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# *Iodophanus carneus* and *I. testaceus* (Ascomycota-Pezizales): Independent taxonomic identity or synonymy? A study of their morphology and isozymes*

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**Summary:** The aim of this study was to delimit two *Iodophanus* species: *I. carneus* and *I. testaceus*, based on morphological characteristics and electrophoretic patterns of their intracellular isozymes. Twenty monosporic strains were used, including five belonging to *I. granulipolaris* as a control. Fourteen isozyme systems were tested, and the five having the best resolution selected: aspartate aminotransferase, esterases, alkaline phosphatase, glutamate dehydrogenase, and superoxide dismutase. These analyses confirmed the similarity between *I. carneus* and *I. testaceus*, since they both produced the same band patterns, which were in turn different from the band pattern of *I. granulipolaris.* So, as we couldn´t find any character wich permit us to classify the isolated studied during this work in defferent species, we believe that *I. testaceus* shoul be consider as a synonym of *I. carneus*.

**Key words:** fungi, *Iodophanus*, isozymes, synonymy, taxonomy.

**Resumen:** *Iodophanus carneus* e *I. testaceus* (ASCOMYCOTA-PEZIZALES): ¿Identidades taxonómicas independientes o sinonimia? Estudio morfológico e isoenzimático. El objetivo del presente trabajo fue la delimitación taxonómica de dos especies del género *Iodophanus*: *I. carneus* e *I. testaceus* a partir de caracteres morfológicos y de los patrones electroforéticos de isoenzimas intracelulares. Para ello se utilizaron veinte cepas monospóricas, cinco de las cuales pertenecientes a *I. granulipolaris* que fueron utilizadas como control. Se probaron catorce sistemas isoenzimáticos y se eligieron los cinco con mejor resolución: aspartato amino transferasa, esterasa, fosfatasa alcalina, glutamato dehidrogenasa y superóxido dismutasa. El análisis de los patrones isoenzimáticos corroboró la silimitud existente entre *I. carneus* e *I. testaceus*, ya que los patrones de bandas obtenidas para estas dos especies fueron iguales y diferentes de *I. granulipolaris.* Entonces, al no encontrar ningún caracter que nos permita separar a los aislamientos estudiados en este trabajo en dos especies distintas, proponemos a *I. testaceus* como un sinónimo de *I. carneus.*

**Palabras clave:** hongos, *Iodophanus*, taxonomía, isoenzimas, sinonimia.

## Introduction

The genus *Iodophanus* Korf belongs to the family Pezizaceae (Ascomycota-Pezizales). The distinctive features of *Iodophanus* species are amyloid asci, eight hyaline ascospores per ascus, and exosporium with callose pectic ornamentations. This kind of ornamentation and having *Oedocephalum* as the conidial state of some species are typical characteristics of the family Pezizaceae. Therefore, *Iodophanus* species, formerly assigned to the family Ascobolaceae, have been later assigned to the family Pezizaceae.

*Iodophanus* species have been found on various different substrates, such as herbivore droppings, soil, paper, clothes, cardboard and a wide range of decomposing plant matter. They are probably cosmopolitan, since they have been reported in North America, South America, Europe, southeast Asia, Australia and Africa (Kimbrough *et al*., 1969; Gamundí & Ranalli, 1964; Schumacher, 1992; Kimbrough, 1970; Seaver, 1916; Cinto & Dokmetzian, 2006; Thind & Kaushal, 1978; Jeng & Krug, 1977) .

The most useful characters in classical taxonomy

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of fungi are spore, asci and apothecia dimension, as well as their form and color. In addition to this, Kimbrough *et al.* (1969) suggest that «the most diagnostic feature are the size, shape and ornamentation of ascospores». One major problem regarding *Iodophanus* species delimitation is that the spore and asci dimensions of the different «species» overlap. Moreover, measurements made by different authors for the same species often differ, making species determination even more difficult. In addition to this, the enviromental condition, as well as the quantity and quality of light, among other factors, would have considerable influence on ascospore and asci size, therefore these characters by themselves are not enough to establish species boundaries (Diorio *et al.*, 1995; Cotty & Misaghi, 1985)

According to Harrington & Rizzo (1999), who tried to arrive at a workable definition for fungal species, «it is important that the individuals comprised in a certain species are derived from a common ancestor and that these individuals are reproductively isolated from sympatric populations of related species. The phenotypic characters most valuable as delimiting characters would be those associated with the ecological adaptations that circumscribe the niche of the species in question.» Molecular markers (e.g. RFLPs, RAPDs, AFLPs) often correlate well with the species-delimiting phenotypic characters, thus providing excellent tools for identification, but they are not the final word in species delimitation (Anderson *et al*., 1997; Bruns *et al*., 1991; Kohn, 1992).

Isozymes are phenotypic characters, and as such they can reflect fixed differences for delimiting fungal species. Differences in electrophoretic mobility *in vitro* may differentiate morphologically similar populations and often are congruent with ecological adaptations to specific climatic conditions or pH of substrate. Isozyme analysis has also proved to be very useful for distinguishing asexual species that differ very little morphologically (Zambino & Harrington, 1989).

Kimbrough *et al.* (1969) separate *I. carneus* and *I. testaceus* mainly according to their natural habitat. They sustain that all the *I. carneus* collections found to date on substrates other than dung (e.g. paper, soil, cardboard and other decomposing cellulose substrates) should be considered as *I. testaceus.* In addition to habitat, they find other morphological characteristics to separate these species: *I. carneus* apothecia are quite a lot smaller than *I. testaceus* apothecia, while the latter contain more carotenoid pigments; *I. carneus* asci are clearly claviform and 40-50 µm shorter, its ascospores are 4 µm shorter, and their tips less rounded than in *I. testaceus*; and the fact that they have never found *Oedocephalum* as the conidial state for *I. carneus.* But others authors as Dennis (1968), Thind & Waraitch (1971) and Doveri (2004) do not find enough justification in separating the two species on the basis of substratum alone.

The aim of this study was to delimit two *Iodophanus* species: *I. carneus* and *I. testaceus*, based on morphological characteristics and electrophoretic patterns of their intracellular isozymes.

## Materials and Methods

*Strains*

Twenty monosporic strains obtained from the germination of spores from polysporic isolates were used. The different strains were routinely maintained on PF medium (Gamundí & Ranalli, 1964) at 5ºC. Table 1 shows the location, substrate, collection date and strain number (BAFC) of the different isolates.

*Liquid culture medium*

Synthetic GA (glucose-asparagine) medium: $SO_4Mg.7H_2O$, 0.5 g; $PO_4H_2K$, 0.5 g; $PO_4HK_2$, 0.6 g; $SO_4Cu.5H_2O$, 0.4 mg; $Cl_2Mn.4H_2O$, 0.09 mg; $BO_3H_3$, 0.07 mg; $MoO_2Na.2H_2O$, 0.02 mg; $Cl_3Fe$, 1 mg; $Cl_2Zn$, 10 mg; biotin, 5 µm; HCl-thiamine, 0.1 mg; glucose, 3 g; L-asparagine, 0.2 g; bidistilled water to 1000 mL.

The culture medium was sterilized at 121ºC and 1.2 atm, for 20 minutes.

*Culture conditions*

Cultures were grown in 125 mL flasks containing 50 mL of liquid medium and inoculated with 9 $mm^3$ cubes taken from a 3-4 day old colony growing on water- agar in the dark.

The flasks were incubated in a New Brunswick Psicrotherm G-27 culture chamber, at 23ºC, at constant upper ilumination with four 20W fluorescent tubes, and in constant agitation at 125 rpm.

For morphological studies, the fungus was grown on TD culture medium (Tyndallized dung), incubated at 23ºC, at constant upper ilumination.

Gross morphological obsevation were made with a stereocopic microscope, noting such characters as color, size and shape of apothecia. Asci, ascospore, paraphyses and excipular elements were examinaded in crush mount. Blueing of asci were checked with Melzer´s reagent.

**Table 1.** List of strains with their geographical location, substrate and BAFC number. ER: Entre Ríos Province; CABA: Ciudad Autónoma de Buenos Aires; Arg: Argentina; BAFC #: Buenos Aires, Facultad de Ciencias Exactas y Naturales.

| Strains | Species | Locality | Subst. | Date (dd/mm/yy) | BAFC # |
|---|---|---|---|---|---|
| ER 1 | *I. carneus* | Gueleguaychú, ER. Arg | Cow dung | 24/04/02 | 2965 |
| ER 2 | *I. carneus* | Gueleguaychú, ER. Arg | Cow dung | 24/04/02 | 2966 |
| ER 12 | *I. carneus* | Gueleguaychú, ER. Arg | Cow dung | 24/04/02 | 2967 |
| ER 14 | *I. carneus* | Gueleguaychú, ER. Arg | Cow dung | 24/04/02 | 2968 |
| ER 15 | *I. carneus* | Gueleguaychú, ER. Arg | Cow dung | 24/04/02 | 2969 |
| TJ 1 | *I. carneus* | Hernández, ER, Arg | Cow dung | 24/04/02 | 2970 |
| TJ 2 | *I. carneus* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3016 |
| TJ 3 | *I. carneus* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3017 |
| TJ 7 | *I. carneus* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3018 |
| TJ 10 | *I. carneus* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3019 |
| IG 1 | *I. granulipolaris* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3020 |
| IG 2 | *I. granulipolaris* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3021 |
| IG 3 | *I. granulipolaris* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3022 |
| IG 4 | *I. granulipolaris* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3023 |
| IG 6 | *I. granulipolaris* | Hernández, ER, Arg | Cow dung | 24/04/02 | 3024 |
| IT 1 | *I. testaceus* | Nuñez, CABA, Arg | Old paper | 12/10/02 | 3025 |
| IT 4 | *I. testaceus* | Nuñez, CABA, Arg | Old paper | 12/10/02 | 3026 |
| IT 7 | *I. testaceus* | Nuñez, CABA, Arg | Old paper | 12/10/02 | 3027 |
| IT 8 | *I. testaceus* | Nuñez, CABA, Arg | Old paper | 12/10/02 | 3028 |
| IT 10 | *I. testaceus* | Nuñez, CABA, Arg | Old paper | 12/10/02 | 3029 |

The key proposed by Kimbrough *et al.* (1969) was used for the identification of the species.

*Sampling*

Mycelium grown in liquid GA medium was harvested by filtering it through a Buchner funnel at reduced pressure, on 2 days before they reached each species' maximum growth day, according to its growth curve which was prevoulsy charter using dry weight as growing stimator. In this particulary case all the species tested shown the same maximun growth day (day 12). Later on, micelium was washed three times with bidistilled water, dried on filter paper and stored at –70ºC until it was processed (Dessauer et al., 1984). The filtered mycelium was freezed with liquid nitrogen and ground in a mortar and cell homogenates was removed with extraction buffer (Soltis et al., 1983). Finally it was divided into aliquots and frozen at -70ºC until it was used.

*Electrophoretic conditions and gel preparation*

Horizontal electrophoresis on 7% polyacrylamide gels (Saidman, 1985) was the technique employed. Different buffers were used for preparing the gels and the electrophoretic run, according to the different isozyme systems analyzed (Scandalios, 1969; Selander et al., 1971; Soltis et al., 1983). Fourteen isozyme systems were analyzed. Table 2 shows the tested isozyme systems, and the buffers and enzyme staining protocols used for each one of them. Those systems having good resolution for all the isolates were selected for subsequent analyses.

A comb with teeth 4 mm wide x 2 mm thick, set at 4 mm intervals, was used to make slots at 2-3 cm from one edge of the polymerized gels. Slips of Nº 3 Whatman filter paper measuring 0.5 x 0.2 cm were soaked in the previously thawed homogenates to load the slots. This was done at a low temperature in order to prevent denaturation of the enzymes. Bromophenol blue (4mg/ml) was used as tracking dye. Electrophoresis was run in a cold chamber at 4ºC at 110 to 120 volts for 3 to 4 hours at constant voltage and after that the different zones of enzymatic activity were stained. The Rf values were calculated as the run length of each band divided by the run length of the bromophenol blue front.

**Table 2.** Isozyme systems tested, Nº of EC, abbreviations, gel and run buffers, enzyme staining protocols. **A:** lithium borate, pH 8 #, a: lithium borate, pH 8,2 #, **B:** Tris-citrate, pH 6,5 *, b: Tris-citrate, pH 7 *, **C** and c: Tris-citrate, pH 8 ^ 1: Vallejos (1983), 2: Wendel & Weeden (1989), 3: Manchenko (1994)

| Enzyme System | Abbrev. | Nº EC | Gel Buffer | Run Buffer | Stain |
|---|---|---|---|---|---|
| **Enzymes with good resolution for all isolates** | | | | | |
| Aspartate aminotransferase | AAT | 2.6.1.1 | A | a | 1 |
| Esterases | EST | 3.1.1... | A | a | 2 |
| Alkaline phosphatase | ALP | 3.1.3.1 | B | b | 3 |
| Glutamate dehydrogenase | GDH | 1.4.1.3 | B | b | 2 |
| Superoxide dismutase | SOD | 1.15.1.1 | A | a | 2 |
| **Enzymes with poor or no resolution** | | | | | |
| Isocitrate dehydrogenase | IDH | 1.1.1.42 | B | b | 3 |
| Peroxidase | PER | 1.11.1.7 | A | a | 3 |
| Catalase | CAT | 1.11.1.6 | A | a | 3 |
| Acid phosphatase | ACP | 3.1.3.2 | B | b | 3 |
| Glucose-6 phosphate dehydrogenase | G6PD | 1.1.1.49 | C | c | 3 |
| Shikimic dehydrogenase | SKD | 1.1.1.25 | B | b | 3 |
| Leucyl aminopeptidase | LAP | 3.4.11.1 | A | a | 3 |
| Malate dehydrogenase | MDH | 1.1.1.37 | A | a | 2 |
| Alcohol dehydrogenase | ADH | 1.1.1.1 | A | a | 3 |

# Scandalios, 1969 (modified), * Selander et al., 1971 (modified), ^ Soltis et al., 1983

## Results

*Morphological studies*

*I. carneus* (Pers.). Korf in J. W. Kimbrough and R. P. Korf (1967)

*Apothecia:* superficial, small, gregarious, globose at first, and entirely covered in hyaline hyphae giving it an arachnoid appearance; lenticular to pulvinate when mature, with papillose hymen due to asci emergence, pale pink to bright orange. Diam. 0.5-1.8 mm.

*Asci:* unitunicate, 8-spored, subclaviform, pseudoamyloid when young and with amyloid reaction of the entirely wall when mature; rounded apex and central operculum; 192-298 x 24-32 µm.

*Paraphyses:* simple, broadening slightly at the apex, containing guttules of orange pigments concentrated at the basal portion of paraphysis; 5-8.3 µm diam. at the apex.

*Ascospores:* irregularly biseriate, 1-celled, clustered at the top of the ascus, ellipsoid, hyaline, smooth when young and ornamented towards maturity with small spiny warts, irregularly distributed; 18-24 x 10-16.6 µm.

*Excipulum:* «globulous» texture, formed of cells pigmented at the outer basal zone, 8-10 µm diam.

*Anamorph: Oedocephalum* conidial state.

*Habitat:* on dung of various herbivores.

*Holotype:* not known.

*Material studied:* ARGENTINA, Prov. Entre Ríos, Hernández, on cow dung, 24 april 2002, Giménez 51546 (BAFC); ARGENTINA, Prov. Entre Ríos, Gualeguaychú, on cow dung, 24 april 2002, Giménez 51545 (BAFC)

*I. testaceus* (Moug.) Korf in J. W. Kimbrough and R. P. Korf (1967)

*Apothecia:* superficial, isolated to concrescent, globose. Pale yellowish when young, turning bright pink on maturing. Diam: 0.45-0.75 mm.

*Asci:* Unitunicate, 8-spored, with central operculum. Amyloid, subclaviform; 190.28-230.48 x 29.48-34.84 µm.

*Paraphyses:* simple, regularly septate and broadening at the apex. Highly pigmented, with great accumulation of orange pigments. Diam: 6.7-7.15 µm.

*Ascospores:* ellipsoid to rounded with small, irregularly distributed ornamentation; 16.25-19.5 x 10.4-11.7 µm.

*Excipulum:* made of globose cells, 15.6-26 x 13-22.75 µm.

*Anamorph: Oedocephalum* conidial state.

*Habitat:* not coprophilous, found on a wide range of decomposing cellulose substrates.

*Type specimen:* not known; *type locallity:* probably France.

*Material studied:* ARGENTINA, Ciudad Autónoma de Buenos Aires, on old paper, 12 october 2002, Ranalli 51547 (BAFC)

*Notes:* Quantitative morphological characteristics, such as asci and ascospore sizes, have always been very important to describe a species and can be used to define species phylogenetically. In the case of these species, there is a noticeable overlap between different features, making species boundaries unclear.

*Isozyme systems*

The isozyme systems that provided a good resolution for all isolates are: AAT, EST, ALP, GDH and SOD. The remaining systems (IDH, PER, CAT, ACP, G6PD, SKD, LAP, MDH & ADH) showed poor resolution and were excluded. Monosporic strains of the independent taxonomic unit *I. granulipolaris* were used as a control.

Figure 1 shows the electromorphs for the five systems. Ten electromorphs were detected for the five systems. *I. granulipolaris* showed a characteristic band pattern for each system tested, while the other two species under study always showed a characteristic pattern for each system, but not a single difference was found between them. No isoenzymatic differences were found among strains from the same geographical location. All the systems tested were monomorphic.

## Discussion

During this study, we found *Oedocephalum* as the anamorph form for both species when they were growing at room temperature (28ºC approximately) on TD culture medium. The morphological differences described by Kimbrough et al. (1969) for these species did not match those we found during this study. We found that although there is a large overlap between the measurements of the two species, *I. testaceus* apothecia were altogether considerably smaller than *I. carneus* apothecia, and the same was true for asci and ascospore sizes.

However, as the different measurements did overlap, it is hard to use these characters for

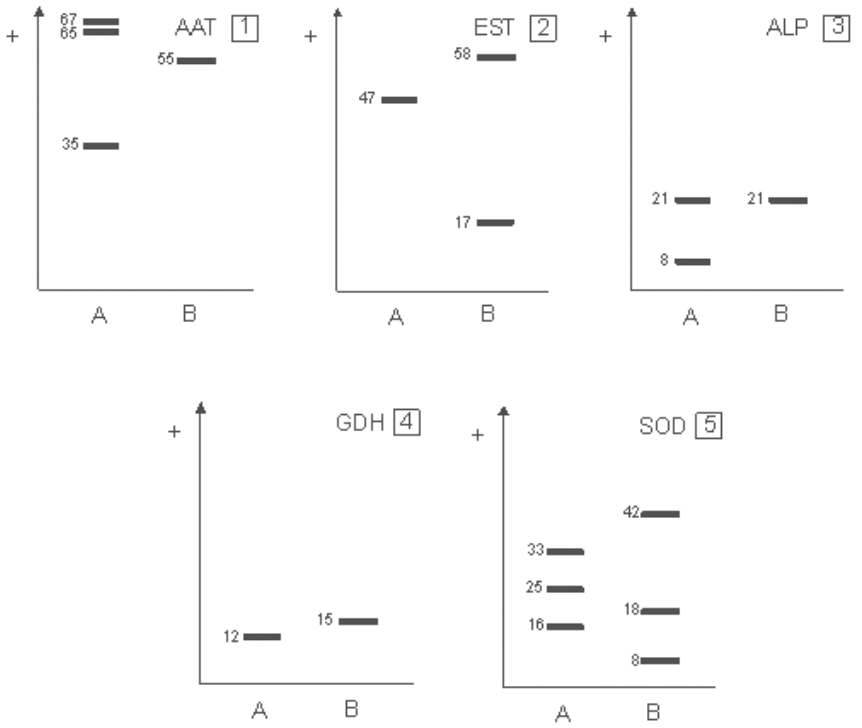


**Fig. 1.** Diagrams of electromorphs (**A-B**) identified in each system. **A:** *I. granulipolaris* (IG); **B:** *I. carneus* (TJ y ER) and *I. testaceus* (IT). **1:** aspartate aminotransferase (AAT); **2:** Esterases (EST); **3:** alkaline phosphatase (ALP); **4:** glutamate dehydrogenase (GDH); **5:** superoxide dismutase (SOD).

identifying species, we consider that the fact of two groups (or population) of organisms develop on different habitats doesn´t seem to be a significant character for species delimitation.

Isozyme analysis of all strains of the same species produced the same pattern. *Iodophanus* species, like many coprophilous species, are homothallic (non-outcrossing), probably as an adaptation to its substrate determining its reproductive isolation. The main source of variation of the homothallic form of a haploid organism is mutation. Thus, while a homothallic organism produces cloned descendants, a heterothallic organism produces variable descendants via recombination (Rayner, 1990). The process of change in homothallic populations will thus be slower because the variation among individuals of the populations, and among populations themselves, will be mainly due to the only source of variation. This may explains why no intraspecific variation was found in our study. Nevertheless, mutation and selection could lead to genetic divergence and increase the level of variability in a homothallic population, but in a longer period of time. The correlation between the degree of enzymatic varability and the type of reproduction and the habitat of organism has been studied by oyhers authors. Ramos (1998) worked with *Saccobolus* and Suárez et al. (2006) worked with *Coprotus*, both homotallic coprophilous fungi, also obtaining low isoenzymatic variability.

During this study we have not found any characteristic enabling a boundary to be established between them. In addition to this, isozyme analysis was useful and appropriate for separating *I. carneus* and *I. testaceus* from *I. granulipolaris,* but no system was found to separate *I. carneus* and *I. testaceus* from each other. So, at the lack of a strong character which permit us to delimit each species, and the impossibility of observe any type specimen we have to agree with those authors that place *I. carneus* and *I. testaceus* in the same taxa.

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