Extensive genetic divergence associated with speciation in filamentous fungi

(Basidiomycotina/fungal evolution/speciation/DNA·DNA hybridization)

R. J. VILGALYS*† AND J. L. JOHNSON‡

*Department of Biology and ‡Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

ABSTRACT   Little is known about genetic differentiation during speciation in fungi. The *Collybia dryophila* complex (Basidiomycetes: Tricholomataceae) contains several closely related groups of fungi at various levels of evolutionary divergence. Mating compatibility studies show there are several intersterile groups within the complex, three of which are distributed over two or more continents. Genetic relationships among five intersterility groups were compared by the method of DNA·DNA hybridization. Clustering techniques were used to reconstruct phylogenetic relationships of these fungi. Genic identity based on DNA·DNA hybridization corresponds well with mating compatibility. Reduced genic identity was observed between geographically isolated populations of a single mating group. This evidence suggests an allopatric mode of evolution for the *C. dryophila* group. These results indicate extensive genic divergence is associated with the appearance of intersterility barriers in basidiomycetous fungi. The levels of divergence observed in these and in other fungi are significantly higher than that observed in many other eukaryotes, and this suggests that the rate of molecular or morphological evolution in fungi may differ from that found in other organisms.

Almost nothing is known about rate and mode of evolution in fungi, particularly at the species level. Although the fungi present difficulties for the study of natural populations, the natural history of many groups is well understood. Most species of fungi in the Basidiomycotina are heterothallic and have multiallelic mating systems, which favor outcrossing (1, 2). Within phenotypically similar fungal groups, mating compatibility tests have been widely used for delimiting biological species (3, 4). A genetic basis for the rigorous prezygotic isolation observed in the higher Basidiomycotina is not understood but has been explained on the basis of heterogenic incompatibility (2, 3). For fungi, which possess a relatively small genome ($\approx$10 times the size of *Escherichia coli*), widespread occurrence of phenotypically similar biological species (sibling species) provides an opportunity to investigate the relationship between intersterility and genetic differentiation in a simple eukaryotic system.

The mushroom *Collybia dryophila* (Bull. : Fr.) Kumm. is a common primary litter decomposer with a cosmopolitan distribution. Although *C. dryophila* has traditionally been viewed as a single polymorphic species, mating studies have shown it to consist of at least four biological species in North America (5), as well as four in Europe (6).

In this paper, we report on mating relationships between allopatric intersterility groups from Europe, North America, and Asia. To assess the level of genetic divergence associated with intersterility and with allopatry, we compared different strains of these fungi by the technique of DNA·DNA hybridization. Our results show that speciation in filamentous fungi

is accompanied by a high degree of genetic divergence, reflected in greatly reduced base sequence complementarity. The reduction in DNA sequence homology also appears to be associated with geographic isolation and suggests that speciation in these fungi is primarily allopatric in mode.

MATERIAL AND METHODS

Strains Used. Monokaryotic and dikaryotic isolates were obtained by dilution plating of basidiospores on 1.5% malt extract agar. Intersterile groups (biological species) were identified by mating-compatibility tests (5). Formation of clamp connections by the newly formed secondary mycelium provided the criterion for compatibility (1, 2, 5).

DNA Isolation and Hybridization. One to several dikaryotic isolates were chosen to represent different biological species within the *C. dryophila* group, as well as several outgroup species of *Collybia* (Table 1). Flask cultures grown in 1.5% malt were harvested by vacuum filtration through Miracloth (Calbiochem) and washed with cold buffer (0.15 M NaCl/0.05 M Tris·HCl/0.05 M Na$_2$EDTA, pH 8.0). If necessary, mycelium was lyophilized and stored at $-20°C$ until use. DNA was isolated as short fragments from hyphae lysed using a French Press and collected on hydroxylapatite (7). Alternatively, high molecular weight DNA was obtained from some strains using a gentle extraction method (8). All DNAs were stored in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0, at $-20°C$ until used.

All DNAs were sheared before hybridization by passage through a French press at 16,000 psi (1 psi = 6.89 kPa) to a modal fragment size of 500 nucleotides (size determined by agarose electrophoresis). DNA was chemically labeled *in vitro* with ^{125}I (9) to an average specific activity of 3 × 10^6 cpm/μg. DNA sequence homology was assayed using S1 nuclease (6, 7). DNA hybridization was carried out in 1.17 M NaCl/1 mM Hepes, pH 7.0, at 65°C for 24 hr. The higher salt concentrations used in our experiments accelerated the rate of reassociation relative to 0.12 M sodium phosphate buffer commonly used by others (10). For comparison with other data, C_0t values attained in our experiments were multiplied by 10 to give equivalent (EC_0t) values. The ratios of unlabeled (driver) DNA to labeled (tracer) DNA in the experiments were in excess of 1000, and the incubations were carried out to a minimum EC_0t of 1000 (except where noted), which provided for essentially complete rehybridizing of homologous DNAs used in the experiments. Radioactivity was counted by using a Beckman model 5500 γ counter. Normalized percentage homology (NPH) was determined as the ratio of heterologous/homologous binding (corrected for self-renaturation of the homologous tracer DNA).

Abbreviation: NPH, normalized percentage homology.
†Present address: Department of Botany, Duke University, Durham, NC 27706.

Table 1. Genetic relatedness among strains in the *C. dryophila* group based on DNA·DNA hybridization

Unlabeled DNA		Reference DNA									
Group*	Strain	RV 149 N-II	M19024 E-II	W14501 E-IV	GB 45 N-IV	BEXC E-I	RV 148 N-I	HC82/15 E-III	OKM18761 N-III	RV 85/3[†] C. maculata	VT 1261[†] C. cirrhata
N-II	RV 149	100	80	68	61[‡]	69	53	49	50	7	6
E-II	M19024	81	100	78	66	63	57	50	44	6	9
E-IV	W14501	73	76	100	73	55	41	54 ·	37	7	6
E-IV	L81/301	73	77	89	76	71	43	55	40	9	10
N-IV	GB 45	61[‡]	63	62	100	107	64	53	50	11	9
E-I	BEXC	ND	ND	ND	ND	100	36	ND	39	ND	ND
E-I	B81/2	53[‡]	57	ND	88	75	35[‡]	46	34	6	5
N-I	RV 148	56	50	64	ND	48[‡]	100	83	38	23	4
N-I	RV 83-180	ND	ND	ND	ND	ND	93	ND	ND	8	6
E-III	HC82/15	47	46	46	51	ND	ND	100	ND	ND	ND
E-III	W14495	62	58	56	67	63	72	97	38	12	12
E-III	M83-11-03	ND	ND	ND	ND	ND	68	ND	ND	12	6
K	OKM2063	ND	ND	ND	ND	ND	67	ND	ND	ND	ND
J	J-8	ND	ND	ND	ND	ND	78	ND	ND	ND	ND
N-III	OKM18761	ND	42	39	42[‡]	52	74	41	100	8	3
C. maculata[†]	RV 85/3	ND	ND	ND	ND	ND	7	ND	1	100	8
C. cirrhata[†]	VT 1216	ND	ND	ND	ND	ND	1	ND	5	6	100
C. iocephala[†]	TB 47	ND	ND	ND	ND	ND	21	ND	9	21	15
C. biformis[†]	RV 83/69	ND	ND	ND	ND	ND	7	ND	1	5	1
C. subnuda[†]	RV 83/88	ND	ND	ND	ND	ND	3	ND	3	15	6
C. luxurians[†]	GB 194	ND	ND	ND	ND	ND	6	ND	0	6	7

The values shown represent normalized percentage hybridization with respect to a given reference DNA. ND, NPH value not determined.
*Intersterility group (biological species) from Fig. 1.
[†]Representative outgroup species of *Collybia* showing little homology with the *C. dryophila* group.
[‡]NPH value based on a single determination. All other values are based on averages of two determinations.

RESULTS AND DISCUSSION

Mating Relationships. Mating compatibility relationships between European, North American, and Asian members of the *C. dryophila* group are summarized in Fig. 1. Matings within a single European or North American biological species (denoted by continent and roman numeral) occur frequently and are under the control of a bifactorial mating system with multiple alleles (1, 2); details concerning intra-group mating compatibility will be presented elsewhere (R.J.V., unpublished data). Different biological species occurring within a single continent are intersterile. Mating relationships among biological species from different continents were as follows: three of the European species are each mating compatible with one of three of the North American biological species; both of the Asian populations are inter-compatible with each other and with an intercompatible pair of European and North American species.

DNA Sequence Homology. The genomes of most fungi studied to date are relatively small, and consist largely (>80%) of unique sequences (11). Because DNA from fungi used in this study also contains relatively little repetitive DNA (unpublished data), we did not separate DNAs into unique and repetitive fractions before labeling them with [125]I.
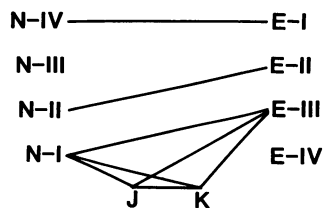

FIG. 1. Mating-compatibility relationships among fungi in the *C. dryophila* group. Intragroup matings occur frequently and are under control of a bifactorial mating system (5). Solid lines indicate mating compatibility between groups from different continents. Groups not connected by a line are incompatible (intersterile).

Since the presence of repeated DNA in our samples could possibly obscure the measure of genetic relatedness (12), a series of low C_0t incubations were included in the study to determine what effect repetitive DNA had on heterologous hybridization. Results of parallel hybridization experiments comparing high C_0t (complete reassociation) and low C_0t (only repeated sequences reassociated) homology values suggest that repetitive DNA affects our measure of genic relatedness, but that this effect is equal for different DNAs (Fig. 2). Although repetitive DNA apparently evolves about twice as fast as single-copy DNA (based on the slope of the data in Fig. 2), correlation of NPH with genetic distance is not affected at high C_0t NPH values above 25%. The effect of repetitive DNA on the measurement of genetic relatedness might be greater when DNAs from more distantly related organisms (e.g., other species of *Collybia*) are compared. At lower NPH values (below 20%), this linear relationship between high and low C_0t homology disappears as the repetitive DNA assumes a higher proportion of the total hybridization detected. For the NPH values observed in this study, however, the small amounts of repeated sequences in our labeled DNAs do not appear to affect the relative degree of genetic similarity observed among species. Similar congruity between homology values for repetitive and single-copy DNA fractions are also reported for *Drosophila* species (13), which also possess little repetitive DNA.

The results of DNA·DNA hybridization experiments between different biological species are summarized in Table 1. The average standard error for replicate measurements was <5%. In general, most reciprocal NPH values between different pairs of reference strains were in agreement. Several nonreciprocal NPH values were observed (e.g., between DNAs of OKM 18761 and RV 148): these nonreciprocal values are probably due to experimental error, but they may also represent differences in genomic DNA content of these species (14, 15).

Phylogenetic Analysis. NPH values reflecting DNA sequence homology among the *Collybia* strains range from 34%

Evolution: Vilgalys and Johnson
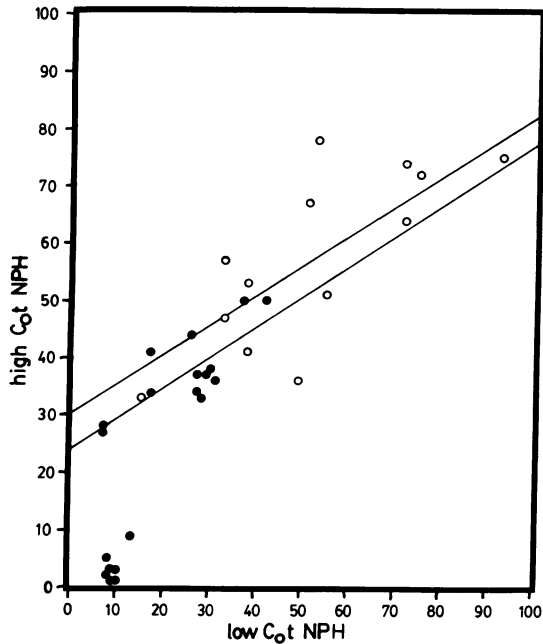
Proc. Natl. Acad. Sci. USA 84 (1987)   2357

FIG. 2. Effect of repeated sequences on the measure of DNA sequence homology between different fungi. Labeled DNAs originating from a more closely related (RV 148; o) and a more distant (OKM 18761; ●) strain were hybridized to heterologous DNAs from other *Collybia* strains. In each experiment, DNAs were allowed to reassociate to a low EC_0t of 10 (only repeated sequences allowed to hybridize) and to a high EC_0t of 1300 (complete renaturation). DNA·DNA hybridization was carried out at 65°C in 1.17 M NaCl/1 mM Hepes, pH 7.0, and assayed using S1 nuclease (6, 7). NPH is the ratio of heterologous/homologous reassociation (corrected for self-renaturation of the labeled DNA). Regression lines for each experiment do not differ significantly for high C_0t NPH values above 20%.

to 107%. Within a single biological species, NPH values ranged down to 75%, but were usually above 90%. Average interspecific NPH values were used to construct a phylogram (Fig. 3) by UPGMA (16). The correlation between the patristic distances shown in the tree and the average NPH values between groups is 0.95 and suggests there is little distortion of the actual phylogenetic relationships due to
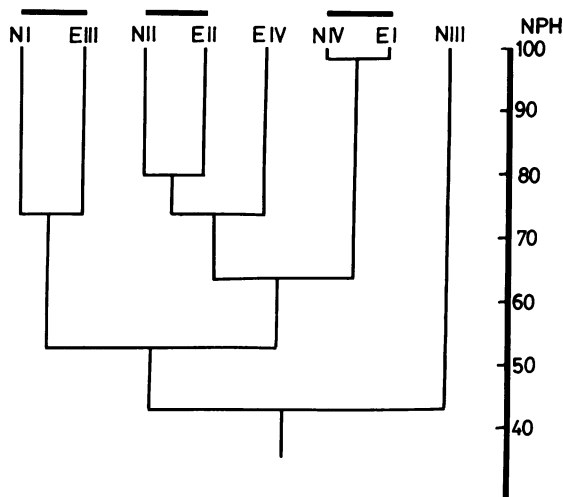

FIG. 3. Phylogram produced using UPGMA showing evolutionary relationships of fungi in the *C. dryophila* group based on average NPH values between North American (N-I through N-IV) and European (E-I through E-IV) biological species. Solid lines above the phylogram indicate mating compatibility between allopatric groups.

experimental errors or other factors (e.g., nonreciprocity, heterogeneous rates of divergence within different lineages). Mating-compatibility relationships among different groups are indicated by a solid horizontal bar above intercompatible species in Fig. 3. Mating compatibility corresponds well with high levels of DNA hybridization and indicates that mating compatibility is indicative of genetic similarity. However, there does not appear to be a single NPH value below which these fungi are found to be intersterile. For example, NPH values between the intercompatible groups E-III and N-I range down to 72%. Similar NPH values exist between group E-IV and two intercompatible groups, E-II and N-II, with which it is intersterile. In fact, no clear pattern of phylogenetic relationship is evident for groups E-IV, E-II, and N-II, since their relative branch lengths fall within the range of experimental error noted for NPH data (17).

Incongruity between genic identity and mating compatibility has also been noted in other fungi. In the yeast genus *Issatchenkia*, fertile ascospore progeny resulted from crosses between strains having as little as 25% sequence complementarity (18). Taken together with our results above, these observations suggest that the development of intersterility barriers during speciation may occur independently of DNA sequence divergence.

Evidence for Allopatric Speciation. Our data demonstrate that mating-compatible strains existing allopatrically are more divergent than when they are sympatric. In each case in which intragroup comparisons were made, NPH values were mostly above 90% between sympatrically derived isolates. Lower NPH values were obtained, however, when European and American mating-compatible strains were compared (examples are N-I with E-III, and N-II with E-II). Reduced sequence homology is also apparent between the two Asian isolates (K-1 and J-8) and the North American reference strain (RV 148) with which they are mating compatible. These observations suggest that intersterile groups in the *C. dryophila* group may have arisen primarily via an allopatric mode of evolution.

The case of groups N-IV and E-I represents the single exception to the trend for higher divergence among allopatric populations. Failure to demonstrate reciprocity for NPH values between DNA hybrids formed between the North American isolate of group N-IV and European isolates from group E-I (107% NPH versus the reciprocal comparison of only 88%) may be attributable to experimental error, since only a limited number of comparisons were made. An alternative explanation might be that species N-IV and E-I are closely related but differ in genome size through polyploidy or aneuploidy.

Molecular Evolution. The level of DNA sequence divergence associated with speciation in these fungi is striking and is considerably greater than that observed in other groups of eukaryotes (17, 19, 20). Since DNA hybridization is highly dependent on reaction conditions used for reassociation (10), data from different laboratories should be compared with caution. The NPH values reported for the *C. dryophila* group ranged down to 37%. Similar NPH values in other eukaryotes correspond to different subfamilies of rodents (14), orders of birds (17), superfamilies of primates (20), species of insects (13), and different sections of a single genus in higher plants (15). This level of divergence is startling for a group conventionally viewed as a single species. DNA hybridization of other outgroup species of *Collybia* with the *C. dryophila* group resulted in NPH values below 23%, indicating negligible homology between these species (Table 1).

Similar high levels of divergence have been reported for interspecific DNA hybrids in other fungi (21–23). Two explanations could account for such a high degree of genetic divergence: (*i*) Rates of molecular evolution may be greater for fungi than for other organisms. Evidence for varying rates

of molecular evolution in different phyletic groups is presented by Britten (24). A high rate of molecular evolution in fungi would contrast with an apparently slow rate of organismal evolution demonstrated by the high degree of morphological similarity of many mushroom species. (*ii*) These groups represent ancient lineages. The scanty fossil data for the Basidiomycotina places them back to the Pennsylvanian, 300 million years before present (25).

The complex mating relationships and range of DNA hybridization values observed in our study suggest that there are several species in the *C. dryophila* group. Although DNA hybridization studies have primarily been used to define taxonomic relationships among fungi (22), data from this study and others also provide insight into fungal evolution by indicating that speciation in fungi is accompanied by tremendous genetic change.

1. Raper, J. (1966) *Genetics of Sexuality in Higher Fungi* (Ronald, New York).
2. Burnett, J. H. (1975) *Mycogenetics* (Wiley, London).
3. Burnett, J. H. (1983) *Trans. Br. Mycol. Soc.* **81**, 1–14.
4. Anderson, J. B. & Ullrich, R. C. (1979) *Mycologia* **71**, 402–414.
5. Vilgalys, R. & Miller, O. K., Jr. (1983) *Mycologia* **75**, 707–722.
6. Vilgalys, R. (1985) Dissertation (Virginia Polytechnic Institute and State University, Blacksburg, VA).
7. Johnson, J. L. (1985) *Methods Microbiol.* **18**, 33–74.
8. Specht, C. A., DiRusso, C. C., Novotny, C. P. & Ullrich, R. C. (1982) *Anal. Biochem.* **119**, 158–163.
9. Selin, Y. M., Harich, B. & Johnson, J. L. (1983) *Curr. Microbiol.* **8**, 127–132.
10. Britten, R. J., Graham, D. E. & Neufeld, B. R. (1974) *Methods Enzymol.* **27E**, 363–418.
11. Dutta, S. K. (1974) *Nucleic Acids Res.* **1**, 1411–1419.
12. Britten, R. J. & Kohne, D. E. (1969) *Science* **161**, 529–540.
13. Entingh, T. D. (1970) *Genetics* **66**, 55–68.
14. Brownell, E. (1983) *Evolution* **37**, 1034–1051.
15. Belford, H. S. & Thompson, W. F. (1981) *Heredity* **46**, 91–108.
16. Rohlf, F. J., Kishpaugh, J. & Kirk, D. (1979) *NT-SYS: Numerical Taxonomy System of Multivariate Statistical Programs* (State Univ. of New York at Stony Brook).
17. Sibley, C. G. & Ahlquist, J. E. (1983) in *Current Ornithology*, ed. Johnston, R. F. (Plenum, New York), Vol. 1, pp. 245–292.
18. Kurtzman, C. P., Smiley, M. J. & Johnson, C. J. (1980) *Int. J. Syst. Bacteriol.* **30**, 503–513.
19. Sibley, C. G. & Ahlquist, J. E. (1984) *J. Mol. Evol.* **20**, 2–15.
20. Benveniste, R. E. & Todaro, G. C. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4513–4518.
21. Bicknell, J. N. & Douglas, H. C. (1970) *J. Bacteriol.* **101**, 505–512.
22. Kurtzman, C. P. (1984) *Microbiol. Sci.* **1**, 44–48.
23. Horgen, P. A., Arthur, R., Davy, O., Moum, A., Herr, F., Strauss, N. & Anderson, J. (1984) *Can. J. Microbiol.* **30**, 587–593.
24. Britten, R. (1986) *Science* **231**, 1393–1398.
25. Dennis, R. L. (1969) *Science* **163**, 670–671.