MOLECULAR IDENTIFICATION AND CULTIVATION OF WILD *Pleurotus* spp. STRAINS FROM VERACRUZ, MEXICO

IDENTIFICACIÓN MOLECULAR Y CULTIVO DE CEPAS SILVESTRES DE *Pleurotus* spp. DE VERACRUZ, MÉXICO

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SUMMARY

Pleurotus species have great potential for commercial cultivation in Mexico; however, the genetic resources of this genus have not yet been comprehensively studied in the country. The objectives of this study were 1) to identify wild strains of the genus *Pleurotus* from Veracruz, Mexico by molecular techniques and 2) to evaluate their productivity. Species identification was carried out by amplifying the internal transcribed spacer region (ITS) and combining their phenotypic characteristics. The production variables considered were biological efficiency, total weight, production rate, production cycles and fruiting body size. Three species were identified. Some strains of *P. ostreatus* presented higher production rates (2.7), with yields higher than those of a commercial strain, with a production rate of 2.2. This study demonstrates the potential for commercial cultivation of *Pleurotus* spp. in tropical areas.

Index words: fruiting bodies, genetic resources, molecular identification, productivity.

RESUMEN

Las especies del género *Pleurotus* tienen un gran potencial para el cultivo comercial en México; sin embargo, los recursos genéticos de este género aún no se han estudiado de manera integral en el país. Los objetivos del presente estudio fueron 1) identificar cepas silvestres del género *Pleurotus* de Veracruz, México mediante técnicas moleculares y 2) evaluar su productividad. La identificación de especies se llevó a cabo mediante la amplificación de la región espaciadora transcrita interna (ITS) y combinando con sus características fenotípicas. Las variables de producción consideradas fueron eficiencia biológica, peso total, tasa de producción, ciclos de producción y tamaño del cuerpo fructífero. Se identificaron tres especies. Algunas cepas de *P. ostreatus* presentaron tasas de producción más altas (2.7), con rendimientos superiores a los de una cepa comercial, con una tasa de producción de 2.2. Este estudio demuestra el potencial para cultivo comercial de *Pleurotus* spp. en áreas tropicales.

Palabras clave: Cuerpos fructíferos, identificación molecular, productividad, recursos genéticos.

INTRODUCTION

Species of the genus *Pleurotus* occupy second place in terms of production of edible mushrooms worldwide. The genus is an important input for the food industry because of its high nutritional value (vitamins, minerals and amino acids) that qualify it as a functional food (Chaurasia *et al.,* 2020; Effiong *et al*., 2024) and both local and global consumers demand it (Royse *et al.,* 2017). Mexico is one of the countries with the largest productions of *Pleurotus* in Latin America. In 2016, *Pleurotus* spp. accounted for 4.7 % of the national production of edible, functional and medicinal mushrooms (Mayett and Martínez-Carrera, 2019).

Despite this, the national production continues to rely on a limited number of strains and most *Pleurotus* strains under commercial cultivation in Mexico originally came from abroad (León-Avendaño *et al.,* 2013; Salmones *et al*., 2020) and generally grow in areas with a temperate climate. One of the challenges for their cultivation is, therefore, their adaptation to local environmental conditions, especially in tropical and subtropical regions (Martínez-Carrera *et al*., 2016). In this sense, wild *Pleurotus* strains growing in tropical regions may contribute to the improvement of commercial crop, since they normally grow at temperatures above 25 °C and could have a great capacity to adapt to tropical climates and, thus, contribute to sustainable livelihoods in these areas (Salmones, 2017; Thawthong *et al.,* 2014).

Wild *Pleurotus* strains constitute an important genetic material in terms of their ecosystems and subsequent use in the genetic improvement of commercial strains. There is, therefore, a need for their characterization and conservation (Aguilar *et al*., 2018). Previous studies have shown that morphological characters can be

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inconsistent and unstable for the purposes of phenotypic characterization (Salmones and Mata, 2017), and a high level of intraspecific polymorphisms can be presented (Aguilar *et al*., 2018; Huerta *et al*., 2010; Zervakis *et al.,* 2019). For this reason, molecular analysis is necessary to clarify the taxonomic and phylogenetic relationships within certain groups of *Pleurotus* (Zervakis *et al.,* 2019). The combined use of molecular and morphological tools is highly recommended in order to achieve the most accurate identification and strain characterization of the species (Salmones and Mata, 2017).

These investigations, both national (Mexico) (Aguilar *et al*., 2018; Guzmán, 2000; Huerta *et al*., 2009; 2010) and international (Menolli *et al*., 2014; Zervakis *et al*., 2019), have not only shown the difficulties in the identification of *Pleurotus*, due to its great variation and distribution, but also reported a broad genetic base which represents a significant advantage for the production and improvement of *Pleurotus*. Therefore, the authors strongly support the continuous review, compilation and study of this genus. Moreover, in tropical regions, species such as *Pleurotus djamor* show promise for production and commercialization in the short term (Salmones, 2017). The commercial exploitation of these strains is an alternative that would imply low resource investment and input of agricultural residues (Gaitán-Hernández and Silva-Huerta, 2016). The objectives of this study were to identify by molecular techniques wild strains of the genus *Pleurotus* from Veracruz, Mexico, and to evaluate their productivity for breeding purposes.

MATERIALS AND METHODS

Collection site

Fresh samples were collected from different localities of the high mountain region in the State of Veracruz, Mexico, where tropical and subtropical climates predominate (Soto *et al.*, 2001). Ten strains (Table 1) were isolated from vegetative tissue following the methodology of Guzmán *et al.* (1993). A commercial strain of *P. pulmonarius* (IE115) was requested from the collection of the Institute of Ecology AC (INECOL) of Xalapa, Veracruz, Mexico for use as a control. All strains were conserved in potato dextrose agar medium (PDA) and preserved in the fungal collection of the Faculty of Biological and Agricultural Sciences (FACBA), Universidad Veracruzana, México.

DNA extraction, polymerase chain reaction (PCR), sequencing

Genomic DNA was obtained with a rapid extraction kit (Norgen®, Biotek Corp., Sigma), following the manufacturer instructions. The internal transcribed spacer region (ITS) was amplified with ITS4/ITS5 primers following the procedure described below:

Initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1:30 min, extension at 72 °C for 1:30 min, with a final extension step at 72 °C for 10 min; the PCR products were maintained at 4 °C. The presence of DNA in the amplified samples was confirmed by electrophoresis on 1 % agarose gels. The PCR products were sent to the Macrogen company (Seoul, South Korea) for sequencing.

Sequence alignment and phylogenetic analyses

A total of 88 sequences were used for phylogenetic analyses. In addition to the 10 sequences produced in this study, 78 sequences were retrieved from GenBank according to previous studies (Aguilar *et al*., 2018; Huerta *et al*., 2010; Li *et al*., 2018; Menolli *et al*., 2014; Zervakis *et al*., 2019). The accession numbers are presented in Figure 1. Sequences were aligned using MAFFT (Katoh and Standley 2013), then manually adjusted in BioEdit v. 7.0.4 (Hall, 2007). Maximum likelihood analysis was performed using RAxMLHPC2 v. 8.2.4 (Stamatakis, 2014), as implemented in the Cipres portal (Miller *et al.*, 2010) under a GTRGAMMA model with 1000 rapid bootstrap (BS) replicates. Bayesian Inference (BI) analysis was performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). Six Markov chains were run for one million generations, with sampling every 100th generation. Burn-in was determined by checking the likelihood trace plots in Tracer v. 1.6 (Rambaut *et al.*, 2014) and subsequently discarded. The outputs were displayed in FigTree v 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Spawn and substrate preparation

Each strain was replicated in PDA medium in Petri dishes. The inoculated Petri dishes were incubated (25 ± 1 °C) in dark conditions for 10 to 13 days (Aditya *et al.*, 2024). Polypropylene bags were filled with 100 g of pre-washed, hydrated and subsequently sterilized sorghum seed (*Sorghum* sp.) (Guzmán *et al.*, 1993). The mycelium developed on PDA was sectioned in order to inoculate 1 cm^2. Four to five fragments were placed on the surface of the sorghum seeds in each bag. The bags were then incubated at 28 °C for 15 to 20 days, until the mycelium completely colonized the seeds.

The spawn was inoculated in oat straw, which was pasteurized by immersion in hot water (45 min at 70 °C). The weight of seed inoculated was 5 % of the total weight of the wet substrate (Guzmán *et al*., 1993). Bags of 4 kg in capacity (5 bags for each strain) were used. After

spawning, the bags were incubated in darkness at 25 °C until complete colonization (7 to 11 days, depending on the strain evaluated). The bags were then transferred to the production area and kept under light conditions at room temperature (25 to 30 °C) and relative humidity of 85-95 %. Humidity was maintained using an automatic humidifier. They were kept in the production area until the fruiting bodies reached commercial maturity. Three flushes were harvested for each strain.

Evaluation of productive characteristics

The productive cycle (PC) was recorded as the period between the day of inoculation and the final day of the third harvest (Royse, 1989). Biological efficiency (BE) was measured as the fresh weight of the fruiting bodies divided by the dry weight of the substrate and multiplied by 100. The production rate (PR) is the result of BE divided by the PC. Total weight (TW) is the fresh weight of fruiting bodies in each bag. The diameters of the harvested fruiting bodies were measured. Fruiting body size was classified into three different groups (G1 ≤ 5 cm, G2 = 5.1-10 cm and G3 > 10 cm) (Gaitán-Hernández and Salmones, 2008).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) to determine the significance of individual differences at P ≤ 0.05 under a randomized complete block experimental design. When statistical differences were found, the Duncan test with P ≤ 0.05 was applied. The statistical package SPSS 22 (for Windows) was used.

RESULTS

Phylogenetic study and species identification

The final ITS alignment contains 714 characters, including gaps. *Hohenbuehelia mastrucata* (Fr.) Singer was used as outgroup. The topologies of the resulting ML and Bayesian trees were highly similar. The ML tree is shown in Figure 1. The 10 strains belonged to three species as follows: seven strains (1182, 1160, 601, MXLD13, MXLD23, MXLD24 and 598) clustered with *P. djamor* with full support (100/1); the strain 1096 formed a fully supported (100/1) clade with *P. albidus*, and two strains (HUV15, 1163) formed a sister clade with *P. eous*. Although ITS sequences differ only at one to three positions, it was chosen to identify the strains (HUV15, 1163) as *Pleurotus* sp. because *P. eous* is known as pink oyster mushroom, but in the fruiting test the studied strains never showed pink tones.

Productivity

The productive characteristics showed significant differences between strains (Table 2). The highest TW and BE were observed in *Pleurotus* sp. (1163), with significant differences between most of the strains. The lowest TW was recorded in *P. djamor* (MXLD24) at 172.6 g and BE of 69 %.

Table 1. Origin of studied strains from the region of the high mountains, State of Veracruz, Mexico.

Code	Collecting site	Collector	Collection date
HUV15	Ayojapa, Zongolica	Maricela Avila	04/09/2016
MXLD24	Amatlán de los Reyes	Jie Chen	05/10/2016
MXLD23	Amatlán de los Reyes	Jie Chen	05/10/2016
MXLD13	Amatlán de los Reyes	Jie Chen	05/10/2016
1182	Ixtaczoquitlán	Miguel Barrales	01/15/2017
1096	Monte Blanco	Miguel Barrales	01/05/2016
601	Tlaquilpa	Miguel Barrales	07/05/2014
1163	Ixtaczoquitlán	Miguel Barrales	09/04/2016
1160	Amatlán de los Reyes	Miguel Barrales	09/03/2016
598	Fortín de las Flores	Miguel Barrales	06/05/2014
IE115	†		

†Commercial strain of *P. pulmonarius* from the collection of the Institute of Ecology (INECOL) of Xalapa, Veracruz, México.

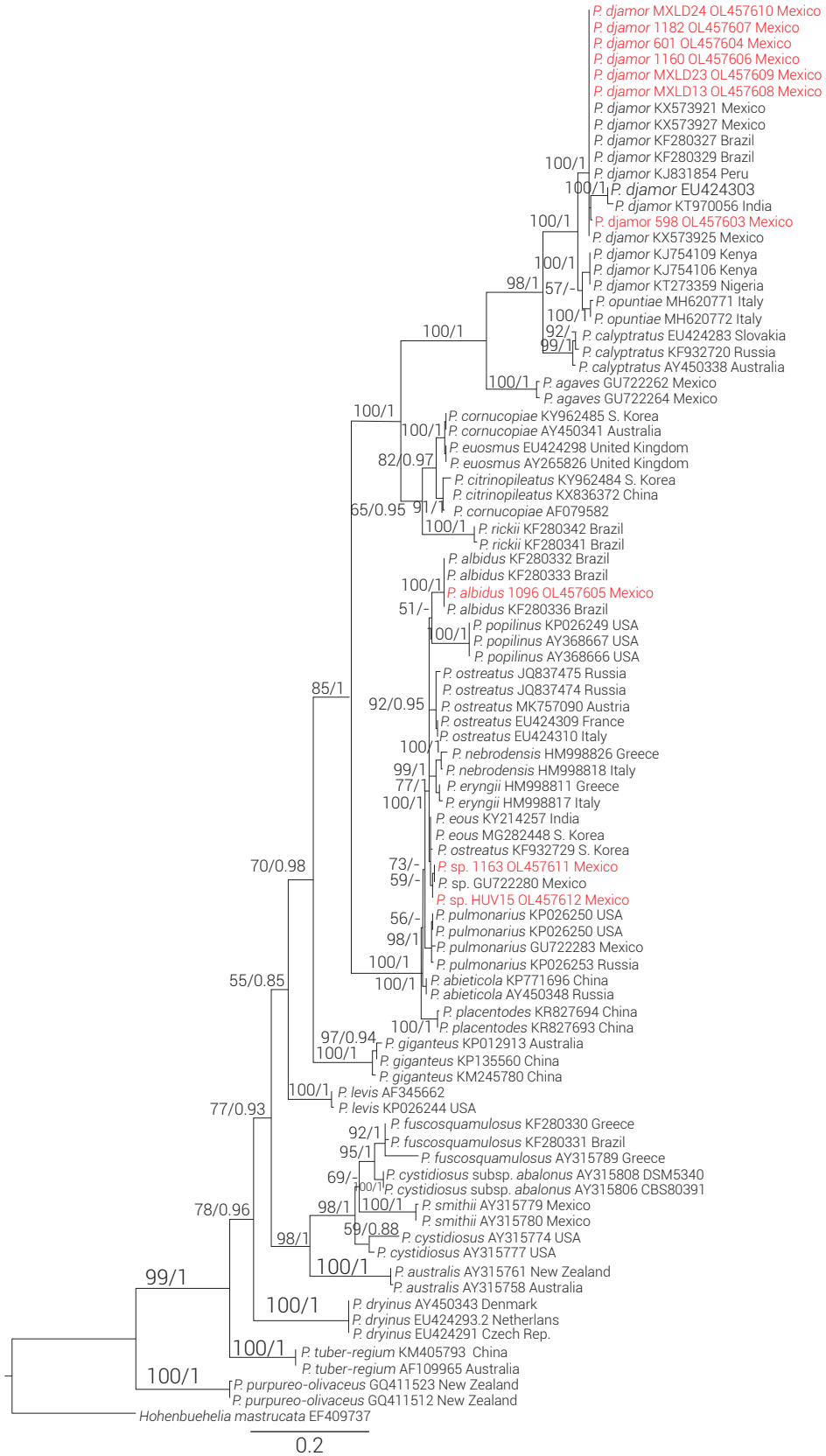

Figure 1. Maximum likelihood phylogram of *Pleurotus* produced from ITS sequence data. ML bootstrap values (BS) greater than 50 % and Bayesian posterior probabilities (PP) greater than 0.9 are shown above and below the branches (BS/PP), respectively. The strains from this study are shown in red.

Strain 1160 was found to be unable to colonize the substrate.

The strains of *P. djamor* (598, MXLD13, 601) and *P.* sp. (1163, HUV15) showed TW, BE, PR and PC values similar to those of strain IE115 (*P. pulmonarius*). These variables are commonly used as indicators to determine the productive capacity of a commercial strain.

Regarding the size of the pileus, *P. albidus* presented a larger number of G1 fruiting bodies, which represent the measurements of smallest diameter (Table 3). Although fruiting bodies classified as G2 (5.1-10 cm) were not predominant in the evaluated strains, the most promising one in terms of obtaining average size (G2) fruiting bodies was strain 598 of *P. djamor*.

Phenotypic differences in fruiting body color and shape were found between the strains studied (Figure 2). Strain 601 (*P. djamor*) presented pink coloration, and the color of the fruiting bodies was more intense when young. As the fruiting bodies grew, the intensity of the pink coloration decreased. The remaining strains of *P. djamor* were cream to whitish in color (Figure 2). Regarding the shape of the fruiting body, HUV15 (*P.* sp) presented a similarity with the control strain IE115 (*P. pulmonarius*), with an elongated eccentric stipe and a flabelliform pileus with smooth edges. The six strains identified as *P. djamor* were characterized by an eccentric stipe smaller than 1 cm in length and almost absent in some fruiting bodies, and a flabelliform pileus with irregular margins. Strain 1096 (*P. albidus*) was distinguished from the others by its infundibuliform and circular pileus and elongated centric stipe with lamellae that extended as edges along much of the length of the stipe.

DISCUSSION

The genus *Pleurotus* is monophyletic and its topologies agree with the results of a previous study (Zervakis *et al*., 2019). In Mexico, seven *Pleurotus* species (*P. agaves*, *P. albidus*, *P. djamor*, *P. levis*, *P. ostreatus*, *P. pulmonarius* and *P. smithii*) have been reported in previous studies (Guzmán, 2000; Huerta *et al.,* 2010; Moreno-Fuentes and Bautista-Nava 2006). With the exception of *P. albidus*, data are available for sequences from wild Mexican strains of more than six species. In the present study, eight strains were assigned to two species: *P. albidus* and *P. djamor*. The two remaining strains (HUV15, 1163) have not yet been identified.

To our knowledge, this is the first report of molecular identification and cultivation of *P. albidus* from Mexico. This species has been recorded by morphological identification in Mexico by Moreno-Fuentes and Baustista-Nava (2006).

Table 2. Productive characteristics of wild *Pleurotus* spp. strains.

Species	Strain	Total weight (g)	Biological efficiency (%)	Production rate	Productive cycle (d)	Appearance of primordia (d)
Pleurotus sp.	1163	260.5^{a}	104.2^{a}	2.7^{a}	39.0^{b}	16.2^{b}
	HUV15	235.6^{b}	94.2^{b}	2.5^{a}	37.6^{b}	17.0^{a}
P. albidus	1096	210.9^{d}	84.3^{d}	1.6^{d}	51.2^{a}	19.4^{a}
P. djamor	MXLD24	172.6^{e}	69.0^{e}	1.8^{c}	37.8^{b}	11.2^{d}
	1182	238.6^{b}	95.4^{b}	1.8^{d}	52.8^{a}	12.2^{d}
	598	230.7^{b}	92.3^{b}	2.7^{a}	33.2^{c}	10.6^{e}
	MXLD23	215.1^{c}	86.1^{c}	2.4^{a}	35.2^{b}	10.0^{e}
	MXLD13	212.6^{b}	85.0^{d}	2.2^{b}	35.8^{b}	13.8^{c}
	601	221.5^{b}	88.6^{b}	2.4^{a}	36.8^{b}	14.0^{b}
P. pulmonarius	IE115 (control)	227.9^{b}	91.1^{b}	2.2^{bc}	41.0^{b}	16.4^{a}

Values represent the mean ± SE (standard error). Means with different letters within the column differ significantly (Duncan, P ≤ 0.05). Data obtained during the evaluation of three harvested flushes.

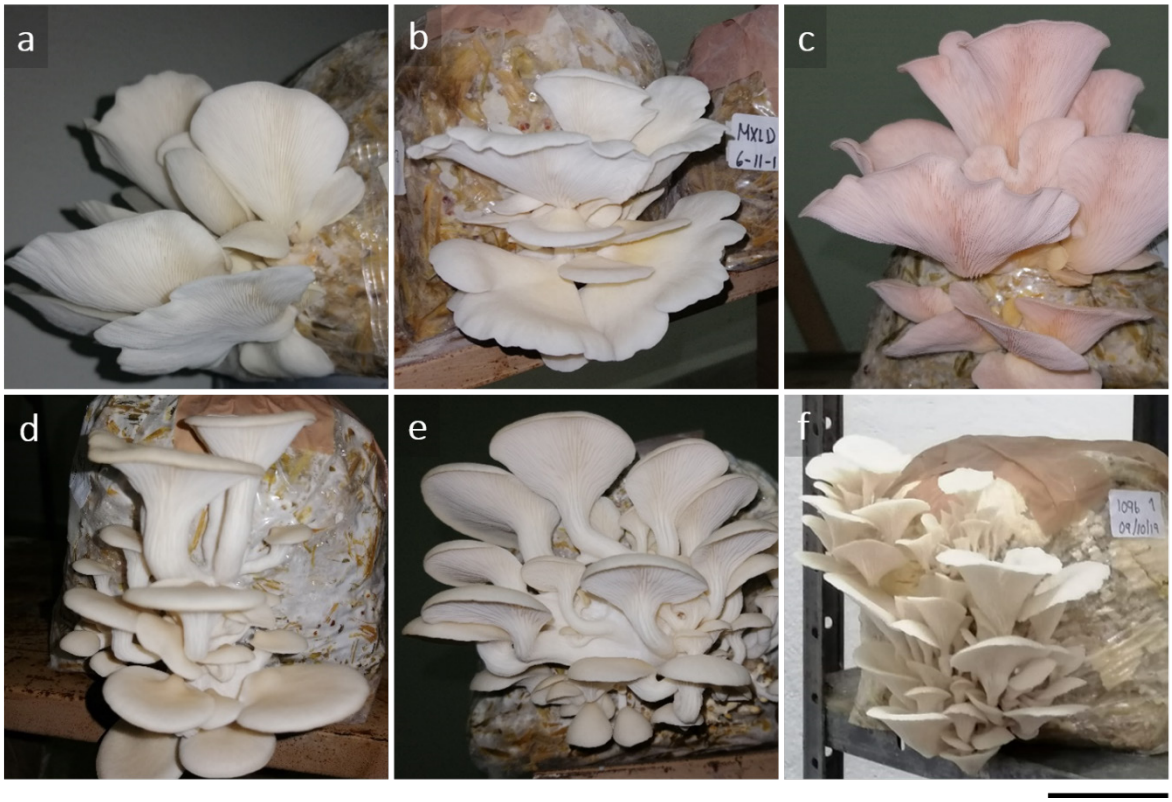

Figure 2. Basidiocarps production in different *Pleurotus* strains during the first harvest. a) MXLD24 strain *P. djamor*, b) MXLD13 strain *P. djamor*, c) strain 601 *P. djamor*, d) IE115 strain *P. pulmonarius*, e) HUV15 strain *Pleurotus* sp., f) 1096 strain *P. albidus*. Bar = 5 cm.

Table 3. Amount of basidiocarps obtained and classified by the size of pileus from *Pleurotus* strains.

Species	Strain	Amount of sporocarps per group		
		G1	G2	G3
Pleurotus sp.	1163	38.4^{b}	16.6^{a}	0.4^{d}
	HUV15	37.0^{c}	10.0^{d}	0.6^{d}
P. albidus	1096	63.0^{a}	10.2^{d}	1.0^{c}
P. djamor	MXLD24	23.0^{c}	16.4^{a}	2.0^{b}
	1182	21.0^{d}	14.2^{b}	3.2^{a}
	598	33.8^{b}	21.2^{a}	1.8^{b}
	MXLD23	28.0^{b}	19.4^{a}	2.6^{b}
	MXLD13	33.2^{b}	19.6^{a}	1.4^{c}
	601	18.0^{e}	12.8^{c}	2.2^{b}
P. pulmonarius	IE115 (control)	23.4^{c}	13.0^{c}	0.0

Values represent the mean ± SE (standard error). Means with different letters within the column are significantly different (Duncan, $P \leq 0.05$). Data obtained during the evaluation of three harvested flushes. G1 ≤ 5 cm, G2 = 5.1-10 cm, G3 >10 cm.

Most of the strains studied correspond to *P. djamor*. This species has been reported in greater abundance in Mexico (Salmones, 2017). According to the present study, strains HUV15 and 1163 are more closely related to *P. eous*; however, the studied strains do not show any pink tones in the fruitification, which is very different from *P. eous*. Although a strain of *P. ostreatus* is observed in the same clade, this and other strains of *Pleurotus* strains fall into a group called *P. ostreatus* OB group (Pánek *et al*., 2019). The authors indicate that commercial strains that have undergone several crossing processes present a high degree of hybridization, which makes genetic identification at the species level difficult, unlike wild species of *P. ostreatus*, which are found in an OA group of *P. ostreatus*. Additional molecular markers, such as TEF1 and RPB2 could be helpful for species delimitation (Li *et al*., 2018).

For the productive evaluation of wild strains, some features of interest may be highlighted, such as the capacity for cultivation at ambient temperatures above 25 °C and short production cycles. Thawthong *et al.* (2014) stated that most tropical and subtropical fungi grow at 25 °C or more, and therefore, can be produced faster than temperate species. *P. djamor* 598 showed the shortest productive cycle (33.2 ± 0.7 days) and the highest production rate (PR) (2.7 ± 0.2). In general, high values of the production rate were observed for *P. djamor* compared to the study by Salmones *et al*. (2004), who evaluated several strains of *P. djamor* strains for breeding, and found production rates from 0.89 to 1.9. In this sense, the studied strains could be an option for growers or serve as a basis for a breeding program.

As for BE and PR, the values presented for the strains of *P.* sp. (HUV15, 1163) in this study (91.1 ± 3.6 and 2.2 respectively) are similar to those reported by Gaitán-Hernández and Salmones (2008), and Gaitán-Hernández *et al*. (2009); the latter cultivated *P. ostratus* strains on barley straw (with maximum values of 98 % for BE and 2.21 for PR).

Furthermore, *Pleurotus* sp. (1163) presented the highest values for production variables (BE = 104.2 ± 1.1, PR = 2.7 ± 0.2). Strain 1163 was better than the control strain IE115. In the case of strain 1163, isolated from wild specimens in Zongolica City, Mexico, the high yield suggests that it is probably a commercial strain that escaped from a cultivation area. Other molecular markers such as SSR could confirm this hypothesis (Li *et al*., 2018).

Commercial exploitation of *P. djamor*, in particular strain 598, would allow a considerable reduction of production cycles (33.2 ± 0.7 days). This is in agrement with Salmones (2017), who reported that the duration of the process in *P. djamor* strains may be shorter than the cultivation cycles of other commercial *Pleurotus* species as *P. pulmonarius* (41 days in this study).

Pleurotus albidus has been recorded in the Caribbean (Trinidad), Central America (Costa Rica and Mexico), and South America (Brazil, Argentina) (Albertó *et al.,* 2002; Moreno-Fuentes and Baustista-Nava, 2006). In this study, *P. albidus* was one of the species that exhibited the lowest values of BE (84.36 ± 1.4) and PR (1.62), as well as longer PC (51.2 ± 1.3); however, Lechner and Albertó (2011) obtained the highest yield with *P. albidus* cultivated on wheat straw (BE = 171.3 %), surpassing the yield of the commercial strain evaluated on the same substrate. This information could be considered to optimize the cultivation of *P. albidus* on different substrates, thereby maximizing its utilization. It is important to highlight its cultivation in the region since it has been proposed for biomass production due to its potential immunomodulatory properties (Castro-Alves *et al.*, 2017; Lechner and Albertó, 2017). Castro-Alves and Oliveira do Nascimento (2018) have suggested that *P. albidus* has potential for use as a functional food.

Relative to the size of fruiting bodies, the strains HUV15, 1163 and 598 showed potential for the production of fruiting body smaller than 5 cm (G1) as well as medium-sized fruiting bodies of 5 to 10 cm (G2). Results of this study are consistent with those reported by Salmones *et al*. (2020), who studied different strains of *Pleurotus* and mostly harvested fruiting bodies on the G1 scale. The optimal harvest size of fruiting bodies depends on the production objective, since small fruiting bodies can be used as snacks while larger fruiting bodies are useful for cutting and industrialization (FAO, 2011). Flores and Contreras (2012) stated that mushrooms smaller than 10 cm constitute a higher quality product and easier to sell. The fruiting bodies obtained from the studied strains can therefore serve for different applications, both for cultivation and as a basis for a breeding program.

CONCLUSION

Eight of the ten *Pleurotus* strains were molecularly identified, with *P. albidus* being molecularly identified for the first time in Mexico. This study demonstrates the potential for commercial cultivation of *Pleurotus* spp. in tropical areas. Strain 1163 showed high potential for cultivation purposes in Veracruz, Mexico. *P. djamor* (598) showed a short production cycle (33.2 ± 0.7 days). *P. djamor* showed phenotypic variability that could be an option for growers or as a basis for a breeding program.

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