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Genetic relationships between Mexican species of *Pleurotus* analyzing the ITS-region from rDNA*

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ABSTRACT

Mexico is considered a country having great biodiversity, but genetic resources of edible mushrooms are as yet poorly studied. Twenty five strains of oyster mushrooms (*Pleurotus*) isolated from tropical, subtropical and temperate regions were studied. Strains were identified sequencing the ITS1-5.8S-ITS2 region from the rDNA. Six species were determined using neighbor-joining and maximum parsimony analyses with high levels of support: *P.* "agaves", *P. djamor*, *P. levis*, *P. ostreatus*, *P. pulmonarius*, and *P. smithii*. Further collections and genetic analysis are needed for *P.* "agaves" and *P. smithii*. Most sequences from Mexican strains were clearly separated in the consensus trees from reference strains of European and North American origin. The exception was the sequence ECS-0156 of *P. pulmonarius*, which was grouped with those of reference strains from North American and European origin, opening the possibility that strains cultivated commercially may have escaped from cultivation. Species identified represent a broad genetic base for breeding programs, and good potential for commercial cultivation.

Key words: *Pleurotus*, ITS region of rDNA, phylogenetic relationships, Mexico.

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INTRODUCTION

Oyster mushrooms (*Pleurotus*) are cultivated commercially worldwide, and show great potential in terms of their functional properties and diverse biotechnological applications[3]. At least fifteen species of *Pleurotus* representing intersterile groups have been recognized[29], and regional studies have become important[11,27], as geographic barriers are associated with patterns of speciation.

In Mexico, many indigenous and peasant communities have traditionally gathered and consumed wild species of *Pleurotus*, and the commercial production of oyster mushrooms in this country started in 1974[13]. The estimated yearly production of *Pleurotus* has increased significantly during the last two decades, from 356 tons in 1991 to 2,920 tons in 2009[14]. However, taxonomic studies in the genus *Pleurotus* are rather scarce, despite its social, economic and ecological importance. Most research work has been focused on morphological and sometimes non-morphological characters leading to debatable identifications[4,5,6,7,8]. Biochemical and molecular tools have also been used recently to identify and characterize Mexican genetic resources of edible mushrooms, including *Pleurotus* [*P. cystidiosus* O. K. Mill., *P. djamor* (Rumph. ex Fr.) Boedijn, *P. levis* (Berk. & M. A. Curtis) Singer, *P. ostreatus* (Jacq.) P. Kumm., *P. pulmonarius* (Fr.) Quél., *P.* sp.] and other genera, such as *Agaricus*, *Lentinula*, and *Ganoderma*[16,22].

In this study, we collected or obtained strains of *Pleurotus* from tropical, subtropical and temperate regions of Mexico. The internal transcribed spacer region (ITS1-5.8S-ITS2) was sequenced for identifying all strains and assessing their genetic relationships by neighbor-joining and maximum parsimony analyses. Corresponding intersterility groups were determined and published by the authors elsewhere[9].

MATERIALS AND METHODS

Strains. A total of 25 strains of *Pleurotus* were studied, and their code and origin are shown in **Table 1**. Ten States from the northern, central, and southern parts of Mexico were represented, namely: Chiapas (5), Hidalgo (1), Jalisco (2), Morelos (4), Nuevo León (2), Puebla (2), Tabasco (1), Tlaxcala (1), Veracruz (5), and Yucatán (2). Strains are deposited at the ECOSUR culture collection, as well as the Centre for Genetic Resources of Edible Mushrooms at COLPOS[22]. They were stored and subcultured in yeast phosphate soluble starch (YPSS/2)[28] and malt extract agar media (MEA, Bioxon). Species authorities are in accord with the Index Fungorum (www.indexfungorum.org).

DNA extraction, amplification, and sequencing. The mycelium of strains studied was grown on YPSS/2 agar medium, pH 5.5, during ten days at 26 C in the dark. The aerial mycelium was harvested using a sterile spatula, placed in Eppendorf tubes (2 ml), dehydrated using a Speed-Vac concentrator (Savant Instruments, Farmingdale, U.S.A.), and freezed at -20 C. DNA minipreparations were performed using CTAB extraction buffer according to Zolan and Pukkila[32]. PCR amplifications of the ITS1-5.8S-ITS2 region were carried out using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), as previously described by White *et al.*[30]. According to Vilgalys and Sun[28], each amplification reaction (17 ml) contained: 2.5 µl 10x buffer (10 mM $MgCl_2$); 3.75 µl ster-

Table 1. Strains of *Pleurotus* from different regions of Mexico, which were selected for this study.

Region	Code	State	Origin	Species	GenBank accession number
North	ECS-0183 (P2)	Nuevo León	UANL, Linares	*P. pulmonarius*	GU722282
	ECS-0184 (CFR)	Nuevo León	UANL, Linares	*P. ostreatus*	GU722280
South	ECS-0123	Chiapas	Talquián, Unión Juárez	*P. djamor*	GU722265
	ECS-0128	Chiapas	CAE Rosario Izapa, Tuxtla Chico	*P. djamor*	GU722266
	ECS-0130	Chiapas	CAE Rosario Izapa, Tuxtla Chico	*P. djamor*	GU722267
	ECS-0150	Chiapas	Los Laureles, Tapachula	*P. djamor*	GU722268
	ECS-0151	Chiapas	Yajalón	*P. djamor*	GU722269
	CP-253	Tabasco	Quintín Arauz, Centla	*P. djamor*	GU722270
	CP-170	Yucatán	Mérida	*P. djamor*	GU722271
	CP-171	Yucatán	Mérida	*P. djamor*	GU722272
Central	ECS-0158 (HEMIM-32)	Puebla	Xonacatlán	*P. pulmonarius*	GU722283
	CP-30	Puebla	Autopista Puebla-Cholula, km 1	*P. levis*	GU722279
	CP-194	Tlaxcala	San Isidro Buensuceso	*P.* "agaves"	GU722262
	CP-98	Hidalgo	UAEH, Pachuca	*P.* "agaves"	GU722263
	ECS-0165 (HEMIM-30)	Morelos	UAEM, Cuernavaca	*P.* "agaves"	GU722264
	ECS-0159 (HEMIM-34)	Morelos	UAEM, Cuernavaca	*P. djamor*	GU722273
	ECS-0162 (HEMIM-44)	Morelos	Santa Catarina de Tepoztlán	*P. djamor*	GU722274
	ECS-0170 (HEMIM-47)	Morelos	Cuernavaca	*P. pulmonarius*	GU722284
East	CP-18	Veracruz	Jardín Botánico Clavijero, Xalapa	*P. smithii*	GU722288
	ECS-0110 (IE-8)	Veracruz	IE, Xalapa	*P. pulmonarius*	GU722285
	ECS-0156 (CP-15)	Veracruz	Carretera Coatepec-Xalapa, vía Las Trancas, km 7	*P. pulmonarius*	GU722286
	ECS-0191 (IE-202)	Veracruz	IE, Xalapa	*P. pulmonarius*	GU722287
	ECS-0176 (IBUG-39)	Veracruz	Coatepec	*P. djamor*	GU722275
West	ECS-0174 (IBUG-7_2)	Jalisco	Guadalajara	*P. djamor*	GU722276
	ECS-01130 (IBUG-3)	Jalisco	Guadalajara	*P. djamor*	GU722277

UANL= University of Nuevo León, Faculty of Forest Sciences. CAE= Agricultural experimental centre. UAEH= University of Hidalgo. UAEM= University of Morelos. IE= Institute of Ecology.

ile distilled water; 2.5 µl de bovine serum albumin; 4 µl dNTP mix (2 mM each); 1 µl of each primer (1 µM); 2 µl template DNA (10 ng/ml); and 0.25 µl of Taq polimerase solution (5 U/µl). Samples were overlaid with mineral oil. The temperature cycling protocol consisted of one initial denaturation cycle at 94 C for 3 min, followed by 35 cycles of 94 C, 1 min; 50 C, 30 s; 72 C, 1 min. Amplification products were analyzed by electrophoresis in 0.8% agarose gels, and purified using Sephadex columns.

DNA sequencing was carried out in an ABI 373 automated sequencer (Perkin-Elmer, U.S.A.) with dye terminator sequencing chemistries, using one initial cycle (96 C, 2 min), followed by 25 cycles (96 C, 30 s; 50 C, 15 s; 60 C, 4 min). The reaction for sequencing amplification products was as follows: 4.0 µl of Big Dye Kit containing ampliTaq DNA polymerase, 20 µl of primer (ITS1 or ITS4), and 4.0 µl of PCR product, being overlaid with mineral oil. Each sample was sequenced in both directions. Chromatograms were processed using software 2.0 from Genes Codes Corp. (U.S.A.).

Phylogenetic analysis. Apart from DNA sequences of Mexican strains studied, other 22 sequences were included as a reference (**Table 2**), which were mainly provided by the Mycology Laboratory, Duke University, N.C., U.S.A. Additional sequences were from the following species: *P.* "agaves" (2) *sensu* Vilgalys *et al.*[29], *P.*

Table 2. Reference ITS sequences of *Pleurotus* included in the phylogenetic analysis, and provided by Duke University and the University of Tennessee, U.S.A.

Species	Strain	Origin and other data	GenBank accession number
P. "agaves"	D2320	RHP6241.8	-
	D2321	RHP6241.6	-
P. calyptratus	F94.4	Germany	-
P. cornucopiae	D383	CBS-276.33, England[28]	U04079, U04118
	D1166	ATCC-38547, Germany[28]	U04059, U04098
P. cystidiosus	D420	ATCC-28599, Indiana, U.S.A.[28]	U04083, U04122
P. djamor	D1847	Mexico[28]	U04071, U04110
	D2324	RHP4326.8, Tabasco, Mexico	-
	D2325	RHP4326.14, Tabasco, Mexico	-
	D2334	RHP6270.6, Xalapa, Mexico	-
	D2335	RHP6270.1, Xalapa, Mexico	-
P. dryinus	MF91	MF91.1116, Germany	-
	ECS-1108	RHP7947.6, U.S.A.	GU722278
P. eryngii	D625	MW85, Europe[28]	U04089
	D1822	ATCC-58080, Asia[28]	U04067
P. levis	D2269	RV94.161.1, North Carolina, U.S.A.	-
P. ostreatus	D261	RV83.233, Tennessee, U.S.A.[28]	U04073
	D403	RLG9960, Arizona, U.S.A.[28]	U04081
	ECS-1102	RHP6689.2, Austria	GU722281
P. pulmonarius	D700	WC152, British Columbia[28]	U04093, U04131
	D2347	RHP4203.11, Sweden	-
	D2349	RHP4203.10, Sweden	-
P. smithii	D478	ATCC-46391, Mexico[28]	U04123
Hohenbuehelia	OK23937	External group	-

calyptratus (Lindblad) Sacc. (1), *P. cornucopiae* (Paulet) Rolland (2), *P. cystidiosus* (1), *P. djamor* (5), *P. dryinus* (Pers.) P. Kumm. (1), *P. eryngii* (DC.) Quél. (2), *P. ostreatus* (2), *P. pulmonarius* (3), *P. levis* (1), *P. smithii* Guzmán (1), and *Hohenbuehelia* (1) as external group. All sequences were aligned using Clustal_X, following standard multiple alignment, as well as visual adjustment. Sequence length was made uniform, and regions showing ambiguous alignments were excluded. Sequences were processed using PAUP, version 4.0b 10x[25], considering empty regions derived from alignments as additional character states. If the empty region was >1, it was recoded as a sole insertion/deletion and considered as a character state. Phylogenetic inferences were based on the genetic distance between sequences according to Tamura and Nei[26]. Maximum parsimony trees were identified using the heuristic search option, and the tree bisection and reconnection (TBR) algorithm[25]. Support for phylogenetic groupings was assessed by bootstrap analysis (1,000 replicates) with random addition of sequences during each heuristic search.

RESULTS

Sequences studied had around 790 characters, from which 667 characters were used for further analysis after alignment and trimming. There were 311 parsimony-informative sites, 171 constant sites, and 185 non-informative sites. The genetic distance matrix derived from neighbor-joining analysis generated a consensus tree from the best 1,104 trees, showing the following scores: tree length (TL)= 1,128 steps, consistency index (CI)= 0.69, homoplasy index (HI)= 0.31, retention index (RI)= 0.87, and rescaled consistency index (RC)= 0.60 (**Fig. 1**). This phylogenetic analysis showed the following groups of *Pleurotus*: a major clade containing *P. ostreatus* (Jacq.) P. Kumm., *P. pulmonarius* (Fr.) Quél., and *P. eryngii* (DC.) Quél.; and another major clade containing *P. djamor* (Rumph. ex Fr.) Boedijn, *P.* "agaves" *sensu* Vilgalys *et al.*[29], and *P. calyptratus* (Lindblad) Sacc. It is possible that differing species within the same clade are grouped together because they have evolved recently. There were independent clades for *P. dryinus* (Pers.) P. Kumm., *P. levis* (Berk. & M. A. Curtis) Singer, *P. cornucopiae* (Paulet) Rolland, and for the group of coremia-forming species *P. smithii* Guzmán and *P. cystidiosus* O. K. Mill. In the latter case, sequences D478 (State of Mexico) and CP-18 (State of Veracruz), both from Mexico, formed the monophyletic group of *P. smithii* close to *P. cystidiosus* of North American origin, which was supported by bootstrap values of 100% and 99%, respectively. *P. smithii* and *P. cystidiosus* shared the position of more than half of the characters studied (56%). The clade of *P. levis* was supported by a bootstrap value of 98%, and its sequences D2269 and CP-30 also shared the position of more than half of the characters studied (58%). All species identified in the clades from Mexican strains conformed intersterility groups[29] having specific morphological characters and yields[9].

DNA sequences belonging to *P. djamor* formed a monophyletic group (bootstrap value= 98%), whose relationships among subgroups are unsolved. A possible explanation can be the presence of vicariance patterns (**Fig. 1**), in which two subpopulations are identified: 1) Sequences of strains from the States of Tabasco (D1847, D2324) and Yucatán (CP-171); and 2) Sequences of strains from the States of Jalisco (ECS-0174,

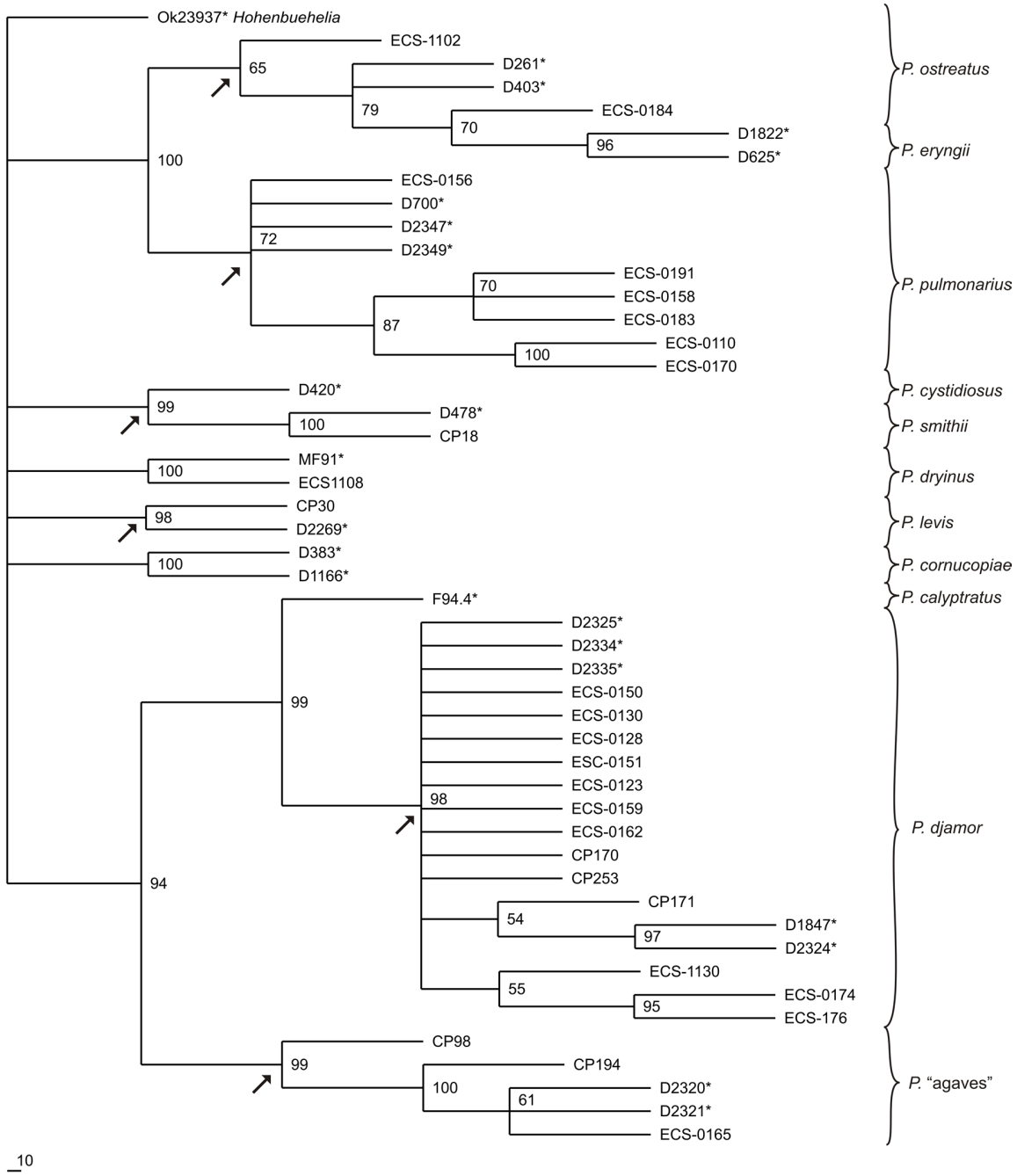

Fig. 1. Consensus tree generated by neighbor joining analysis of the ITS1-5.8s-ITS2 sequences from Mexican and reference strains of *Pleurotus* studied. Tree scores: tree length (TL)= 1,128 steps, consistency index (CI)= 0.69, homoplasy index (HI)= 0.31, retention index (RI)= 0.87, and rescaled consistency index (RC)= 0.60. Bootstrap values are shown above the branches (1,000 replicates). Codes are those from Tables 1-2: ECOSUR culture collection (ECS), and the Centre for Genetic Resources of Edible Mushrooms at COLPOS (CP). *= Reference sequences from Duke University or the University of Tennessee, U.S.A. Clades grouping Mexican strains are indicated by arrows.

ECS-01130) and Veracruz (ECS-0176). All sequences of *P. djamor* shared the position of most characters studied (60-63%).

Sequences of strains CP-98, CP-194, and ECS-0165 integrated another monophyletic group (bootstrap value= 99%), along with reference sequences D2320 and D2321 identified as *P.* "agaves" by Vilgalys *et al.*[29]. All sequences of *P.* "agaves" shared the position of most characters studied (66-68%).

Sequences belonging to the subclade of *P. pulmonarius* (ECS-0191, ECS-0158, ECS-0183, ECS-0110, ECS-0170, ECS-0156) grouped with the reference strains (D700, D2347, D2349) in a monophyletic group (bootstrap value= 72%). A subpopulation grouping sequences from five Mexican strains with high level of support (bootstrap value= 87%) also suggested the presence of vicariance patterns.

The monophyletic group containing *P. ostreatus* included the sequence ECS-0184 (State of Nuevo León, Mexico) and those from the reference strains (D261, D403, ECS-1102), which are separated from the subgroup of *P. eryngii* (bootstrap value= 96%; reference strains: D625, D1822). All sequences from the monophyletic group shared the position of most characters studied (72-73%).

The maximum parsimony analysis of all ITS sequences generated 1,089 trees. The consensus most parsimonious tree confirmed the results from neighbor-joining analysis in all cases, although levels of support differed slightly. The consensus tree showed the following scores: CI= 0.69, HI= 0.31, RI= 0.87, and RC= 0.60 (**Fig. 2**).

DISCUSSION

Neighbor-joining and maximum parsimony analyses of DNA sequences from 25 *Pleurotus* strains studied allowed the identification of six different species as part of the genetic diversity of oyster mushrooms in Mexico: *P.* "agaves" (*sensu* Vilgalys *et al.*[29]), *P. djamor*, *P. levis*, *P. ostreatus*, *P. pulmonarius*, and *P. smithii*. This is in agreement with species and intersterility groups, which have been previously recognized within the genus on the basis of morphological characters, mating compatibility studies, and molecular phylogenetic analyses[18,21,28,29,31]. Most sequences from Mexican strains were clearly separated in the consensus trees from reference strains of European and North American origin. The exception was the sequence ECS-0156, which was grouped with those from the reference strains, opening the possibility that strains cultivated commercially may either have escaped from cultivation or have been deposited in culture collections. The taxonomic status of *P.* "agaves" needs a thorough nomenclature analysis involving morphological, genetic, and molecular studies. At least two closely related species can be considered within this complex, *P. agaves* Dennis and *P. opuntiae* (Durieu & Lév.) Sacc.

There has been controversy regarding the taxonomic status of *P. cystidiosus* and *P. smithii*, as several authors consider them either as separate[24,31] or the same species[1,10]. Similar cases of partial compatibility between separate biological species have been reported within the genus *Pleurotus*[20]. In this study, the sequences CP-18 and D478 from Mexican strains formed the monophyletic group of *P. smithii*, a minor independent subclade but close to *P. cystidiosus*. Further molecular studies are needed involving a larger number of strains from diverse regions, as well as genetic analysis of hybrids and their progeny, in order to elucidate the relationships between these populations.

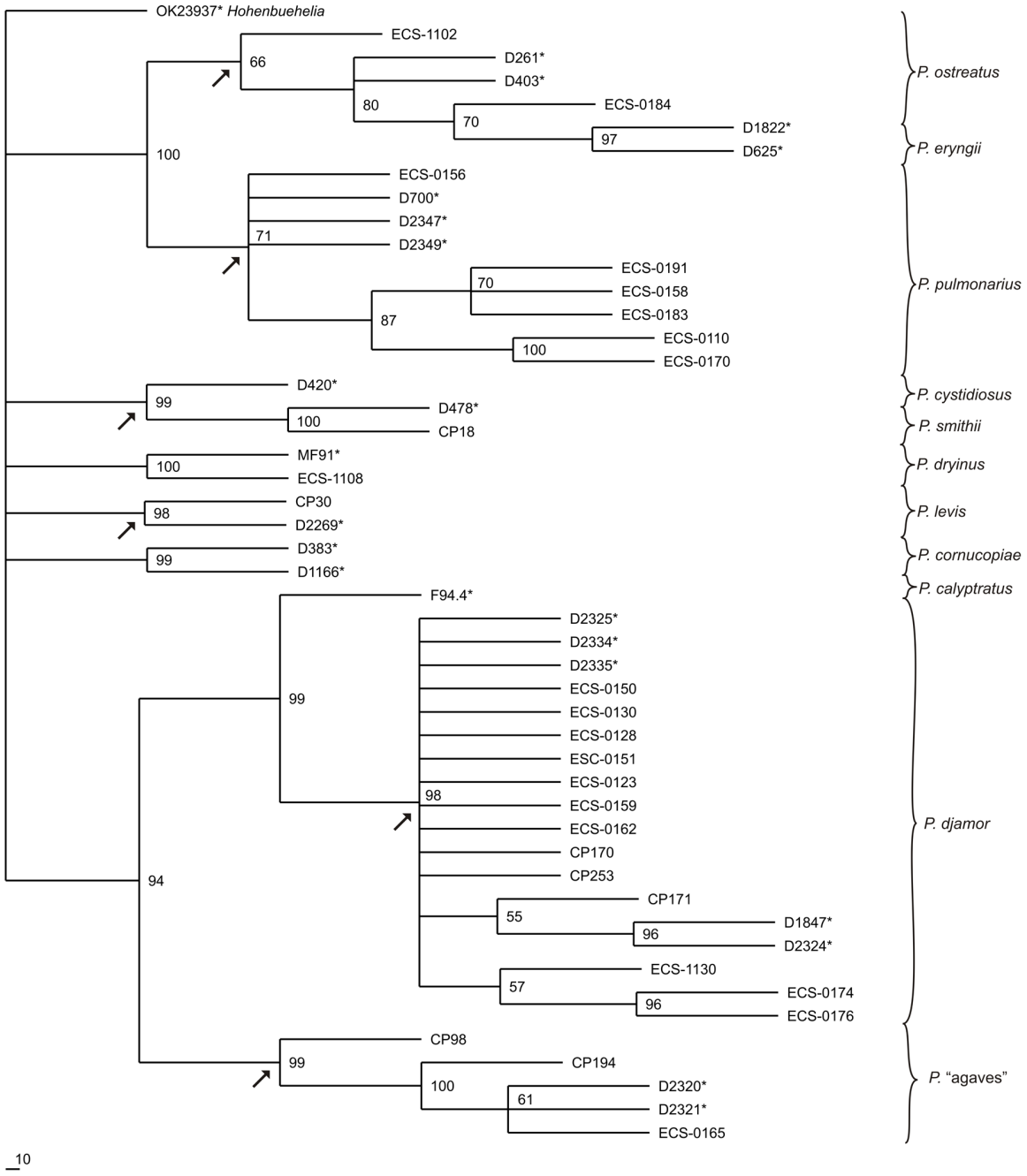

Fig. 2. Consensus tree from the most parsimonious 1,089 trees, which was generated analyzing the ITS1-5.8s-ITS2 sequences from Mexican and reference strains of *Pleurotus* studied. Tree scores: consistency index (CI)= 0.69, homoplasy index (HI)= 0.31, retention index (RI)= 0.87, and rescaled consistency index (RC)= 0.60. Bootstrap values are shown above the branches (1,000 replicates). Codes are those from Tables 1-2: ECOSUR culture collection (ECS), and the Centre for Genetic Resources of Edible Mushrooms at COLPOS (CP). *= Reference sequences from Duke University or the University of Tennessee, U.S.A. Clades grouping Mexican strains are indicated by arrows.

Major lineages comprising *P. djamor*, *P.* "agaves" *sensu* Vilgalys *et al.*[29], and *P. calyptratus* (intersterile groups: V, XI), as well as *P. ostreatus*, *P. pulmonarius*, and *P. eryngii* (intersterile groups: I, II, VI), are in agreement with further phylogenetic analysis of intersterile groups carried out by Vilgalys *et al.*[29] using the nuclear-encoded large subunit (LSU) RNA gene. In the case of *P. djamor*, a species of known phenetic plasticity[19], the subclade included intercompatible white and pink forms belonging to the same biological species[9], which had previously been considered as separate species or varieties[2,6]. Similar was the case for *P. pulmonarius*, whose strains may have been considered as *P. ostreatus sensu lato* in studies of mushroom breeding and cultivation many years ago[12,15,17]. Another case was *P. levis*, which was previously identified as *Lentinus levis* on the basis of morphological characters[23]. Considering the origin of strains studied, it is possible that *P. djamor*, a species widely distributed in tropical and subtropical regions in Mexico, overlaps geographically with *P. ostreatus* and *P. pulmonarius* in temperate highlands. However, this assumption requires confirmation derived from a larger number of authentic indigenous strains, as the large-scale cultivation of oyster mushrooms has allowed the uncontrolled introduction of commercial strains to Mexico.

The species of *Pleurotus* identified in this study are a broad genetic base for systematic breeding programs, and represent good potential for commercial cultivation. However, further studies are needed, particularly in the northern, southern, and eastern regions, in order to have a better assessment of the genetic diversity of *Pleurotus* species in Mexico.

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