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Characterization of three species of the genus *Coprotus* (Ascomycota) by isozyme analysis

María Eugenia Suárez[1], María Esther Ranalli, Diana Ana Dokmetzian & Araceli Marcela Ramos[2]

[1] *euge@bg.fcen.uba.ar* [2] *araceli@bg.fcen.uba.ar*
Depto. Biodiversidad y Biología Experimental, FCEN, Universidad Buenos Aires Pab. II Ciudad Universitaria, Buenos Aires, C1428EHA, Argentina

Abstract—Identification of *Coprotus* species has never been an easy task. Their morphological and cultural characteristics are very similar and this often makes species delimitation very difficult. In this study we first identified 44 monosporic strains of three species of the genus (*C. lacteus, C. niveus, C. sexdecimsporus*) by using exclusively morphological and cultural characters; then, an extensive isozyme analysis was performed as an additional taxonomical technique. Eleven isozyme systems were tested. Six of them were chosen for the following analysis. The phenogram (UPGMA) and the 3D graphic (ordination technique) clearly separated the three species. The results of this study support the utilization of isozyme patterns as a valuable additional tool in delimiting *Coprotus* species based on traditional taxonomical methods.

Keywords—fungi, taxonomy, phenetics

Introduction

The genus *Coprotus* Korf ex Korf & Kimbr. comprises homothallic species previously placed in *Ascophanus* Boud. and *Ryparobius* Boud. (Kimbrough 1966, Kimbrough et al. 1972). It was originally placed in the tribe *Theleboleae* (Bref.) Kimbr. (=*Pseudoascoboleae* Boud.) of the *Pezizaceae* Dumort., but in more recent studies the tribe was raised to family rank (Kimbrough & Gibson 1980). Since 1974, when Kish suggested transferring *Coprotus* to *Pyronemataceae* Corda, based on cytological and developmental studies, many other arguments have been found that strongly ratify this movement.

Coprotus includes those species of coprophilous discomycetes with non-amyloid operculate asci containing hyaline, smooth, elliptic ascospores that usually develop one de Bary bubble. Apothecia are small, superficial, sessile, white to bright orange, and pulvinate to discoid in shape. Paraphyses are always septate, simple or branched, and usually curved in the apex. Traditional identification of *Coprotus* species is exclusively based on cytological and

morphological characters, such 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. However, difficulties often arise while attempting to identify *Coprotus* species, as they are morphologically very similar and characters frequently overlap.

In the last few decades, there has been a clear tendency towards the utilization of biochemical and molecular characters as a complement to the classic methods of fungal species identification. Morphological, cytological and developmental characters are not always sufficient to allow clear species identification, especially in taxonomical groups with overlapping characters, or simply in polymorphic fungi that change the size, shape and pigmentation of their structures according to the variation of environmental factors.

Isozymes are multiple forms of an enzyme that share a common substrate and catalyse the same reaction (Markert & Moller 1959). They can exist in the same individual or in different individuals of the same species or taxon, and catalyse reactions either in separate cellular compartments or tissues, or in different metabolic conditions (Markert 1975).

Isozyme analysis is one of the most commonly employed techniques to evaluate genetic variation at population and species level. This technique may provide essential data to clarify evolution and taxonomical problems, and it is particularly useful in classifying problematical groups, such as synmorphic species (Ferreyra 2000). In the past twenty years, isozyme analysis has been satisfactorily employed to delimit fungal taxa and to identify unknown fungi at species or subspecies level (Micales et al. 1992). Several authors have delineated fungal species using isozymes: *Puccinia* (Burdon et al. 1983, Newton et al. 1985), *Penicillium* (Cruickshank & Pitt 1987), *Rhizopogon* (Ho & Trappe 1987), *Agaricus* (Kerrigan & Ross 1988), *Glomus* (Hepper et al. 1988), *Phytophthora* (Erselius & de Vallavieille 1984, Bielenin et al. 1988, Blaha et al. 1994, McHau & Coffey 1995), *Pleurotus* (Boisselier-Dubayle 1983, May & Royse 1988), *Tremella* (Hanson & Kenneth 1991), *Arthrobotrys* (Araújo et al. 1997), *Ganoderma* (Gottlieb et al. 1998), *Saccobolus* (Ramos et al. 1999, Ramos et al. 2000), *Mucor* (Vagvolgyi et al. 2001), *Fusarium* (Laday & Szecsi 2002, Aly et al. 2003), *Polyporus* (Borges da Silveira et al. 2003), *Zygosaccharomyces* (Duarte et al. 2004), and *Ascobolus* (Dokmetzian et al. 2005).

Taking into account the difficulties that frequently arise when identifying *Coprotus* species by traditional methods, as well as the fact that it has been well proved that isozymes are useful for delimiting fungal species, we performed an extensive isozyme analysis to characterize three species of the genus. The particular purposes of this analysis were to establish the degrees of intra- and interspecific similarity and also to evaluate the phenetic relations between strains in order to confirm our previous species identification.

Materials and methods

Monosporic strains

Forty-four monosporic strains of three species of the genus *Coprotus* (*C. lacteus* (Cooke & W. Phillips) Kimbr. et al. (1972), *C. niveus* (Fuckel) Kimbr. et al. (1972) and *C. sexdecimsporus* (P. Crouan & H. Crouan) Kimbr. & Korf (1967) were obtained from individual ascospore germinations, following the procedure indicated by Gamundí & Ranalli (1964). They were all deposited in the BAFC Herbarium & Culture Collection of the Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Table 1 shows a list of the strains with their geographical location, substrate and BAFC number. Cultures of all of the monosporic strains were regularly kept in PF medium (yeast extract, 3 g; agar 18 g; distilled water, 1000 ml; a slice of filter paper) (Ranalli & Forchiassin 1974) at 5°C.

Table 1. List of strains with their geographical location, substrate and BAFC number.

Strain	Geographical location	Substrate	BAFC	Strain	Geographical location	Substrate	BAFC
Coprotus lacteus				***Coprotus niveus***			
lacA1	Agronomía	cow dung	874	nivE1	Bahía Ensenada	cow dung	1956
lacA2	Agronomía	cow dung	1936	nivE2	Bahía Ensenada	cow dung	982
lacA3	Agronomía	cow dung	1937	nivE3	Bahía Ensenada	cow dung	1957
lacA4	Agronomía	cow dung	1938	nivE4	Bahía Ensenada	cow dung	1958
lacA5	Agronomía	cow dung	1939	nivE5	Bahía Ensenada	cow dung	1959
lacA6	Agronomía	cow dung	1940	nivC2	Campana	cow dung	1960
lacA10	Agronomía	cow dung	1941	nivC3	Campana	cow dung	1961
lacA13	Agronomía	cow dung	1942	nivC4	Campana	cow dung	1962
lacA14	Agronomía	cow dung	1943	nivC5	Campana	cow dung	1963
lacL1	Villa Lugano	cow dung	1944	nivU1	Ciudad Universitaria	horse dung	1964
lacL3	Villa Lugano	cow dung	1945	nivU3	Ciudad Universitaria	horse dung	1965
lacL4	Villa Lugano	cow dung	1946	nivU6	Ciudad Universitaria	horse dung	1966
lacL6	Villa Lugano	cow dung	1947	nivU7	Ciudad Universitaria	horse dung	1967
Coprotus sexdecimsporus				nivU8	Ciudad Universitaria	horse dung	1968
sexG1	Los Gigantes	cow dung	1948	nivBC1	Bahía Craft	cow dung	1969
sexG2	Los Gigantes	cow dung	873	nivBC2	Bahía Craft	cow dung	1970
sexG3	Los Gigantes	cow dung	1949	nivBC3	Bahía Craft	cow dung	1971
sexG4	Los Gigantes	cow dung	1950	nivBC4	Bahía Craft	cow dung	1972
sexG7	Los Gigantes	cow dung	1951	nivL1	Villa Lugano	cow dung	1973
sexU1	Ciudad Universitaria	horse dung	1952	nivL3	Villa Lugano	cow dung	1974
sexU2	Ciudad Universitaria	horse dung	1953	nivL4	Villa Lugano	cow dung	1975
sexU4	Ciudad Universitaria	horse dung	1954	nivL5	Villa Lugano	cow dung	1976
sexU5	Ciudad Universitaria	horse dung	1955				

Bahía Craft = Villa La Angostura, Neuquén province; *Bahía Ensenada* = Tierra del Fuego province; *Campana* = Buenos Aires province; *Los Gigantes* = Córdoba province. *Agronomía, Ciudad Universitaria* and *Villa Lugano* are different locations in Buenos Aires city.

Identification of species

Morphological and cultural studies were carried out in order to identify the species. The former included diverse characteristics of apothecia, ascospores, asci, and paraphyses characteristics, whereas the latter emphasized the time and percentage of NaOH, incubation time at 37ºC and the time in ET and solid GA media required for ascospore germination.

The key proposed by Kimbrough et al. (1972) was used for the identification of the species.

Growth media and culture conditions

Erlenmeyer flasks containing 50 ml of liquid growth medium GA (glucose, 10 g; asparagine, 4 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $CuSO_4.5H_2O$, 0.4 mg; $MnCl.4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $NaMoO_2.2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 10 mg; biotine, 5 µg; thiamine-HCl 0.1 mg; bidistilled water to complete 1 litre) (Galvagno 1976), were inoculated with a 5 mm^2 squares taken from a 5 to 10-day-old colony of monosporic strains growing in solid GA medium (glucose, 10 g; agar, 18-20 g; L-asparagine, 1.35 g, $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $CuSO_4.5H_2O$, 0.4 mg; $MnCl.4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $NaMoO_2.2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 10 mg; biotine, 5 µg; thiamine-HCl 0.1 mg; bidistilled water to complete 1 litre) (Galvagno 1976).

Liquid and solid cultures were both incubated in a New Brunswick Psicrotherm G-27 chamber, at 23°C, permanently lit by four fluorescent tubes of 20 W each; liquid cultures were placed in a rotary shaker at 125 rpm during incubation.

Growth media were sterilized at 121°C and 1.2 atm for 20 minutes.

Preparation of extracts

Mycelia were harvested from liquid cultures one to two days before they reached maximum growth, which was established by growth curves previously charted for each species.

Mycelia were vacuum filtered in a Buchner funnel, through Whatman GP filter paper, washed several times with bi-distilled water, dried with filter paper and stored at –70°C until used (Dessauer et al. 1984).

Extracts were prepared by freezing the mycelia with liquid nitrogen and crushing it several times in a steel mortar, and crushing it once again adding extraction buffer (0.1 M Tris-HCl buffer, pH 7.5); 0.1% v/v 2-mercaptoethanol; 0.001 M ethylenediaminetetraac etic acid (EDTA); 0.01 M KCl; 0.01 M $MgCl_2.6H_2O$; 10% p/v polyvinyl polypyrrolidone (PVP) 10.000) (Soltis et al., 1983). Homogenates were divided into small fractions and stored at –70°C (Dessauer et al. 1984).

Electrophoresis and enzymatic dying

A horizontal electrophoresis technique (Beckman & Johnson 1964) was performed to test eleven isozyme systems. Native gels were prepared using a 7% concentration of polyacrylamide (Saidman 1985). Table 2 shows a list of the eleven isozyme systems tested with their abbreviation and EC number as stated in IUPAC-IUB, Enzyme Nomenclature (1984).

Buffer solutions (gel buffer (a) and electrode buffer (b)) varied according to the specific isozyme system tested. Buffer: (a) Lithium borate pH 8.1 and (b) Lithium borate pH 8.5 (Scandalios 1969, modified by Saidman 1985) was used for AAT, EST and SOD; Buffer: (a) Tris-citrate pH 6.5 and (b) Tris-citrate pH 7 (Selander et al. 1971, modified by Saidman 1985) was employed for ACP, ALP, G6PD, GDH and IDH; and Buffer: (a) and (b) Tris-citrate pH 8 (Soltis et al. 1983) was chosen for the LAP, MDH and SKD systems.

Rectangles of 2 x 4 mm of Whatman N°3 paper were soaked in the protein extracts after thawing the samples, and were introduced into grooves made in the gel (20 per gel). Bromophenol-blue (4 mg/ml) was used as dye marker. Electrophoreses were carried out at 4°C and 100 volts for three to four hours, until the dye marker was at 3-4 cm from the end of the gel.

Staining procedures were performed according to Manchenko (1994) for ACP, ALP, G6PD, IDH and SKD; Soltis et al. (1983) for LAP; Wendel & Weeden (1989) for EST, GDH, MDH and SOD; and Vallejos (1983) for AAT. Once stained, gels were photographed and fixed with a solution of ethanol/ water/ acetic acid (5: 5: 1). Gels were finally transferred to a plastic bag, heat-sealed and kept at room temperature.

The relative position (Rf) of each band of enzymatic activity was determined as the ratio between the migration distance of each band from origin and the migration distance of the dye marker from origin.

Electrophoresis was repeated at least twice for every isozyme system for each strain. Electromorphs were drawn with the average Rf for each band.

Table 2. Isozyme systems tested, their abbreviation and EC number

Isozyme system	Abbreviation	EC number
Aspartate aminotransferase	AAT	2.6.1.1
Acid phosphatase	ACP	3.1.3.2
Alkaline phosphatase	ALP	3.1.3.1
Esterases	EST	3.1.1…
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49
Glutamate dehydrogenase	GDH	1.4.1.3
Isocitrate dehydrogenase (NADP)	IDH	1.1.1.42
Leucine aminopeptidase	LAP	1.4.1.9
Malate dehydrogenase (NAD)	MDH	1.1.1.37
Shikimate dehydrogenase	SKD	1.1.1.25
Superoxide dismutase	SOD	1.15.1.1

Numerical analysis

Statistical analyses were performed using the NTSYS-PC version 1.8 program (Rohlf 1993). The nine geographical groups of strains (groups of strains of the same species from the same geographical location) constituted the operative taxonomic units (OTUs), as no isoenzymatic differences were found between monosporic strains from the same geographical location.

Table 3. Morphological and cultural characters of the three *Coprotus* species

Character / Species	*C. lacteus*	*C. sexdecimsporus*	*C. niveus*
1. Apothecia			
Type of growth in the substrate	Solitary or gregarious	Solitary or gregarious	Solitary or gregarious
Colour when young	Translucid to white	Translucid to whitish	White or translucid
Colour when mature	Yellowish	Yellow to orange	Slightly yellowish
Form	Discoid to cupulate	Pulvinate	Discoid to cupulate
Diameter (µm)	200-500	500-1000	200-500
Other characteristics	Superficial, sessile, glabrous	Superficial, sessile, glabrous	Superficial, sessile, glabrous
2. Apothecial excipulum			
Texture	Angularis to globulosa	Globulosa	Globulosa to angularis
Staining	Cyanophilous and dextrinoid	Dextrinoid	Slightly cyanophilous
3. Asci			
Length (µm)	65-85	106-123	86-164
Width (µm)	15-20	23-28	29-41
Form	Clavate cylindrical	Clavate	Broadly clavate
Number of spores	8	16	64
Apex	Round or cupulate, central operculum	Round or cupulate, central operculum	Round, with a prominent central operculum
4. Ascospores			
Length (µm)	9.5-11	11.7-12.35	9.1-12.7
Width (µm)	5.8-6.5	7.8-9.1	5.9-7.3
Form	Elliptical	Elliptical	Elliptical
Colour	Hyaline	Hyaline	Hyaline
Exosporium	Smooth	Smooth	Smooth
Arrangement within the asci	Uniseriate or biseriate	Regularly biseriate, sometimes in threes in the apex	Irregularly arranged in the apical portion or occupying all the volume in small asci
5. Paraphyses			
Diameter (µm)	1.5-2	1.7-2.6	1.8-2.7
Form	Filamentous	Club	Filamentous
Branching	Simple or branched	Simple or bifurcated below	Simple or branched
Apex	Curved	Hooked	Curved
Other characteristics	Septate, slightly inflated	Hyaline, septate	Septate, hyaline, without oil droplets
6. Ascospore germination			
Optimum NaOH %	0.3	0.3	0.4
Treatment time with NaOH	30 minutes	30 minutes	20 minutes
Incubation time at 37°C	48-72 hours	48 hours	48 hours
7. Culture			
Mature fructifications in ET media	15 days	15-16 days	15 days
Mature fructifications in GA media	10 days	15-16 days	12 days

A data matrix was constructed by coding the presence (1) and absence (0) of bands (characters). A similarity matrix was then obtained by using the Simple Matching Coefficient (Sneath & Sokal 1973). Both a clustering method (Unweighted pair-group method using arithmetic averages, UPGMA) and an ordination technique (Principal Coordinates) were performed. With the former method, a phenogram was obtained, and the distortion produced during the grouping analysis was calculated with the cophenetic correlation coefficient (r) (Sokal & Rohlf 1962). A three-dimensional graphic was obtained with the ordination method.

Results

Morphological and cultural studies

Table 3 gives the morphological characters and cultural aspects observed for each *Coprotus* species. Overlapping of several qualitative and quantitative characters can be clearly seen in this table. For this reason, in many cases considerable difficulty was experienced in identifying the strains. A greater similarity between *C. lacteus* and *C. niveus* was observed.

Isozyme analysis

Only six of the eleven systems tested showed a good activity band resolution for every strain: AAT, ALP, EST, G6PD, IDH and SOD. The remaining systems (ACP, GDH, LAP, MDH and SKD) showed poor resolution, or none at all, and were therefore excluded from the following statistical analyses.

No isoenzymatic differences were found between strains from the same geographical location, and only EST revealed differences between geographical groups of the same species, thus proving the existence of a high intraspecific similarity.

Seventeen electromorphs were detected for the six systems chosen. Photographs of gels and zymograms of each electrophoretical phenotype are shown in Figures 1 and 2.

Two electromorphs were found for the AAT, IDH and SOD systems. In each case, all geographical groups of *Coprotus lacteus* and *C. niveus* shared an electromorph (A), while the two geographical groups of *C. sexdecimsporus* displayed a different pattern (B). Only one activity-band characterized the two electromorphs of AAT and IDH, while four bands were revealed in the two patterns found for SOD.

The ALP system was the only one that revealed a diagnostic pattern for each species (A-C), the three of them with only one band of enzymatic activity.

The G6PD system did not show differences between geographical groups or between species. Only one electromorph was found, with one band of Rf 16.

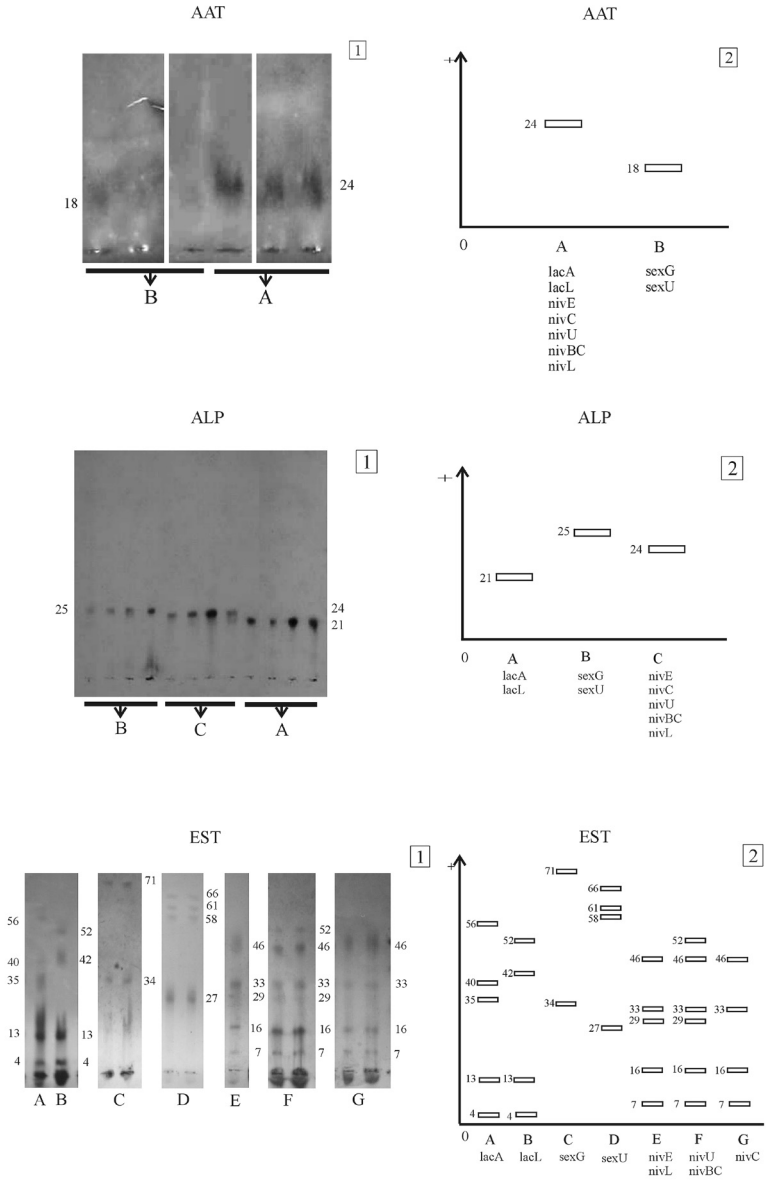

Figure 1. Gels photographs (1) and zymograms (2) of each electrophoretic phenotype found for AAT, ALP and EST isozyme systems. The geographical groups corresponding to each electromorph are indicated in (2).

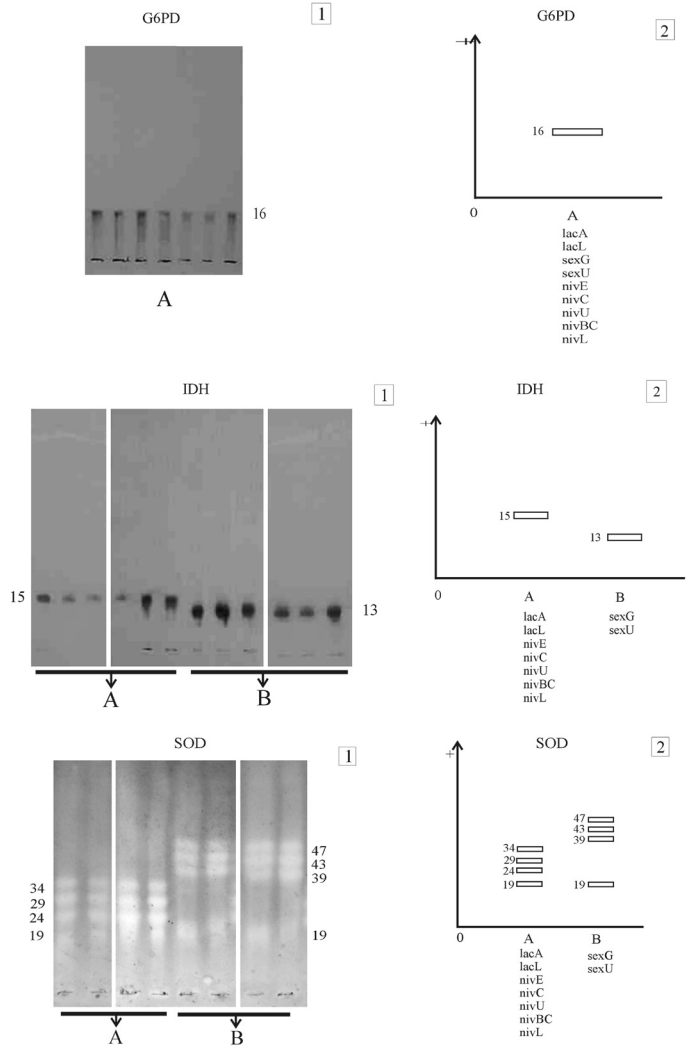

Figure 2. Gels photographs (1) and zymograms (2) of each electrophoretic phenotype found for G6PD, IDH and SOD isozyme systems. The geographical groups corresponding to each electromorph are indicated in (2).

On the contrary, EST was the system that revealed the greatest number of activity-bands and also the only one that allowed us to distinguish between geographical groups of the same species. The two geographical groups of *C. lacteus*, as well as the two of *C. sexdecimsporus*, showed a characteristic band pattern for EST (patterns A to D). The strains of *C. niveus* from Bahía Ensenada and Villa Lugano displayed another band pattern (E), and the same happened with those from Ciudad Universitaria and Bahía Craft, characterized by another electromorph (F). The group of strains from Campana revealed the seventh pattern (G) found for esterases for this species.

The phenogram obtained using the UPGMA clustering method is shown in Figure 3. Little distortion occurred while constructing this dendrogram, as implied by the value of the cophenetic correlation index (r=0.992). The three species are clearly separated in the phenogram. Apart from that, two main clusters of OTUs are distinctly seen: one of them covers the two geographical groups of *C. sexdecimsporus* with a similarity index of 80%, while the other includes all of the geographical groups of *C. niveus* and *C. lacteus*. This result reveals a higher isoenzymatic resemblance between these two species, which are associated by an index of 63%. The group of *C. sexdecimsporus* strains is associated to the other species by a remarkably low degree of similarity (33%). All the geographical groups corresponding to *C. niveus* proved to be practically identical, as in the phenogram they are related by a similarity index of 95%. The geographical groups of *C. lacteus* are associated to each other by an index of 84%, evidence of further isoenzymatic differences.

The three-dimensional graphic produced by the ordination technique (Figure 4) shows the same relations between different geographical groups and between species as the phenogram. It displays three main sets of OTUs separated in axes 1 and 2. The first one includes the two geographical groups of *Coprotus lacteus*, a little differentiated in axis 1 but very closely attached in the other two axes. The second set shows the five geographical groups of *C. niveus* joined closely together in the three axes, thus revealing a high degree of similarity. The third and last main set of OTUs comprises the two geographical groups of *C. sexdecimsporus*, which are very close to each other in axes 1 and 2, but are largely separated in axis 3. The differences among species as a unit also agree with the associations obtained with the phenogram: *C. lacteus* and *C. niveus* separate from each other in axes 1 and 2, but in axis 3 they are practically at the same level. *C. sexdecimsporus* separates itself from the other two species in the three axes, thus proving a higher isoenzymatic differentiation.

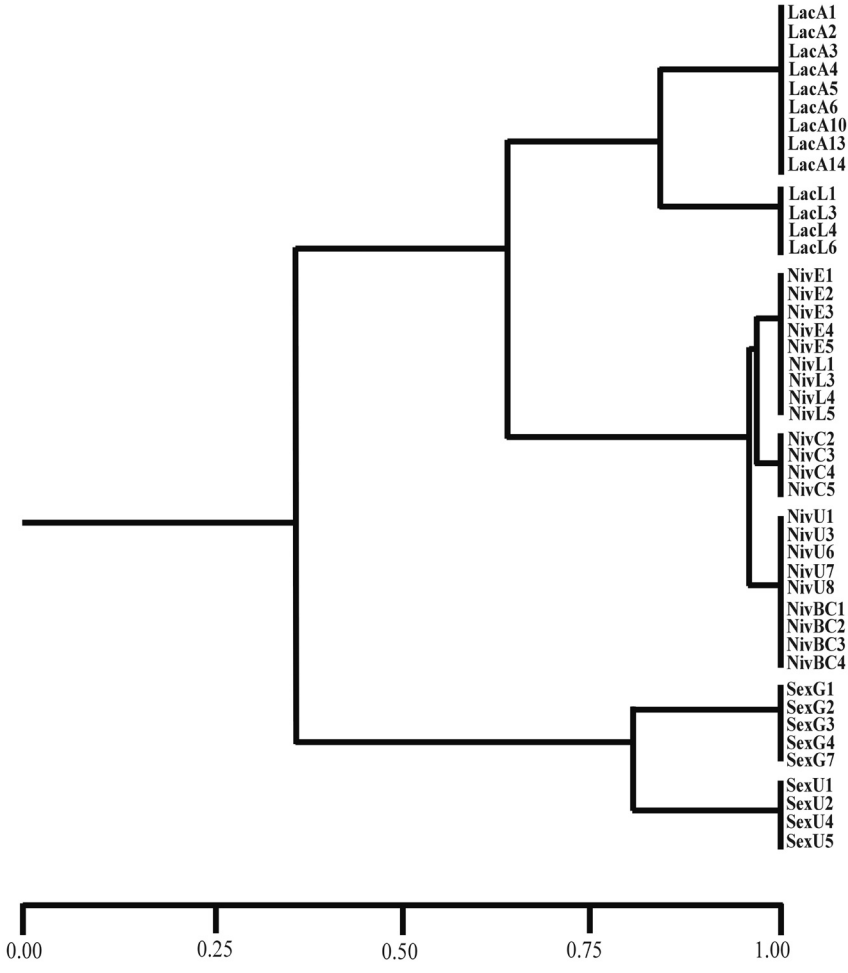

Figure 3. Phenogram obtained using UPGMA clustering method. For details on the strains, see Table 1.

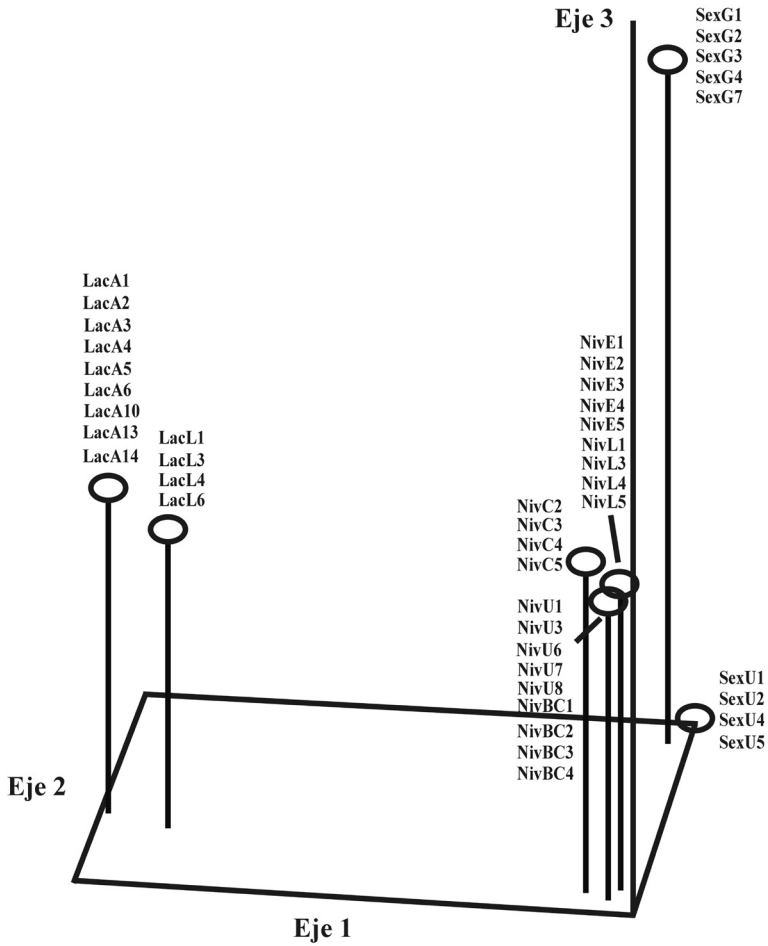

Figure 4. Three-dimensional graphic obtained with Principal Coordinates ordination technique. For details on the strains see Table 1.

Discussion

Although identification of the species was possible, morphological and cultural characterization of the strains proved the high similarity and coincidences in many of the characters traditionally used for identifying *Coprotus* species. Harrington & Rizzo (1999) suggest that the most important diagnostic characters to delineate fungal species would be those phenotypic characters

associated with the ecological niche, as they would play a decisive role in developing and maintaining fungal species through evolution. Hence, species should be delineated considering not only morphological but also other phenotypic characters, such as physiological and biochemical characteristics, including isozymes.

Another interesting result from cultural and morphological observations is that both qualitative and quantitative characters show that *C. lacteus* is more similar to *C. niveus* than to *C. sexdecimsporus*.

The isoenzymatic results confirmed the previous identification of the strains using morphological and cultural characters. Both the phenogram and the ordination graphic showed the same three clearly separated clusters of OTUs (geographical groups), each of them corresponding to one of the three species. The phenogram also showed that there is a greater isoenzymatic resemblance between *C. lacteus* and *C. niveus*, and this result is consistent with previous morphological observations.

The scarce intraspecific variability encountered during isozyme analysis highlighted interspecific differences. This was crucial for our work, because when high intraspecific variability exists it overshadows the differences between species, thus reducing the efficiency of the technique used to separate them. Studies using enzymes that detect high levels of intraspecific variability are incapable of distinguishing species (Racine & Langley 1980). Different types of enzymes show different levels of intraspecific variation according to the selection forces they are subjected to (Johnson 1974). Regulatory enzymes of the energetic metabolism, and even the enzymes that regulate the intermediate metabolism, generally evidence a lower variability than non-regulatory enzymes such as esterases. Despite the slight overestimation of the differences they may cause between OTUs, isozyme systems that generally reveal intraspecific variability, such as EST, are also crucial in achieving correct species characterization. Both types of enzymes (regulatory and non-regulatory) are therefore necessary to prevent overestimation and underestimation of isozyme variability within and between populations.

ALP was the only system that showed a diagnostic electromorph for each species. Hence, this was the most useful system in confirming our previous species identification. In any study that uses isozymes to identify species this is the expected kind of result, as they reveal a clear and easy species distinction.

The existence of band patterns shared by two or more species is consistent with the high overlapping in morphological characters associated with them. In addition, the fact that only one band for each species in the majority of the systems analyzed was obtained is in accordance with the fact that *Coprotus* species are haploid fungi with only one locus per enzyme (Ramos 1998).

The generally low intraspecific variability found may be related to habitat and the type of sexual reproduction of the *Coprotus* species. They are homothallic organisms, which is not unusual among coprophilous fungi. As they live in pieces of dung (island substrate), they undergo reproductive isolation. In these cases, homothallism allows them to complete their biological cycle and to reproduce sexually without requiring another thallus. Mutations are the principal source of genetic variation in a haploid homothallic organism, which explains the relatively scarce intraspecific isoenzymatic variability found in this study. The correlation between the degree of enzymatic variability and the type of reproduction and the habitat of organisms has been studied by several authors in recent decades. Burdon et al. (1983) found that there was no isoenzymatic variability in *Puccinia graminis* f. sp. *tritici* when it reproduces asexually. The same behaviour was observed in *Phakopsora pachyrhizi* (Bonde et al. 1988) and *Puccinia striiformis* (Newton et al. 1985), both pathogens that do not reproduce sexually. Harrington et al. (1996) observed very low isoenzymatic variability in homothallic *Ceratocystis* species and a much higher variability in those heterothallic species of the genus. Ramos (1998) worked with *Saccobolus* species, homothallic coprophilous fungi, also obtaining low isoenzymatic variability. This was the case for Dokmetzian (1999) while working with *Ascobolus*: as these are heterothallic and coprophilous fungi, the low variability may be due in this particular case to homogeneous environmental conditions rather than to the type of reproduction. In a heterogeneous environment, the optimum evolutionary strategy for enzymes would be the existence of multiple forms of the enzyme, rather than only one alternative with high capacity (Johnson 1974). Heterogeneity of enzymes provides organisms with metabolic versatility, thus generating a higher biological efficiency in heterogeneous environments (Zeidler 2000). As regards coprophilous environments, which are often quite homogeneous, it would seem that coprophilous fungi do not require a high isoenzymatic variability to survive. However, the dung microhabitat is slightly conditioned by climatic factors, plant cover and soil properties, which determine the temperature and humidity of the substrate, factors that in turn indirectly influence metabolic activities and the competitive capacity of organisms (Wicklow 1981). Therefore, having different isozyme systems, at least in non-regulatory systems (such as EST), is always a benefit for these fungi.

Principal Coordinates analysis revealed the same groupings as the phenogram and even the same intraspecific differences. This provides much greater reliability in the relationships among species and among populations found in this study.

Considering that isozyme characterization of the three *Coprotus* species allowed us to clearly identify each geographical population and each species, and that it confirmed our previous identification, this technique may be considered

an important additional tool to the traditional taxonomic methods, particularly in problematical groups as *Coprotus*, in which overlapping of characters and high interspecific similarity usually make species identification difficult.

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