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AFLP characterization of three argentine *Coprotus* species

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Abstract — AFLP methodology was applied to characterize three *Coprotus* species (*C. lacteus, C. niveus, C. sexdecimsporus*) so as to estimate the levels of polymorphism within species, to analyze the phenetic relationships among them, and to contrast the AFLP findings to those of a previous RAPD study. The high number of AFLP bands obtained with the six assayed primers allowed us to detect intra-specific variability. The genetic variability within species obtained using AFLP (measured in terms of percentage of polymorphic loci) was two to three times higher than those obtained by RAPD. The phenograms generated by AFLP markers grouped all strains of the same species into three defined clusters, and a higher association between *C. lacteus* and *C. sexdecimsporus* was also observed. The AFLP technique could become a powerful tool for genera such as *Coprotus*, in which a high intra-specific homogeneity does not allow detection of genetic variability using other PCR-based markers.

Key words — *Ascomycota*, molecular markers, coprophilous

Introduction

The fungal genus *Coprotus* Korf & Kimbr. comprises coprophilous discomycetes, which belong to the *Pyronemataceae* Corda. Species of this genus are homothallic, probably due to an adaptation to the substrate, which determines their sexual isolation (Wicklow 1981). Sexual reproduction produces ascospores dispersed by effective discharge methods.

A homothallic mating system can be very difficult to distinguish from clonality using some molecular markers, since both systems produce progenies that do not show evidence of segregation for these markers and yield linkage disequilibrium at the population level over the long term (Billiard et al. 2012).

Coprotus species have been traditionally distinguished by such cytological and morphological characters (Kimbrough et al. 1972) as the number of ascospores per ascus, the presence or absence of pigments in paraphyses and excipulum, and the size and shape of asci, ascospores and sterile elements.

Different concepts have been used to define fungal species. The phenotypic concept is the classic approach based on morphology, and *Coprotus* species have traditionally been classified on cytological and morphological characters (Kimbrough et al. 1972). However, difficulties often arise while attempting to identify *Coprotus* species, as they are morphologically very similar, characters frequently overlap, and some vary greatly according to the culture conditions (Dokmetzian et al. 2005). A polythetic concept, which circumscribes species based on a combination of characters (Kohn 1992, Guarro et al. 1999), could prove helpful in characterizing the *Coprotus* genus.

RAPD [random amplified polymorphic DNA] analysis was performed on *Coprotus* species by Ramos et al. (2008). In that study, the RAPD technique confirmed the previous identification of strains using morphological characters (Dokmetzian et al. 2005) and provided a greater number of species-specific bands, but genetic variability within species (in terms of percentage of polymorphic loci) was very low. All strains had the same geographic origin and showed identical patterns for the six primers assayed on the three *Coprotus* species studied. Therefore, it would seem that to detect variability among strains, other PCR markers, such as AFLP, should be used.

The amplified fragment length polymorphism (AFLP) method (Vos et al. 1995) is a DNA-based fingerprinting technique, which requires no prior sequence information on the organism under scrutiny. This method is used to determine the degree of similarity among isolates and offers certain advantages over other techniques, i.e., high level of identified polymorphism, high reproducibility, and relative technical simplicity (Briad et al. 2000). AFLP analysis has been successfully used to estimate the amount of genetic variation within fungal species. Given that a large number of amplicons can be screened, AFLP is especially useful for characterizing clonal lineages and for establishing phenetic relationships among species with little morphological differentiation. AFLP has a clear advantage over other PCR based markers such as RAPDs, not only in terms of reproducibility but also because more loci can be screened in each reaction.

In our present study we applied the AFLP methodology to characterize three *Coprotus* species—*C. lacteus* (Cooke & W. Phillips) Kimbr., *C. niveus* (Fuckel) Kimbr., and *C. sexdecimsporus* (P. Crouan & H. Crouan) Kimbr. & Korf—so as to estimate the levels of polymorphism within species, to analyze the phenetic relationships among them, and to contrast the findings of our AFLP study to those of a previous RAPD study.

Table 1. Fungal strains of *Coprotus* species used, with their Argentinian collection localities and BAFC numbers

Strain	Geographical location *	BAFC
C. lacteus		
lacA1(11), lacA3(12), lacA6(13), lacA10(14), lacA13(15)	Agronomía, CABA	874, 1937, 1940, 1941, 1942
lacL1(16), lacL 3 17), lacL4(18), lacL6 (19)	Villa Lugano, CABA	1944, 1945, 1946, 1947
C. niveus		
nivBC2 (25), nivBC3 (27), nivBC4 (30)	Bahia Craft, Villa La Angostura, NQ	1970, 1971, 1972
nivE1 (48), nivE2 (49)	Bahía Ensenada, TF	1956, 982
nivC2 (42), nivC3 (38), nivC4 (45), nivC5 (46)	Campana, PBA	1960, 1961, 1962, 1963
nivU1 (36), nivU3 (37), nivU6 (39), nivU7 (40), nivU8 (41)	Ciudad Universitaria, CABA	1964, 1965, 1966, 1967, 1968
nivL1 (34), nivL3 (35)	Villa Lugano, CABA	1973, 1974
C. sexdecimsporus		
sexU1 (6), sexU2 (7), sexU4 (8)	Ciudad Universitaria, CABA	1952, 1953, 1954
sexG1 (5), sexG4 (21), sexG7 (23)	Los Gigantes, C	1948, 1950, 1951

* C = Córdoba province:, CABA = Buenos Aires city; NQ = Neuquén province; PBA = Buenos Aires province; TF = Tierra del Fuego province.

Materials & methods

Isolation and maintenance of monosporic strains

The characters used to identify the *Coprotus* species were those used by Kimbrough et al. (1972). The isolation and maintenance of monosporic strains followed the procedure indicated by Suárez et al. (2006). Thirty-one monosporic strains, from nine geographical locations, were used (Table 1). All strains were deposited in the Herbarium and Culture Collection of the Department of Biodiversity, Faculty of Natural & Exact Sciences, University of Buenos Aires, Argentina (BAFC).

DNA extraction

Mycelium was obtained as in Ramos et al. (2000) and ground to powder in liquid nitrogen using a sterile pestle. Genomic DNA was extracted following Gottlieb & Lichtwardt (2001). Quality control and quantification of genomic DNA was carried out by agarose gel (0.8% w/v) electrophoresis and by comparison with a DNA molecular-size standard (Lambda EcoRI/HinDIII, Promega Corp.). Ethidium bromide gels were photographed under UV light.

AFLP

The AFLP methodology was carried out on 250 ng of genomic DNA using the AFLP® Analysis System for Microorganisms Primer Kit (Invitrogen) as described in the instructions manual with minor modifications (Gottlieb et al. 2005). Selective primers were combined as in Table 2.

Table 2. AFLP primers used in this study

Primers	Sequence 5′-3′
E+ACG	GAC TGC GTA CCA ATT CAC G
E+AAC	GAC TGC GTA CCA ATT CAA C
E+AAG	GAC TGC GTA CCA ATT CAA G
M+A	GAT GAG TCC TGA GTA AA
M+G	GAT GAG TCC TGA GTA AG
M+C	GAT GAG TCC TGA GTA AC
M+T	GAT GAG TCC TGA GTA AT

* E stands for *EcoRI* and M stands for *MseI*.

All PCR amplifications were performed in a TECHNE PROGENE thermal cycler. Polyacrylamide gel electrophoresis conditions followed Gottlieb et al. (2005). A 30–330 bp AFLP® DNA Ladder (Invitrogen) size marker was included twice in each electrophoresis, and the size of AFLP bands scored ranged from 90 to 330 bp. AFLP bands were visualized using the SILVER SEQUENCE TM DNA Sequencing System (Promega).

Statistical methods

The data were extracted as a table and marked as either present (1) or absent (0). Monomorphic bands (bands present in all individuals of a species) were discriminated within each species and across the entire data set. The binary matrix was analyzed with the program NTSYS-PC version 2.02 (Rohlf 1993). The unweighted pair-group arithmetic mean method (UPGMA) cluster analysis was performed on the simple matching (SM) association coefficient (Sneath & Sokal 1973), and the same program generated the phenogram showing similarity relations. The distortion produced during the grouping analysis was calculated using the correlation cophenetic coefficients (r) (Sokal & Rohlf 1962) using the NTSYSPC version 2.02 (Rohlf 1993) program. A cophenetic correlation (r) of 0.8 was considered a good fit. A three-dimensional graphic was obtained with the principal coordinated ordination method (Gower 1966).

The percentage of polymorphic loci (P = (number of polymorphic loci/ number of loci analyzed) × 100) for each primer combination was calculated.

Results

The analysis of the six primers that produced clear and reproducible bands by AFLP amplification yielded a total of 926 bands that appeared consistently in all experiments among the amplified fragments of the 31 isolates. Although the amplified bands ranged from 50 to 550 bp, we analyzed only bands from 90 to 330 bp.

Table 3 describes the total number of AFLP (polymorphic + monomorphic) bands and the percentage of polymorphic loci obtained for each selective primer combination in the three assayed fungal species. All primers detected polymorphic bands within species and a high percentage of polymorphic loci

Table 3. Total number of AFLP (polymorphic + monomorphic) bands detected for each selective primer combination per *Coprotus* species assayed.

Primer combination	Species	Total N° of bands	Polymorphic bands	Monomorphic bands	P%
M+T/E+ACG	*C. lacteus*	50	16	34	32
	C. niveus	27	7	20	26
	C. sexdecimsporus	72	60	12	83
M+C/E+AAG	*C. lacteus*	101	28	73	28
	C. niveus	108	10	98	9
	C. sexdecimsporus	163	141	22	86
M+A/E+AAG	*C. lacteus*	75	12	63	16
	C. niveus	90	38	52	42
	C. sexdecimsporus	152	135	17	89
M+C/E+ACG	*C. lacteus*	77	23	54	30
	C. niveus	129	75	54	58
	C. sexdecimsporus	156	144	12	92
M+G/E+ACG	*C. lacteus*	79	26	53	33
	C. niveus	80	36	44	45
	C. sexdecimsporus	100	74	26	74
M+A/E+ACG	*C. lacteus*	74	24	50	32
	C. niveus	70	14	56	20
	C. sexdecimsporus	143	130	13	90

were obtained for *C. sexdecimsporus* and a reasonable percentage for *C. niveus* and for *C. lacteus.*

Genetic variability within species, measured in terms of percentage of polymorphic loci was variable: P = 87 % for *C. sexdecimsporus,* P= 36 % for *C. niveus* and, P= 28 % for *C. lacteus.*

The primer group M+C/E+ACG was more effective in detecting polymorphisms (Table 3).

Degree of similarity and cluster analysis

The phenogram obtained using the UPGMA method was constructed based on Simple Matching (SM) coefficients. Little distortion occurred while constructing this phenogram, as implied by the value of the correlation cophenetic coefficient (r=0.991). There was no direct correlation between molecular genotype and geographic origin.

The UPGMA phenogram (Fig. 1) grouped all strains in three defined clusters that correspond to the three *Coprotus* species.

The degree of similarity among species was low. Between *C. lacteus* and *C. sexdecimsporus*, the coefficient of similarity was S = 0.5. The group formed

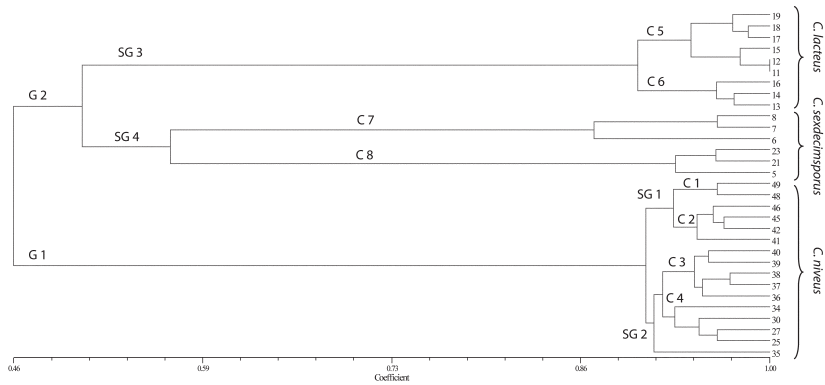

Figure 1. UPGMA phenogram showing relationships among *Coprotus lacteus, C. sexdecimsporus,* and *C. niveus* isolates based on the simple matching (SM) association coefficient estimated from AFLP loci.

by the isolates identified as *C. niveus* clustered with the other two species with a coefficient of similarity of S = 0.46.

The main group (Fig. 1, group G1) comprised two subgroups (SG1 and SG2) formed by all isolates of *C. niveus*. Subgroup SG1 is formed by two sets (C1 and C2) and SG2 subgroup comprised C3 and C4 sets and one isolate separated from the rest. A second group (Fig. 1, G2) included two subgroups (SG3 and SG4). Subgroup SG3 is formed by two sets (C5 and C6) that included all isolates of *C. lacteus*. The other subgroup (SG4) comprised C7 and C8 sets, formed by all isolates of *C. sexdecimsporus.*

Intra-specific variability detected for *C. lacteus* and *C. niveus* was low. This fact is reflected by the association coefficient: SM = 0.91 among isolates of *C. lacteus* and SM = 0.92 among *C. niveus* isolates. Only two pairs of isolates presented 100% of similarity (association coefficient 1.0), and they belonged to *C. lacteus*. *Coprotus sexdecimsporus* showed a higher degree of variability than the other species, with SM = 0.54 among all isolates of this species.

The ordination of isolates through the principal coordinated method allowed us to recognize three groups (Fig. 2, groups 1–3) in three-dimensional dispersion revealing nearly the same relations between isolates as the phenogram (Fig. 1). The first group included all isolates of *C. lacteus*, very closely attached in the three axes. The second set showed *C. sexdecimsporus* isolates differentiated in axis 1 but very closely in the other two axes. The third comprised isolates of *C. niveus* closely together in the three axes, thus revealing a high degree of similarity.

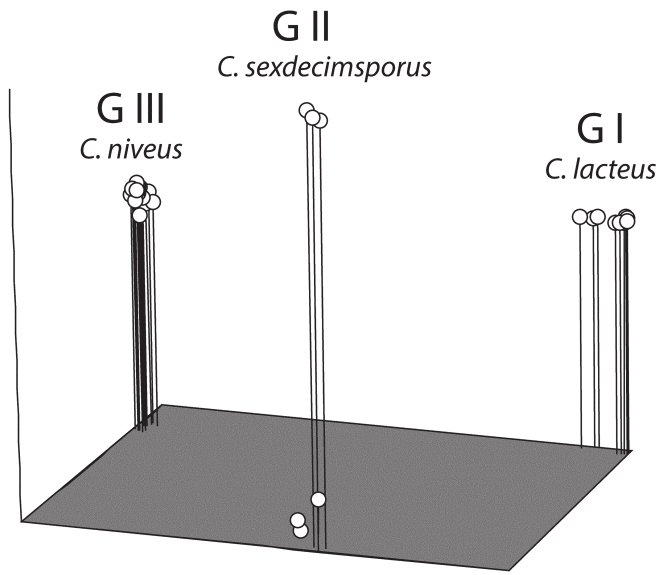

Figure 2. Three-dimensional graphic of *Coprotus* species obtained with the Principal Coordinates ordination technique.

Discussion

In the present study the high number of AFLP bands obtained with the six primers assayed allowed us to detect intra-specific variability.

Previous biochemical and RAPD studies detected a high intra-specific homogeneity (Suárez et al. 2006, Ramos et al. 2008), which could be related to the fact that *Coprotus* is homothallic and produces clonal offspring (Rayner 1994). In the present study the genetic variability within species, in terms of percentage of polymorphic loci, was two to three times higher than those obtained by RAPD. AFLP fingerprinting is more effective for detecting genetic diversity in species with high intra-specific homogeneity (e.g., *Coprotus*), because it generated a very large number of polymorphic loci.

Genetic diversity could be a potential indicator of the relative abundance of sexual and asexual reproduction (Chen & McDonald 1996). Furthermore other alternatives for genetic interaction among fungi besides sexual recombination are heterokaryosis and mitotic recombination (Esser & Kuenen 1967). In this

sense, natural selection could lead to genetic divergence and therefore increase genetic variability levels in the homothallic fungi as *Coprotus* (non-out crossing populations). The AFLP technique allowed us to detect such intraspecific variability.

In the phenograms generated by AFLP and RAPD markers, all strains of the same species group into three defined clusters. Besides, both phenograms showed very similar phenetic relationships among species.

The phenogram obtained from AFLP datasets showed a higher association between *C. lacteus* and *C. sexdecimsporus*. This result is consistent with that obtained by means RAPD markers. This agreement might be related to the nature of these markers since AFLP and RAPD are usually considered neutral markers. Sampling and genotyping the progeny within sexual structures in natural populations should allow determining whether haploid self-mating system actually occurs in nature (Billiard et al. 2012).

The AFLP technique could become a powerful tool for researching genera with homothallic mating systems such as *Coprotus*, in which a high intraspecific homogeneity does not allow detection of variability using other PCR-based markers.

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