	0.33	ns	0.47	0.49	0.03
OPA4:800	0.53	0.53	0.63	ns	0.49	0.49	0.01
OPA10:250	1.00	0.73	1.00	ns	0.11	0.16	0.29
OPA10:300	0.90	1.00	1.00	ns	0.06	0.06	0.10
OPA10:400	0.27	0.36	0.33	ns	0.43	0.44	0.01
OPA10:425	1.00	0.93	1.00	ns	0.04	0.05	0.09
OPA10:440	0.10	0.23	0	ns	0.17	0.20	0.13
OPA20:420	1.00	1.00	0.93	ns	0.04	0.05	0.09
OPA20:520	1.00	0.97	0.97	ns	0.04	0.04	0.02
OPA20:560	0.77	0.66	0.69	ns	0.41	0.41	0.02
OPA20:600	0.13	0.17	0.07	ns	0.21	0.22	0.03
OPA20:650	0.70	0.57	0.30	ns	0.42	0.50	0.17
OPA20:700	1.00	0.93	0.97	ns	0.06	0.07	0.06
OPA20:800	0.83	0.43	0.62	ns	0.39	0.47	0.17
OPA20:900	1.00	1.00	0.97	ns	0.02	0.02	0.03
OPA20:1020	0.97	0.90	1.00	ns	0.08	0.08	0.07
OPA20:1260	0.66	0.70	0.93	ns	0.32	0.36	0.12
OPA20:1650	0.07	0.13	0.60	ns	0.22	0.39	0.43
Average					0.22	0.26	0.14
SE					0.03	0.03	0.03

ns = not significant.

* Significantly different at the $P < .05$ level.

While the analysis of genotypic diversity, the hierarchical analysis performed by AMOVA, and the cluster analysis all revealed that most of the variation in the population is accounted for among individual fungal isolates, these analyses also revealed significant differentiation among isolates from each of the plants at the three sites and among the three sites. According to the AMOVA, only 12% and 10% of the total genetic variation were accounted for at the between-plant and between-site levels, yet both are highly significant ($P < .001$), indicating that isolates from each site are appropriately referred to as genetic subpopulations and populations, respectively. In diploid or dikaryotic organisms, regional population structure of this nature is usually the result of more frequent interbreeding events among isolates within a site than between sites (Gryta et al. 2000). Given that *A. niger* is an exclusively asexual fungus known to readily perform parasexuality in the laboratory (but not previously demonstrated in natural populations), we propose that parasexuality may, in part, account for the extreme levels of variation we observed in these subpopulations and populations.

While fungal populations that reproduce sexually are often more genetically variable than those that reproduce asexually (Burdon and Roelfs 1985), evidence is accruing that many asexual fungi have higher than expected levels of genetic variation and are undergoing some means of genetic exchange. However, only two of these studies have experimentally proved that parasexuality is occurring under controlled field conditions (Souza-Paccola et al. 2003; Zeigler et al. 1997), and studies have assessed neither the degree to which parasexuality occurs in natural populations nor the significance of such asexual horizontal gene transfer as an adaptive mechanism relative to migration and genetic drift (Correll and Gordon 1999).

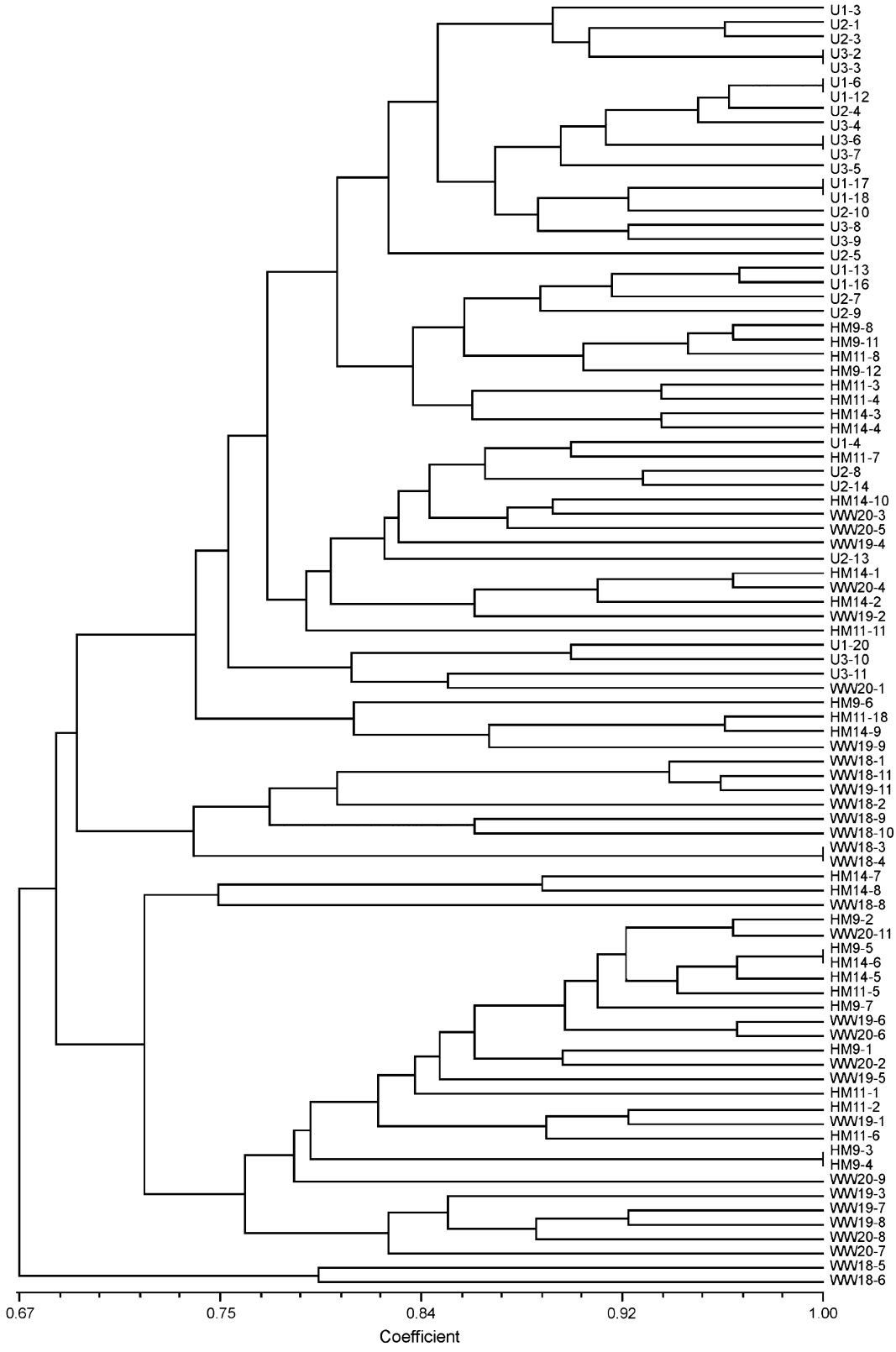

Figure 2. One of 64 phenograms generated using unweighted pair group method with arithmetic mean, based on the similarity matrix using Jaccard's algorithm. Data used are 35 RAPD markers for *Aspergilllus niger* isolates (1–10) from each of three *Welwitschia mirabilis* plants at Urinindes (U), Hope Mine (HM), and Welwitschia Wash (WW). Phenograms differed only in the placement of isolates that were more than 93% similar.

Table 3. Average genetic diversity estimates (+/−SE) of *Aspergilllus niger* isolates among three *Welwitschia mirabilis* plants within each site (Welwitschia Wash, Hope Mine, and Urinindes). Genetic parameters were corrected for small, unequal sample sizes according to Nei and Chesser (1983)

Sites	No. of loci	H_S	H_T	G_{ST}
HM	23	0.26 (0.03)	0.31 (0.03)	0.17 (0.04)
WW	27	0.24 (0.03)	0.32 (0.03)	0.21 (0.04)
U	20	0.22 (0.03)	0.33 (0.05)	0.25 (0.07)

Previous studies of other asexual *Aspergillus* species have been unable to make definitive conclusions about the level of genetic variation attributable to parasexual recombination because of uncertainty as to whether variation was caused by recombination or the introduction of inoculum from other populations (Bayman and Cotty 1990). As with these other studies of asexual fungi that exhibit higher than expected levels of variation, the relative contributions of gene flow and parasexual recombination in structuring these *A. niger* populations are not known. However, the relatively low level of genetic differentiation among all 89 isolates ($G_{ST} = 0.14$) indicates that gene flow is indeed occurring across distances ranging from 6 to 500 km and is homogenizing the populations. Among the 29–30 isolates within each population, our study also suggests that gene flow occurs among subpopulations occupying separate plants ($G_{ST} = 0.17$–0.25). More intensive sampling of fungi at the plant level is needed to determine whether these differences in levels of gene flow within the different sites reflect actual differences in the manner in which these populations are exchanging genetic material.

While the results of a spatial autocorrelation study by Cooper-Driver et al. (2000) favor local transmission by a dispersal agent (e.g., *P. sexpunctatis)* with a limited range that would account for gene flow within *A. niger* populations, the authors do not preclude transmission by other biotic and abiotic agents, some with the potential to cross great distances between *W. mirabilis* populations. An obvious possibility is the wind dispersal of the dark, thick-walled conidia. In addition, other potential agents of transmission include pollinator flies (Wetschnig and Depish 1999); animals which shelter under or feed on *W. mirabilis* such as Grays lark, snakes, lizards, arthropods, oryx, and springbok; and the tires of four-wheel drive vehicles (Cooper-Driver et al. 2000).

Table 4. AMOVA for 89 *Aspergilllus niger* isolates from three *Welwitschia mirabilis* plants from each of three sites (n = 9–10 isolates/plant, 29–30 isolates/site)

Sources of variation	df	SSD	MSD	Variance component	Percent total variance	*P*
Between sites	2	428.1	214.1	0.15	10.18	<.001
Plants/site	6	367.2	61.2	0.17	12.01	<.001
Individuals/ plant	3106	3493.1	1.1	1.12	77.81	<.001

Another potential explanation for this high level of gene flow between sites is that the highly ubiquitous *A. niger* may be infecting the surrounding vegetation (e.g., *Euphorbia*, *Commiphora*, and *Arthraerua* species, or annual grasses) and dispersing by wind to nearby *W. mirabilis* plants. Indeed, *A. niger* is a common soil saprophyte in many environments (Abarca et al. 2004; Raper and Fennel 1965), and while the organic content of the Namib Desert soil is exceptionally low, it is possible that this fungus is a common soil saprophyte here as well. This has not been previously considered because it was assumed that the *A. niger* variant on *W. mirabilis* was unique and occupied a small niche on *W. mirabilis* alone. Alternatively, if the fungus is endemic to *W. mirabilis* in the Namib Desert, it may be more common in soils and on vegetation in the higher rainfall regions to the east of the Namib Desert. Strongly directional winter east winds could potentially transport conidia from the more mesic inland savanna to the arid coastal desert.

Any efforts taken to control fungal infections of *W. mirabilis* should bear in mind the high levels of genetic diversity found by this study and the possibility that surrounding soils and vegetation and prevailing winds from upland savannas may be serving as sources of inoculum of this apparently common fungus. Before any control measure can be put in place, efforts should be made to identify all means of conidia dispersal and sources of inoculum, as well as the identity of the pathogen relative to the known phylogeny of this group (Abarca et al. 2004). Given the inherent difficulties in attempting to control such a potentially common and ubiquitous pathogen, further studies should address whether this fungus really is reducing plant recruitment at higher than normal levels. Changes in the size and frequency of the large precipitation events (50 mm) required for seed germination may be an equally important factor affecting plant recruitment. Our observations of plants at 12 sites throughout the plant's range suggest that there have been only three to five recruitment events over the last 150 years resulting in cohorts of plants all of approximately the same size. Healthy populations of *W. mirabilis* produce seeds each year, and seeds are long lived (at least 10 years—personal observations). It is thus entirely possible that seed recruitment, albeit with a heavy loss to fungal infection, is adequate to take advantage of the uncommon high rain events that facilitate germination and survival.

Acknowledgments

We gratefully acknowledge the Namibian Ministry of Environment and Tourism for granting permission to sample infected cones from *W. mirabilis* in the Namib-Naukluft and Skeleton Coast Parks. Thanks also to the Desert Research Foundation of Namibia and Mary Seely for critical logistical support for the fieldwork, Grinnell College and the Woodrow Wilson Foundation for financial support, and Thomas Harrington and three anonymous reviewers for helpful comments on the manuscript.

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Received July 18, 2005
Accepted February 7, 2006

Corresponding Editor: Reid Palmer